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ORIGINAL PAPER

Genetic variants of the glucagon-like receptor-1 in obesity

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ABSTRACT

Introduction and aim. Dysfunction of the glucagon-like peptide 1 (GLP-1)/GLP-1 receptor (GLP-1R) axis promotes obesity and metabolic disorders. The aim was to study the associations of the single nucleotide variants (SNV) *GLP1R* gene with pro-inflammatory cytokines and metabolic disorders in children with various obesity phenotypes.

Material and methods. 252 children with obesity aged 6-18 years were examined. The first group (n=152) was represented by children with metabolically unhealthy obesity (MUO). The second group (n=100) consolidated of children with metabolically healthy obesity (MHO). Whole genome sequencing (CeGat, Germany) was performed in 52 children.

Results. An association with the development of obesity was noted for T alleles rs61754624 (t=3.33) and rs10305457 (t=2.06); with MUO – for C alleles rs1042044 (t=2.23), rs1126476 (t=2.63), rs2235868 (t=2.82); T alleles rs61754624 (t=3.33), rs10305457 (t=2.06) *GLP1R*, p<0.05. In the MHO group, a correlation was found with the levels of pro-inflammatory markers IL-1β, IL-6 in the presence of the GA genotype SNV rs3765468; with hyperglycemia - GA genotype SNV rs6923761, CC genotype SNV rs1042044, AA rs6918287; hyperinsulinemia - GA genotype SNV rs3765468, GG rs10305421; triglyceridemia - AA rs6918287 of *GLP1R*.

Conclusion. SNV rs1042044, rs3765468, rs6923761, s6918287, and rs rs10305421 *GLP1R* are associated with the development of MUO in individuals with MHO.

Keywords. analysis of single nucleotide gene variants, children, glucagon-like peptide-1 receptor, metabolically healthy obesity, metabolically unhealthy obesity

Introduction

The spread of obesity and associated metabolic disorders in populations of both adults and children in the last 50 years has reached epidemic levels throughout the world.[1-5]

Obesity significantly increases the risk of developing diseases such as type 2 diabetes mellitus, metabolically associated fatty liver disease, arterial hypertension, myocardial infarction, stroke, osteoarthritis, obstructive sleep apnea and some types of cancer, thereby contributing to a decrease in both quality and life expectancy.[6-8]

It has now been demonstrated that among the various molecular systems involved in the regulation of energy balance and eating behavior, the glucagon-like peptide-1 (GLP-1) and GLP-1 receptor (GLP-1R) axis plays one of the key roles. Dysfunction of the GLP-1/GLP-1R axis contributes to the development of obesity and metabolic disorders.[9-11]

A gastrointestinal GLP-1 peptide that, in response to direct food stimulation, is released from intestinal enteroendocrine cells and excites GLP-1R, which is expressed by various body cells. The *GLP1R* gene (HGNC:4324) is located on the short arm of chromosome 6 (6p21). The GLP-1R molecule consists of 463 amino acid residues and contains 7 transmembrane domains. The GLP-1R receptor belongs to the family of G protein-coupled receptors. Excitation of GLP-1R afferent vagal neurons leads to a decrease in appetite and

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a feeling of satiety; β-cells of the islets of Langerhans of the pancreas – to increase insulin secretion; α-cells of the islets of Langerhans of the pancreas – to inhibition of glucagon production; hepatocytes – to suppress the release of glucose and accumulation of glycogen; muscle cells – to increase the activity of glucose uptake and oxidation, adipocytes – to increase the activity of glucose uptake and suppression of lipolysis processes.[12-15]

According to the modern concept, obesity is considered as a disease that occurs with the development of a chronic inflammatory reaction with a low level of activity, called meta-inflammation. The molecular features of overweight-induced meta-inflammation are of particular practical interest in the context of the obesity pandemic in the human population. Adipose tissue, being in conditions of increased concentration of free fatty acids, which can induce a TLR-mediated inflammatory response, contains cellular anti-inflammatory mechanisms, the main component of which is a population of macrophages with the M_1 phenotype.[16] Of interest is the anti-inflammatory role of activation of the GLP-1/GLP-1R axis in the regulation of the immune response and the prevention of meta-inflammation. Both innate and innate-like cells express GLP-1R. The interaction of GLP-1R and its ligands activates several signaling pathways including PKA/STAT, PI3K/Akt, MAPK and NFκB.[17] GLP-1 and its analogs (GLP-1A) can directly polarize macrophages to the M_2 phenotype, also indirectly promote M_2 polarization by inhibiting M_1[18] and potentiating regulatory T cells (Treg).[19] Activation of the JNK/STAT3 signaling pathway by GLP-1 results in decreased c-Jun N-terminal kinase (JNK) phosphorylation and its signaling through the cyclic adenosine monophosphate/protein kinase A (PKA) signaling pathway, while STAT3 phosphorylation is increased, which additionally induces polarization of macrophages towards M_2.[20] Under the influence of GLP-1, activation of the MAPK/ERK and PKA signaling pathway suppresses fatty acid synthase (FASN), IL-6 production, and eliminates endothelial progenitor cell (EPC) dysfunction that is induced by high glucose.[21] Experimental administration of GLP-1A to rats inhibits the activation of nuclear factor kappa-B (NF-κB) and IL-1β, and thus reduces inflammation.[22] In addition, GLP-1RA may function via the phosphoinositide-3-kinase (PI3K)/Akt pathway to protect the microvascular endothelium from oxidative stress in cardiometabolic disorders.[23]

The meta-inflammation that drives the metabolically unhealthy obesity (MUO) phenotype is clearly associated with dysfunction of innate immune control, including the local intestinal intraepithelial lymphocyte (IEL)-GLP-1R signaling network. Therapy with GLP-1RA (liraglutide, semaglutide, and others) in patients with MUO leads to a decrease in body weight due to a decrease in visceral fat, and suppresses the activity of manifestations of metabolic disorders.[11, 24-28]

A decrease in the level of GLP-1 reception, which is caused by single nucleotide variants (SNVs) of the *GLP1R* gene, can induce the development of obesity and metabolic disorders.[29] However, the study of associations with MUO was carried out only for some SNVs of the *GLP1R* gene.

Aim

The research was aimed to study the associations of the SNV *GLP1R* gene with pro-inflammatory cytokines and metabolic disorders in children with various obesity phenotypes.

Material and methods

Ethical approval

Participants provided written informed consent, and research protocols and procedures were approved according to the ethical standards of the Helsinki Declaration 2013 and by the Human Research Ethics Committee of Dnipro State Medical University, Ukraine (meeting minutes No. 7 of December 11, 2019 and minutes from meeting No. 4 of September 2, 2020). We obtained formal written informed consent from the parents of the children to participate in the study. Time of data collection: January 2020–February 2023.

Study design

Study design: observational, analytical, longitudinal, cohort study.

Inclusion criteria: children with polygenic obesity (BMI≥97th percentiles) 6-18 years old. Exclusion criteria: children with monogenic and/or syndromic obesity, pregnancy.

252 children with obesity aged 6–18 years were examined. The first group (n=152) was represented by children with MUO. The second group (n=100) consolidated of children with metabolically healthy obesity (MHO). For inclusion in the first observation group, the presence of abdominal obesity and two of the presented criteria were taken into account: 1). Fasting glycemia ≥ 5.6 mmol/L and/or according to the recommendations of the IDEFICS Study, the level of basal insulinemia is more than 90 percentile; 2).[30,31] High density lipoprotein cholesterol (HDL-C) ≤ 1.03 mmol/L or less than 10th percentile of the age norm; 3).[32] Triglycerides (TG) ≥1.7 mmol/L or more than the 90th percentile of the age norm; 4) Systolic blood pressure (SBP) and Diastolic blood pressure (DBP) above the 90th percentile for a given age, gender and height.[33]

The abdominal type of obesity was determined according to the consensus of the International Diabetes Federation (IDF), based on the excess of the waist circumference over the 90th percentile for children 6-15 years old or more than 94 cm for boys aged 16–18 years and more than 80 cm for girls 16–18 years old.[34]

To study carbohydrate metabolism disorders, the level of basal glycemia and insulinemia was determined by the immunochemical testing method with electrochemiluminescent detection (ECLIA), in the certified Synevo Laboratory (Dnipro, Ukraine), followed by the calculation of the generally accepted marker of insulin resistance (HOMA-IR).[30,31]

To study lipid metabolism disorders, the level of HDL-C and TAG was determined by the enzymatic-colorimetric method using kits from Roche Diagnostics (Switzerland) on a Cobas 6000 analyzer in the certified Synevo Laboratory (Dnipro, Ukraine).

To study the role of pro-inflammatory markers in the development of meta-inflammation in children with obesity, the serum levels of interleukin-1β (IL-1β), interleukin-6 (IL-6) were determined in the certified Synevo Laboratory (Dnipro, Ukraine). IL-1β was detected by the immunochemical method with chemiluminescence immunoassay (CLIA). Analyzer and test – system: Immulite (Siemens AG), Germany. The reference value of IL-1β level was 0-5 pg/mL. IL-6 was determined by an enzyme-linked immunosorbent assay (ELISA) using a Cobas 6000/Cobas 8000 kit provided by Roche Diagnostics (Switzerland). The reference value of IL-6 level was 1.5–7 pg/mL.

From the first and second groups, 52 samples for WGS were selected by limited randomization for an unbalanced distribution with a distribution coefficient of 1.5 between baseline and selective subgroups with different obesity phenotypes.[35] The sample population examined by whole genome sequencing (NGS, Illumina CSPro®, CeGat, Germany) consisted of 31 children of the first and 21 children of the second group and was qualitatively homogeneous in relation to the general population).

Material for research: venous blood. Starting material: dried blood spot cards. For DNA extraction from blood cards, we use the following protocol: Sbeadex DNA Purification Kit, customized CeGaT version (Biosearch Technologies, LGC). Average amount of DNA (μg) in samples – 0.875. Library Preparation: Quantity used 50 ng. Library Preparation Kit: Twist Human Core Exome plus Kit (Twist Bioscience). Sequencing parameters: NovaSeq 6000; 2 x 100 bp.

Bioinformatic analysis – demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (version 2.20). Adapters were trimmed with Skewer, version 0.2.2. DNA-Seq: Trimmed raw reads were aligned to the human reference genome (hg19-cegat) using the Burrows-Wheeler Aligner, BWA – mem version 0.7.17-cegat.[36,37] ABRA, version 2.18 and GenotypeHarmonizer v.1.4.20 were used for local restructuring of readings in target regions to improve more accurate detection of indels in the genome during mutagenesis.[38,39]

We used ClinVar Version 20200316, InterVar gnomeAd Version 3.0 and dbnsfp Version 35c for clinical and functional variant annotation and GWAS catalog database annotation.[33,34,40,41]

Reference sequence obtained from the National Center for Biotechnology Information RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/).[44]

Statistical analysis

Statistical analysis of the obtained results was carried out using a package of application programs Statistica 6.1 (No AGAR909E415822FA) with help a personal computer based on an Intel processor Pentium 4.

For statistical processing of the materials studied, the normality of the distribution of signs was rechecked according to the Shapiro-Wilk test (SW-W), the evenness of the dispersions - according to the Fisher test (F). The arithmetic mean with the error of the mean value (M±m) was used to describe quantitative traits with a normal distribution; the standard deviation (SD) to describe the variation in the traits and the 95% confidence interval (95% CI), sequential Wald analysis with calculation of Relative Risk (RR), to define the range of the population means.

The relationship between indicators was determined using Spearman correlation analysis. The reliability assessment of the difference of means in multiple comparisons for quantitative traits with a normal distribution was carried out by one-way analysis of variance (ANOVA) with a posteriori pairwise comparisons according to the Tukey test. Intergroup comparisons of statistical characteristics were performed taking into account the law of distribution using parametric and non-parametric criteria: assessment of the probability of differences in means for unrelated samples – according to Student's criteria (t) in the Welch modification (R Studio, Version 1.0.136, 2016). Only statistically significant results were taken into account ($p<0.05$).

Results

The average age of children in the first observation group (n=152) was 13.64±0.43 years, while in the second group (n=100) it was 11.05±0.6, $p<0.005$. The number of children under 12 years old in the first group was 13%, in the second group – 48%. In this regard, the RR=4.67±0.46 (95% CI 1.87–11.16) of MUO in children over 12 years old was 4.67 times higher than in children under 12 years old, $p<0.05$. The number of boys in the first group – 42% (65/152), in the second group – 48% (48/100), $p>0.05$. RR of MUO in girls was 0.99±0.13 (95% CI 0.8–1.2), $p<0.05$.

The results of clinical and paraclinical examinations (Table 1) of children with various obesity phenotypes revealed the most frequent clinical associations of markers of the complicated course of obesity (dyslipidemia,

hyperglycemia, pro-inflammatory orientation of the immune response).

Table 1. Data of clinical and paraclinical examination of children with different obesity phenotypes

Significative	MUO (n=152), M±m	MHO (n=100), M±m	p
BMI in percentiles, %	99.54±0.21	98.74±0.29	0.12
Presence of extreme obesity 2nd stage (120-139% over the 95th percentile), %	19±3.92	16.1±3.68	0.06
Presence of extreme obesity 3rd stage (over 140% out of 95th percentile), %	32.3±4.66	0	0.00001
WC in percentiles	96.65±0.42	93.38±0.82	0.0004
SBP in percentiles	83.77±3.05	71.38±3.96	0.014
DBP in percentiles	87.48±2.75	66.33±4.09	0.0006
HDL in percentiles	30.83±4.04	32.81±2.79	0.68
TAG in percentiles	87.7±2.28	83.33±3.63	0.3
Fasting blood glucose, mmol/L	4.15± 0.37	3.36±0.48	0.2
Basal insulin, mcU/mL	29.47±1.14	12.53±1.44	0.00001
HOMA-IR	5.32±0.3	2.13±0.12	0.00001
IL-6, pg/mL	3.4±0.82	1.04±0.22	0.007
IL-1β, pg/mL	3.6±0.63	1.78±0.17	0.008

In children with obesity examined by whole genome sequencing, 14 SNVs of the *GLP1R* gene were identified: rs761386, rs1042044, rs1126476, rs2235868, rs3765468, rs61754624, rs6918287, rs6923761, rs10305420, rs10305421, rs10305457, rs10305492, rs10305493, rs1472308929. The distribution of genotype frequencies was in Hardy-Weinberg equilibrium in both groups.

Molecular genetic characteristics of the identified SNVs of the *GLP1R* gene are presented in Table 2.
Among the identified SNV of the *GLP1R* gene, the highest CADD was observed in three nonsynonymous variants rs10305493, rs10305421, rs10305492 (26.1; 25; 22.5, respectively).

Associations of SNV GLP1R gene with obesity phenotypes in children

The frequency of occurrence of SNV of the *GLP1R* gene in children with different obesity phenotypes is presented in Table 3.

In obesity, the AF of the minor T alleles for SNV rs61754624 (t=3.33) and rs10305457 (t=2.06) of the *GLP1R* gene was significantly higher than the allele frequency of these polymorphisms among healthy Europeans of non-Finnish origin, p<0.05.

In individuals with MUO, the AF of the minor C alleles of SNV rs1042044 (t=2.23, p<0.05), rs1126476 (t=2.63, p<0.05), rs2235868 (t=2.82, p<0.05); T alleles of SNV rs61754624 (t=3.33, p<0.05) and rs10305457 (t=2.06, p<0.05) of the *GLP1R* gene were significantly higher than the allelic frequency of these polymorphisms among healthy non-Finnish Europeans.

Among probands with MUO, the AF of the minor T allele rs761386 and A allele rs10305492 (t=2.29, p<0.05) was significantly higher compared to the allele frequency of these SNV gene *GLP1R* among children with MHO.

Table 2. Characteristics of SNV types of the *GLP1R* gene[a]

SNV	Variant name and GRCh38 reference sequence file identifier (HGVS)	GnomAD _maxPOP	Ref	Alt	Consequence	Base Change	CADD	RawScore	Clinical significanse (ClinVar)
rs6918287	6:39065826A>G (NM_002062.5: c.399A>G)	EAS	A	G	synonymous	c.399A>G	9.35	0.49	not reported
rs6923761	6:39066296G>A (NM_002062.5: c.502G>A	NFE	G	A	missense	c.502G>A	16.12	1.47	not reported
rs761386	6:39079095C>T (NM_002062.5: c.955-17C>T)	AMR	C	T	intronic	c.955-17C>T	4.32	0.10	not reported
rs1042044*	6:39073726A>C (NM_002062.5: c.526A>C)	AMR	A	C	missense	c.526A>C	14.9	1.25	not reported
rs1126476*	6:39080715A>C (NM_002062.5: c.1200A>C)	AMR	A	C	synonymous	c.1200A>C	11.53	0.75	not reported
rs2235868*	6:39072878A>C (NM_002062.5: c.526A>C)	AMR	A	C	synonymous	c.526A>C	12.35	0.85	not reported
rs3765468	6:39065817G>A (NM_002062.5: c.390G>A)	EAS	G	A	synonymous	c.390G>A	8.41	0.40	not reported
rs61754624*	6:39066295C>T (NM_002062.5: c.501C>T)	AMR	C	T	synonymous	c.501C>T	0.11	-0.47	likely benign
rs10305420	6:39048860C>T (NM_002062.5:c.20C>T)	NFE	C	T	missense	c.20C>T	13.38	0.99	not reported
rs10305421	6:39048899G>A (NM_002062.5: c.59G>A)	NFE	G	A	missense	c.59G>A	22.5	2.49	not reported
rs10305457*	6:39066319C>T (NM_002062.5: c.509+16C>T)	AMR	C	T	intronic	c.509+16C>T	0.43	-0.28	not reported
rs10305492	6:39079018G>A (NM_002062.5: c.946G>A)	NFE	G	A	missense	c.946G>A	25	3.51	not reported
rs10305493	6:39079155C>G (NM_002062.5: c.998C>G)	OTH	C	G	missense	c.998C>G	26.1	3.77	not reported
rs1472308929	6:39066202C>T (NM_002062.5: c.408C>T)	NFE	C	T	synonymous	c.408C>T	9.32	0.49	not reported

[a] HGVS – Human Genome Variation Society;[45] GnomAD_maxPOP – the frequency distribution of *GLP1R* mutations. AFR, NFE represent African, Non-Finnish European; Ref – reference allele; Alt – alternative allele; Consequence –functional consequence of the variation in relation to the transcript. The nucleotide change and position relative to the coding sequence of the affected transcript in HGVS nomenclature: c. CDS Position Reference Base > Alternative Base. Example: c.223A>T (c. - interpretation for DNA coding sequence: first nucleotide of the translation start codon of the coding DNA reference sequence).[46] This column is empty if the variant is intergenic; CADD – combined annotation dependent depletion; *- SNV *GLP1R* associated with MUO

Table 3. The frequency of occurrence of SNV *GLP1R* gene in children with different obesity phenotypes[a]

SNV	gnomAD browser		The frequency of occurrence of major and minor options (%)				The value of Student's t-test in Welch's modification		
			MHO (n=21)		MUO (n=31)				
	Popmax AF (HET/HOM^P), %	AF NFE, (HET/HOM^P), %±m	(HOM^N), % (n)	(HET/HOM^P), %±m (n)	(HOM^N), % (n)	(HET/HOM^P), %±m (n)	t_1	t_2	t_3
rs6918287	98	99±2.18	5 (1)	95±2.18 (20)	6 (2)	94±2.37 (29)	0.31	1.67	1.94
rs6923761	32	33±4.7	57 (12)	43±4.95 (9)	55 (17)	45±4.97 (14)	0.28	1.46	1.75
rs761386	19	3±2.18	95 (20)	5±2.18 (1)	100 (31)	0	2.29*	0.72	1.76
rs1042044	56	56±4.96	38 (8)	62±4.85 (13)	29 (9)	71±4.54 (22)	1.35	0.86	2.23*
rs1126476	50	50±5	52 (11)	48±5 (10)	32 (10)	68±4.66 (21)	2.93*	0.28	2.63*
rs2235868	46	52±5	48 (10)	52±5 (11)	29 (9)	71±4.54 (22)	2.82*	0	2.82*
rs3765468	8	7±2.55	90 (19)	10±3 (2)	90 (28)	10±3 (3)	0	0.76	0.76
rs61754624	0.7	0.6±0.77	90 (19)	10±3 (2)	100 (31)	0	3.33*	3.33*	3.33*
rs10305420	37	39±4.88	48 (10)	52±5 (11)	48 (15)	52±5 (16)	0	1.86	1.86
rs10305421	0.2	0.5±0.71	100 (21)	0	97 (30)	3±1.71 (1)	1.76	0	0
rs10305457	18	9±2.86	81 (17)	19±3.92 (4)	77 (24)	23±4.21 (7)	0.7	2.06*	2.06*
rs10305492	1	1±0.99	95 (20)	5±2.18 (1)	100 (31)	0	2.29*	1.67	1.67
rs10305493	0	0.01±0.1	100 (21)	0	97 (30)	3±1.71 (1)	1.76	0	0
rs1472308929	-	-	100 (21)	0	97 (30)	3±1.71 (1)	1.76	-	-

[a] HOM^P – homozygous variant (biallelic single nucleotide substitution), HET – heterozygous variant (single allelic single nucleotide substitution), HOM^N – homozygous variant (absence of nucleotide substitutions); Popmax AF – Maximum population allele frequency in the genome (gnomAD browser); AF NFE – Allele frequency for Non-Finnish Europeans in the genome (gnomAD browser); * – Critical value of Student's t-test modified by Welch >1.97, number of degrees of freedom f=198, at which the differences in the compared groups are significant, $p<0.05$; t_1 – Student's test of significance in the MUO and MHO comparison groups; t_2 – Student's test of significance in the comparison groups MHO and healthy Non-Finnish Europeans; t_3 – Student's test of significance in the comparison groups MUO and healthy Non-Finnish Europeans; m – relative indicator mean error

Associations of SNV GLP1R gene with inflammatory activity

Correlations of heterozygous SNV phenotypes rs6923761, rs3765468, rs10305420 of the *GLP1R* gene with the level of pro-inflammatory cytokines in blood serum were observed in children with MHO. Thus, rs6923761 and rs10305420 of the *GLP1R* gene were inversely proportional to the levels of IL-1β, IL-6 (r=-0.38 rs6923761 – IL-1β); r=-0.33; -0.48 (rs10305420 – IL-1β, IL-6), respectively), and rs3765468 of the *GLP1R* gene is directly proportional to the level of IL-1β, IL-6 concentration in blood serum (r=0.30; 0.72, respectively), $p<0.05$.

One-way analysis of variance (ANOVA) revealed the influence of SNV rs3765468 genotype of the *GLP1R* gene in children with MHO on the pro-inflammatory variant of the immune response in the form of increased IL-6 (F=5.77; p=0.05). A pairwise comparison of indicators depending on the genotype revealed the formation of a pro-inflammatory immune response in the form of an increase in IL-6 with the AA rs3765468 genotype ($p<0.05$ for pairwise comparisons of AA rs3765468 genotypes with others according to the Tukey test).

While in patients with MUO, there was no association of the level of pro-inflammatory cytokines in the blood serum with any SNV of the *GLP1R* gene.

Associations of SNV GLP1R gene with disorders of carbohydrate metabolism

It has been established that SNVs rs6923761, rs1042044, rs1126476, rs2235868, rs3765468 of the *GLP1R* gene are associated with the mechanisms of regulation of carbohydrate metabolism in children with MHO. It was shown that the GA genotype SNV rs6923761 (RR=1.39) and the CC genotype rs1042044 (RR=1.35) of the *GLP1R* gene are associated with the level of glycemia (r=0.35; 0.33, respectively), $p<0.05$. Whereas the CC SNV genotypes rs1126476 and rs2235868, as well as the GA genotype rs3765468 of the *GLP1R* gene, are associated with basal serum insulin levels (r=0.48; 0.51; 0.56, respectively), $p<0.05$.

In contrast to children with MHO, in children with MUO carbohydrate metabolism disorders were not associated with SNV of the *GLP1R* gene. In addition, the presence of the GG genotype SNV rs6918287 of the *GLP1R* gene prevented a decrease in carbohydrate tolerance (r=-0.43), and the presence of the GA genotype SNV rs10305421 of the *GLP1R* gene prevented the development of insulin resistance (r=-0.72), $p<0.05$. The results of ANOVA also showed that the genotype SNV rs6918287 and rs10305421 of the *GLP1R* gene affects the level of basal glycemia and insulin resistance in children with obesity (respectively F=6.26 and F=5.62; $p<0.05$). A pairwise comparison of the indicators of the formation of basal hy-

perglycemia and insulin resistance among themselves, depending on the genotype, revealed statistically significant higher levels of indicators in AA rs6918287 and GG rs10305421 genotypes ($p<0.05$ for pairwise comparisons of AA rs6918287 and GG rs10305421 genotypes with others according to the Tukey test).

Associations of SNV GLP1R gene with lipid metabolism disorders

Correlation analysis made it possible to establish that in children with MHO, the presence of CT genotype SNV rs10305420, GA genotype rs10305421 of the *GLP1R* gene is accompanied by a lower level of atherogenicity of the blood serum lipid spectrum (r=-0.43, -0.35, respectively), $p<0.05$. Whereas in children with MUO, the presence of the GG genotype SNV rs6918287 of the *GLP1R* gene is associated with a lower level of triglyceridemia (r=-0.49), $p<0.05$.

According to the results of ANOVA, significant differences in the influence of genotypes of SNV rs6918287 of the *GLP1R* gene in children with MHO on the level of triglyceridemia were found (respectively, F=51.34; p=0.05). A pairwise comparison of indicators of the formation of hypertriglyceridemia among themselves, depending on the genotype, revealed statistically significant higher levels of indicators with the AA rs6918287 genotype ($p<0.05$ for pairwise comparisons of the AA rs6918287 genotype with others according to the Tukey test).

Discussion

Considering the key role of the GLP-1/GLP-1R axis in maintaining the body's energy balance and regulation of carbohydrate metabolism, it is assumed that impaired GLP-1 reception or GLP-1R functioning will contribute to the development of obesity and metabolic disorders.[26] Currently, rs1042044 (Leu260Phe), rs10305420 (Pro7Leu), rs6923761 (Gly168Ser), and rs3765467 (Arg131Gln) are considered the most common nonsynonymous SNVs of the *GLP1R* gene.[28] We did not find the rs3765467 variant from the SNV data group of the *GLP1R* gene in obese individuals.

This study demonstrates that healthy children in the European population with at least one copy of the minor allele T rs61754624, rs10305457 have a higher risk of obesity, and with the presence of one or two copies of the minor allele C rs1042044, rs1126476, rs2235868 or minor T alleles rs61754624, rs10305457 *GLP1R* genes have a higher risk of developing MUO than children with null copies of the above alleles.

We have shown for the first time that SNV rs3765468 of the *GLP1R* gene in children with the MHO obesity phenotype is associated with pro-inflammatory status, and SNV rs6923761, rs10305420 of the *GLP1R* gene with anti-inflammatory status. In all likelihood, given that GLP-1R is expressed by various immune cells, such as monocytes, macrophages, and T cells, SNV-mediated changes in GLP-1R activity may predetermine the level of production of pro-inflammatory cytokines.[47]

The GLP-1/GLP-1R axis is known to be a key regulator of carbohydrate metabolism. We have shown that the SNVs rs6923761, rs1042044, rs1126476, rs2235868, rs3765468 of the *GLP1R* gene introduce specific features into the functioning of carbohydrate metabolism in children with MHO.

It was found that two nonsynonymous SNVs rs6923761, rs1042044 of the *GLP1R* gene contribute to the development of glycemia. The most common polymorphism of the *GLP1R* gene is the genetic variant rs6923761, which, according to our data and the results of other researchers, is moderately associated with the level of glycemia.

The rs6923761 (G>A/C) variant is a missense variant, which is accompanied by the substitution of a glycine for a serine residue at position 168 (Gly168Ser) of the GLP-1R molecule. According to the ACMG classification, SNV rs6923761 was classified as a benign variant. At the same time, Michałowska et al. revealed a tendency to hyperglycemia in carriers of the AA rs6923761 genotype, compared with carriers of the AG rs6923761 genotype, but found no association with the metabolic syndrome, which also coincided with the results of our studies.[48] Sathananthan et al.[49], also Daniel Antonio de Luis et al. demonstrated that GLP-1R in heterozygotes for SNV rs6923761 of the *GLP1R* gene (GA genotype) has a low receptor affinity for GLP-1, which leads to relatively reduced insulin secretion in response to GLP-1 infusion and, as a result, promotes the development of hyperglycemia. Individuals with the A allele of SNV rs6923761 of the *GLP1R* gene and morbid obesity have higher levels of triglycerides, insulin, and insulin resistance.[50] Interestingly, carriers of the GG SNV rs6923761 genotype show a weaker response to treatment with liraglutide than carriers of the non-wild-type allele.[51] Individuals with the genotype AA rs6923761 are at higher risk of becoming overweight.[48]

The missense mutation rs1042044 (A>C,G,T) of the *GLP1R* gene is a variant that is accompanied by the substitution of a leucine for a phenylalanine residue at position 260 (Leu260Phe), which is accompanied by a decrease in receptor excitation and the development of glycemia.[52-55]

Li et al.demonstrated that the nonsynonymous SNV rs10305492, which leads to the replacement of an alanine by a tryptophan residue at position 318 (Ala316Thr) of the GLP-1R molecule, is also accompanied by a decrease in insulin secretion by β-cells.[56] However, in our study, we did not find any correlation between the presence of the minor allele and the level of glycemia or insulin in the blood serum. Wessel et al. also found no association

of SNV rs10305492 with fasting insulin levels or incretin response. [57] According to our data, synonymous SNVs rs1126476 (A>C), rs2235868 (A>C,G,T), rs3765468 (G>A) of the *GLP1R* gene are associated with a greater ability of basal insulin secretion by β-cells in children with MHO, which is consistent with the data other researchers.[58] The absence of associations of these SNV data of the *GLP1R* gene in children with MUO with hyperglycemia is probably due to the fact that the development of hyperglycemia in children with MUO is mainly due to insulin resistance induced by meta-inflammatory factors.

We have shown for the first time that in children with MHO, the presence of the missense mutation rs10305420 (C>T), which is accompanied by the replacement of proline with a leucine residue in position 7 (Pro7Leu), the missense mutation rs10305421 (G>A), which is accompanied by the replacement of arginine by lysine the residue at position 20 (Arg20Lys) of the GLP-1R receptor molecule prevents the occurrence of atherogenic disorders of lipid metabolism.

While in children with MUO, the GG SNV rs6918287 genotype of the *GLP1R* gene protects against the development of triglyceridemia. We did not find any relationship between SNV rs6923761 and lipid metabolism disorders. At the same time, de Luis et al.[12] demonstrated that individuals with the wild GG genotype, compared with individuals with the AA SNV rs6923761 genotype of the *GLP1R* gene, had a significantly lower HDL-C level and a higher serum triglyceride level.

In this work, we demonstrated the role of SNV *GLP1R* in the formation of a pro-inflammatory immune response and metabolic disorders with the possibility of the formation of certain MUO and MHO phenotypes among the European population. However, to determine the significance of SNV *GLP1R* gene polymorphisms and taking into account the limitation of their influence in the development of MUO, further study of their clinical associations in large cohorts of individuals with different obesity phenotypes is required.

Conclusion

Variants of the *GLP1R* gene in children with the MHO phenotype determine the level of inflammatory status (GA/AA SNV rs3765468), carbohydrate tolerance (GA rs6923761, CC rs1042044 and AA rs6918287), insulin resistance (GA/AA SNV rs3765468, CC rs10305421), and serum lipid spectrum atherogenicity blood (AA rs6918287) and thus predetermine its transformation into MUO.

From a practical point of view, the determination of the SNV genotype of the *GLP1R* gene will make it possible to predict the likelihood of MUO and personalize the trajectory of the development of various metabolic disorders associated with obesity in children.

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Declarations

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Author contributions

Conceptualization, A.N.; Methodology, A.N.; Software, A.N.; Validation, A.N.; Formal Analysis, A.N.; Investigation, A.N.; Resources, A.N.; Data Curation, A.N.; Writing – Original Draft Preparation, A.N.; Writing – Review & Editing, A.N.; Visualization, A.N.; Supervision, A.N.; Project Administration, A.N.; Funding Acquisition, A.N.

Conflicts of interest

The author declare no competing interests.

Data availability

The datasets used and/or analyzed during the current study are open from the corresponding author on reasonable request.

Ethics approval

Human Research Ethics Committee of Dnipro State Medical University, Ukraine (meeting minutes No. 7 of December 11, 2019 and minutes from meeting No. 4 of September 2, 2020).

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ORIGINAL PAPER

Evaluation of salivary alpha amylase activity in smokers with periodontitis, Khartoum state, 2023

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ABSTRACT

Introduction and aim. Smoking widely affect oral health, including its role in the development of periodontitis. Saliva contains an antioxidant system and various enzymes. The study was designed to evaluate the activity of salivary alpha amylase among individuals who smoke and have periodontitis.

Material and methods. A total of 100 participants were included, with 50 cases (cigarette smokers with periodontitis) and the remaining 50 nonsmokers with healthy periodontium as the control group. Saliva samples were collected to measure salivary alpha amylase activity.

Results. Smokers with periodontitis had significantly higher levels of salivary alpha amylase compared to the control group (177.96±14.5 vs 94.04±19.6 IU/mL, $p<0.001$). Additionally, there was a weak negative correlation between the level of alpha amylase and the age of the patients ($p=0.01$, $r=0.376$). However, no correlation was found between the level of alpha amylase and the duration of smoking ($p=0.584$, $r=0.079$).

Conclusion. There is a significant increase in salivary alpha amylase levels among smokers with periodontitis. No correlation was found between age and salivary alpha amylase levels. However, a weak positive correlation was observed between the duration of smoking and salivary alpha amylase activity.

Keywords. alpha-amylase, periodontitis, saliva, smokers

Introduction

Periodontitis is an inflammatory condition that causes damage to the periodontal tissue and ultimately results in tooth loss. It can affect people of all ages but is more prevalent in adults.[1] Lifestyle factors, such as smoking and oral hygiene habits, can influence the severity and progression of chronic periodontitis, in addition to bacterial plaques.[2,3]

Smoking poses a significant risk to oral health, playing a major role in the development of cancerous and precancerous lesions, as well as periodontal disease. It is considered an independent risk factor for these cancers due to the presence of toxic compounds in cigarettes, including aldehydes, carbon monoxide, hydrogen cyanide, benzopyrene, and oxygen radicals.[4,5]

Saliva serves as a protective barrier for the mouth. It consists of a complex system primarily composed of water, but it also contains low-molecular-weight enzymes, hormones, antibodies, antimicrobial ingredients, and growth factors. Some of these components are produced locally by the salivary gland, while others are transported from the bloodstream through diffusion processes, such as active transport and ultrafiltration. Saliva provides insight into overall bodily function.[6] Saliva contains various enzymes, such as lipase, peptidase, and hydrolase. Notably, the most abundant protein in hu-

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man saliva is alpha-amylase, which functions as a digestive enzyme.[7]

Alpha-amylase plays a crucial role in the breakdown of starch into dextrin and malt oligosaccharides that contain α-D-(1, 4) linkages. It also facilitates the breakdown of isomaltooligosaccharides with alpha-D-(1,6) linkages, as well as the trisaccharide maltotriose and the disaccharide maltose.[8]

The addictive nature of nicotine in tobacco and the role of smoking as a preventable risk factor for periodontitis have been well-established. Hence, the measurement of salivary biomarkers can be a valuable tool for identifying individuals who may be at risk of developing or worsening periodontal disease.[9] Nevertheless, the silent nature of periodontitis poses a challenge for identifying individuals at risk until the disease has already progressed to a severe stage. Saliva offers several advantages over blood as a diagnostic fluid, including its non-invasive nature, ease of collection and storage without specialized equipment, and suitability for individuals who have difficulty with blood collection. Therefore, the current study was conducted to evaluate the activity of salivary alpha-amylase in smokers with periodontitis.[10]

Material and methods

This prospective analytical cross-sectional study was conducted at the Khartoum Dental Teaching Hospital in Khartoum, Sudan, from January to April 2023.

The aim of this study was to compare the activity of salivary alpha-amylase enzymes between smokers with periodontitis and nonsmokers without periodontitis. This research consisted of both clinical and laboratory investigations. A total of 100 participants who met the inclusion criteria and expressed interest in participating were selected from the pool of individuals.

Fifty individuals were smokers and had periodontitis (case group), whereas the control group consisted of 50 participants who had a healthy periodontium and were non-smokers. Individuals with concurrent chronic conditions such as hypertension or diabetes mellitus, as well as those taking medications, currently undergoing antimicrobial therapy, or who had received periodontal treatment within the past six months, were excluded from the study.

The study was approved by Ethical Committee of University of Medical Sciences and Technology (No. UMST/EG/2023/18), and before the clinical examination, participants were provided with a verbal explanation of the study's objectives and procedures. They were then requested to sign a formal consent form.

Periodontitis was defined as having a probing pocket depth of ≥4 mm and a clinical attachment level of ≥2 mm. The stage of the disease was not taken into consideration.

The participants were given specific instructions before collecting unstimulated whole saliva for analysis. They were instructed to wait for a minimum of 30 minutes after eating, drinking, smoking, or chewing gum. After this waiting period, they were required to rinse their mouth with water several times and wait for 1-2 minutes until the water was clear before proceeding with saliva collection. After collection, the samples were centrifuged at 4000 rpm for 10 minutes and then frozen at -20°C until α-amylase analysis was conducted. Salivary α-amylase analysis was performed using α-amylase liquicolor, a colorimetric test, from Demeditec Diagnostics GmbH in Kiel, Germany, following the manufacturer's instructions.

The data was entered and organized in a Microsoft Office Excel 2010 spreadsheet. The Statistical Package for the Social Sciences software (version 22.0; IBM SPSS Inc.) was used for analysis. The information collected from the questionnaire was coded as variables. The normality of the data was tested using the Kolmogorov-Smirnov test. Descriptive and inferential statistics, including analysis of independent variables, were then conducted.

Results

A total of 100 participants were included in the study, with a mean age of (36±7.9) years for the case group and (35±8) years for the control group. This information is presented in Table 1. The mean salivary alpha amylase was significantly higher in the cases group (177.962±14.5 IU/mL) compared to the control group (94.042±19.6 IU/mL), with a p<0.001.

Table 1. Distribution of the study group according to age (case vs. control), n=100

Age	Mean ± SD	Minimum	Maximum	p
Case (n=50)	36 ± 7.9	22	52	0.207
Control (n=50)	35 ± 8.0	19	49	

Table 2. Mean difference of amylase levels among case and control group, independent t-test, n=100

Study population	α-amylase (IU/ml)		p
	Mean	SD	
Case group (n=50)	177.96	14.5	<0.001
Control group (n=50)	94.04	19.6	

Table 3. Correlations between α-amylase activities and age, Pearson's correlation, n=50

Correlation		Age	Duration
α-amylase	n	50	50
	Correlation coefficient (r)	0.376	0.079
	p	**0.01**	**0.584**
	Strength	Weak	Weak
	Direction	Positive	Positive

There was a weak negative correlation between the salivary alpha amylase level and the age of the participants (r=-0.376). However, this correlation was found to be statistically insignificant (p=0.01, Table 2).

There was a weak positive correlation between salivary alpha amylase activity and duration of use (r=0.79). However, the correlation was not statistically significant (p=0.584, Table 3).

Discussion

Extensive research has been conducted on the oral health of smokers, particularly in relation to periodontal disease. Salivary proteins have been found to play a crucial role in the body's natural defenses against these diseases.[11]

Traditionally, periodontitis has been diagnosed through clinical examination and radiographic assessment, which can lead to measurement errors. However, changes in specific markers in oral fluids serve as valuable diagnostic tools for assessing severity and identifying individuals who are susceptible.[12] The concept that saliva reflects the overall health of the body remains valid. In recent years, researchers have utilized saliva analysis to monitor the onset, response to treatment, and outcomes of various diseases.[13] Therefore, in this study, salivary alpha amylase levels are compared between smokers with periodontitis and non-smokers with healthy periodontium.

When compared to the control group, the current study found a significant increase in salivary amylase levels in the case group. This result was consistent with the research conducted by Parlak et al. and Rashid et al., which demonstrated that periodontitis can lead to an elevated production of salivary proteins, such as mucin and amylase.[14,15] Patients with moderate to severe periodontitis had higher concentrations of these two forms of proteins. According to a study by Papacosta, salivary alpha-amylase serves as the initial defense mechanism, which aligns with the observed increase in alpha-amylase levels in periodontitis. Rohleder claims that this enzyme protects against pathogens entering the body through the mucosal surface. It may serve as the most significant marker of mucosal immunity in the oral cavity by inhibiting the attachment of bacteria.[16,17]

In contrast, some of the findings of this study contradicted those reported previously by Sequeira et al. and found no evidence that smoking was a risk factor for periodontal disease.[18] This could be attributed to the smaller sample size of 24 patients and the diverse study locations, as well as the limited availability of information regarding the severity of periodontitis and inaccurate assumptions.

Furthermore, the study revealed a weak positive correlation between the level of alpha amylase and the age of patients. These findings are consistent with a study conducted by Parlak et al., which reported a significant correlation between the age of participants and the activity of salivary enzymes.[14]

Additionally, the study showed that there was no significant correlation between alpha amylase levels and the duration of smoking. These findings are inconsistent with the previous studies. The study has limitations, such as a short duration and the absence of a periodontitis stage. We suggest developing programs to educate the community about the impact of smoking on oral health. Additionally, it is important to manipulate salivary biomarkers for the early detection and monitoring of chronic periodontitis. Furthermore, it is recommended to conduct additional studies that assess a broader range of salivary biomarkers.

Conclusion

In conclusion, the study revealed a significant increase in salivary alpha amylase activities in individuals who smoke and have periodontitis. Duration of smoking was not found to be linked to salivary alpha amylase activity. However, a weak positive correlation was observed between age and salivary alpha amylase levels.

Declarations

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Author contributions

Conceptualization, H.A. and F.A.; Methodology, F.A.; Software, H.A.; Validation, H.A., and F.A.; Formal Analysis, H.A.; Investigation, H.A.; Resources, F.A.; Data Curation, H.A.; Writing – Original Draft Preparation, F.A.; Writing – Review & Editing, H.A.; Visualization, H.A.; Supervision, H.A.; Project Administration, H.A.; Funding Acquisition, F.A.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

All datasets are available upon request from the corresponding author.

Ethics approval

The study was approved by the ethical committees of the University of Medical Sciences and Technology No. UMST/EG/2023/18. Informed consents were obtained from all participants, and approval was granted by the hospital administration through the office of the medical director.

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ORIGINAL PAPER

Haemocytometric profile and plasma levels of selected cytokines in patients at various stages of cervical cancer

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ABSTRACT

Introduction and aim. Reports have shown that there is alteration in haematological and inflammatory processes in patients with cervical cancer. However, there is the dearth of information on the pattern of alteration in Nigerian patients with cervical cancer at various stages of the disease. Therefore, haemocytometric profile and plasma levels of interleukin-6 (IL-6) and IL-12 were determined in Nigerian patients with cervical cancer at various stages of the disease.

Material and methods. Eighty-nine adults consisting of 49 patients with cervical cancer and 40 apparently healthy controls were enrolled into this study. Haemocytometric profile was determined using automated haematology analyzer while the plasma levels of interleukin-6 (IL-6) and IL-12 were determined using ELISA.

Results. Of the participants with cervical cancer, 6.12%, 24.49%, 53.06% and 16.33% were in stages I, II, III and IV respectively. The mean plasma IL-6 level was significantly higher in patients at stage IV of the cancer compared with those in stages I, II and III. No significant differences were observed in the mean plasma IL-12 level, and the haemocytometric profile when patients in different stages of the cancer were compared with one another. Plasma IL-6 had significant positive correlation with the lymphocytes count and cancer stage but had significant negative correlation with packed cell volume (PCV), haemoglobin and total white blood cells count (WBC) in patients with cervical cancer.

Conclusion. Interleukin-6 appears to play an important role in the progression of cervical cancer and could be involved in cervical cancer-associated alteration in haemocytometric profile.

Keywords. cancer stage, cervical cancer, haemocytometry, interleukin-6, parity

Introduction

Cervical cancer is the fourth most common cancer in women and the fourth leading cause of cancer death in women globally.[1,2] Although cervical cancer is more common in elderly women, reports have shown that the proportion of young women with the disease has risen from 10 to 40% in the last three decades.[3]

In 2020, an estimated 604,000 new cases and 342,000 cervical cancer-related deaths were reported. Unfortunately, about 90% of the new cases and deaths occurred in poor resource countries.[4] In Nigeria, about 53.3 million women are estimated to be at risk of developing cervical cancer.[5,6] This disproportionately high incidence of cervical cancer in poor resource countries

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has been attributed to myriads of factors including poverty, poor access to healthcare, limited availability of human papilloma virus (HPV) screening service, and limited access to preventative measures. Similarly, late presentation and limited access to quality healthcare are key factors responsible for the high incidence of cervical cancer-related mortalities in the developing countries.[2,7]

Significant alteration in the haemocytometric profile of cervical cancer patients has been reported.[8-10] The report of Hamad[8] showed that cervical cancer patients had elevated leukocyte count, and decreased haemoglobin level and haematocrit compared with healthy women. Similarly, Tavares-Murta et al.[9] reported that leukocytosis, lymphopenia, neutrophilia, and elevated neutrophil-lymphocyte ratio (NLR) were more frequent at advanced stages of cervical cancer compared with the preinvasive neoplasia stage and early-stage. They also suggested that neutrophilia could be a good indicator of cancer invasiveness. In addition, Kose et al.[11] showed that there is a correlation between invasion of cervical cancer and NLR as well as platelet-lymphocyte ratio (PLR).

Antitumor response by the immune system is dependent on production of cytokines that promote a Th1 cytotoxic response.[12] These cytokines mediate proinflammatory activities which are vital in antineoplastic processes. Reports have shown that serum levels of IL-6 are elevated in lung, colorectal, breast, brain, liver, and gynecological cancers.[13-15] Similarly, the reports of Tjiong et al.[16] and Tavares-Murta et al.[17] showed that IL-6 level is elevated in the cervicovaginal washings and in the serum of patients with intraepithelial neoplasia and cancer of the cervix, and that the elevation is associated with invasive cervical cancer and metastasis. It was also shown in an experimental study that there is a significant correlation between local cervico-uterine and serum levels of IL-6 with increasing grades of cervical intraepithelial neoplasia (CIN) and metastasis.[18]

Interleukin-12 (IL-12), a 74kDa heterodimeric glycoprotein, is a potent pro-inflammatory cytokine with antitumor properties.[19,20] Its antitumor activities are mediated by induction of Th1 cell differentiation, increased cytotoxic activities of T, NK and NKT cells, and reprogramming of immunosuppressive cells.[20-25] Reports have shown that IL-12 heightens the production of IFN-γ, a cytostatic, cytotoxic and anti-angiogenic cytokine which can upregulate MHC I and II expression on tumour cells thereby enhancing recognition and lysis of the cells.[26-30] These antitumour activities of IL-12 have been explored in various preclinical and clinical studies where the results showed notable antitumor effects of IL-12 against various malignancies.[24, 31-35]

Aim

Although reports have shown that there is alteration in haematological and inflammatory processes in cancer patients, information on the pattern of alteration in cervical cancer patients at various stages of the disease is sparse in Nigeria. This study was thus designed to determine the haemocytometric profile and serum levels of selected cytokines; IL-6 and IL-12 in Nigerian women with cervical cancer.

Material and methods

Ethics approval

Ethical approval was obtained from the University of Ibadan/University College Hospital (UI/UCH) Joint Ethics Review Committee (UI/EC/17/0019) before the commencement of the study. Also, written informed consent was obtained from each study participant after detailed explanation on the purpose and significance of the study.

Study participants

Ninety-two women consisting of 49 histologically confirmed cervical patients and 49 age-matched apparently healthy women, who served as controls, were enrolled into this study. The patients were enrolled from the Radiation Oncology Clinic, University College Hospital, Ibadan. The controls were randomly selected amongst women who came for routine pap smear test at the Department of Obstetrics and Gynaecology, University College Hospital, Ibadan.

Patients with history of other malignancies and those who were critically ill were excluded from the study. Also, women who have had hysterectomy with the removal of the cervix were not included among the controls.

Data collection

Clinical history and information on demography and risk factors for cervical cancer were obtained using a semi-structured questionnaire.

Blood sample collection

Venous blood sample (10 mL) was aseptically obtained from each study participant; 5 mL each of the blood sample was dispensed into EDTA-containing bottle and lithium heparin containing bottle for haematology and biochemical analysis respectively. Blood samples dispensed into the lithium heparin containing bottles were centrifuged and plasma samples obtained were stored at -20°C until analysed.

Laboratory analyses

Haemocytometric profile was determined using an automated haemocytometer (URIT: 5160E-01262, China) while the plasma levels of IL-6 and IL-12 were determined using ELISA kits following the manufacturer's

instructions (Invitrogen, USA). The immunoplate was read at 450 nm using an Absorbance Microplate Reader (SpectraMax® Plus384). Analytical sensitivity of the kit for IL-6 was <1 pg/mL while that of IL-12 was 0.2 pg/mL.

Statistical analysis

Data were analysed using the Statistical Package for Social Science SPSS (IBM, Armonk, NY, USA) software, version 21.0. Two group mean comparisons were carried out using the Student's t-test for parametric variables whereas; non-parametric variables were compared using the Mann-Whitney U test. Analysis of variance (ANOVA) was used for comparison of more than two groups for parametric variables while Kruskal Wallis test was used for non-parametric variables. Correlations were tested using Spearman rank correlation. Statistical significance was set at $p<0.05$. Results are presented as mean±standard deviation (SD) when parametric and median (interquartile range) when non-parametric.

Results

The distribution of the cancer stages of the cervical cancer patients is shown in Figure 1. More than half of the patients (53.06%) were in stage III while only a few of the patients were in stage I (6.12%) of the cancer (Fig. 1). Histologically, 97.96% of the patients had SCC while 2.04% had AD. NO patient had histological report indicating ADCC.

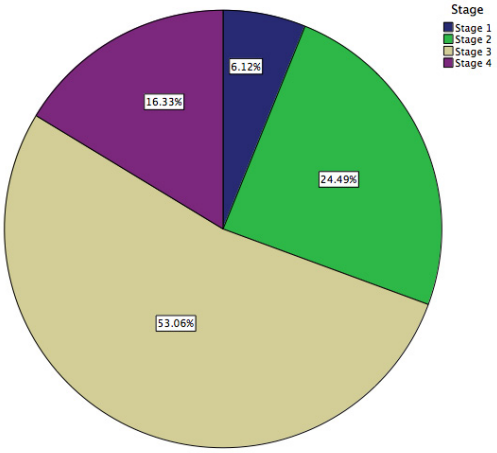

Fig. 1. Distribution of cancer stages in cases

In Table 1, selected characteristics of the study participants, haemocytometric profile, and plasma levels of the cytokines are shown. There was no significant difference in the mean age of patients with cervical cancer compared with the controls. However, parity was significantly higher in patients with cervical cancer compared with the controls.

The mean PCV, haemoglobin (Hb), total white blood cell count (WBC) and platelet count were significantly lower while parity and the mean plasma IL-6 level were significantly higher in patients with cervical cancer compared with the controls (Table 1). The mean neutrophil count, lymphocyte count and serum IL-12 level in patients with cervical cancer and the controls were not significantly different (Table 1).

Table 1. Age, haemocytometric profile and plasma cytokine levels in patients with cervical cancer and controls[a]

Variables	Cases (n=49)	Controls (n=40)	p
Age (years)	52.94±10.64	50.55±1.89	0.321
Parity	5.22±2.06	3.25±1.19	<0.001*
PCV (%)	35.02±3.87	39.63±2.96	<0.001*
Haemoglobin (g/dL)	11.41±1.32	13.22±1.31	<0.001*
WBC (cells/μL)	5126.53±1286.43	6445.0±1031.04	<0.001*
Neutrophil (%)	54.51±6.98	55.98±6.18	0.303
Lymphocytes (%)	42.55±7.87	40.93±5.24	0.266
Platelet (cells/μL)	172571±24298	256673±33738	<0.001*
IL-6 (pg/mL)	18.74 (13.12–61.31)	2.18 (1.79–2.86)	0.001*
IL-12 (pg/mL)	10.08 (6.36–21.61)	11.43 (7.53–16.73)	0.102

[a] * – significant at $p<0.05$; PCV – packed cell volume; WBC – white blood cells count; IL – interleukin

As shown in Table 2, there was significant increase in parity of the cancer patients with increasing stage of the cancer. The mean plasma IL-6 level was significantly higher in patients at stage IV of the cancer compared with those in stages I, II and III. No significant differences were observed in the mean plasma level of IL-12, PCV, haemoglobin, WBC, neutrophil, lymphocyte, and platelet counts when patients in different stages of the cancer were compared with one another (Table 2).

Table 2. Parity, haemocytometric profile and plasma levels of cytokines in patients with cervical cancer at various cancer stages[a]

Variables	Stage I (n=3)	Stage II (n=12)	Stage III (n=26)	Stage IV (n=8)	p
Parity	3.33±1.20	4.75±0.25[a]	5.15±0.40[a,b]	6.88±0.93[a,b,c]	0.035[#]
PCV (%)	32.67±1.45	35.17±1.31	35.31±0.75	34.75±1.26	0.738
Haemoglobin (g/dL)	10.90±0.49	11.29±0.38	11.62±0.30	11.09±0.22	0.654
WBC (cells/μL)	4900±100	5067±325	5169±296	5162±380	0.986
Neutrophil (%)	53.33±0.88	53.75±2.18	55.08±1.54	54.25±1.67	0.942
Lymphocytes (%)	47.0±1.0	40.75±3.14	42.62±1.41	43.38±2.19	0.656
Platelet (cells/μL)	164666±7859	176417±8982	163375±6946	172571±3471	0.6
IL-6 (pg/mL)	28.19 (21.28–29.73)	21.20 (12.71–29.52)	16.30 (12.58–41.51)	197.27 (13.13–559.48)[a,b,c]	0.037[#]
IL-12 (pg/mL)	8.70 (5.80–11.42)	20.15 (7.41–30.21)	8.13 (6.16–18.87)	12.36 (5.62–29.96)	0.777

[*] [#] – significant at $p<0.05$; [a] – compared with stage I; [b] – compared with stage II; [c] – compared with stage III; PCV – packed cell volume; WBC – white blood cells count; IL – interleukin

As shown in Table 3, the plasma IL-6 level had significant positive correlation with the plasma IL-12 level, lymphocytes count and cancer stage but had significant negative correlation with PCV, haemoglobin and WBC in patients with cervical cancer (Table 3). In the controls, the plasma IL-6 level had significant positive correlation with neutrophil count but a significant negative correlation with lymphocytes count. Also, IL-12 had significant positive correlation with PCV, haemoglobin and platelets count (Table 3).

Table 3. Correlation between the cytokine levels, haematological indices and cancer stages in patients with cervical cancer and controls[a]

Correlating pair		Cases (R, p)	Controls (R, p)
IL-6	PCV	-0.307, 0.032*	-0.243, 0.131
	Haemoglobin	-0.296, 0.039*	-0.298, 0.062
	WBC	-0.314, 0.028*	0.145, 0.372
	Neutrophil	-0.227, 0.117	0.327, 0.040*
	Lymphocytes	0.302, 0.035*	-0.446, 0.004*
	Platelet	-0.207, 0.153	0.265, 0.1113
	Cancer Stage	0.372, 0.009*	
IL-12	IL-6	0.587, <0.001*	0.252, 0.116
	PCV	-0.070, 0.635	0.444, 0.004*
	Haemoglobin	-0.068, 0.640	0.417, 0.007*
	WBC	-0.069, 0.639	-0.320, 0.044
	Neutrophil	-0.183, 0.209	0.279, 0.082
	Lymphocytes	0.049, 0.740	-0.094, 0.564
	Platelet	-0.041, 0.777	0.531, <0.001*
	Cancer Stage	0.096, 0.513	

[a] * – significant at $p<0.05$

Discussion

The continuous rise in the proportion of patients with cervical cancer especially, in young women, is of public health concern.[3] This disproportionate high incidence highlights the need for proper understanding of the dynamic changes in haemocytometric profile and cytokines levels during the course of the disease with a view to improving on patients' management.

Iron deficiency and tumour-associated bleeding are common causes of anaemia in cervical cancer.[36] Anaemia seen in cervical cancer has the characteristics of anaemia of chronic disorder associated with low PCV. In this study, the mean PCV and haemoglobin count were significantly lower in patients with cervical cancer compared with the controls. This observation corroborates previous reports which showed that haemoglobin concentration was significantly lower in cervical cancer patients compared with healthy controls.[10, 37-41] Our observation could be due to several factors including poor nutrition due to anorexia associated with cancers generally, haemorrhage, metastasis to the bone marrow thereby causing suppression of erythropoiesis and tumour-associated infections.[42]

Reports have shown that tumour-related leukocytosis (TRL) occurs in 1% to 10% of patients with non-haematopoietic malignancies.[43] This has been attributed to upregulation in haematological growth factors expression. Previous reports have shown that WBC and platelet counts are elevated in cervical cancer patients compared to healthy controls.[8,10,41] Observation from our study was in contrast to these previous reports as WBC and platelet counts were observed to be significantly lower in patients with cervical cancer compared with the controls. The observed lower WBC count in the patients with cervical cancer may be an indication of bone marrow involvement leading to suppressed haematopoiesis, decreased production of haematological growth factors or increased rate of WBC lysis.

Platelets have been reported to play a multifaceted role in cancer progression and metastasis through complex interactions between the platelets and tumour cells resulting in tumour growth, aberrant angiogenesis, invasion, and metastasis.[44,45] The report of Seretis et al. suggested that a normal platelet count could conceal the presence of highly hypercoagulative and pro-inflammatory cancer phenotypes in the presence of efficient compensatory mechanisms.[46] The observed reduction in platelet count in patients with cervical cancer, in this study, may suggest highly hypercoagulative and pro-inflammatory cancer phenotypes with an inefficient compensatory mechanism. This observation may be associated with the cancer stage as most of the study participants with cervical cancer were at stages III and IV.

Alteration in cytokines levels has been associated with most tumours and this alteration is suggested to play a role in cell transformation, cancer cell proliferation, survival, invasivity and metastasis.[47] In this study, the plasma level of IL-6 was significantly higher in patients with cervical cancer compared with the controls. This observation supports previous studies which reported cancer related inflammation with a corresponding increase in circulatory levels of IL-6 in patients with cervical cancer.[48-50] Similarly, studies have shown that there is elevated IL-6 level in vaginal fluid of cervical cancer patients.[51,52] Although IL-6 is a pleotropic cytokine that can either function as a pro-inflammatory or anti-inflammatory cytokine, its potential to promote inflammation in cancer has been demonstrated. Previous studies suggested that IL-6 and its soluble receptor (sIL-6Ra) appear to have a key role in the transition from an acute to a sustained or even chronic inflammation by decreasing neutrophil and favouring mononuclear-cell accumulation.[53-55] During cancer development, an initial acute inflammatory response usually entails neutrophils infiltration of tumour site, but a more sustained population of mononuclear cells later replaces the neutrophils in a process orchestrated by the release of IL-6.[53] Through its soluble receptor (sIL-6Ra), IL-6 activates endothelial cells to produce monocyte chemotac-

tic protein-1, thus stimulating monocyte recruitment,[55] while inducing polymorphonuclear-cell apoptosis.[55,56] This results in attraction of macrophages toward the tumour where they are integrated and thus, represent the major inflammatory component of the stroma as tissue-associated macrophages (TAMs).[57] The TAMs predominantly comprise an M2 population which promotes angiogenesis, tissue remodelling and repair that propagate cancer progression.[58] The study of Wei et al.[59] showed that IL-6 promotes in vivo tumour growth of human cervical cancer C33A cells. Therefore, our observed elevation in IL-6 levels in patients with cervical cancer could be indicative of chronic inflammation which tends to further promote tumour growth and angiogenesis. Therefore, plasma IL-6 level could serve as a diagnostic marker of cervical cancer, however further studies are required to assess its diagnostic performance and its inhibition as a possible immunotherapeutic strategy.

Late presentation by cancer patients is usually associated with high morbidity and mortality. In this study, more than 50% of the patients with cervical cancer were in stage IV of the disease. This observation underscores the need for increased advocacy on voluntary screening for cervical cancer with the view to enhancing early diagnosis which ultimately results in reduced morbidity and mortality.

The observed higher level of IL-6 in patients presenting at late stages (stages III and IV) compared with patients presenting at early stages (stages I and II) corroborates the report of Song et al.[60] which showed that expression of IL-6 is associated with progression and prognosis of human cervical cancer. In the study, IL-6 expression was assessed in cervical cancer tissues histologically obtained. This represents a highly invasive procedure that would be difficult to implement for routine examination. Hence, our observed significant differences in the plasma IL-6 levels between patients at the late and early stages of cervical cancer as well as a positive correlation between plasma IL-6 level and stage of cervical cancer shows promise of a more convenient prognostic marker of cervical cancer that can be implemented for routine monitoring of the course of cervical cancer.

The observed significant negative correlation between the plasma IL-6 level and PCV, haemoglobin and WBC suggests that plasma IL-6 suppresses the process of haematopoiesis. This further confirms our earlier observed lower WBC count in the patients with cervical cancer. Akchurin et al.[61] reported that IL-6 contributes to development of anaemia via induction of hypoferremia, aggravation of renal fibrosis, and alteration of the erythropoietin axis in juvenile chronic kidney disease.

Significant positive correlation between IL-6 levels and stage of gastric cancer as well as colorectal cancer has been reported.[62,63] Similar result was observed in this study as the plasma IL-6 level had significant positive correlation with the cancer stage in patients with cervical cancer. This observation also confirms our observed higher level of IL-6 in patients presenting with stages III and IV cervical cancer compared with patients presenting with stages I and II cervical cancer. These observations indicate that the plasma level of IL-6 increases as the cancer stage progresses.

An association between high parity and risk of cervical cancer has been reported.[64] This has been attributed to high rate of pregnancy-associated cervical abnormalities, increased rate of HPV infection and local changes to cervical cells due to birth-associated traumas.[65] In this study, parity was significantly higher in patients with cervical cancer compared with the controls. Our observation together with the previous reports indicate that there is the need for improved understanding of the interplay between high parity and the risk of developing cervical cancer. This could further enhance our knowledge on cervical cancer preventive strategies.

Conclusion

In conclusion, alteration in haemocytometric profile is a prominent clinical feature in patients with cervical cancer. Also, plasma IL-6 level appears to play an important role in alteration of the haemocytometric profile and the progression of disease in patients with cervical cancer. Therefore, IL-6 could be further explored as an immunotherapeutic intervention in patients with cervical cancer. However, the mechanisms through which parity increases the risk of cervical cancer require further studies.

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Declarations

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Author contributions

Conceptualization, M.A.J., A.A.O. and F.M.A.; Methodology, M.A.J., A.A.O., F.M.A., V.F.E., S.K.R., and O.G.A.; Investigation, M.A.J., A.A.O., F.M.A., V.F.E., S.K.R., and O.G.A.; Data Curation, M.A.J., V.F.E. and S.K.R.; Data Analysis, V.F.E. and S.K.R.; Writing – Original Draft Preparation, M.A.J., A.A.O., F.M.A., V.F.E., S.K.R., and O.G.A.; Writing – Review & Editing, M.A.J., A.A.O., F.M.A., V.F.E., S.K.R., and O.G.A.; Supervision, M.A.J., A.A.O and F.M.A.,

Conflicts of interest

The authors have no competing interest to declare.

Data availability

Data are available upon request from the correspondence author.

Ethics approval

Ethical approval was obtained from the University of Ibadan/University College Hospital (UI/UCH) Joint Ethics Review Committee (UI/EC/17/0019).

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ORIGINAL PAPER

Plasma level of beta endorphin in seborrheic dermatitis patients

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ABSTRACT

Introduction and aim. Defects in the epidermal barrier, changes in sebum secretion and its composition, *Malassezia* spp. overgrowth, endocrine, immune, and neurological disorders are the main pathogenesis items of seborrheic dermatitis (SD). "The opioid system of the skin" was considered a new target in the diagnosis and treatment of SD. The study aimed to determine beta-endorphin (BE) levels in adult patients with seborrheic dermatitis and correlate them with the severity of symptoms and itching.

Material and methods. 26 healthy and 62 SD people were examined. SEDASI scale were used to estimate the severity of symptoms and intensity of itching. The determination of the beta-endorphin level was carried out by the ELISA method with the test system Human BE NBP2 (78774 Novus Biologicals).

Results. BE in the SD group was higher compared to the control group (35.5 pg/mL, 22. pg/mL, p<0.001). The level of BE in seborrheic patients did not depend on age and sex but was rising with severity of symptoms. Positive correlations were found between the level of BE and the SEDASI was 0.42 (p<.001), between the level of BE and itching was 0.332 (p=0.009).

Conclusions. SD patients have an increased level of BE that positively correlates with itching and disease severity.

Keywords. beta-endorphin, itch, seborrheic dermatitis

Introduction

The development of seborrheic dermatitis (SD) includes three main prerequisites: hypersecretion of sebum, overgrowth of *Malassezia* fungus, and an immune system response. Pathogenesis is described in the following phases. Sebaceous glands secrete lipids onto the skin surface. Then *Malassezia* funguses colonize areas covered with lipids and consume saturated fatty acids, leaving behind skin-irritating unsaturated fatty acids (like oleic). Those acids induce desquamation and skin barrier disruption. Because of the increased growth and activity of funguses, T-lymphocytes produce cytokines that stimulate keratinocyte proliferation and differentiation. Immune response and products of fungal metabolism damage the skin surface, resulting in erythema, pruritus, and scaling.[1] This pathogenic chain gives a logical explanation of the inflammatory process, but some facts are contradictory. Nearly 80% of SD patients are colonized with *Malassezia spp.*,[2] and hyphae are more invasive than yeast forms, but they colonize a third of all inflamed sites in SD patients.[3] *Staphylococcus* and *Streptococcus spp.* are cultivated from skin specimens more often than pathogenic fungi.[4] Also, patients with SD may not have oily skin.[5] Additional skin regulatory mechanisms should be considered to provide a more flexible pathogenesis theory (Fig. 1).

Neuroendocrine regulation of derma metabolism and the immune response are combined into the term "opioid system of the skin". Opioid receptors and their ligands are part of this system, allowing the skin to respond to various biological, chemical, and physical stresses.[6] The cutaneous opioid system is mainly responsible for nociception and inflammation, playing an essential role in

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skin homeostasis, regeneration, wound healing, and aging.[7] Unmyelinated nerve fibers in the derma, epidermis and keratinocytes express μ-opiate receptors that respond specifically to β-endorphin (BE). BE may be absorbed from the blood or synthesized directly by nerve endings, keratinocytes, and sebaceous glands. The keratinocytes that produce BE are clustered around the terminal ends of the unmyelinated nerve fibers and can influence nerve fibers directly. On the other hand, nerve fibers also secrete BE and influence the differentiation, migration, and cytokine production of keratinocytes.[8]

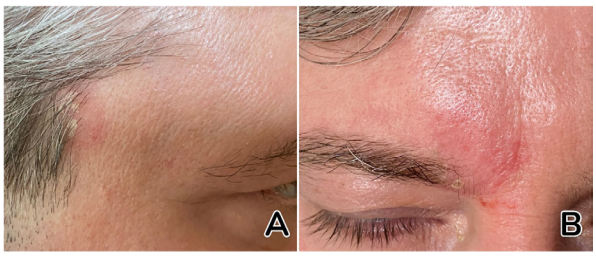

Fig. 1. Mild form of seborrheic dermatitis: the temple area (A); forehead area (B). Patient washed away the flakes of dry skin and used skin moisturizer to conceal the inflamed areas

After the skin damage, the opioid system is activated via a pain stimulus. BE has a strong antinociceptive effect.[9] In low dosages, it's excreted immediately after the skin damage. Concentration rises in the first two hours and reaches its peak in the fourth hour. BE level rises due to the high threshold of sensitivity of the opioid receptors (OR). Typically, action lasts no longer than a day.[10] The decrease in sensitivity of OR due to constant exposure to BE makes its action dosage-dependent. Chronic exposure to BE opioid use leads to tolerance, defined as a decrease in the drug response.[9] It is possible to reproduce in vitro such a phenomenon when cellular models expressing OR are exposed to agonists; in that situation, a decrease in signaling is observed and is designated as OR desensitization.[11] Some reports distinguish the OR desensitization from the cellular tolerance. When rats are chronically exposed to morphine, examination of μ-OR activity on the outward potassium current shows a reduction compared to naive animals, which is not reversible even after 6 hours in morphine-free medium.[12]

Aim

The aim of the study was to determine the levels of beta-endorphin in adult patients with seborrheic dermatitis and correlate them with the severity of symptoms and itching.

Material and methods

Patients with seborrheic dermatitis were recruited from the Transcarpathian regional clinical dermato-venereological center and the Center of Family Medicine of Uzhgorod city during the 2020–2022 years. The study was carried out within the framework of "Alternative methods of treatment of opportunistic infections using medicinal and non-medicinal means," subject code 12A-2021, state registration number 0121U110174, and "Health and recreation. Peculiarities of the clinical and epidemiological courses of infections and parasitosis characteristic of the Transcarpathian region", state registration number 0117U00283, subject code 02070832. The Ethics Committee of the Medical Faculty at Uzhgorod National University approved the scientific evaluation and study protocol (No. 318/4-6, dated September 16, 2020). Before the examination, the patients were informed about the research design, developed within the framework of the Helsinki Declaration of the World Medical Association "Ethical Principles of Medical Research with the Participation of a Person as an Object of Research," the Convention of the Council of Europe on Human Rights and Biomedicine, and the legislation of Ukraine, and signed the informed consent. People with mild-to-moderate seborrheic dermatitis were asked to participate in the study. Patients were included according to the following criteria: age between 18 and 55 years and diagnosis of SD based on the symptom scale of seborrheic dermatitis (SSSD). The control group was chosen from healthy medical workers with no skin diseases. Participants were excluded if they were diagnosed with viral hepatitis, HIV+, atopic dermatitis, acne, psoriasis, autoimmune, oncological, or rheumatological diseases that require corticosteroid medication use. The following diseases were taken as most commonly associated with SD to exclude their impact on the severity of SD.[13]

The seborrheic dermatitis area and severity index (SEDASI)[14] scale was used to estimate the severity of symptoms: 1–14 mild; 15–29 moderate; 30–44 severe; 45–60 very severe. A visual analog scale where zero is no itch (or sleeplessness) and 10 is the worst imaginable itch (or sleeplessness) was used to represent the intensity of itching. 0 = no pruritus, > 0-< 4 points = mild pruritus, ≥ 4-< 7 points = moderate pruritus, ≥ 7-< 9 points = severe pruritus, and ≥ 9 points = very severe pruritus.[15]

The determination of the beta endorphin level was carried out by the ELISA method with the test system Human BE NBP2 – 78774 Novus Biologicals (sensitivity of the test: 9.38 pg/mL. detection range 15.63 -1000 pg/mL)

Fasting blood samples were collected between 8 and 9 a.m. Blood was collected in a Vacuette tube with a plasma coagulation activator (CAT serum cloth activator) and left to rest at room temperature for 2 hours. The tubes were centrifuged for 20 minutes at 1000xg at 8°C. Frozen plasma was stored at -18°C for 2 weeks. Defrosted samples were tested by given protocol of enzyme-linked immunosorbent assay for quotative detection.

Statistical analysis was carried out by Jamovi v. 1.6 (Sydney, Australia). The chi-square test was used to de-

termine the difference in gender distribution between the SD and control groups. The Mann-Whitney t-test for independent samples was used to determine the difference in age between groups. The normality of the distribution of quantitative data was analyzed by the Shapiro-Wilk test. The Kruskal-Wallis test was used to compare levels of BE in SD and control groups. The Dwass-Steel-Critchlow-Flinger pairwise comparison test was used to compare levels of BE at different severity levels. Spearman's correlation was used to determine the relationship between the level of BE, SEDASI and itching scores. The strength of the correlation was evaluated using the Chaddock scale. α=0.05 was considered the critical level of reliability.

Results

88 people participated in the study, including 62 people with SD (the main group) and 26 people without SD (the control group). There were 35 (56.5%) men and 27 (43.5%) women in the main group, 10 (38.5%) men, and 16 (61.5%) women in the control group. The BE level of one of the participants in the main group exceeded the average group level by more than 10 times and therefore was evaluated as an outlier, being excluded from further analysis. The average age of the main group participants was 32.9±1.55, and the average age of the control group participants was 34.88±1.79 years; the groups did not differ in age (p>0.05). Men and women in the main group did not differ in age (34.57±2.49 vs. 30.65±1.42; p>0.2).

Table 1. Comparison of BE level in healthy individuals and seborrheic dermatitis patients*

BE (pg/mL)	SD group	control group	χ^2	W	p
Men under 30 years	36.5±10.1	22±2.8	8.44	4.11	0.004
Men over 30 years	31.9±9.78	21.1±6.07	5.03	3.17	0.025
Men of all ages	34.1±10.1	22.6±9.61	12.3	4.95	0.001
Women under 30 years	36.6 ±8.3	18.4±6.58	8.58	4.14	0.003
Women over 30 years	39.1±8.03	24.1±10.5	8.54	4.12	0.003
Women of all ages	37±6.4	18.5±3.15	17.5	5.95	0.001
All people under 30 years	35±6	21.1±5.53	17.8	5.96	0.001
All people over 30 years	34.7±9.67	23.3±5.44	12.8	5.05	0.001
All men	34.1±10.1	22.6±9.61	12.3	4.95	0.001
All women	37.0±6.92	18.5±2.98	17.5	5.95	0.001
Group total	35.0±5.37	22.0±3.69	29.8	7.72	0.001

* under 30 – age from 18 to 29 years; over 30 – age from 30 to 55 years

The level of BE in the main group was significantly higher compared to the control group (35.5 pg/mL, 22 pg/mL, p<0.001). BE in SD patients did not differ significantly between males and females and did not depend on age (Table 1). A comparison of males and females under and over 30 showed a significant difference between SD patients and the healthy control group. The SEDASI score did not depend on sex (χ^2=1.2756, p=0.982) or age (χ^2=0.2367, p=0.627). Itching score also had no signific difference between males and females (χ^2=0.8100, p=0.235) and did not depend on age of the patients (χ^2=0.4496, p=0.503).

The level of BE depended on the severity of the SD. The average of BE in plasma of patients with mild forms of SD (n=13) was 26±6.13 pg/mL (the amount of BE is rounded to whole numbers); average of SEDASI score in mild form group was 11±2.0; itch intensity 3±0.725. BE in moderate form group (n=29) was 35±3.97 pg/mL, SEDASI 20±3.5, pruritus 6±1. BE in severe SD group (n=20) was 43±5.24 pg/mL, SEDASI 36±3, pruritus 6±1. Very severe SD patients (n=4) had the average of BE of 46±1.22 pg/mL, pruritus 6±1.5. A statistical difference was found in BE level of mild and severe form (p=0.006) (Table 2).

Table 2. BE pairwise comparisons between different degrees of severity of SD

Severity of SD	W	p
Mild vs moderate	4.014	0.024
Mild vs severe	4.598	0.006
Mild vs very severe	3.538	0.060
Moderate vs severe	2.619	0.249
Moderate vs very severe	2.290	0.368

Correlation analysis showed that the level of endorphins increases with SEDASI score, intensity of itching and does not depend on age in patients with seborrheic dermatitis (Table 3).

Table 3. Correlation between BE, intensity of itching, severity of disease and age of SD patients*

	BE		Pruritus vs BE*		SEDASI vs BE*		SEDASI vs pruritus *	
	R	p	R	p	R	p	R	p
All patients (n=67)	-0.074	0.569	0.332	0.009	0.42	<0.001	0.533	<0.001
All patients under 30 years (n=32)	0.078	0.671	0.307	0.087	0.415	0.018	0.612	<0.001
All patients over 30 years (n=29)	-0.039	0.842	0.362	0.054	0.421	0.422	0.41	0.027
Man of all ages (n=35)	-0.192	0.27	0.35	0.039	0.424	0.011	0.511	0.002
Man under 30 years (n=17)	0.098	0.709	0.3	0.242	0.546	0.023	0.563	0.019
Man over 30 years (n=18)	-0.025	0.920	0.412	0.09	0.202	0.423	0.443	0.065
Woman of all ages (n=26)	0.219	0.282	0.277	0.171	0.391	0.048	0.573	0.002
Woman under 30 years (n=15)	0.041	0.884	0.326	0.235	0.221	0.43	0.695	0.004
Woman over 30 years (n=11)	0.205	0.546	0.254	0.45	0.6	0.601	0.44	0.176

* data represents result of correlation between level of beta endorphin and intensity of itching or SEDASI score in a group divided by age and sex

The correlation in the whole group of SD patients (n=67) between the level of BE and the SEDASI score

was 0.42 ($p<0.001$), between the level of BE and itching was 0.332 ($p=0.009$). Positive correlation was found between BE level and SEDASI score in a group of SD patients younger than 30 years ($p=0.018$), males younger than 30 years ($p=0.023$) and man and woman of all ages ($p=0.011$, $p=0.048$). Positive correlation of mild intensity between SEDASI score and intensity of itching was found in all most all age groups of SD patients ($p=0.027$, $p<0.001$). There was found no significant correlation between the age of SD patients and level of BE ($p>0.27$). No significant correlation between BE and age was found in a control group ($R=0.141$, $p=0.491$).

Discussion

In this work, we first determined that seborrheic dermatitis patients have increased levels of BE in the blood compared to healthy individuals. The average level was 35 (28.3; 43.8) pg/mL. To accurately determine a difference in the level of BE at different ages, the amount of patience needs to be increased. Males and females older than 30 years had different mean levels of BE (31.9±9.78; 39.1±8.03) compared to a group level 34.7±9.67, but no signific statistical difference was found ($p=0.52$). The level of BE rises with the severity of seborrheic dermatitis and is strongly associated with the intensity of itching. BE level correlates with the area of skin damage. There is a significant increase in plasma BE in burned patients that correlates positively with the extent of the burn areas.[10] Classical itching mechanisms involve histamine release by IgE activation of mast cells in response to allergens. SD patients have a significant increase in histamine[16] and cathepsin S in the blood.[17] High levels of histamine increase BE in cerebrospinal fluid,[18] but the direct influence of BE on histamine release remains unclear. Although opioid analgesics like codeine provoke mast cell degranulation,[19] morphine was reported to have dose-dependent itching intensity.[20]

Histamine-unrelated theories of skin itch are based on a decrease in BE sensitivity. The unmyelinated nerve fibers in the epidermis are stretched and therefore thinner and less sensitive to itching stimuli. The opiate receptors on nerve endings in inflamed skin are downregulated, which suggests dosage-dependent mechanisms of chronic itch development.[21] In addition to this theory, it was described that intravenous administration of naloxone, an opioid receptor antagonist, significantly reduces chronic itching.[22]

Psoriasis patients have twice the normal levels of BE, and about 50% have both atopic dermatitis (AD) and systemic sclerosis, compared with healthy individuals. BE rises following long-lasting and actively spreading psoriatic plaques and decreases after treatment and in the remission stage. The study reports that men with psoriasis and itch have comparatively lower amounts of BE than non-itch males, but there is no difference in female patients.[23] The other study compared the level of BE in children with AD. They found that kids with aggravation of atopic dermatitis have higher levels of BE and more intense itch. Kids in the remission stage had similar to control group levels of BE.[24]

A new trichoscopic sign of SD was described. The vascular conglomerate "Dandelion," which looks like a yellow dot, is surrounded by glomerular and comma-shaped vessels. The cumulation of sebum and keratin in the hair infundibulum forms the yellow center.[25] Interestingly, opioid growth factor (the met-encephalin molecule) inhibits angiogenesis, including mesenchymal and endothelial vessels.[26] According to these data, endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling.[27] Keratinocytes produce BE, which binds to opioid receptors, and this has been associated with the intensity of subjective itch in patients with AD.[28] Also, activation of the cannabinoid receptor-2 (CB2R) in skin keratinocytes by endocannabinoids is the mechanism underlying circulating BE elevation in patients with obstructive jaundice.[29] The "itchscriptome" analysis was made via RNA sequencing to identify itch-related mediators and receptors in patients with AD and psoriasis. Cytokines such as IL-17A, IL-23A, IL-31, and BE had elevated gene transcript levels in both itchy atopic and psoriatic skin. However, the administration of BE may dose-dependently enhance scratching, which can be inhibited by μ-opioid peptide antagonists.[30] The effect of BE was studied on nonhuman primates. Administration of BE intensified the itch and attenuated inflammatory pain by binding to the mu-opioid peptide receptor. An anti-itch effect was achieved after dynorphin A blockage on kappa-opioid peptide receptors.[31]

The increase in BE levels in SD patients is associated with the ability of CD4+ T lymphocytes to produce opioids.[32] The skin of SD patients is overpopulated with *Staphylococcus epidermidis* and *Cutibacterium acnes*.[33] Excessive bacterial growth causes CD4+ T lymphocytes to produce BE upon antigen priming in draining lymph nodes[34] and correlates with the stimulatory potency of antigen-presenting cells.[35] BE-induced B-lymphocyte suppression was observed too. Exposition of *S. aureus*-stimulated peripheral blood-derived mononuclears to BE resulted in a dose-dependent inhibition of immunoglobulin-secreting cell (ISC) formation. It was found that IgG-ISC was suppressed more than IgA-ISC or IgM-ISC. In contrast to these results, BE was found to be unable to suppress *S. aureus*-induced immunoglobulin secretion.[36]

Endogenous opioid BE inhibits the transcription of IL-2 in T lymphocytes and activates the transcription factors AP-1, NFAT, and NF-kappaB, which transactivate IL-2. Incubation of T-cells with opioids causes a marked increase in cAMP and further enhancement of the ton-

ic inhibition of the leukocyte-specific protein tyrosine kinase, thereby blocking the initiation of T-cell receptor signaling.[37] Also, there is a strong induction of interleukin 4, a cytokine that induces differentiation of naive helper T cells.[38] On the other hand, BE, acting through a nonopioid beta-endorphin receptor, may modulate immunocompetence by stimulating T-cell proliferation and counteracting the inhibitory effects of prostaglandin E1.[39] The strain-dependent opposing effects of BE on inflammation are mediated through delta and kappa opioid receptors and involve changes in the production of reactive oxygen species by inflammatory cells.[40]

T-lymphocytes were described as being able to switch on analgesia by accumulating near the sites of injured nerves and utilizing BE to activate local antinociception receptors. This effect was demonstrated in wild-type and severe combined immunodeficiency (SCID) mice. In wild-type mice, T-lymphocytes that infiltrated the injured nerve expressed BE and receptors for corticotropin-releasing factor (CRF), which associate with the release of opioids from leukocytes. In SCID mice, T-cells expressing BE and CRF receptors were absent. The decreased antinociception was fully restored after transferring T-lymphocytes from wild-type mice. Also, antinociception was reversed after BE-antibodies injection.[41] In mice, CD4+ T-lymphocytes lose analgesic opioid-mediated activity when there is an encephalin deficiency.[32]

BE enhances NK cell activity. It is, however, not known whether it influences NK cell activity by recruiting effector cells, increasing adhesion (the number of effector cell-target cell conjugates), or enhancing the lytic step.[42] The IL-1 family directly influences the anterior pituitary cells and thereby induces the production of BE.[43] IL-31 is an inflammatory cytokine that triggers cell-mediated immunity against pathogens. IL-31 stimulates BE production by keratinocytes and correlates with the intensity of pruritus in inflamed skin.[44]

Conclusion

To conclude, both keratinocytes, sebaceous glands, and nerve endings possess μ-opioid receptors and can be activated by blood or self-produced BE. Dosage-dependent opioid receptors become less sensitive after prolonged exposure to high dosages of BE, which leads to an addiction mechanism and chronization of inflammation in the skin. Normally, opioid peptide is needed no longer than 4 hours after wounding to start antinociception and inflammation. Increased opioid receptor tolerance involves higher dosages of BE. Instead of activating the immune response in a wound, a high BE level inhibits T-cell maturation and IgG production, stimulates sebaceous glands to produce more fat, provokes pathological itching as well as new vessel growth and hyperkeratosis, and enhances NK cell activity. A better understanding of SD pathogenesis leads to a personalized treatment approach, including pharmacological or biological via antibodies regulation of epidermal μ-opioid BE specific receptors.

Declarations

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Author contributions

Conceptualization, V.V. and G.M.; Methodology, V.V.; Software, V.V.; Validation, V.V.; Formal Analysis, V.V.; Investigation, V.V.; Resources, V.V.; Data Curation, V.V.; Writing – Original Draft Preparation, V.V.; Writing – Review & Editing, G.M.; Visualization, V.V.; Supervision, G.M.; Project Administration, V.V.; Funding Acquisition, V.V.

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets used and/or analyzed during the current study are open from the corresponding author on reasonable request.

Ethics approval

The study was carried out within the framework of "Alternative methods of treatment of opportunistic infections using medicinal and non-medicinal means," subject code 12A-2021, state registration number 0121U110174, and "Health and recreation. Peculiarities of the clinical and epidemiological courses of infections and parasitosis characteristic of the Transcarpathian region", state registration number 0117U00283, subject code 02070832. The Ethics Committee of the Medical Faculty at Uzhgorod National University approved the scientific evaluation and study protocol (No. 318/4-6, dated September 16, 2020). All participants were provided with a study protocol and signed the informed consent.

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ORIGINAL PAPER

Predictors of adverse perinatal outcomes in women at 40 weeks or more of pregnancy

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ABSTRACT

Introduction and aim. To evaluate the clinical features of women at ≥40 weeks of pregnancy and the utility of obstetric Doppler indices in predicting adverse perinatal outcomes in these pregnancies.

Material and methods. This prospective study was conducted at a single academic medical center between 2020 and 2022. Women aged 18 years and older with no risk factors who were at ≥40 weeks of pregnancy and delivered their babies in our hospital were included in the study. The fetal biometry, placental maturity grading, and doppler velocytometry indices of the pregnant women were evaluated. The cases were divided into two groups according to the development of adverse perinatal outcomes. The relationship between clinical features and adverse perinatal outcomes was evaluated.

Results. Adverse perinatal outcomes developed in 19.6% (42) of the 214 cases. The multiple logistic regression analysis was performed to identify factors affecting perinatal outcomes. Accordingly, a maternal age of ≥35 years (odds ratio [OR]: 1.74, 95% confidence interval [CI]: 1.29–3.96, p=0.038), nulliparity (OR: 1.42, 95% CI: 1.13–4.63, p=0.040), and grade 3 placental calcification (OR: 1.98, 95% CI: 1.11–4.53, p=0.029) were independent predictors of adverse perinatal outcomes.

Conclusion. Care should be taken in terms of adverse perinatal outcomes in the presence of nulliparity, a maternal age of ≥35 years, and grade 3 placental calcification in ≥40 week pregnancies.

Keywords. adverse perinatal outcomes, doppler velocytometry, placental maturity grading, prolonged pregnancy

Introduction

Prolonged pregnancy causes a significant increase in maternal and perinatal mortality and morbidity and has an incidence of 3-14%.[1] The majority of prolonged pregnancy cases have no known cause, but many risk factors, such as nulliparity, advanced maternal age, post-term pregnancy history, male fetus, and maternal obesity, have been implicated in their etiology.[2] One of the most important determinants of perinatal outcomes is the gestational week. It has been found that perinatal adverse outcomes during prolonged pregnancies are especially associated with changes in the placenta (e.g., fatty degeneration of the placenta, placental infarction, and multiple placental calcifications).[3] Prolonged pregnancy has been found to be related to conditions such as stillbirth, oligohydramnios, macrosomia, uteroplacental insufficiency, dysmaturity, meconium aspiration, and a low APGAR score.[4]

Although it is known that the continuation of pregnancy after the expected delivery time increases perinatal mortality, the time to start fetal monitoring and the gestational week to intervene remain controversial is-

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sues.[5] Doppler velocimetry is a non-invasive method for evaluating uteroplacental circulation. Many uterine artery Doppler studies have shown a relationship between increased wave resistance in uterine artery flow and preeclampsia and/or fetal growth retardation in the second trimester of pregnancy.[6,7] However, there are only limited data concerning whether Doppler flow changes can predict adverse outcomes in ≥40-week pregnancies.

Aim

Therefore, this study aimed to evaluate the clinical features of women at ≥40 weeks of pregnancy and the utility of obstetric Doppler indices in predicting adverse perinatal outcomes in these pregnancies.

Material and methods

Study design and participants

This prospective observational study was conducted between March 1, 2021, and March 1, 2023 at a tertiary university hospital. The study population consisted of women aged over 18 years with no risk factors who were at a gestational age of ≥40 weeks and delivered their babies in our hospital. All patients were evaluated in terms of gestational age, last menstrual period, and previous ultrasounds. Approval for the study was obtained from the Clinical Research Ethical Committee of Ahi Evran University Faculty of Medicine with a protocol number of 2021-02/23. All women were informed about the study, and their written consent was obtained before participating in the study.

Pregnant women aged under 18, those who delivered their babies before the 40 gestational week, cases in which there was no heartbeat on ultrasound, high-risk pregnant women (those with diabetes mellitus, hypertension, multiple pregnancy, or intrauterine growth retardation), pregnant women with fetal anomalies, macrosomic fetuses, oligohydramnios, or polyhydramnios, and those who withdrew their consent or wanted to withdraw from the study were excluded.

Data collection and process

Age, gravida, parity, body mass index, and mode of delivery were recorded, and fetal biometry, the amniotic fluid index, and placental location and presentation were evaluated in each woman. The Grannum classification (grades 0, 1, 2, and 3) was used for the grading of placental maturity: grade 0, a smooth chorionic plate and homogeneous tissue; grade 1: placental tissue with undulations and scattered echoic areas in the chorionic plate; grade 2, linear hyperechoic plates (calcifications) in the basal plate; grade 3: calcifications along the contour of the cotyledons.[8] The blood flow patterns of the umbilical artery (UA), uterine artery (UtA), ductus venosus (DV), and middle cerebral artery (MCA) were evaluated using Doppler ultrasound. Ultrasonographic examinations were performed using the Samsung RS85 Prestige, ultrasonography device equipped with a CA1-7A convex probe, with the patients placed in the supine position, slightly turned to the left side. The UA pulsatility index (UA-PI), UA resistive index (UA-RI), MCA-PI, MCA-RI, DV-RI, the average UtA-PI (of the right and left UtA-PI values), and UtA-RI were recorded. The cerebroplacental ratio (CPR) was calculated by dividing MCA-PI by UA-PI.

Outcome measures

The primary outcomes of the study were adverse perinatal outcomes, including cesarean section due to fetal distress, a fifth-minute Apgar score of <7, meconium-stained amniotic liquor or meconium aspiration, neonatal intensive care unit (NICU) admission, and perinatal mortality. The secondary outcome was the relationship between clinical features and adverse perinatal outcomes.

Statistical analysis

Statistical analysis was obtained using the Statistical Package for the Social Sciences (SPSS) version 21 (Chicago, IL). In the statistical evaluation of the data obtained from the study, categorical data were expressed as frequencies (n) and percentages (%), and continuous data were expressed as mean ± standard deviation and median (25^{th}-75^{th} percentile) values. The conformity of the data to the normal distribution was analyzed with the Kolmogorov-Smirnov test. Student's t-test was used to compare normally distributed parametric data, and the Mann-Whitney U test to compare non-normally distributed data. Pearson's chi-square or Fisher's test was used to compare categorical variables. Univariate and multivariate logistic regression analyses were conducted to determine the relationship between adverse perinatal outcomes and clinical variables. Variables that were found significant in the univariate logistic regression analysis were included in multivariate logistic regression analysis. Odds ratios (ORs) and their 95% confidence intervals (CIs) were also calculated. $p<0.05$ was considered statistically significant in all tests.

Results

The study included 214 pregnant women. The rate of adverse perinatal outcomes in women at ≥40 weeks of pregnancy was 19.6% (42/214). The mean age of the pregnant women was 25.9±6.2 years in the group with adverse perinatal outcomes and 27.5±6.2 years in the group without adverse perinatal outcomes. The mean gestational age of the patients at the time of delivery was 284 (281–286) days in the group with adverse perinatal outcomes and 284 (282–286) days in the group without adverse perinatal outcomes. The demographic and clinical features of the cases are shown in Table 1.

Table 1. Demographic and obstetric characteristics of the sample*

Variables	Adverse perinatal outcomes		p
	Present (n=42)	Absent (n=172)	
Age, years	25.9±6.2	27.5±6.2	0.097
Age ≥ 35 years	9 (21.4%)	14 (8.1%)	0.013
Parity			
Nulliparity	34 (81%)	107 (62.2%)	0.022
Multiparity	8 (19%)	65 (37.8%)	
Gravidity	1.38±0.85	1.50±0.76	0.113
Body mass index (kg/cm^2)	26.9±3.6	25.5±3.4	0.062
GA at delivery, days	284 (281–286)	284 (282–286)	0.221
Placental grading			
Grade 0	11 (26.2%)	66 (38.4%)	0.14
Grade 1	8 (19.0%)	48 (27.9%)	0.242
Grade 2	10 (23.8%)	34 (19.8%)	0.561
Grade 3	13 (31.0%)	21 (9.8%)	0.003
Doppler test			
UA-PI	0.92 (0.83–0.98)	0.88 (0.72–1.12)	0.838
UA-RI	0.59 (0.45–0.78)	0.59 (0.51–0.65)	0.624
MCA-PI	1.61±0.51	1.29±0.57	0.122
MCA-RI	0.73 (0.65–0.89)	0.70 (0.63–0.82)	0.285
CPR	1.61±0.75	1.37±0.5	0.318
UtA-PI	0.94±0.28	0.93±0.23	0.845
UtA-RI	0.99 (0.65–1.03)	0.73 (0.59–0.91)	0.041
DV-RI	0.82±0.23	0.89±0.26	0.492

* Data are presented as mean ± standard deviation, median and 25th–75th percentiles, or n (%). GA – gestational age; UA – umbilical artery; MCA – middle cerebral artery; UtA – uterine artery; DV – ductus venosus; CPR – cerebroplacental ratio (MCA-PI/UA-PI); PI – pulsatility index; RI – resistive index

Nulliparity (81%) and a maternal age of ≥35 years (21.4%) were found at a higher rate in the group with adverse perinatal outcomes. Grade 3 placental calcification and MCA-RI were statistically significantly higher in the group with adverse perinatal outcomes compared to the group without adverse perinatal outcomes. Table 2 shows the distribution of the adverse perinatal outcomes in pregnancies over 40 weeks.

Table 2. Type and rate of adverse perinatal outcomes in the sample*

Adverse perinatal outcome	Number of cases[a] (%)
Cesarean delivery due to fetal distress	13 (6.1%)
Presence of meconium stained liquor or meconium aspiration	15 (7%)
Fifth-minute Apgar score < 7	8 (3.7%)
NICU admission	11 (5.1%)
Perinatal mortality	0

* NICU – neonatal intensive care unit; [a] – some women experienced more than one adverse outcome; therefore, the total of all adverse outcomes exceeds the number of women who experienced adverse outcomes (n=42)

The most common adverse perinatal outcomes were the presence of meconium-stained liquor or meconium aspiration (7%) and cesarean delivery due to fetal distress (6.1%). Statistically significant parameters were included in a regression model (Table 3).

Table 3. Univariate and multivariate analyses of predictive factors for adverse perinatal outcomes*

Variables	Univariate logistic regression			Multivariate logistic regression		
	OR	95% CI	p	OR	95% CI	p
Age ≥ 35 years	2.81	1.63–4.24	0.013	1.74	1.29–3.96	0.038
Nulliparity	2.04	1.49–5.12	0.022	1.42	1.13–4.63	0.04
UtA–RI	2.32	1.17–4.61	0.041	1.23	0.57–2.89	0.582
Placental grading (grade 3)	2.66	1.20–5.96	0.003	1.98	1.11–4.53	0.029

* UtA-RI – uterine artery resistive index; CI – confidence interval; OR – odds ratio

According to univariate logistic regression analysis, a maternal age of ≥35 years, grade 3 placental calcification, UtA-RI, and nulliparity were important predictors of adverse perinatal outcomes. Multiple logistic regression analysis was performed to determine factors affecting adverse perinatal outcomes, and a maternal age of ≥35 years (OR: 1.74, 95% CI: 1.29–3.96, p=0.038), grade 3 placental calcification (OR: 1.98, 95% CI: 1.11–4.53, p=0.029), and nulliparity (OR: 1.42, 95% CI: 1.13–4.63, p=0.040) were found to be independent predictors of adverse perinatal outcomes.

Discussion

In this study, adverse perinatal outcomes developed at a rate of 19.6% in women at ≥40 weeks of pregnancy. When the pregnant women were compared according to the development of adverse perinatal outcomes, nulliparity, a maternal age of ≥35 years, and the presence of grade 3 placental calcification were determined to be associated with adverse perinatal outcomes.

Perinatal morbidities, such as growth retardation, hypoglycemia, polycythemia, meconium aspiration, and pulmonary hypertension, have a higher incidence in postmature babies and present with a higher rate of neurodevelopmental complications.[9] There are many options in fetal monitoring, including the non-stress test (NST), contraction stress test, biophysical profile, and modified biophysical profile (NST and amniotic fluid evaluation). Doppler velocytometry evaluation provides additional information concerning fetal status. Although antepartum fetal monitoring is required at ≥41 0/7 weeks of gestation, there are not sufficient data to define the most appropriate test type and frequency. In addition, only a few studies have investigated Doppler flow changes in post-term pregnancies and reported conflicting Doppler data concerning the in-

crease in placental insufficiency findings. In a prospective study including women at a gestational age of 40 to 42 weeks, Maged et al. showed that women with adverse perinatal outcomes had higher UA-PI and lower MCA-PI values and a lower CPR compared to those with normal perinatal outcomes.[10] In addition, the authors reported that women with adverse perinatal outcomes had a higher rate of cesarean section due to fetal distress and a higher rate of induced vaginal delivery due to oligohydramnios. In contrast, recent studies indicate that the Doppler indices UA, UtA, MCA, and DV are not useful in the follow-up of post-term pregnancies or in predicting and preventing adverse fetal and perinatal outcomes.[5,11] Consistent with these studies, we found that Doppler indices were not predictors of adverse outcomes in women at ≥40 weeks of pregnancy.

It is known that post-term pregnancy is associated with increased fetal and perinatal risks, with most complications developing as a result of excessive fetal growth and placental insufficiency.[12] Placental calcification, characterized by calcium deposits in the placenta, is a very common condition in prolonged pregnancy. Placental calcification is a physiological process associated with a decrease in placental function during prolonged pregnancy.[13] Studies have found that preterm placental calcification is associated with maternal and perinatal adverse outcomes (e.g., preeclampsia, at least one abnormal Doppler index, obstetric cholestasis, placental abruption, intrauterine growth retardation, maternal intensive care unit admission, low-birth-weight infants, and low perinatal APGAR scores) oligohydramnios, perinatal mortality, hypoxia due to placental insufficiency, asphyxia, and cesarean section are also seen at increased rates in post-term pregnancies compared to term pregnancies.[1,14-16] Although the etiology of post-term pregnancies is not yet fully known, it has been reported in the literature that there are many risk factors for the development of a post-term pregnancy, such as obesity, primiparity, advanced maternal age, and low education level.[17,18] In our study, the presence of grade 3 placental calcification seemed to negatively affect perinatal outcomes in women at ≥40 weeks of pregnancy.

A study examining the relationship between advanced maternal age (≥40 years) and pregnancy outcomes in late and post-term pregnancies found that advanced maternal age was associated with adverse pregnancy outcomes (stillbirth, perinatal death, meconium aspiration syndrome, fifth-minute Apgar score<7, NICU admission, and sepsis).[19] In a retrospective study conducted in late and post-term pregnancies, the authors reported that maternal and perinatal adverse risks increased in primiparous women compared to multiparous women.[19] Our study showed a significant relationship between adverse perinatal outcomes and nulliparity and a maternal age of ≥35 years in women at ≥40 weeks of pregnancy.

Our study has certain limitations, with the first and most important being the single-center design. Another limitation concerns the low number of cases. Multicenter studies with a larger patient population will further contribute to the results obtained from the current study.

Conclusion

Every pregnant woman at advanced gestational age is at a potential risk for adverse perinatal outcomes. We consider that care should be taken in terms of adverse perinatal outcomes in the presence of nulliparity, a maternal age of ≥35 years, and grade 3 placental calcification in ≥40-week pregnancies.

Declarations

Funding

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Author contributions

Conceptualization, Z.S.S. and E.T.S.; Methodology, A.E.D. and Z.S.S., Software, Z.S.S. and E.A.; Validation, A.E.D., E.A. and Z.S.S.; Formal Analysis, E.T.S.; Investigation, Z.S.S.; Resources, Z.S.S.; Data Curation, Z.S.S.; Writing – Original Draft Preparation, Z.S.S.; Writing – Review & Editing, Z.S.S.; Visualization, Z.S.S.; Supervision, Z.S.S.; Project Administration, Z.S.S.; Funding Acquisition, Z.S.S.

Conflicts of interest

No conflict of interest was declared by the authors.

Data availability

Data will be provided if necessary.

Ethics approval

This study protocol was approved by Clinical Research Ethical Committee of Ahi Evran University Faculty of Medicine with a protocol number of 2021-02/23 and conducted in accordance with the Declaration of Helsinki and Good Clinical Practices.

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ORIGINAL PAPER

Challenges of the COVID-19 pandemic for children and their families during home quarantine in Turkey

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ABSTRACT

Introduction and aim. The COVID-19 pandemic has caused significant changes in human life. As a result of these changes, it is important to determine the effects on the child and family life. This study was conducted to determine the challenges experienced by children and their families while home quarantine in the pandemic.

Material and methods. The study was conducted with parents who had children aged 6-18, use social media, and willing to participate in the study. A total of 450 parents participated in the study. *Online survey was used.*

Results. It was determined that some of parental daily activities decreased (doing sports, communicating with friends, engaging in hobbies) and some of them increased (personal hygiene, internet, and playing games with their children). The decreased daily activities of children (doing sports and communicating with friends) and increased activities (eating, personal hygiene, internet, and social media use, playing with toys and technological devices) were detected. There was an increase in some of the family activities (watching movies, playing games, and studying).

Conclusion. It was determined that the home quarantine in the pandemic led to some negative changes in the daily living habits, lifestyles, and feelings of the family.

Keywords. child, COVID-19, family health, nursing, pandemics, quarantine

Introduction

Considerable changes have occurred in human life with the COVID-19 pandemic, which has had a worldwide effect. The physical, social, economic, and psychological consequences of the measures taken by governments to reduce the spread of the pandemic have affected all segments of society. The measures taken during the quarantine period have brought about some difficulties and had negative effects on the family life, which is the smallest unit of society, especially the children.[1]

As in the rest of the world, measures have also been taken to prevent the spread of the pandemic in Turkey. Some of these measures were lockdowns for individuals under the age of 20 and over the age of 65, the closure of schools and other educational institutions, the suspension of flights and public transportation, the restriction of intercity travels, the closure of workplaces serving in sectoral areas, such as cafes and restaurants, and carrying out of some services by working from home (education, public services, etc.).[2] Although these measures were taken to protect society, they had a significant negative impact on all its segments. Economic losses and unemployment, difficulties in transition to distance education, problems experienced by parents in the care of children at home, and failure to follow up, treat, and provide care for individuals with

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chronic health problems led to physical and psychosocial health problems due to staying at home.[3,4]

With the onset of home quarantine, children's group activities at schools, team sports, or access to playgrounds disappeared. Many parents had difficulties keeping their children busy and safe at home. In particular, working parents with inadequate social support resources had problems caring for their young children.[5] The loss of jobs or income among parents who had been working in the private sector or were self-employed negatively affected family processes. The economic crisis in the family and the crowded household made this situation even worse.[6] In this process, the increase in domestic violence, divorce, and abuse made children vulnerable to the exploitation and abuse by online risk groups.[4,6] It is stated that 65.7% of child neglect and abuse cases in the USA were determined by employees in institutions serving children, 19.4% by teachers, and 9.6% by health workers.[7] As children did not go to school during the pandemic, it became difficult to detect such cases.[8]

The rapid spread of the virus at a speed that threatens human health around the world and the restrictive measures taken caused individuals to feel intense stress, isolation, loneliness, and fear. Studies indicated that there was an increase in the level of children's stress, fear, and internet addiction and that they experienced posttraumatic stress disorder, fear of infection, lack of attention, anger, and stigma problems as a result of long-term isolation.[5,9] This process caused children and parents who struggled with the difficulties of life and had inadequate coping to experience psychological problems.[10,11]

The closure of schools caused children to have limited contact with their classmates, physical activities to decrease, and eating habits to change.[9,11] During the COVID-19 pandemic, inactivity, changes in eating habits, increased food intake, and unhealthy food choices were detected in children due to the quarantine process. This increased the risk of obesity in children.[12]

Although experts report that COVID-19 has a mild course in children and the mortality rate is low, it is important to accept that children are a risk group affected by this process.[13] Nurses, who are one of the occupational groups that work closely with children and their families, have responsibilities for the solution of biopsychosocial problems. For this reason, they must identify the difficulties experienced by children and families they work with. The lack of enough studies in the literature for determining the difficulties experienced by children and their families during the COVID-19 pandemic guided the planning of this research.

Aim

This study was designed to determine the difficulties of the COVID-19 pandemic for children and their families during home quarantine.

Research questions

1. What were the changes in the daily living habits of children and parents who were in home quarantine during the pandemic?

2. Was there any change in the activities that the family did together during the home quarantine in the pandemic?

3. Was there any change in the lifestyle of children and families who were in home quarantine during the pandemic?

4. Was there any change in the emotional state of children and parents who were in home quarantine during the pandemic process?

Material and methods

Study design, setting and participants

The study was planned in a retrospective, descriptive and cross-sectional design. The study sample consisted of parents who lived in Turkey and had children between the ages of 6-18. G*Power 3.1.9.7 was used to calculate the required sample size. Based on the study findings of sleep anxiety mean scores found by Liu et al., it was calculated that 439 people were required to conduct the research, according to the 0.01 significance level, 99% power and low effect size (0.24). Considering the 10% loss, it was determined that 483 people should be taken. As a result, we reached 450 parents. An online questionnaire (a Google Docs.) developed by the researchers in line with the literature, was shared with the participants via various social media platforms (Facebook, Instagram, blogs, and forums). The inclusion criteria applied for participation in the study were as follows: The children of the parents must be between the ages of 6 and 18, lived in Turkey during lockdown period, be able to read and write in Turkish, use social media, and voluntarily agree to participate in the study.

As a result of the post-hoc analysis calculated on the basis of child and parent eating behaviors as the main variable in the study, the power of the study was calculated as 99% when a significance level of 0.01 and an effect size of 0.3 were taken. As a result, it was determined that the number of samples was sufficient.

Data collection instruments

Descriptive information form

This form consisted of 13 questions about the descriptive characteristics of the children and their families (age, gender, number of children, education level, parents' job, income level, place of residence, working status, whether family members lived together, etc.).

Data form for determining challenges of the COVID-19 pandemic for children and their families during home quarantine

This form, which was developed by the researchers in line with the literature, was used to determine the difficulties faced by the children aged 6-18 and their families in home quarantine during the COVID-19 Pandemic.[6,8-11,14] The form consisted of a total of 60 items about the daily living habits of children and parents who were in home quarantine during the pandemic (total 27 items; 12 for parents and 15 for children), family activities (8 items), lifestyles (5 items), and emotion changes (20 items). The form items are evaluated as "decrease", "no change" and "increase" by the participants' self-evaluation of themselves and their children.

Content validity of the questionnaire was evaluated by taking the evaluations of the expert pediatric nursing academicians and the questionnaire was finalized. The form had internal consistency of .87 (subitems for parents .77, for children .76 and for family acrivities .76).

Data collection and analysis

A total of 450 parents were involved in the study. Completed data collection forms were controlled by two researchers independently, and incomplete forms were excluded from the study. Data were analyzed on the IBM SPSS 24.0 software package (Armonk, NY, USA). Counts, mean scores, and percentage values were used in the analysis of the descriptive data.

Ethics approval

Written permissions were obtained from the Non-Interventional Research Ethics Committee of a University (No: 61351342/2020-510, date: 29.10.2020). At the outset, the participants were informed about the purpose, method, and plan of the study by the researchers, and their informed consent was obtained.

Results

The mean age of the 450 parents participating in the study was 37.23±5.62, the majority of them were between the ages of 35–44 (58.2%), and 94.2% were mothers. Most of the parents (50.2%) had two children, and 94.9% were married. Some of the parents had high school (33.3%) and university (31.8%) education, and more than half of them had equal income and expenses (57.8%). During the pandemic period, the majority of the mothers (64.7%) were not working, 66.7% of the fathers continued to go to work, 10.9% worked from home, and 6.2% were on unpaid leave. It was determined that 93.8% of the family members lived together during the pandemic process.

The changes in the daily living habits of the children and parents during the pandemic are given in Table 1. There was a decrease in children's engagement in sports (66%) and communication with friends (90%), while there was an increase in eating (46.9%), personal hygiene (81.8%), time spent on the Internet (69.8%), social media use (48.7%), playing with toys (52.7%), and playing games with technological devices (68.9%). On the other hand, there was a decrease in parents' engagement in sports (65.6%), communicating with friends (91.1%), and hobbies (48.2%), whereas there was an increase in the duration of their personal hygiene (85.8%), housework (79.6%), Internet activities (71.3%), and playing games with their children (48%).

Table 1. Changes in the daily living habits of the children and parents during the pandemic process (n=450)

Children	Decrease		No change		Increase	
	n	%	**n**	%	**n**	%
Eating	36	8	203	45.1	211	46.9
Sleep time	104	23.1	198	44	148	32.9
Sports	297	66	117	26	36	8
Personal hygiene	2	0.4	80	17.8	368	81.8
Doing housework	9	2	226	50.2	215	47.8
Communication with friends	405	90	28	6.2	17	3.8
Time spent on the Internet	26	5.8	110	24.4	314	69.8
Social media use	41	9.1	190	42.2	219	48.7
Receiving online courses	62	13.8	217	48.2	171	38
Reading books	104	23.1	208	46.2	138	30.7
Playing with toys	69	15.3	144	32	237	52.7
Playing games with technological devices	30	6.7	110	24.4	310	68.9
Time allocated for hobbies	131	29.1	197	43.7	122	27.1
Parents	**Decrease**		**No change**		**Increase**	
	n	%	**n**	%	**n**	%
Eating	34	7.6	214	47.6	202	44.9
Sleep time	131	29.1	205	45.6	114	25.3
Sports	295	65.6	77	17.1	40	8.9
Personal hygiene	4	0.9	60	13.3	368	85.8
Doing housework	1	0.2	89	19.8	358	79.6
Communication with friends	410	91.1	26	5.8	14	3.1
Time spent on the Internet	21	4.7	108	24	321	71.3
Workload	135	30	231	51.3	84	18.7
Time allocated for hobbies	217	48.2	125	27.7	108	24
Reading books	99	22	203	45.1	148	32.9
Listening to music	79	17.6	223	49.6	148	32.9
Playing with the child	45	10	189	42	216	48

The change in activities performed with children during the pandemic is given in Table 2. The parents stated that there was an increase in watching movies (59.8%), playing games (49.8%), and studying (57.8%) with their children during the pandemic process.

The change in the lifestyles of the children and parents during the pandemic is given in Table 3. The parents stated that there was an increase in their children's body weight (48.2%), their sleep patterns were impaired (49.8%), and that their anger behaviors in-

creased (49.1%). When the changes in the family lifestyles were questioned, there was an increase in marital problems (19.3%), communication problems among family members (22.4%), and cigarette/alcohol/substance use (16%).

Table 2. Changes in activities performed with the children during the pandemic (n=450)

Activities	Decrease		No change		Increase	
	n	%	n	%	n	%
Having meals together	31	6.9	228	50.7	191	42.4
Cooking	27	6	237	52.7	186	41.3
Doing housework	24	5.3	221	49.1	205	45.6
Watching movies	29	6.4	152	33.8	269	59.8
Playing games	42	9.3	184	40.9	224	49.8
Listening to music	45	10	252	56	153	34
Taking videos	46	10.2	263	58.4	141	31.3
Studying	36	8	154	34.2	260	57.8

Table 3. Changes in the lifestyle of the children and families during the pandemic (n=450)

Children	Decrease		No change		Increase	
	n	%	n	%	n	%
Body weight	23	5.1	210	46.7	217	48.2
Disruption in sleep patterns	39	8.7	187	41.6	224	49.8
Tidying up their room	62	13.8	254	56.4	134	29.8
Taking home responsibilities	49	10.9	227	50.4	174	38.7
Anger behaviors	23	5.1	206	45.8	221	49.1
Smoking/alcohol use	23	5.1	415*	92.2	12	2.7
Arguing with family members	23	5.1	264	58.7	163	36.2
Arguing with friends	109	24.2	316	70.2	25	5.6
Arguing with girlfriend/ boyfriend	88	19.6	333	74	29	6.4
Course success	129	28.7	251	55.8	70	15.6
Family	**Decrease**		**No change**		**Increase**	
	n	%	n	%	n	%
Marital problems	27	6	336	74.7	87	19.3
Communication problems of family members	43	9.6	306	68	101	22.4
Smoking/alcohol/substance use	27	6	351	78	72	16
Domestic violence	29	6.4	378	84	43	9.6
Divorce	23	5.1	405	90	22	4.9

* parents who gave this answer stated that their children did not smoke/use alcohol

The effect of the pandemic on the feelings of the children and parents is given in Table 4. The parents stated that their children had increased feelings of fear (55.1%), anxiety (63.6%), sadness (57.3%), and restlessness (58%). It was also determined that there was an increase in parents' feelings of fear (66%), anxiety (78.2%), sadness (67.3%), restlessness (68.9%), and doubt (63.1%).

Discussion

This research was carried out to determine the difficulties of the pandemic for children and their families during home quarantine. Due to the rapid global spread of COVID-19 infection, it was declared a pandemic on March 11, 2020.[14] The pandemic has affected people of almost all nations, continents, races, and socioeconomic groups. Daily life has suddenly changed due to quarantine in many countries.[15] Education was suspended and outdoor leisure activities were disrupted, which caused individuals to spend more time at home and their daily living habits to change.[16]

Table 4. The effect of the pandemic on the feelings of the children and parents (n=450)

Children	Decrease		No change		Increase	
	n	%	n	%	n	%
Fear	11	2.4	191	42.4	248	55.1
Anxiety	13	2.9	151	33.6	286	63.6
Sadness	14	3.1	178	39.6	258	57.3
Restlessness	13	2.9	176	39.1	261	58
Depression	14	3.1	277	61.6	159	35.3
Doubt	13	2.9	222	49.3	215	47.8
Joy	153	34	232	51.6	65	14.4
Happiness	135	30	245	54.4	70	15.6
Obsession	22	4.9	298	66.2	130	28.9
Introversion	26	5.8	303	67.3	121	26.9
Parents	**Decrease**		**No change**		**Increase**	
	n	%	n	%	n	%
Fear	8	1.8	145	32.2	297	66
Anxiety	6	1.3	92	20.4	352	78.2
Sadness	7	1.6	140	31.1	303	67.3
Restlessness	9	2	131	29.1	310	68.9
Depression	11	2.4	231	51.3	208	46.2
Doubt	9	2	157	34.9	284	63.1
Joy	166	36.9	234	52	50	11.1
Happiness	156	34.7	246	54.7	48	10.7
Obsession	17	3.8	251	55.8	182	40.4
Introversion	23	5.1	315	70	112	24.9

COVID-19 infection spreads through breathing and physical contact. The World Health Organization recommends the use of masks, disinfection, social isolation, and quarantine practices for the prevention of the disease.[14] In the study, it was determined that there were changes in the daily living habits and lifestyles of children and parents who were quarantined at home during the pandemic period. These changes, it was determined that there was an increase in the eating and personal hygiene habits of children during home quarantine. In addition, about half of the children evaluated within the scope of this study had gained weight according to the parents' reports. Similarly, some studies have reported an increase in body weight in children, adolescents, and young adults.[17,18] This situation is thought to be related to changes in dietary habits, increased food intake, and

increased consumption of unhealthy foods during the COVID-19 pandemic. Decreased physical activity due to home quarantine is also considered an important risk factor for weight gain.[12,17]

The COVID-19 virus can be neutralized with hand hygiene and disinfection practices. It is extremely important to follow the hygiene rules to protect from the virus during the pandemic process and prevent its spread. It can be said that school children and adolescents are more cognitively competent in understanding the importance of hygiene rules such as hand washing and wearing a mask and adhering to them.[19] In our study, it was determined that there was an increase in the hygiene habits of the children and parents. Similar results were found in a study.[20]

The school has an important place in children's daily physical activities. They spend a very active time doing sports in physical education classes, playing games, and dancing with their friends during breaks at school. In addition, children play active games in playgrounds and parks. School closure causes limited interaction among classmates and decreased physical activity in children.[9] In a study, it was determined that 60% of children spent less time doing physical activities.[21] In this study, too, the sports and exercise status of children and their parents were questioned, and it was determined that there was a decrease in the physical activity levels of both parents and their children.

One of the negative effects of the COVID-19 pandemic on children has been predicted as sleep disorders. In this study, it was determined that the sleep duration of more than half of the parents and their children had changed. Some studies in the literature reported an increase in sedentary life and sleep time of children as a result of home quarantine.[22,23]

Play is one of the most important means of meeting the spiritual, mental, social, and physical needs of children. It is an important method for facilitating the child's coping with stress.[24] In the study, it was found that there was an increase in the duration that children played with their toys and technological devices, as well as the duration that parents played with their children. In the study, it was determined that there was a change in family activities being made together in home quarantine. The participants spent more time watching movies and studying with their children during the pandemic. Some studies in the literature indicated that during the COVID-19 pandemic, parents spent more time with each other and with their children, played games at home, searched the Internet for games that could be played at home, and tried to spend quality time as much as they could.[25,26] It is thought that staying at home constantly during the COVID-19 process can strengthen the bonds between family members as it has increased physical presence and contact.[27] In this process, the increase in the time that children spend on games and the time parents spend with their children can be interpreted as a positive result.

In the study, it was determined that children and parents spent more time on the Internet but that the time they spent with their friends decreased. Similarly, some studies found that internet use increased during the COVID-19 process.[28,29] However, it was also found that individuals' feelings of loneliness increased along with the COVID-19 quarantine practices.[30,31] In a study conducted by Ellis et al. with 1054 high school students in Canada, it was determined that individuals spent more time using social media during the pandemic process and that especially those with depressive symptoms had more online connections with their friends.[23] In the study, it was determined that there was a change in the emotional states of children and parents who were quarantined at home during the pandemic. It was found that depressive feelings increased in parents (about half) and children (about one third). In a study conducted on 2330 primary school students in Wuhan, China, it was determined that the students in the study had higher depressive symptoms than students from other regions. In the same study, it was stated that the decrease in outdoor activities and social interaction might be related to the increase in children's depressive symptoms.[32] Supporting children's online conversations with their friends during the quarantine period may be important in terms of protecting their mental health.

The spread of the virus at a speed that threatens human health around the world and the restrictive measures taken have led to intense stress, isolation, loneliness, and fear in individuals.[1,10,11] In the COVID-19 pandemic, monotony in the lifestyle of children and parents, lack of social interaction, and other emerging familial problems have affected their lives adversely, and this may cause mental health problems.[11] In a study by Patrick et al., it was determined that the mental health of 27% of parents deteriorated and behavioral disorders developed in the children of the 14%.[33] Some studies indicated that there was an increase in stress, fear, and internet addiction in children and that they experienced posttraumatic stress disorder, fear of infection, attention deficit, anger, and stigma problems due to the long-term isolation.[5,9] Similarly, it was determined in this study that there was an increase in the feelings of fear, anxiety, sadness, and restlessness in both parents and children during the pandemic process.

Conclusion

In this study, it was determined that the children and their families who were in home quarantine during the COVID-19 pandemic were affected physically, psychologically, and socially. There were some negative changes in the daily living habits, lifestyles, and feelings of the children and their families. It was determined that parents

spent less time doing sports and communicating with friends and that there was an increase in the duration of personal hygiene, internet use, and playing games with their children. It was also found that children spent less time doing sports and communicating with their friends and that the duration of eating, personal hygiene, internet use, social media use, and playing games with toys and technological devices increased. The family members were found to spend more time watching movies, playing games, and studying together. The parents stated that there was an increase in their children's weight gain and deterioration in their sleep patterns. It was determined that there was an increase in children's and parents' feelings of fear, anxiety, sadness, and restlessness.

During the COVID-19 pandemic, nurses and other health professionals should increase the physical activity levels of parents and children, support their healthy eating habits, direct them to social activities, improve their sleep patterns, and guide family members to spend quality time. Thus, children and parents can cope with emotions, such as anxiety, sadness, and fear.

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Author contributions

Conceptualization, B.A., N.Ç.Ç. and S.A.; Methodology, B.A. and N.Ç.Ç.; Software, B.A. and N.Ç.Ç.; Validation, B.A.; Formal Analysis, B.A. and N.Ç.Ç.; Investigation, B.A. and N.Ç.Ç.; Resources, B.A., N.Ç.Ç. and S.A.; Data Curation, B.A. and N.Ç.Ç.; Writing – Original Draft Preparation, B.A., N.Ç.Ç. and S.A.; Writing – Review & Editing, B.A., N.Ç.Ç. and S.A.; Visualization, B.A. and N.Ç.Ç.; Supervision, B.A. and N.Ç.Ç.; Project Administration, B.A. and N.Ç.Ç.

Conflicts of interest

No potential conflict of interest was reported by the author(s).

Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Written permissions were obtained from the Non-Interventional Research Ethics Committee of a University (No: 61351342/2020-510, date: 29.10.2020). At the outset, the participants were informed about the purpose, method, and plan of the study by the researchers, and their informed consent was obtained.

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ORIGINAL PAPER

The effect of a physical therapy programme on the condition of upper limb muscles in patients with rheumatoid arthritis

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ABSTRACT

Introduction and aim. Rheumatoid arthritis is a chronic progressive systemic disease of the connective tissue affecting the joints, mainly small, of the erosive-destructive polyarthritis type, and frequent systemic inflammatory damage to internal organs. The purpose of the research is to evaluate the effectiveness of physical therapy on the muscles of upper limbs in patients with rheumatoid arthritis by the dynamics of muscle strength and strength index.

Material and methods. To determine the strength of the muscles of the affected upper limbs were used dynamometry, calculated strength index as a percentage to assess the functional ability of the affected limb. All patients were divided into control (n=92) and main (n=96) groups, taking into account the functional insufficiency of the joints.

Results. After 6 months of physical therapy, muscle strength indicators in the main group of patients with 1st degree of functional joints insufficiency (FJI) in the affected right limb increased by 3.1 kg, in the left by 3 kg; with 2nd degree FJI – in the right limb by 4.2 kg, in the left – by 3.7 kg, significantly exceeded the same indicators in patients of the control group ($p<0.05$). Similarly, there was an increase in the strength index in the patients of the main group.

Conclusion. Physical therapy technology has been developed and implemented effectively influenced the increase in muscle strength and strength index of affected upper limbs of the main group of patients with rheumatoid arthritis.

Keywords. dynamometry, muscles strength, physical therapy, rheumatoid arthritis, strength index

Introduction

Rheumatoid arthritis is a chronic progressive systemic disease of the connective tissue with damage to the joints, mainly small ones, a type of erosive-destructive polyarthritis and frequent systemic inflammatory lesions of the internal organs, registered in all countries of the world with a frequency of 0.4 to 1.5%.[1,2]

This disease is one of the four large-scale medical problems of humanity, and more than 14 million people around the world suffer from it.[3]

In Ukraine, the prevalence of rheumatoid arthritis is 340 cases per 100,000 of adult population, and the disease mainly affects people of working age (20–50 years), which leads to frequent and long-term hospitalization, and often to disability. According to generalized statistical data, women suffer from rheumatoid arthritis 3-4 times more often than men. At the same time, the peak incidence of rheumatoid arthritis occurs in women aged 40-50.[4,5] The disease leads to frequent and long-term hospitalization of patients, a decrease in their working capacity, disability and large economic costs.[6-8] The mortality rate in patients with rheumatoid arthritis is 2 times higher than in the general population. This indicator worsens every year.[6,9]

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Autoimmune inflammatory process, chronic pain syndrome has a steadily progressive nature. All this, without proper treatment, leads to the destruction of articular cartilage in the form of erosions and destruction of the bones that form the joint. In the future, the joints are deformed and their functional ability is impaired.[10-12]

As a result of the inflammatory process affecting the upper limbs of patients with RA, there is a decrease in the amplitude of movements in the joints, and in muscle strength.[13] An early and permanent sign of rheumatoid arthritis is progressive muscle atrophy, which leads to sharp decline in strength, muscle weakness and is accompanied by a significant decrease or cessation of the patient's physical activity.[14,15]

Numerous clinical data indicate a high degree of reduction in physical activity of patients with rheumatoid arthritis due to impaired mobility and functional insufficiency of joints.[1,10,11,16,17]

According to most authors, in order to increase the effectiveness of drug treatment, a special role is given to physical therapy, which should be an integral part of the comprehensive restorative treatment of patients with rheumatoid arthritis.[6,9,13,14,18,19]

As of today, there is a need for the development and implementation of effective physical therapy technology, which would include an individual approach to the use of innovative restorative physical therapy measures, objective methods of evaluating the effectiveness of the measures and predicting the results.[4,15,20,21]

The lack of such works devoted to a personalized approach to physical therapy of patients with rheumatoid arthritis determined the relevance of the presented work.

Aim

The purpose of research is to evaluate the effectiveness of physical therapy on the muscles of upper limbs in patients with rheumatoid arthritis by the dynamics of muscle strength and strength index.

Material and methods

The research was conducted on the basis of the rheumatology department, the department of restorative treatment with traditional and non-traditional methods of the Municipal Enterprise "Rivne Regional Clinical Hospital named after Yuriy Semenyuk". Accumulation of research results was carried out as patients were admitted to hospital treatment. 188 patients with rheumatoid arthritis were examined, 156 (83%) were women, 32 (17%) were men, and their average age was 44.9±7.6 years. All patients were randomly divided into control (n=92, men – 16 (17.4%), women – 76 (82.6%)) and main (n=96, men – 16 (16.6%), women – 80 (83.4%)) groups. In the control group, there were 34 patients with functional joint disability of the first degree, 58 patients with functional joint disability of the second degree; in the main group, 32 patients with functional joint disability of the first degree, and 64 patients with functional joint disability of the second degree.

The inclusion criteria were: the presence of a confirmed diagnosis of rheumatoid arthritis based on the criteria of ACR/ EULAR 2010.[20] and in accordance with the Order of the Ministry of Health of Ukraine of 11.04.2014 № 263 "Unified Clinical Protocol of Primary, Secondary, Tertiary Medical Care and Medical Rehabilitation of Patients with Rheumatoid Arthritis".[8]

The exclusion criteria: age over 60 years, IV radiological stage of joint damage, the third stage of inflammation, acute inflammatory pain, the patient's refusal to participate in the research. The joints-exceptions included distal interphalangeal, the first wrist-heel, the first shoulder-phalangeal joints.

The research was carried out in compliance with the main provisions of the "Rules of Ethical Principles of Conducting Scientific Medical Research with Human Participation", approved by the Declaration of Helsinki (1964-2013), ICH GCP (1996), EU Directive No. 609 (from November 24, 1986) orders of the Ministry of Health of Ukraine No. 690 of 09/23/2009, No. 944 of 12/14/2009, No. 616 of 08/03/2012. Patients participated in the research completely of their own free will, which is confirmed by personally signing the appropriate informed consent.

Patients were carried out anthropometric (BMI, goniometry, dynamometry), X-ray examinations, MMT, VASH scale, laboratory parameters were studied, joint index was determined.

To determine the strength of the muscles of the flexor of the hand of the affected upper limbs, dynamometry was used (with the help of a carpal dynamometer). In the initial standing position, the patient moved his straight hand to the side and squeezed the carpal dynamometer. At the same time, the free hand was relaxed and lowered down. The dynamometric measurement was conducted alternately with both hands in three attempts, with the best possible outcomes for each hand. The results of the measurements, their comparison with the initial data and the evaluation were carried out three times: before the beginning of the course of physical therapy, after 3 months and after 6 months in the course of rehabilitation activities. In addition, the strength index of the muscles of the hand (relative strength index) was calculated in percentages, which is of great importance for assessing the functional capacity of the affected limb. The calculation was performed using the formula: hand strength index = hand strength (kg): weight (kg) • 100. The average hand strength index for men is 65–75%, for women – 50–60%.

Science-based and developed rehabilitation technology intervention with the use of physical therapy

products with a personalized approach to patients with rheumatoid arthritis and in accordance with the ICF domains and taking into account the factors affecting the level of functional disorders and the quality of their life. For the main group, a 6-month physical therapy programme was developed and implemented, which included the following elements: therapeutic exercises, therapeutic massage and self-massage, physiotherapy procedures, hydrotherapy, orthotics, kinesiotaping of the upper extremities, mechanotherapy and psychological support.

The statistical description of the samples was carried out by determining the arithmetic mean (M) and its error (m). The type of distribution of parameters in the variational series was determined by the Shapiro-Wilk test. The significance of differences between samples was assessed using non-parametric methods for dependent and independent samples (Wilcoxon T-test, Mann-Whitney U-test). The criterion of the reliability of the estimates was the level of significance with an indication of the probability of a false estimate (p). The difference in means was considered significant at $p<0.05$. The resulting digital material was statistically processed using the statistical analysis package Statistica 10 (Serial Number: STA999K347150-W, StatSoft Inc. 2017, Tulsa, OK, USA).

Results

The results of the initial examination of patients with rheumatoid arthritis indicated that the long-term inflammatory process, pain syndrome significantly reduced muscle strength.

During the initial examination of patients with rheumatoid arthritis (n=188), there was a deviation of the initial indicators of dynamometry of the affected upper limbs from normal values of muscle strength. Thus, a decrease in muscle strength was noted in the right affected limb to 27.1±6.2 kg, in the left – to 23.1±6.7 (x ±S) kg, which indicated the development of muscle atrophy in patients. A decrease in the strength of the affected hands was also observed in the examined patients, which was confirmed by the low indicators of the strength index, which are presented in the Table 1.

After the initial examination, all patients were randomly divided to a control group (92 people), 34 of them with functional joint insufficiency (FJI) of the I degree, 58 patients with functional insufficiency of the joints of the II degree, and the main group (96 people), of which there are 32 patients with functional insufficiency of the joints of the 1st degree, 64 patients with functional insufficiency of the joints of the II degree; proportionally, as they were admitted to the hospital.

All patients were examined by a standard clinical, laboratory and functional examination. They were treated in accordance with the regulatory protocol of the Ministry of Health of Ukraine and were under the supervision of doctors.[8] Against the background of drug therapy, physical therapy measures were provided for patients, according to the severity of the disease.

Table 1. Indicators of dynamometry of the affected limbs in examined patients with RA before the course of physical therapy (n=188)*

Upper limb	Statistical indicators	
	$\bar{x}$	S
Dynamometry (kg)		
Right hand	27.1	6.2
Left hand	23.1	6.7
Strength index (%)		
Right hand	37.5	8.6
Left hand	31.8	9.3

* $\bar{x}$ – arithmetic mean value; S – standard deviation

Patients of the control group underwent rehabilitation in accordance with the recommendations of the regulatory document of the Ministry of Health of Ukraine[8] (appendix 1), according to which standard rehabilitation measures (exercises, massage, physical physiotherapy) were used.

The patients of the main group were engaged in the proposed technology of physical therapy measures, which involved the development of an individual program of physical therapy for each patient for a long term, with the necessary correction, which included inpatient and outpatient stages. Phase control was carried out after 3 and 6 months.

The rehabilitation measures included: therapeutic exercises taking into account the period of the disease and the functional insufficiency of the joint (position treatment, static, passive and active exercises with assistance, without assistance, with resistance); exercises with objects, special exercises to improve the amplitude of movements in the affected joints and muscle strength. Attention was focused on aerobic exercises, since they are characterized by lower intensity and longer duration and, in our opinion, are the most appropriate type of motor activity for patients with rheumatoid arthritis. It was recommended to perform exercises for extension and abduction of the limbs to increase the tone of the muscles that perform flexion and adduction and to reduce the tone in the muscles that extend and abduct the limb.

Therapeutic massage was applied and the patient was taught how to perform self-massage, physiotherapy procedures, hydrotherapy, orthotics, kinesiotaping of the upper limbs, mechanotherapy and psychological support were carried out. Hydrotherapy was used to improve circulation, reduce joint pain and muscle spasm. The method of mechanotherapy was differentiated depending on the features of the clinical forms of joint damage to improve the amplitude of movements, stretch

and improve the elasticity of muscles and ligaments, restore muscle strength and motor function of the joints of the upper limbs.

The study of the obtained results, their comparison with the initial data and the assessment were carried out three times: before the beginning of the course of physical therapy, after 3 months, and after 6 months in the process of carrying out rehabilitation measures.

At the beginning of the research, we found out that the patients of both groups with FJI (functional joints insufficiency) I and II degree had reduced indicators of dynamometry and strength index of the upper limbs. Weak muscle strength was noted in the joints of the hand and fingers of both the right and left hand, symmetrically affected upper limbs. The development of functional insufficiency of the joints was facilitated by early growing muscle atrophy, which was related to the affected joint. In patients with rheumatoid arthritis with FJI I and II degrees, a decrease in muscle tone, deterioration of the strength characteristics of muscles with subsequent stiffness and pain in the joints was observed.

Thus, at the beginning of the research, the patients of the main group with FJI I degree, there was a decrease in muscle strength in the right affected limb to 28.2±3.3 kg, in the left – to 24.3±3.2 kg; respectively, in patients of the control group with FJI I degree, in the right affected limb – up to 29.4±2.7 kg, in the left – up to 25.1±2.7 (x ±S) kg.

Similarly, in patients of the main group with the II degree of FJI, a more significant decrease in muscle strength was observed in the right affected limb – up to 25.5±3.1 kg, in the left – up to 21.6±3.4 kg; in patients of the control group with FJI of the II degree, in the right affected limb – up to 27.1±3.0 kg, in the left – up to 22.8±3.1 kg, which is a deviation from the normal values of muscle strength according to dynamometry indicators (x±S) (Table 2).

Both standard treatment and physical therapy after 3 months contributed to the improvement of dynamometry indicators in the affected upper limbs (both right and left) of all patients, indicating the results shown in Table 2. However, in patients of the main group, the changes were more pronounced than in the patients of the control group, where the indicators were significantly lower. So, in general, after 3 months of physical therapy in patients of the main group with FJI I degree, the strength of the muscles in the affected right limb increased from 28.2±3.3 kg to 34.4±3.4 kg (x±S), which is significantly higher than the figure of the control group of patients with FJI of the 1degree, at repeated examination: from 29.4±2.7 kg to 32.1±2.9 kg (x±S) ($p<0.05$). The strength of the muscles of the affected left limb in the main group with FJI 1 degree, also increased from 24.3±3.2 kg to 30.3±3.2 kg (x±S), the indicator of patients of the control group with FJI 1 degree, was comparatively lower: from 25.1±2.7 kg to 27.3±2.8 kg (Table 2) (x±S).

Accordingly, in the patients of the main group with FJI of the II degree, an advantage was also observed in terms of increasing dynamometry indicators: muscle strength in the affected right limb increased from 25.5±3.1 kg to 32±3.4 kg, in the left – from 21.6±3.4 to 27.6±3.3 kg; in patients of the control group, muscle strength in the affected right limb increased from 27.1±3 kg to 29.9±3.1 kg, in the left – from 22.8±3.1 to 24.9±3 kg, which demonstrates significantly lower dynamometry indicators (Table 2) (x±S).

As a result of the use of the recommended means of physical therapy in the main group, the indicators of the strength index increased significantly. In particular, in the dynamics of the strength index of the affected right limb of the main group of patients with FJI of the 1 degree, increased on average by 4.2%, in the left – by 4.1% (x±S) ($p<0.05$). At the same time, patients of the control group with FJI I degree the strength index of the affected right limb increased by only 2.1%, in the left one by 2% (Table 2) (x±S).

Accordingly, the strength index of the affected right extremity of the main group of patients with the II degree of FJI, increased on average by 4.3%, in the left –

Table 2. Dynamics of dynamometry indicators and strength index in both groups of patients before and 3 months after the course of physical therapy*

Upper limb	Before the course of physical therapy				After 3 months			
	Main group		Control group		Main group		Control group	
	FJI I (n=32)	FJI II (n=64)	FJI I (n=34)	FJI II (n=58)	FJI I (n=32)	FJI II (n=64)	FJI I (n=34)	FJI II (n=58)
	$\bar{x}$±S		$\bar{x}$±S		$\bar{x}$±S		$\bar{x}$±S	
Dynamometry (kg)								
Right	28.2±3.3	25.5±3.1	29.4±2.7	27.1±3	34.4±3.4#	32.0±3.4#	32.1±2.9	29.9±3.1
Left	24.3±3.2	21.6±3.4	25.1±2.7	22.8±3.1	30.3±3.2#	27.6±3.3#	27.3±2.8	24.9±3
Strength index (%)								
Right	39.4±5	37.1±4.6	39.5±6	35.7±5.6	43.6±5.2#	41.4±4.8#	41.6±5.9	37.9±5.5
Left	33.4±4.8	31.4±4.4	33.7±5.6	30.1±5.3	37.5±4.7#	35.4±4.2#	35.7±5.8	32.1±5.4

* $\bar{x}$ – arithmetic mean value; S – standard deviation; # – $p<0.05$ between the indicators of the main and control groups

Table 3. Dynamics of dynamometry indicators and strength index in both groups of patients 3 and 6 months after a course of physical therapy*

Upper limb	Indicators after 3 months				Indicators after 6 months			
	Main group		Control group		Main group		Control group	
	FJI I (n=32)	FJI II (n=64)	FJI I (n=34)	FJI II (n=58)	FJI I (n=32)	FJI II (n=64)	FJI I (n=34)	FJI II (n=58)
	$\bar{x}$±S		$\bar{x}$±S		$\bar{x}$±S		$\bar{x}$±S	
Dynamometry (kg)								
Right	34.4±3.4	32.0±3.4	32.1±2.9	29.9±3.1	37.5±4.2#	36.2±3.9#	34.6±2.8	32.5±3.3
Left	30.3±3.2	27.6±3.3	27.3±2.8	24.9±3.0	33.3±3.1#	31.3±3.5#	29.6±3.1	27.1±3.2
Strength index (%)								
Right	43.6±5.2	41.4±4.8	41.6±5.9	37.9±5.5	50.2±5.4#	48.2±4.7#	46.8±5.7	43.1±5.7
Left	37.5±4.7	35.4±4.2	35.7±5.8	32.1±5.4	43.4±4.9#	41.5±4.9#	40.1±5.9	36.4±5.5

* $\bar{x}$ – arithmetic mean value; S – standard deviation; # – p<0.05 between the indicators of the main and control groups

by 4% (x±S) (p<0.05). In patients of the control group with FJI of the II degree. the strength index of the affected right limb increased by 2%, in the left limb by 2.0%, which shows significantly lower indicators than in patients of the main group (Table 2) (x±S).

The analysis of the dynamometry indicators of the affected joints of the hand in rheumatoid arthritis after 6 months of the course of physical therapy indicated their probable improvement in the examined patients of the main group. So, muscle strength in patients with FJI of the 1 degree, in the affected right limb increased from 34.4±3.4 kg to 37.5±4.2 kg, in the left – from 30.3±3.2 kg to 33.3±3.1 kg (Table 3) (x±S) (p<0.05). In patients of the control group with FJI of the I degree an increase in muscle strength was also noted in both affected limbs (in the right – from 32.1±2.9 kg to 34.6±2.8 kg, in the left – from 27.3±2.8 kg to 29.6 ±3.1 kg), however, their dynamometry indicators were significantly lower, compared to the indicators of the main group, which is presented in the Table. 3.

Similarly, the indicators of muscle strength improved in patients of the main group with FJI of the II degree in the affected right limb – from 32±3.4 kg to 36.2±3.9 kg, in the left – from 27.6±3.3 kg to 31.3±3.5 kg and significantly exceeded the same indicators in patients of the control group with FJI of the II degree: muscle strength in the right limb increased from 29.9±3.1 kg to 32.5±3.3 kg, in the left – from 24.9±3 kg up to 27.1±3.2 kg (Table 3) (x±S) (p<0.05).

The increase in the muscles strength of the right and left hand as a result of the use of a 6-month course of physical therapy contributed to the increase in the strength index of the affected upper limbs in the patients of the main group. In particular, the strength index of the right limb in patients of the main group with FJI of the I degree, increased from 43.6% to 50.2%, the left – from 37.5% to 43.4%, which was significantly better than in the patients of the control group with 1degree of FJI – from 41.6% to 46.8% (right limb) and from 35.7% to 40.1% (left limb) (Table3) (x±S) (p<0.05).

Accordingly, the strength index of the affected right limb of the main group of patients with the FJI of the II degree, increased on average from 41.4% to 48.2%, in the left – from 35.4% to 41.5% (x±S) (p<0.05). In patients of the control group with FJI of the II degree the strength index of the affected right limb increased from 37.9% to 43.1%, in the left – from 32.1% to 36.4%, which shows significantly lower indicators than in patients of the main group (Table 3) (x±S).

Thus, the above results of dynamometry and strength index in the main group, which significantly exceed the same indicators of the control group, testify to the effectiveness of the developed physical therapy technology for the patients of the main group.

This was expressed in an increase in the functional capacity of the upper limbs, an improvement in the bending of the fingers of the hand, the elasticity of muscles and ligaments, and a restoration of the strength of the muscles of the upper limbs at the end of the study.

Discussion

A group of scientists analyzed the general kinematics of the upper limbs in 3D while performing the "can opening movement" in rheumatoid arthritis and compared them with healthy people.[22] 24 women (12 healthy and 12 with rheumatoid arthritis) were included. Assessment was performed using the JAMAR dynamometer, the Health Assessment Questionnaire, and 3D kinematic analysis of the upper limb during the "can opening motion". As a result of the study, it was found that there was a statistical difference between the groups: compared to healthy people, women with rheumatoid arthritis have slower movements, more elbow flexion, and less hand grip strength.

Researchers Sferra da Silva, G., de Almeida Lourenço, M. and de Assis assessed disease activity in patients with rheumatoid arthritis.[23] Body weight and height were measured for all participants, questionnaires were used on patients' functional abilities, and

wrist dynamometry was performed. Research results have shown that hand strength in patients with rheumatoid arthritis is strongly correlated with function, but not with disease activity. This confirms our research on the formation of hand muscle atrophy in patients with rheumatoid arthritis, which negatively affected the functional capacity of the affected upper limbs and significantly reduced muscle strength.

According to the results of a number of authors, it was established that the endurance and grip strength of patients with rheumatoid arthritis is related to the functions of the upper limbs and their quality of life. This result demonstrates that the assessment of hand grip endurance can be a reference for scientists who develop a physical therapy program for the upper limbs of patients with rheumatoid arthritis.[24]

The research results presented above confirm the hypothesis that in patients with rheumatoid arthritis, as a result of a long-term inflammatory process, pain syndrome, and a significant decrease in muscle strength, the function of the upper limbs deteriorates and, accordingly, their quality of life.[15,23-25]

Conclusion

The developed and implemented physical therapy programme effectively increased muscle strength and strength index of the affected upper limbs of patients with rheumatoid arthritis, in contrast to conventional restorative treatment. This contributed to the improvement of finger flexion, hand grip endurance, muscle and ligament elasticity, and restoration of upper extremity muscle strength in the main group at the end of the study, which confirms the effectiveness of the implemented physical therapy programme.

Prospects for further research are to study the functional status of patients with rheumatoid arthritis using the Stanford Health Assessment Questionnaire HAQ Disability Index (HAQ-DI).

Declarations

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Author contributions

Conceptualization, I.G. and A.N; Methodology, I.G.; Software, A.N.; Validation, I.G.; Formal Analysis, I.G.; Investigation, A.N.; Resources, I.G.; Data Curation, A.N.; Writing – Original Draft Preparation, A.N.; Writing – Review & Editing, A.N.; Visualization, I.G.; Supervision, I.G.; Project Administration, I.G.; Funding Acquisition, I.G.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The research was carried out in compliance with the main provisions of the "Rules of Ethical Principles of Conducting Scientific Medical Research with Human Participation", approved by the Declaration of Helsinki (1964-2013), ICH GCP (1996), EU Directive No. 609 (from November 24, 1986) orders of the Ministry of Health of Ukraine No. 690 of 09/23/2009, No. 944 of 12/14/2009, No. 616 of 08/03/2012.

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ORIGINAL PAPER

A study on the mycobacterial burden and phenotypic drug resistance pattern with reference to the GeneXpert Cycle Threshold values in pulmonary tuberculosis

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ABSTRACT

Introduction and aim. Tuberculosis (TB) remains a significant global health challenge. Early and accurate diagnosis is crucial to prevent further transmission. The present study aimed to correlate cycle threshold values with smear microscopy and culture positivity, and determine cut-off cycle threshold values for levels of smear grade and culture positivity.

Material and methods. Forty presumptive cases of pulmonary TB were included and subjected to Ziehl- Neelsen stain, culture on Lowenstein Jensen media, CBNAAT and drug susceptibility testing for first line anti-tubercular drugs.

Results. Our study predicts 3+, 2+, and 1+ sputum smear grade at a cut-off of Ct value ≤ 16.74, ≤19.68, and ≤ 22.32 respectively. A strong positive correlation was found between time to culture positivity and Ct value. A cut-off of Ct value ≤22.32 predicts culture positivity with a sensitivity of 92%, and a specificity of 67%. None of the isolates showed rifampicin resistance by 1% proportion method.

Conclusion. Understanding the appropriate utilization of CBNAAT Ct values and their correlation with smear microscopy grade, culture, and drug susceptibility testing can assist clinicians in early identification and prompt initiation of appropriate treatment. This knowledge can contribute to the prevention of drug resistance, reduced transmission, and a decreased disease burden associated with TB.

Keywords. CBNAAT, cycle threshold values, rifampicin resistance, smear microscopy, tuberculosis

Introduction

Tuberculosis (TB) continues to affect millions of people worldwide despite intensified standard TB control measures. Many patients with active TB, especially in TB and HIV endemic areas remain undiagnosed and continue to spread the disease in the community.[1] Mycobacterial load, measured as smear positivity grades (scanty, 1+, 2+, and 3+) in accordance with Revised National TB Control Programme (RNTCP) is used to determine infectiousness and severity of the disease.[2] Another measure of mycobacterial burden is the time to culture positivity, a culture-based method routinely used to assess the bacillary burden in TB.[3] However, conventional solid culture has a slow turnaround time of up to 10–12 weeks, while smear microscopy has low sensitivity and quality control issues.[4] Liquid culture techniques were developed for the early detection of mycobacterial growth, but the turnaround time is still quite long for a diagnostic test to control transmission.[5] The cartridge-based nucleic acid amplification test (CBNAAT), an automated molecular test for the detection of *Mycobacterium tuberculosis* (MTB) has been recommended

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by WHO as a first line TB diagnostic test as an alternative to smear microscopy.[6] The GeneXpert is based on real-time PCR technique that simultaneously detects DNA of *Mycobacterium tuberculosis* and rifampicin resistance.[7] The diagnostic techniques for the detection of resistance in *M. tuberculosis* isolates include phenotypic methods like 1% proportion method, absolute concentration method and resistance ratio methods, which are based on assessment of growth of *M. tuberculosis* in culture media containing a critical concentration of specific anti-TB drugs. Genotypic or molecular methods for detection of drug resistance are the Xpert MTB/RIF assay and two commercial line probe assays, the MTB-DR plus assay and the Nipro NTM plus MDR-TB detection kit 2 which detects resistance to rifampicin alone or in combination with isoniazid. Molecular methods are highly efficacious and provide faster results.[8]

Emerging technologies aim to maintain the trend of highly accurate TB tests that have been made possible by advancements in molecular TB diagnostics over the past few years. Despite notable scientific advancements, TB is still not properly diagnosed and monitored. Concerns with improper diagnosis of latent cases and the alarming emergence of drug resistance continue.

Aim

Hence, the present study aimed to correlate cycle threshold values with RNTCP smear microscopy grades and culture positivity, and also to determine cut-off cycle threshold values to predict the different levels of smear grade and culture positivity in pulmonary TB. We further wanted to study the phenotypic drug resistance pattern to first line antitubercular drugs among various levels of cycle threshold values in pulmonary TB.

Material and methods

All presumptive cases of pulmonary tuberculosis diagnosed by Ziehl-Neelsen (ZN) staining for acid fast bacilli and/or CBNAAT. All the general and COVID-19 precautionary measures were taken before sample collection. Two sputum samples (one spot and one early morning) were collected for each patient. The study was conducted in the Direct Observation Treatment Short-course (DOTS) Centre of University College of Medical Sciences and Guru Teg Bahadur Hospital, New Delhi from November 2019 to October 2021. One sample was subjected to CBNAAT, while the other to microscopy and culture. Smears were made from the growth and identified on ZN staining, and further confirmed by MPT64 antigen detection assay. Drug susceptibility testing was performed by 1% Proportion method to Isoniazid, Rifampicin and Ethambutol as per RNTCP guidelines. We utilized the *H37RV* strain as a control to assess the drug susceptibility testing method for first-line drugs.

Ethical approval

Informed written consent was taken from the study subjects prior to conducting the study. Participation was fully voluntary based including the right to withdraw from the study at any time without presenting an explanation for their withdrawal. Data confidentiality was maintained through anonymity by avoiding any personal identifiers. Clearance for the study was taken from IEC-HR Institutional Ethics Committee-Human Research of the University College of Medical Sciences (IEC-HR/ 2019/41/68).

Data management and statistical analysis

MS Excel spreadsheet program was used to record the data. SPSS v23 (IBM Corp., Armonk, NY, USA) was used for data analysis. Descriptive statistics were elaborated in the form of means/standard deviations and medians/IQRs for continuous variables. Frequencies and percentages were used for categorical variables. Data were presented in a graphical manner wherever appropriate for data visualization using histograms/box-and-whisker plots/column charts for continuous data and bar/pie charts for categorical data. Coefficient of correlation (Spearman correlation coefficient) for Ct value with smear grade, time to culture positivity, serum adenosine levels and clinical scores were calculated. The optimum value of sensitivity and specificity of Ct value were calculated at an optimum cut-off after obtaining the ROC curve. Statistical significance was kept at p value <0.05.

Results

Our study had population range between 18 to 53 years, with male to female ratio 1.6:1. Out of the 40 samples tested, 2 were smear-negative, 12 (30%) had 1+ sputum smear grade, 15 (37.5%) had 2+, while 11 (27.5%) had 3+ sputum smear grade as per RNTCP sputum smear grading. Our study reported 37 (92.5%) of the total samples to be culture positive. Out of the total 37 culture positive isolates, 8.10% were culture positive at the end of 3 weeks, 18.90% were positive at 4 weeks, followed by 13.50% at 5 weeks, 24.30% at 6 weeks, 16.20% at 7 weeks and 18.90% at 8 weeks. The mean (SD) of time to culture positivity was 5.78 (1.6) weeks. The time to culture positivity ranged from 3-8 weeks. Eleven samples (27.5%) of the total had a high CBNAAT category, while 22 (55%) and 7 (17.5%) had medium and low CBNAAT categories respectively. None (0%) showed rifampicin resistance.

Correlation of Ct value (mean) and sputum smear microscopy (RNTCP sputum smear grade)

Table 1 depicts the comparison of the 4 subgroups of sputum smear microscopy grades in terms of the Ct value (mean). There was a significant difference between

the 4 groups of sputum smear grade in terms of Ct value (mean) (χ^2=30.799, p<0.001), and the strength of association (Kendall's Tau) was 0.72 (large effect size).

Table 1. Comparison of the 4 Sputum smear microscopy grades in terms of Ct value (mean) (n=40)

Ct value (Mean)	Sputum smear microscopy grade				Kruskal Wallis Test	
	Negative	1+	2+	3+	χ^2	p
Mean (SD)	20.67 (2.45)	22.25 (1.63)	19.39 (1.05)	14.53 (1.84)	30.799	<0.001
Median (IQR)	20.67 (19.8–21.53)	21.86 (21.09–23.89)	19.26 (18.98–19.49)	14.9 (14.71–15.09)		
Range	18.94–22.4	19.5–24.56	17.12–21.58	11.08–16.74		

The area under the ROC curve (Fig. 1) predicts 3+ sputum smear grade at a cutoff of Ct value (Mean) ≤ 16.74, with a sensitivity of 100%, and a specificity of 100%, thus demonstrating an excellent diagnostic performance. It was statistically significant (p<0.001). The odds ratio (95% CI) for smear grade 3+ when Ct value (mean) ≤ 16.74 was 413 (15.58–10944.98), while the relative risk (95% CI) was 30 (6–169.25).

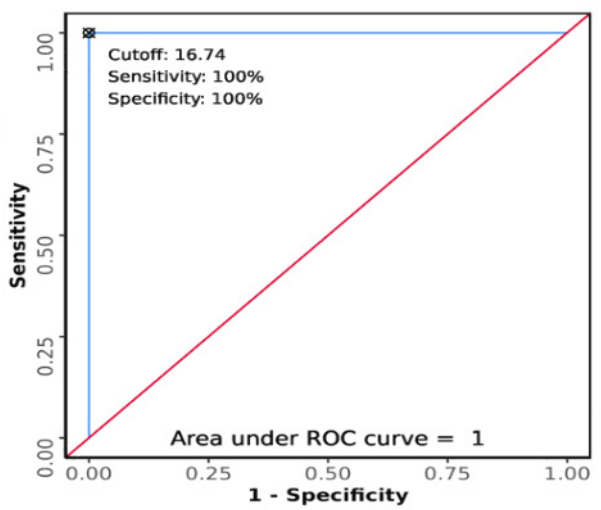

Fig. 1. ROC curve analysis showing diagnostic performance of Ct value (Mean) in predicting 3+ sputum smear grade (n=40)

The area under the ROC curve (Fig. 2) predicts 2+ sputum smear grade at a cutoff of Ct value (mean) ≤19.68, with a sensitivity of 92%, and a specificity of 86%, thus demonstrating excellent diagnostic performance. It was statistically significant (p = <0.001). The odds ratio (95% CI) for sputum smear grade 2+ when Ct value (mean) ≤19.68 was 46 (6.74–313.92), while the relative risk (95% CI) was 4.6 (2.01–13.12).

Figure 3 depicts the ROC curve analysis showing the diagnostic performance of Ct value (Mean) in predicting 1+ sputum smear grade. At a cut-off of Ct value (mean) ≤ 22.32, it predicts sputum smear grade 1+, with a sensitivity of 90%, and a specificity of 50%.

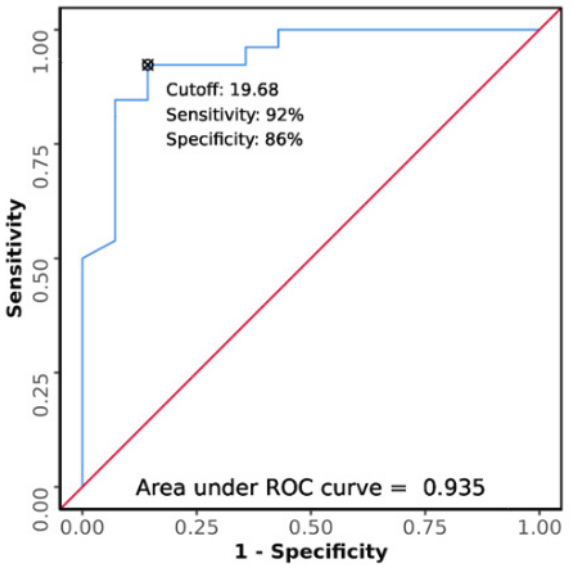

Fig. 2. ROC curve analysis showing diagnostic performance of Ct value (Mean) in predicting 2+ sputum smear grade (n=40)

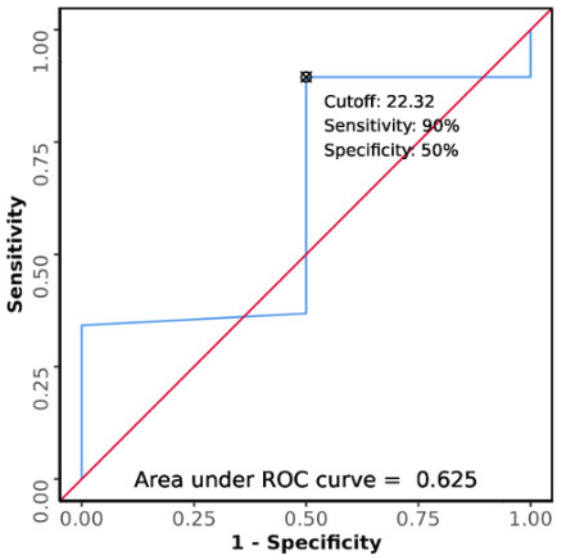

Fig. 3. ROC curve analysis showing diagnostic performance of Ct value (Mean) in predicting 1+ sputum smear grade (n=40)

Correlation of Ct value (mean) and culture positivity

Table 2depicts the correlation of Ct value (mean) and culture positivity. The mean (SD) and median (IQR) of Ct value (mean) in the culture positive group were 18.73 (3.31) and 19.26 (16.62–21.1) respectively.

Correlation of Ct value (mean) and time to culture positivity

Scatterplot in the figure (Fig. 4) shows a strong positive correlation between time to culture positivity (weeks) and Ct value (Mean), and this correlation was statistically significant (rho=0.88, p<0.001). For every 1 unit increase in time to culture positivity (weeks), the Ct Value (mean) increases by 1.75 units.

Table 2. Comparison of the 2 Subgroups of Culture in terms of Ct value (mean) (n=40)

CT Value (Mean)	Culture		Wilcoxon-Mann-Whitney U Test	
	Positive	Negative	W	p
Mean (SD)	18.73 (3.31)	21.97 (2.83)		
Median (IQR)	19.26 (16.62–21.1)	22.4 (20.67–23.48)	26.500	0.143
Range	11.08–24.34	18.94–24.56		

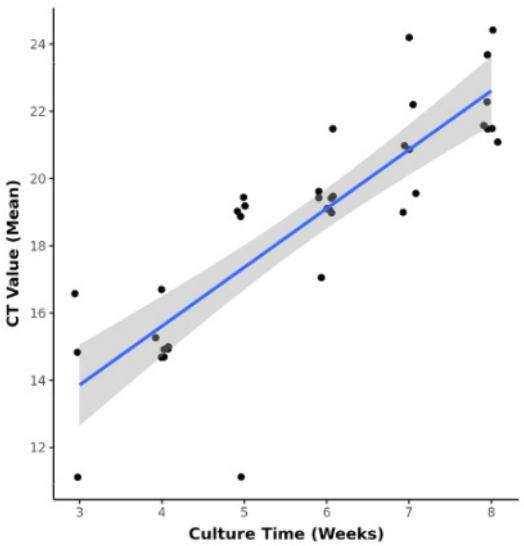

Fig. 4. Correlation between time to culture positivity (weeks) and Ct value (mean) (n=37)

The area under the ROC curve (Fig. 5) for Ct value (mean) in predicting culture positive vs culture negative was 0.761 (95% CI: 0.365–1), thus demonstrating fair diagnostic performance. At a cutoff of Ct value (mean) ≤ 22.32, it predicts culture positivity with a sensitivity of 92%, and a specificity of 67%.

Table 3. Phenotypic DST pattern to 1st line anti-tubercular drugs (n=37)

Rifampicin resistance by CBNAAT	Number of isolates resistant to 1st line anti-tubercular drug in DST by 1% proportion method		
	Isoniazid	Rifampicin	Ethambutol
0	1	0	0

Phenotypic drug susceptibility testing (DST) pattern to 1st line anti-tubercular drugs

Table 3 shows the phenotypic drug susceptibility pattern to 1st line anti-tubercular drugs. Out of the 37 culture positive isolates, none showed resistance to rifampicin and ethambutol by 1% proportion method. However, 1 (2.7%) isolate showed isoniazid mono-drug resistance. No significant association was found between isoniazid mono-resistance and the various other patients' parameters like smear microscopy grade, culture positivity and Ct value (mean).

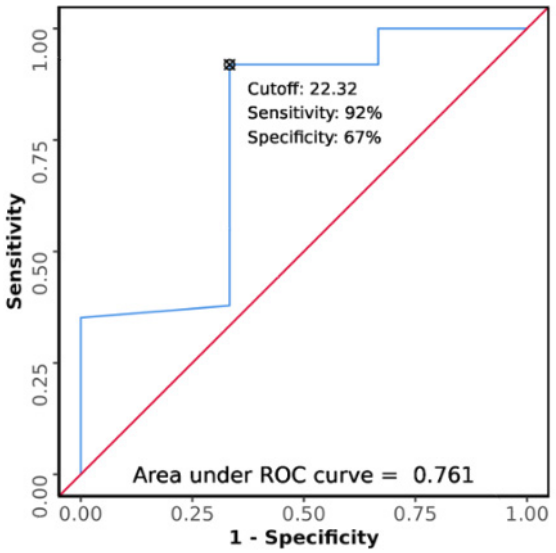

Fig. 5. ROC curve analysis showing diagnostic performance of Ct value (mean) in predicting culture positivity (n=37)

Discussion

Lack of adequate diagnostic facilities for TB is one of the key barriers to TB control in the majority of high-burden countries. The steadily rising number of cases of drug-resistant TB emphasizes the critical need for precise and rapid diagnostic techniques for their detection.[9] The laboratory detection turnaround time should be as short as possible with a large capacity in order to reach the large number of patients efficiently.

Our study has demonstrated an excellent diagnostic performance of Ct value in predicting the various grades of smear positivity, with higher sensitivity as well as specificity. We also found a significant difference between sputum smear grade and Ct value (mean) (χ^2=30.799, p<0.001), with the strength of association (Kendall's Tau) of 0.72 (large effect size). A study conducted in Uganda among pulmonary TB patients found Xpert Ct values comparable with smear microscopy, similar to our findings. Their study reported a cut-off of 23.62, which had the highest sensitivity and specificity in predicting +1 smear grade, comparable to our study findings.[10]

In a study conducted by Kassa et al. on pulmonary drug-resistant tuberculosis patients, 16.35% (95% CI: 13.40, 19.79) were smear-negative, which is a slightly higher proportion compared to our study (5%).[11] Furthermore, the distribution of smear grades also varied. While our study showed a higher proportion of 1+ smear grade (30.0%) compared to Kassa et al. (18.27%), their study reported a higher percentage of 3+ smear grade (34.42%) compared to our study (27.5%). It is evident that there are differences in the proportions of smear-negative cases and the distribution of smear grades which could be attributed to the variations in

study populations, sample sizes, and different geographical locations.[11]

Our study found a strong positive correlation (Spearman correlation coefficient=0.9) between time to culture positivity and Ct value (Mean), and this correlation was statistically significant (rho=0.88, p=<0.001). For every 1 unit increase in time to culture positivity (in weeks), the Ct value (mean) increases by 1.75 units. Najjingo et al. found a correlation of 0.37 between the Xpert Ct values and time to culture positivity in their study on pulmonary TB patients.[10] Their study has also shown a linear relationship between time to culture positivity and Ct values; the Ct values were found to increase by 2.57 for every unit increase in days to culture positivity, which is comparable to our study findings. However, their study compared the time to culture positivity of liquid culture media with Ct values, in contrast to solid culture media used in our study.[10]

Our study showed 37 (92.5%) of the total samples to be culture positive. Out of the total 37 culture positive isolates, 8.10% were culture positive at the end of 3 weeks, 18.9% were positive at 4 weeks, followed by 13.50% at 5 weeks, 24.30% at 6 weeks, 16.20% at 7 weeks and 18.9% at 8 weeks. The mean (SD) of time to culture positivity was 5.78 (1.6) weeks. The time to culture positivity ranged from 3–8 weeks. Palange et al. in his study found that the mean of time to culture positivity on Lowenstein Jensen media for pulmonary samples was 31.32 days which ranged from 30.8–31.64 days.[12] Among 7 low CBNAAT category detected samples in our study, culture growth was not seen in 3 samples. However, one sample among the culture-negative samples showed 1+ sputum smear grade; rest two were smear-negative. Prakash et al reported similar discordance in his study in the very low category of CBNAAT.[13] In his study, only two samples out of 34 samples in the very low category were smear positive, one of which was culture negative, whereas growth was seen in 10 samples only. The two samples which were smear positive in the very low category did not show growth on culture which is comparable to our study findings. On the contrary, ten samples which showed growth on culture had delayed time to culture positivity and were AFB smear negative.[13] Najjingo et al. in his study concluded that majority of low and very low Ct values quantified by Xpert were negative by smear microscopy.[10] This poses a higher risk of TB transmission in the community.

In our study, the following patients' parameters were significantly associated (p< 0.05) with Ct value (mean): sputum smear microscopy findings, culture time, and CBNAAT category. The mean (SD) of Ct value (mean) in smear-negative group was 20.67 (2.45), whereas the mean (SD) of Ct value (mean) in 1+, 2+ and 3+ grades were 22.25 (1.63), 19.39 (1.05) and 14.53 (1.84) respectively. The Ct value (mean) ranged from 18.94–22.4 in the smear-negative group, whereas it ranged from 19.5–24.56, 17.12–21.58, and 11.08–16.74 in 1+, 2+ and 3+ grades respectively. Najjingo et al. reported that the median Ct values were 25.4, 23.8, 18.2, 20.1 and 16.6 for negative, scanty, 1+, 2+ and 3 +smear grades respectively.[10] A recent study reported that the smear negative samples had a median Ct of 28.4 (IQR 24.2–31.44) compared to 18.7 (15.8–22.6) for smear positive samples.[14] Fradejas et al. reported that the samples from smear-negative patients had a significantly higher mean Ct value than those from smear-positive patients 20.9(±5.8) vs. 16.9 (±4.9), which is comparable to our study findings.[6] Another study reported a statistically significant difference in the mean Ct value between sputum smear-positive and sputum smear-negative patients (17.8±4.8 and 22.3±6.7, respectively; p=0.002).[15] This could possibly be due to variable sample size in the various studies described above. Moreover, the co-morbid conditions and various other factors associated with the study population also varied in different studies.

Conclusion

Several correlations were found between CBNAAT Ct values and various microbiological parameters. Various cutoff levels to determine different grades of smear and culture positivity were also established. Phenotypic drug resistance pattern was also analyzed. The knowledge of the proper utilization of Ct values by the treating physicians and the drug resistance epidemiology will bridge the gap between the delay in diagnosis and management of TB with further prevention of transmission of drug resistant strains.

Declarations

Funding

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Author contributions

Conceptualization, B.K. and K.S.; Methodology, K.S.; Software, K.Si.; Validation, B.K., K.S. and P.H; Formal Analysis, K.S.; Investigation, K.S.; Resources, P.H.; Data Curation, K.Si.; Writing – Original Draft Preparation, K.S.; Writing – Review & Editing, B.K.; Visualization, K.S.; Supervision, B.K.; Project Administration, B.K.

Conflicts of interest

The authors have no conflict of interest. There is no relationship of interest with any company in the study we are responsible for. No support was received from any project or company for the research.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Institutional Review Board of the Institutional Ethics Committee-Human Research of the University College of Medical Sciences (IEC-HR/ 2019/41/68).

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ORIGINAL PAPER

Salivary microbial diversity – an investigation on possible biomarkers for polycystic ovarian syndrome from eastern India

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ABSTRACT

Introduction and aim. Polycystic ovarian syndrome (PCOS) is one of the major reproductive health issues, thought to be multifactorial, needs serious attention as a dual burden (health and economic) mainly for developing countries like India, due to its rapid rise (30%) in the last couple of years. Therefore, widespread and liberal screening for this disorder towards prognosis, diagnosis and intervention seems to be an urgent area of research. In this background, the present study attempts to unravel the association of salivary microbial diversity and PCOS.

Material and methods. To achieve the purpose 100 clinically diagnosed PCOS individuals and 110 age matched non-PCOS participants from Bengalee Hindu caste population, West Bengal, India was considered. Obtained salivary samples were identified with 16S rDNA amplification and microbial diversity were determined by Alu I restriction enzyme digestion.

Results. The present study revealed an explicit pattern of DNA fragment lengths varied between 200 bp and 225 bp in PCOs in comparison to the non-PCOS group.

Conclusion. The cardinal feature of the present study as the first attempt from India envisaged, utilization of salivary microbial diversity as an additional potential and economizing biomarker for PCOS that stimulate new horizon of research in 21st century's anthropology – the anthropology of microbes.

Keywords. diagnostic biomarker, PCOS, reproductive health, salivary microbiome, well-being

Introduction

Polycystic Ovarian Syndrome (PCOS) is one of the major reproductive health issues, currently recognized to be a multifactorial, complex endocrine disorder of less known etiology with an intricate pathophysiology.[1] It is a familial, multifaceted condition associated with different clinical manifestations (Fig. 1) such as, ovarian dysfunction, infertility, irregular menstrual cycles, anovulation, hyperandrogenism, hirsutism, acne, obesity, hypertension, diabetes mellitus, and cardiovascular abnormality, dyslipidemia, elevated pro-inflammatory cytokines, metabolic abnormalities and many conditions of the metabolic syndromes (MetS), depending on genetic background which is influenced by environmental factors.[2-10]

Furthermore, women with PCOS have a potential risk of gynecological cancer morbidities such as; endometrial cancer, ovarian cancer and breast cancer.[11,12] Thus, PCOS is considered to be not only a reproductive endocrinopathy, but also a metabolic disorder.[13] Apart from physiological maladies, studies have suggested that women with PCOS often show psychologi-

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cal issues including; symptoms of negative body image perception, low self-esteem, depression, and decreased quality of life.[14]

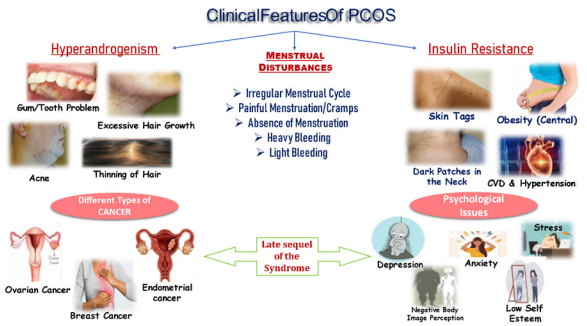

Fig. 1. Clinical manifestations of PCOS

PCOS needs serious attention because it emerges as one of the biggest health issues all over the world.[15] Globally, prevalence this syndrome is highly variable ranging from 2.2% to as high as 26%.[16,17] The prevalence of PCOS was estimated in different populations, primarily in Caucasians, black races, and a significant difference was noted in white and black females, i.e., 8% and 4%, respectively.[18-21] In Greek women, it is 6.8%. among the Chinese (East Asia) prevalence is 5.6%, while most of the studies reported among Indians (South Asia) it is nearly 9.13% to 36%. India, a developing country has witnessed a sudden rise of about 30% in PCOS cases in the last couple of years and that is only the tip of the iceberg.[22] This syndrome needs serious attention not only because it emerges as one of the biggest health issues all over the world but also as an economic burden, billions of dollars are spent annually in the United States to screen the disease.[15,23] In Indian context, it is appropriately pointed in a study that "the health budget of India is unlikely to meet the costs posed to tackle the associated multiple consequences of PCOS", needs more widespread and liberal screening for the disorder in terms of earlier diagnosis and intervention for the amelioration and prevention of early and late sequel of the disease.[23,24] Although the disease displays a wide variety of characteristics, for the diagnosis of PCOS an internationally accepted criteria is used known as, Rotterdam Criteria which requires the presence of two out of three of the following criteria: clinical/biochemical hyper androgenism, oligo-/anovulation, and polycystic ovaries.[25] Although it is a universal diagnostic criterion but it is very important to uncover some other diagnostic biomarkers along with this as this criterion is unable to discriminate the cardinal features of PCOS from various other clinical symptoms and is used when the women already developed this syndrome and suffering from its consequences but to prevent this syndrome investigation of various biomarker is much more needed to make earlier diagnosis.

Microbial population in an individual has a great impact on his overall physiological characteristics. Thus, for many clinical purpose and diagnosis, microbiota of several locations in the body has been studied and a direct correlation between a certain disease/disorder with the type, amount of microbial diversity has been established.[26] The two main components of the microecosystem are oral flora and intestinal flora.[27] Microbiome of the digestive tract has already been studied and proved to be a possible indicator of PCOS.[28] Although recent studies in abroad shows that salivary microbiome profiles are very similar with those present in gut microbiome, therefore can be used as an indicator of bacterial dysbiosis in systemic disease study.[29,30] Female Sex hormonal changes is a very important feature of PCOS and these changes are also likely to influence the salivary levels of putative periodontal pathogens (Fig. 2) causing periodontal disease like gingivitis.[31-34]

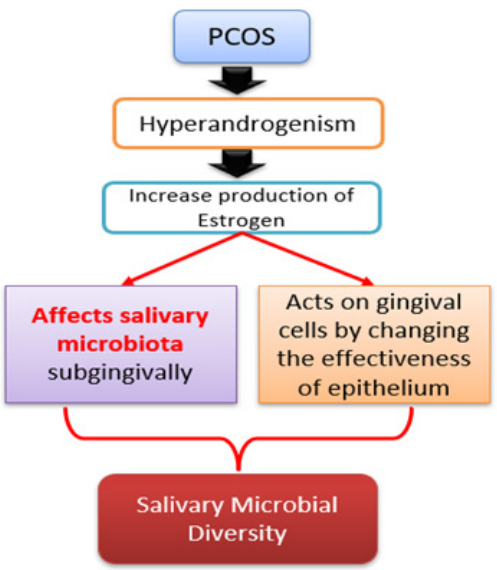

Fig. 2. Salivary microbial diversity; a biomarker of PCOS

Therefore, the levels of most of the studied putative periodontal pathogens can be studied as a biomarker of PCOS. Although there are still limited studies in this regard, but a contemporary study from abroad reported PCOS could quantitatively affect the composition of the salivary microbiota and the relative abundance of salivary Actinobacteria was reduced in PCOS patients compared with healthy controls.[27,30,31] However, no such report has been found from India yet. The anthropology of microbes can expand ethnographic analyses to include investigations of "indigenous" microbial populations (microbiota) and shaping human health and their possible impacts in clinical practice.[35] Thus, apart from the conventional Rotterderm Criteria salivary microbial diversity study can also be taken as a useful diagnostic biomarker for PCOS studies.

Aim

In this backdrop, the present study, to best of the knowledge is the first attempt, to discern the association of salivary microbial diversity between PCOS and non PCOS individuals.

Material and methods

Present study was conducted among one hundred clinically diagnosed PCOS patients, selected from 'Ramkrishna Mission Seva Pratisthan (Shishumangal)', Kolkata, under the assistance of Gynecology department and one hundred and ten (110) non PCOS individuals from the Department of Microbiology, Department of Biochemistry and Department of Anthropology, University of Calcutta, Ballygunge campus. Prior to the study verbal and written consent were obtained from the participants.

The saliva samples were collected from all the 210 persons (PCOS and non PCOS) using commercially available Sterile Cotton Swab Stick. Then the samples were inoculated into L-B broth (2%). Total DNA was extracted from saliva samples using a standardized protocol[30] with slight modification. A PCR reaction was performed to amplify the V3–V4 region of the bacterial 16S rRNA gene using the primers 27 F primer (AGAGTTTGATCMTGGCTCAG) and 1492 R primer (GGTTACCTTGTTACGACTT) with initial denaturation at 95^{0}C for 5 minutes, followed by 35 cycles of denaturation at 95 ^{0}C for 30 seconds, annealing at 50^{0}C for 30 seconds, and extension at 72^{0}C for 1.5 minutes, one cycle of final extension at 72^{0}C for 10 minutes and final cooling step to 4^{0}C. The PCR samples were run on 1% agarose gel to visualize the bands of 16S rRNA (V3–V4) region under UV transilluminator. For genotyping, RFLP were done using a tetra cutter restriction endonuclease - Alu I which cuts at the sequence 5' AG$^{\vee}$CT 3'. Then the amplified 16S DNA were subjected to restriction digestion and gel electrophoresis was performed using 2% agarose gel, visualized under UV Trans illuminator.

Results

The graphical representation using the fragment lengths of PCOS and non PCOS after treating them with Alu I shows that the respective restriction enzyme cut the fragments of length 200-225 bp (Fig. 3) for those with PCOS while, women with no signs of polycystic ovary showed no particular pattern of DNA fragment lengths. Their fragment lengths varied from 200 bp to 225 bp (Fig. 4). Almost 70% showed a similar fragment length (100 bp and 200 bp) indicating significant pattern (Fig. 5) which establishes the fact that there might be a prevalence of a particular group of bacteria in the oral cavity of women having PCOS.

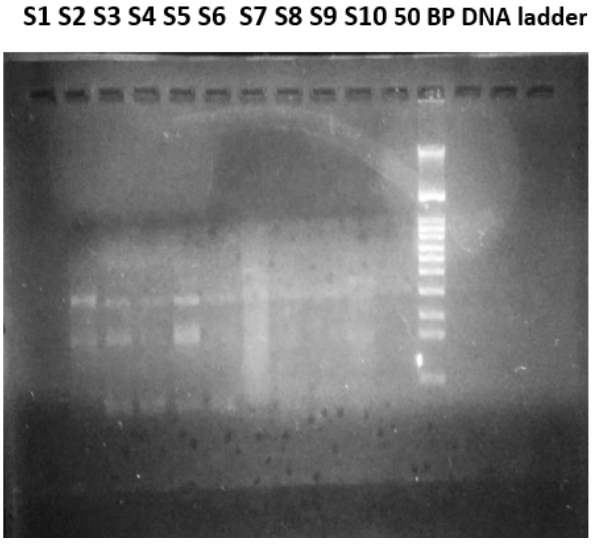

Fig. 3. DNA fragments of PCOS samples after being digested with Alu I

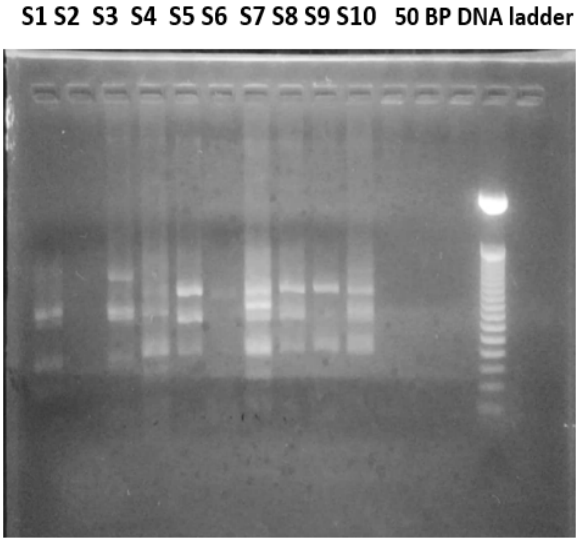

Fig. 4. DNA fragments of NON-PCOS samples after being digested with Alu I

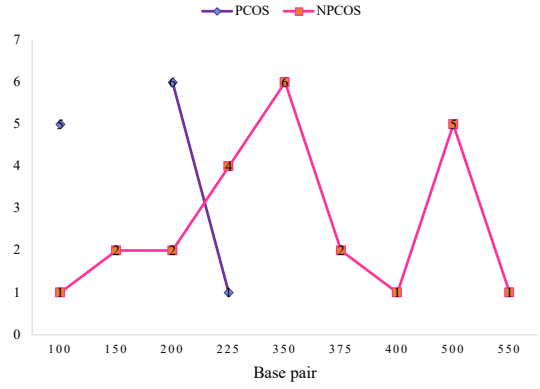

Fig. 5. Relationship between different fragment lengths of PCOS and Non-PCOS after treatment with Alu I

Discussion

It is apparent from the foregoing study, PCOS is an endocrine disorder affecting reproductive-aged women, but the cause remains unclear. However, in a recent systematic review and meta-analysis on current knowledge of the microbes across body sites (oral cavity, blood, vagina/cervix, gut)" in PCOS, it was reported that there is growing evidence links oral and salivary microbial composition to PCOS.[30,31,27,37] Most studies (around 75%) discerned low-risk bias, but incidentally, all the studies were conducted from abroad and eventually, the present study is perhaps the single and first reported evidence from India. Female sex hormone levels have been associated with oral microbiome composition linked to oral pathologies, such as periodontal disease, which indicates the plausible effect of oral microbial organization.[36] However, interestingly, lower alpha oral microbial diversity in PCOS was noticed compared with controls and the present study also in consistency when, Alu I showed that the respective restriction enzyme cut the fragments of length 200-225 bp among the PCOS, in comparison to much diverse as from 100 bp to 550 bp for non-PCOS.[27] Since entire participants of the present study were from a single ethnic population, therefore, minimize the confounder effects of endogenous and exogenous factors, such as oral hygiene, diet, smoking (none), drinking habit (none) was found to have in similarity in the PCOS and without PCOS participants. Most human studies have determined differences in microbial taxa in women with and without PCOS.[38,39] As mentioned earlier the systematic review and meta-analysis reported a significant pooled Standardized Mean Difference (SMD) value (detected for Shannon diversity index), indicating a significantly lower bacterial richness in PCOS individuals compared with the non-PCOS control group and the present study, best of the knowledge, the first report from India found in corroboration of lower oral microbiome diversity in PCOS compared to the non-PCOS group counterpart.[37] Therefore, the salivary microbial diversity study can be taken as a useful diagnostic biomarker of reproductive health studies.

Conclusion

This is the first report of the salivary microbiome diversity in PCOS sample from India. This data may become helpful for clinical studies and diagnosis of PCOS prior to USG. As, analyzing salivary microbiome is more convenient than gut microbiota. This study opens a new door to the medical field to carry forward the research on polycystic ovary syndrome which is largely affecting almost every other woman in this world. However, several other studies should be conducted with other digestive enzymes and large sample size to ensure this result and to further clarify the relationship between salivary microbiota and PCOS.

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Declarations

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Author contributions

Conceptualization, K.G., S.C., D.C. and A.R.B.; Methodology, K.G. and S.C.; Software, K.G. and S.C.; Validation, K.G., S.C. and A.R.B.; Formal Analysis, K.G., S.C. and A.R.B.; Investigation, K.G., S.C. and A.R.B.; Resources, K.G., S.C. and A.R.B.; Data Curation, K.G. and S.C.; Writing – Original Draft Preparation, K.G., S.C. and A.R.B.; Writing – Review & Editing, K.G., S.C., D.C., and A.R.B.; Visualization, K.G. and S.C.; Supervision, A.R.B.; Project Administration, A.R.B. and D.C.; Funding Acquisition, A.R.B..

Conflicts of interest

No conflict of interest.

Data availability

Data available on request from the authors.

Ethics approval

Institutional Ethical Committee for Bio Medical and Health Research involving Human Participants, University of Calcutta & Secretary, UCSTA, C.U., Ref no.: CUIEC/02/15/2022-23.

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ORIGINAL PAPER

An in vitro study for the evaluation of morphological and biochemical characteristics of absorbable sutures coated with genistein and nicotine

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ABSTRACT

Introduction and aim. Inflammation, cell proliferation, matrix deposition, and tissue remodeling are all elements of the well-structured and well-coordinated process of wound repair. The aim of this study was to analyze the effect of genistein and nicotine on polyglycolic acid (PGA) and vicryl sutures.

Material and methods. Genistein and nicotine were isolated and solution was prepared and the suture material PGA and vicryl were immersed in the solution and dried. They were tested for their tensile strength and degradation values after immersion in artificial saliva (on the first day and on the 14th day). The sutures were also seen under a scanning electron microscope (SEM) for its uniform coating and the mixture formulation of genistein and nicotine were tested for their anti-inflammatory and anti-oxidant activity using protein denaturation assay and 2,2-diphenyl-1-picrylhydrazyl assay respectively.

Results. Nicotine has a high anti-inflammatory activity on the suture material, whereas Genistein has an insignificant anti-inflammatory effect. The mixture formulation has a relatively similar anti-inflammatory effect when compared to the control. The SEM analysis shows a uniform coating of the formulation on the PGA and vicryl sutures. In comparison, PGA has shown lesser tensile strength and hence higher degradation ability.

Conclusion. Nicotine and Genistein affect the tensile strength and degradation properties of the sutures.

Keywords. absorbable sutures, antiinflammatory, antioxidant, genistein, nicotine, tensile strength

Introduction

Inflammation, cell proliferation, matrix deposition, and tissue remodeling are all elements of the well-structured and well-coordinated process of wound repair. Until the wound develops enough tensional strength to prevent dehiscence, sutures serve to maintain tissue proximity.[1] The success of the surgical treatment is influenced by the proper closure and stabilization of the surgical wound margins. The behavior of sutures used in oral and maxillofacial surgery depends on the quality of the tissues involved, the presence of saliva, and particular bacteria. They serve as a conduit connecting the tissues' internal and exterior regions, which affects how well wounds heal.[2] It has been noted that general characteristics of

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the patients (namely, gender and age) and of the wounds (that is, length and site) seemed to be the two principal risk factors responsible for local wound complications, rather than suture materials and different surgical techniques. A suitable suture material shouldn't obstruct connective tissue organization or cellular growth.

For the majority of oral surgical procedures, suturing a previously raised flap is used for initial wound closure. A number of suture materials are available for this purpose and can be categorized based on their origin (organic and synthetic) or on how long they will last in the host tissues (absorbable and nonabsorbable).[3] A strong suture that generates stable knots is the perfect suture. It does not stimulate infection and just mildly inflamed the tissue. Although no single suture combines all of these characteristics at once, careful suture selection aids in obtaining better outcomes. In order to avoid scarring and poor wound healing and to achieve good results, proper suturing technique is crucial.[4] The aforementioned elements can be enhanced and regulated to create a setting that is close to optimal, although research is still being done to find the "ideal" suturing material. Naturally, the best suturing material will vary depending on the clinical setting. The so-called ideal suture material has characteristics like appropriate approximation, support, and low immunogenicity, which are significant. Suture material must have the following characteristics in order to function properly: knot safety, stretch capacity, tissue reactivity, and wound safety.[5] Along with the chosen surgical and suturing methods, the choice of suture material may also have an impact on how quickly the soft tissues that were incised, recover. Three case reports of complications following the use of a subepithelial connective tissue graft, where an abscess developed after the first healing phase, were documented in Vastardis and Yukna's study.[6] According to this study's findings, the abscesses may have been brought on by a stitch abscess or a reaction to the suture material utilized for the submerged sutures. Thus, when establishing a treatment strategy for oral surgical operations, the choice of suture material should be taken into account.[6]

In tobacco leaves, nicotine, a natural substance, serves as a botanical insecticide. It is the main tobacco alkaloid, making up roughly 95% of the overall alkaloid content and appearing in commercial cigarette tobacco to the level of about 1.5% by weight.[7] While cigars and chewing tobacco only have roughly half the nicotine concentration of cigarette tobacco, oral snuff and pipe tobacco have nicotine concentrations that are comparable to cigarette tobacco.[8]

The medical research world has recently given genistein, an isoflavone derived from soy, a great deal of attention. It was discovered that this substance was a powerful agent in the prevention and treatment of cancer as well as other chronic diseases.[9] The ATP-using enzymes tyrosine-specific protein kinases, topoisomerase II, and those involved in phosphatidylinositol turnover are all inhibited by genistein at the molecular level. An estrogen receptor-mediated mechanism is one way that genistein can work.[10] Genistein affects cells at a level one step higher, or at the cellular level, where it induces apoptosis and differentiation in cancer cells, suppresses osteoclast and lymphocyte activity, reduces cell proliferation, modifies cell cycle, has antioxidant effects, and inhibits angiogenesis.[11] The key benefits of genistein as a possible medication are because of its numerous actions in living cells and its extremely low toxicity.

A synthetic, multifilament, and absorbable suturing material is called vicryl (polyglactin 910). The substance most frequently used in dentistry is this one. Due to the fact that it prevents plaque from adhering and is convenient for handling, it has been demonstrated to be clinically effective.[12] Additionally, it does not have a strong local reaction. Vicryl rapide helps human wounds heal more quickly by reducing the chances of dehiscence and causing milder local reactions.[13]

Aim

The aim of this study was to:

- prepare a genistein and nicotine extract and coat the sutures with the formulation
- to test the antioxidant and anti-inflammatory activity of the solution
- to analyze the coating of genistein and nicotine on PGA and vicryl sutures
- to test the effect of these components on the tensile strength of the suture samples at day 1.
- to test the tensile strength of the suture samples on the 14th day of immersion in artificial saliva

Material and methods

Preparation of extract

Here in this study the Genistein is taken in the form of soybean (Fig. 1). Soya powder and nicotine was bought from an organic shop. 30 g of soybeans powder was weighed using a weighing machine and added 80 mL of water and 200 mL of ethanol to it by measuring in the 100 mL measuring cylinder in a conical flask. Then it was kept in an orbital shaker (Sisco India, Mumbai, Maharashtra, India) for 24 hrs. 30 g of nicotine was also diluted with 40 mL ethanol in a conical flask and then kept in an orbital shaker for 24 hrs.

Preparation of the suture materials

The extract of genistein and nicotine prepared was taken in two different labeled test tubes. PGA (Surucry|, Gujarat, India) and vicryl sutures (Ethicon, Mumbai, India) were used for the study.

The sutures were divided for analysis into the following groups:

- group I - control PGA - uncoated
- group II - control vicryl - uncoated
- group III - vicryl (4.4 pH) - coated with genistein and nicotine
- group IV - vicryl (7.2 pH) - coated with genistein and nicotine
- group V - PGA (4.4 pH) - coated with genistein and nicotine
- group VI - PGA (7.2 pH) - coated with genistein and nicotine

The sutures (except control group) were immersed in the genistein and nicotine extract for 6 hours (Fig. 2 and 3) and placed in an orbital shaker for a uniform coating of extract over the sutures followed by air drying in a Petri dish for 3-4 hours (Fig. 4).

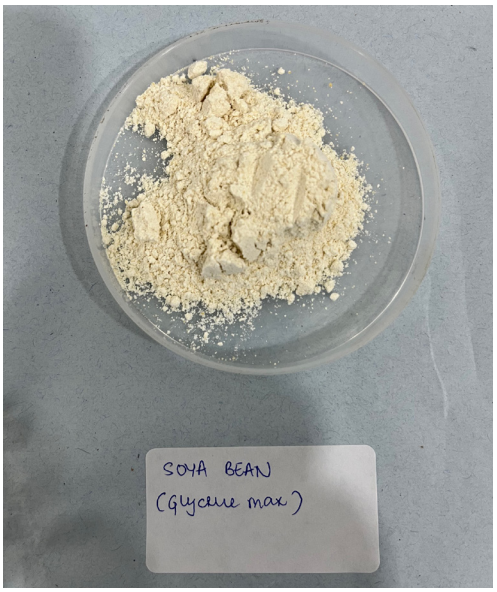

Fig. 1. Showing soybean powder used to obtain genistein extract

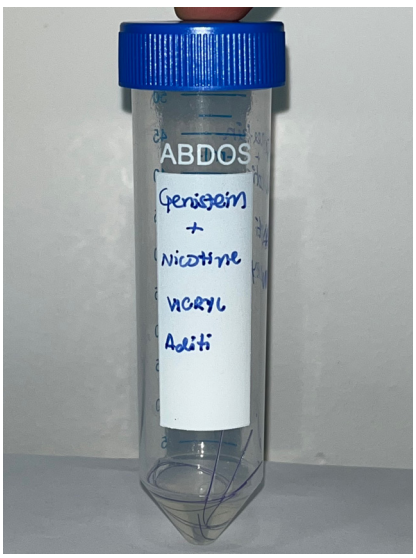

Fig. 2. Showing the Vicryl suture materials immersed in the genistein nicotine mixture

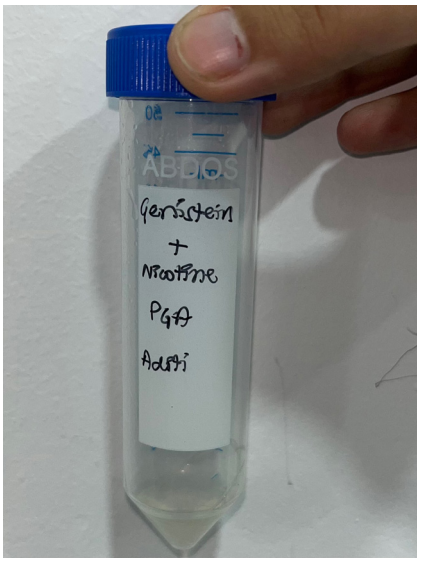

Fig. 3. Showing the PGA suture materials immersed in the genistein nicotine mixture

Fig. 4. Showing the air drying of the suture materials post immersion

Preparation of artificial saliva

Before testing the samples for tensile strength, and degradation study, the suture materials were kept in artificial saliva for 24 hours. The artificial saliva of 1000 mL was prepared by the mixture of 8.035 g of sodium chloride, 0.355 g of sodium bicarbonate. 0.225 g of potassium chloride, 0.231 g of potassium hydrogen phosphate and 0.311 g of magnesium chloride, was added to 40 mL of 1 M hydrochloric acid along with 0.292 g of calcium chloride, 0.072 g of sodium sulfate, 6.118 g of Trizma Base and 1 M hydrochloric acid. The prepared artificial saliva was divided into 2 separate beakers of 500 mL with 7.4 pH and 500 mL with pH 4.4.

Analysis

Anti-inflammatory activity of the formulation was done using protein denaturation assay - the formulation of

genistein and nicotine, genistein extract, nicotine extract were screened for anti-inflammatory activity by using inhibition of protein denaturation assay. The 500 µg of control drug and test compounds (formulation of genistein and nicotine, genistein extract, nicotine extract) were dissolved in a minimum amount of dimethyl formamide (DMF) and diluted with a phosphate buffer (0.2 M, pH 7.4). Final concentration of DMF in all solutions was less than 2.5%. Test solution (1 mL) containing different concentrations of drug was mixed with 1 mL of 1 mM albumin solution in a phosphate buffer and incubated at 27±°C in the BOD incubator for 15 min. Denaturation was induced by keeping the reaction mixture at 60±10°C in a water bath for 10 min. After cooling, the turbidity was measured at 660 nm (UV-Visible Spectrophotometer, Konica Minolta CM 5, Gujarat, India). Percentage of inhibition of denaturation was calculated from control where no drug was added. The diclofenac sodium was used as a standard drug. Percent inhibition was calculated using the following formula:

$$\% \text{ of inhibition} = ((\text{control OD} - \text{sample OD}) \cdot 100)/(\text{control OD})$$

where: control OD – optical density of control; sample OD – optical density of test sample

Antioxidant activity of the formulation was done using DPPH assay – the DPPH (1,1-diphenyl-2-picryl-hydrazil) free radical scavenging activity of formulation of genistein and nicotine, genistein extract and nicotine extract was determined. 50 µg of 4 different extracts (genistein, nicotine, genistein + nicotine formulation and control) were taken in 4 test tubes and mixed with 1 mL of 0.1 mM DPPH in methanol solution and 450 µL of 50 mM Tris-HC1 buffer (pH 7.4) and incubated for 30 minutes. After incubation, the reduction in the number of DPPH free radicals was measured based on the absorbance at 517 nm. BHT was used as control. The percentage of inhibition was calculated from the following equation:

$$\% \text{ of inhibition} = ((\text{absorbance of control} - \text{absorbance of test sample}) \cdot 100) / (\text{absorbance of control})$$

Extract coated suture threads were evaluated for their morphological characterization using Scanning electron microscope (Jeol, JSM-IT800 NANO SEM, USA).

Tensile strength of the coated and uncoated (control) suture samples was assessed using INSTRON Universal Testing Machine E-3000 on day 1 of immersion in artificial saliva. All the data generated by the measurements were represented as mean and standard deviation. SPSS software version 23.0 (IBM, Armonk, NY, USA) for Windows was used for the statistical analysis. Control and coated groups were analysed with a one way ANOVA and p value <0.05 was considered to be statistically significant.

Degradation analysis was done by immersing the suture samples in artificial saliva at two different pH i.e. 7.2 and 4.4 for 14 days. This reflects 4.4 for the pediatric patients whereas 7.2 is for adult patients. Their tensile strength is tested again on day 14 of immersion to assess the degradation property of the sutures.

Results

Figure 5 represents the antioxidant effect of genistein and nicotine and the mixture formulation showed that when compared to the control ascorbic acid (90%), genistein (64%) showed a relatively higher level of antioxidant potential whereas nicotine (31%) showed very less. As a mixture (18%), nicotine will reduce the antioxidant activity of genistein as well. Figure 6 represents the anti-inflammatory effect of genistein and nicotine and the mixture formulation. Nicotine (92%) showed the highest activity when compared to the control group that is diclofenac (90%). Since genistein (63%) had a lower anti-inflammatory activity, it lowered the anti-inflammatory capacity of the genistein-nicotine mixture (81%) as well. Figures 7–10 are the SEM images revealing uniformly coated with the genistein- nicotine mixture on the suture materials. This increased the surface area of the suture, which in turn increased the healing effects of the suture on the tissues.

In Table 1, it can be seen that the Group II suture sample had the highest tensile strength (1451.71 MPa) at the maximum force of 9.3N. In comparison, the Group I suture sample had a lesser tensile strength (993.16 MPa) at a lesser maximum force of 6.38N.

The coated suture samples of vicryl and PGA at both pH values (4.4 and 7.2) have shown a lesser tensile strength when compared to the control group. This means that the coated samples will break more easily post application of force as compared to the control group.

In Table 2, it can be observed that the Group II suture sample had the lowest probability of degradation, since it had the highest tensile strength (1430.79 MPa) on the 14th day of immersion in artificial saliva. In comparison, the Group I suture sample had a lesser tensile strength (959.07 MPa) and hence degraded faster. The coated suture samples showed lesser tensile strength and therefore higher degradation values at 4.4 and 7.2 pH. While comparing the pH, it can be observed that the coated vicryl suture degraded faster at a lower pH (4.4) whereas the coated PGA suture degraded faster at a higher pH (7.2).

Pair wise comparison of PGA and vicryl groups through one way ANOVA analysis was done (Table 3). PGA sutures showed p value as 0.0363 which is statisti-

cally significant. ANOVA analysis of the Vicryl sutures showed p value as 0.0278 which is also statistically significant.

Antioxidant activity

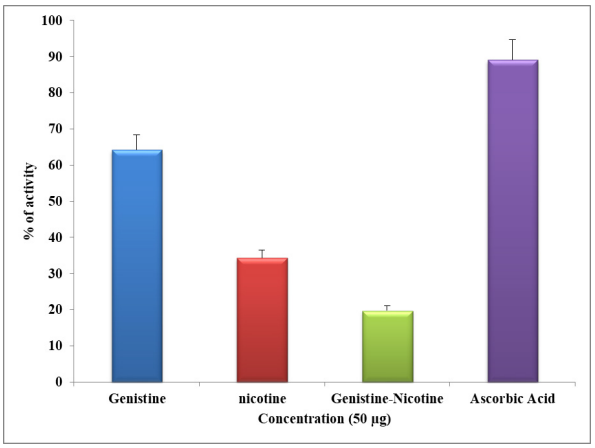

Fig. 5. Graphical representation of the antioxidant activity of the suture samples

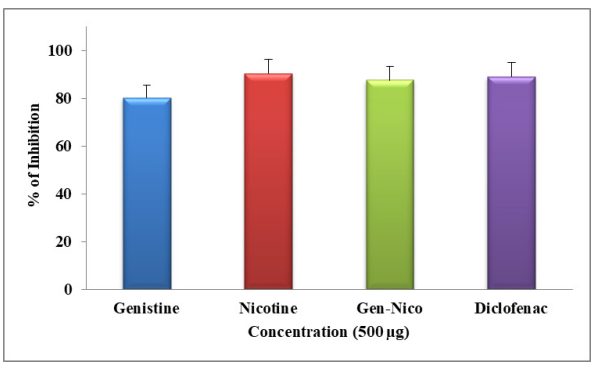

Fig. 6. Graphical representation of the anti-inflammatory activity of the suture samples

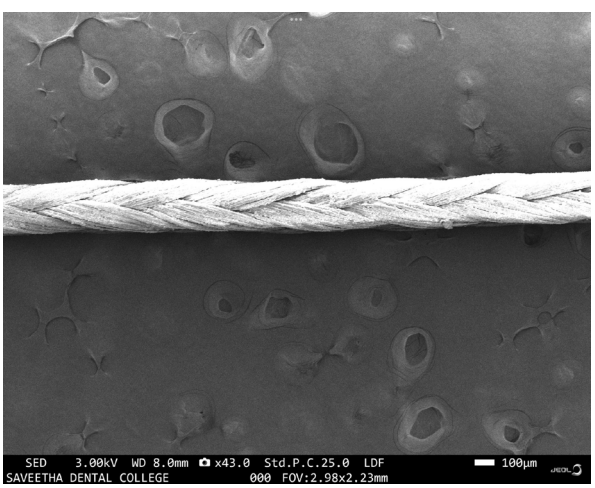

Fig. 7. Shows the SEM analysis of PGA at 37x magnification. It shows a uniform layer of the genistein and nicotine mixture on the PGA suture

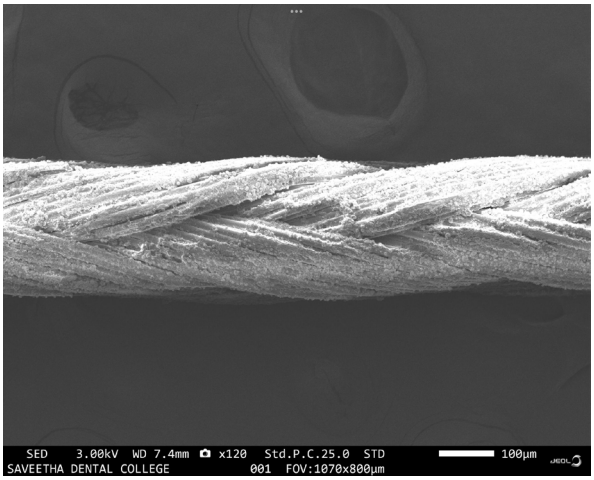

Fig. 8. Shows the SEM analysis of PGA at 120x magnification. It shows a homogenous and even coating of the genistein and nicotine formulation

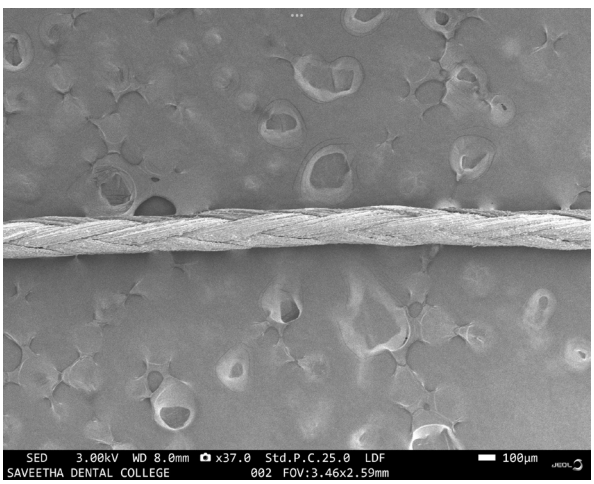

Fig. 9. Shows the SEM analysis of vicryl at 37x magnification. It shows a uniform layer of the genistein and nicotine mixture on the vicryl suture

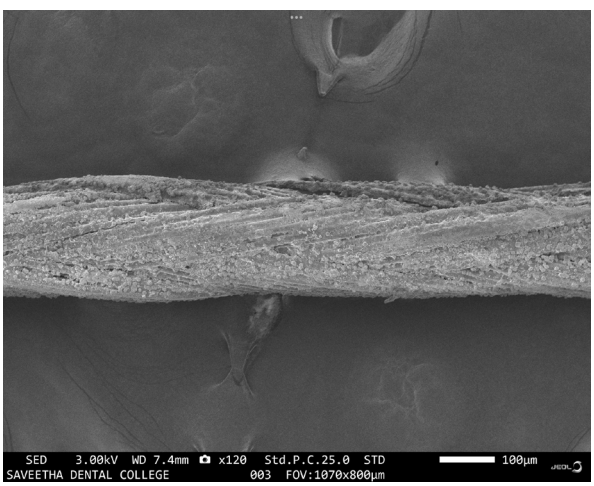

Fig. 10. Shows the SEM analysis of vicryl at 120x magnification. It shows a homogenous and even coating of the genistein and nicotine formulation

Table 1. Tabular representation of tensile strength of the coated suture samples at different pH values (4.4 and 7.2) compared to the control uncoated values. The values compared are maximum force (N), tensile stress at tensile strength (MPa) and tensile strain (displacement) at break (standard)

Sample group	Specimen label	Maximum force (N)	Tensile stress at tensile strength (MPa)	Tensile strain (displacement) at break (standard) (%)
Group I	PGA (uncoated) (control)	6.38±0.51	993.16±1.21	11.78±0.93
Group II	Vicryl (uncoated) (control)	9.30±0.83	1451.71±2.51	22.94±1.22
Group III	Vicryl (coated) (pH 4.4)	7.88±0.61	1231.48±1.73	12.68±0.87
Group IV	Vicryl (coated) (pH 7.2)	6.87±0.24	95.68±1.82	16.00±0.94
Group V	PGA (coated) (pH 4.4)	4.09±0.48	639.73±1.49	7.39±0.38
Group VI	PGA (coated) (pH 7.2)	5.26±0.75	817.92±2.31	7.60±0.42

Table 2. Tabular representation of degradation results (on the 14th day of immersion in artificial saliva) of the coated suture samples at different pH values (4.4 and 7.2) compared to the control uncoated groups

Sample groups	Specimen label	Tensile stress at break (standard) (MPa) (degradation at 14th day)
Group I	PGA (uncoated) (control)	959.07±2.45
Group II	Vicryl (uncoated) (control)	1430.79±3.27
Group III	Vicryl (coated) (pH 4.4)	1190.87±2.91
Group IV	Vicryl (coated) (pH 7.2)	1067.11±2.34
Group V	PGA (coated) (pH 4.4)	635.21±1.85
Group VI	PGA (coated) (pH 7.2)	818.98±2.27

Discussion

In the present study, it can be observed that nicotine has a high anti-inflammatory activity on the suture material, whereas genistein has an insignificant anti-inflammatory effect. The genistein-nicotine mixture formulation has a relatively similar anti-inflammatory effect when compared to the control. In accordance with the antioxidant effect, genistein has shown to have high results whereas nicotine shows the opposite. The mixture formulation shows a relatively low antioxidant effect when compared to the control. The SEM analysis shows a uniform coating of the formulation on the PGA and vicryl sutures. Tensile strength testing concludes that the vicryl control group has the tensile strength and hence the lowest degradation capacity. The coated vicryl suture samples at different pH have also shown similar results. In comparison, PGA has shown lesser tensile strength and hence higher degradation ability.

Table 3. Tabular representation of pairwise comparison and statistical significance of PGA (group I, V, VI) and vicryl (group II, III, IV) groups through one way ANOVA analysis*

Sample group	Specimen label	Tensile stress at tensile strength (MPa)
Group I	PGA (uncoated) (control)	991.0±4.839
Group II	Vicryl (uncoated) (control)	1454±17.72
Group III	Vicryl (coated) (pH 4.4)	1235±10.69
Group IV	Vicryl (coated) (pH 7.2)	93.23±4.895
Group V	PGA (coated) (pH 4.4)	639.4±7.925
Group VI	PGA (coated) (pH 7.2)	820.0±8.934

Pair wise comparison of PGA and vicryl groups through One Way ANOVA analysis

Sample groups	Specimen label	p [a]
Group I, V, VI	PGA groups	0.0363
Group II, III, IV	vicryl groups	0.0278

* [a] – p value<0.05, therefore statistically significant

For dental and medical surgical procedures, a variety of suture materials are available. However, it is crucial for surgeons to understand the suture material's properties, the biologic processes of healing, and how the suture material interacts with the surrounding tissues.[14] The need for a suture that will hold its strength until the tissues of the previously raised surgical flaps regain enough strength to bind the incision edges together makes this a crucial challenge.[15] Research information on the efficacies of various materials is still disputed and conflicting as of this writing. The goal of the current study was to analyze the suture responses to various food materials.

For many years, silk has been the suture material of choice for dentistry and other surgical treatments. The authors contend that silk should not be regarded as a "material of choice" for oral surgical treatments, despite the fact that it is less expensive and easier to handle than other nonabsorbable suture materials.[16] Studies on the effects of sutures on oral tissue have demonstrated significant inflammatory responses, which are more pronounced with silk and cotton and less pronounced with other materials such nylon, polyester, ePTFE, polyglecaprone 25, PGA, and vicryl.[17]

In a study conducted by Varma et al., silk and polyamide sutures were coated with hyaluronic acid and compared to the control (chlorhexidine) and tested for their tensile strength. They used the Tinius Olsen Universal Testing Machine for testing the tensile strength. As a result, polyamide showed better stability than silk

and hyaluronic acid did not alter the tensile strength of either suture material pre and post immersion.[18] This is contradictory to our study, where this is a significant difference in the tensile strength of the suture material after coating. In another study the physical and functional properties of polyglactin 910 suture with and without triclosan.[19] The authors concluded that the addition of triclosan did not affect the handling properties or performance characteristics of either suture, and the breaking strength was same for both sutures, ranging from 79% on day 14 to 5% on day 35. In our study, the tensile strength decreased considerably from day 1 of immersion to day 14 of immersion, when the breaking point of the sutures were tested. A study done by Wu et al. concluded that a surface coating of polylactic acid and polycaprolactone on commercially degradable polydioxanone suture maintained 20% of its original tensile strength after 12 weeks of implantation. In addition, in vivo results of PCL-coated sutures also showed less inflammatory cell infiltration and less surface inflammation.[20] In the present study, we have similar results in which the tensile strength is reduced after immersion in the Genistein and nicotine solution. In another study, two biodegradable polymers with different degradation rates, such as polycaprolactone (PCL) and PGA, were applied to carry the drug of tea polyphenol (TP). The drug-loading finishing solution which is made of PCL/PGA carriers and TP, was coated on the PLA suture. With the increasing of PCL in drug-carriers, the strength of suture gradually increases. At 70/30 of PCL/PGA, the fracture elongation of suture reaches the highest point.[21] This is contradictory to the present study as we have found a gradual decrease in the tensile strength of PGA and vicryl post immersion.

The limitations of this study include small sample size and short span of testing for tensile strength post immersion in the extract and artificial saliva. This study can be further continued by analyzing the effect of genistein and nicotine on absorbable sutures in vivo, via animal studies.

Conclusion

From our study we can conclude that the secondary metabolites nicotine and genistein, present in our daily lives, have a significant effect on absorbable sutures such as PGA and vicryl. Genistein has shown to have a good antioxidant activity and hence will help in the healing of the surrounding tissues. Nicotine has a significant anti-inflammatory activity but since it is a carcinogenic substance, it has to be used in a limited amount. Nicotine and genistein also affect the tensile strength and degradation of the sutures. This concludes that sutures can be coated with genistein and nicotine in the future for their antioxidant and anti-inflammatory effect. Future studies need to be conducted to test the coated sutures for their antibiotic properties and their performance in vivo.

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Declarations

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Author contributions

Conceptualization, An.C. and R.E.; Methodology, R.E.; Software, Ad.C.; Validation, R.S.J., Ad.C. and An.C.; Formal Analysis, Ad.C.; Investigation, An.C. and R.E.; Resources, Ad.C.; Data Curation, R.S.J.; Writing – Original Draft Preparation, Ad.C.; Writing – Review & Editing, An.C. and R.S.J.; Visualization, An.C. and R.E.; Supervision, R.S.J.; Project Administration, R.E.; Funding Acquisition, Ad.C.

Conflict of interest

The authors declare that there are no conflicts of interest in the present study.

Data availability

All the data in this study is completely incorporated in the manuscript.

Ethical approval

Not applicable.

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ORIGINAL PAPER

Serum levels of IFN-γ and IL-4 in hospitalised COVID-19 patients – evidence of reduced cytokine storm in discharged patients

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ABSTRACT

Introduction and aim. Coronavirus disease 2019 is characterised by cytokine storm and it was managed with repurposed drugs, however the effect of this treatment on cytokine storm is unknown. The aim of the study was to investigate the effect of repurposed management on serum Th1 pro-inflammation cytokine (IFN-γ) and Th2 anti-inflammation cytokine (IL-4) in COVID-19 patients.

Material and methods. The levels of IFN-γ and IL-4 were determined in sera from 45 COVID-19 patients at admission followed-up till discharge after repurposed treatment using ELISA. The mean levels and proportions above normal reference ranges of IFN-γ and IL-4 were compared in COVID-19 at admission and discharge.

Results. The mean values of IFN-γ and IL-4 were significantly higher in COVID-19 patients at admission compared with discharged COVID-19 patients whereas IFN-γ:IL-4 ratio was significantly higher in discharged COVID-19 patients compared with admitted COVID-19 patients. Significantly higher proportion of COVID-19 patients at discharge had IFN-γ within the normal reference ranges compared with COVID-19 patients at admission whereas the proportions of COVID-19 patients at discharge and COVID-19 patients at admission having IL-4 within the normal reference ranges were the same.

Conclusion. Cytokine storm was evidenced in COVID-19 patients at admission and repurposed treatment suppressed pro-inflammation cytokine (IFN-γ) in most discharged COVID-19 patients.

Keywords. cytokine storm, re-purposed treatment, SARS-CoV-2

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been a global disaster since December 2019.[1] As at July 08, 2023, there was 266,675 COVID-19 cases, 3155 death by COVID-19 and 259,953 patients recovered.[2] The mortality rates caused by COVID-19 in different regions of the world varied with demographic variables, presence or absence of comorbidities, immune status, exposure to malaria parasites or use of anti-malaria drugs, blood group and lifestyle.[1,3-7] COVID-19 severity have been associated with hyperinflammation caused by elevated levels of proinflammatory cytokines such as interleukin (IL)-6, nitric oxide (NO), C-reactive protein (CRP), deranged blood cell counts and lymphopenia.[6-11]

SARS-CoV-2 exhibits clinical characteristics ranging from asymptomatic to severe acute respiratory dis-

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tress syndrome, septic shock, and/or multiple organ dysfunctions, leading to death.[12,13] Specific treatment for COVID-19 is not known but many therapeutic strategies involved the use of anti-inflammatory-, antiprotozoan-, antioxidant- agents, antibiotics, moderate exercises and protein rich diets.[13-16] This study predicted that repurposed treatment adopted in our IDC would reduce the cytokine storm and improve the clinical indices of patients with COVID-19. However, because of limited resources and unavailability of data from hospitalised patients at admission and discharge in Nigeria, it was difficult to ascertain the effectivenss of prescribed repurposed treatment (a process of identifying new therapeutic use(s) for old/existing/available drugs) in our setting. Also, because of our concern about the disproportionate immunopathologic burden of COVID-19, we aimed to elucidate the Th1-Th2 cytokine parameters in admitted and discharged patients with COVID-19, which can help distinguish immune signature or explain immune regulation leading to determining better COVID-19 treatment. In addition, a study to ascertain proportions of COVID-19 patients in Nigeria having cytokine levels above normal ranges is yet to be performed. In line with that, this study analysed proportion of individuals with COVID-19 having cytokine levels above normal ranges.

Immunological biomarkers have been suggested as primary drivers of morbidity and mortality with COVID-19. Several cytokines have been correlated with COVID-19 severity. Most notably, elevated IL-4 and IL-6 while reduced IFN-γ were detected in hospitalized patients, especially critically ill patients.[17] Increased IL-2R, IL-8, IL-10, and GM-CSF have been associated with disease severity but studies are limited.[18] Conflicting results regarding IL-1β and IL-4 were reported.[19] Elevated cytokine concentrations of IL-6, IL-10, IL-18 and IFN-γ have been widely described in COVID-19 patients but these did not seem to have prognostic values.[17] IL-4 was associated with impaired lung lesions, but some reports point to a potential mediator effect.[17-19]

Cytokines that enhances inflammatory responses(pro-inflammatory cytokines) include tumor growth factor-beta (TGF-β), gamma interferon (IFN- γ), IL-1, IL-8, IL-6, TNF-α, while some other cytokines function to reduce the inflammation (anti-inflammatory cytokines) like IL-4 and IL-10.[7] Therefore, it becomes important to analyse the cytokine load (IFN-γ and IL-4) in the sera of patients with COVID-19 in comparison to reference normal ranges and treatment success or effectiveness.

Aim

In this longitudinal study, the serum levels of IFN-γ and IL-4 were measured through ELISA in sera from patients with COVID-19 at admission and discharge compared with normal reference ranges in order to decipher the cellular mechanism behind the treatment of coronavirus. To our knowledge, this is the first report regarding the effectiveness of repurposed treatment using cytokine release in COVID-19 patients.

Material and methods

Ethical approval was granted by the Institutional Ethical Committee with approval number UI/EC/20/0283. The participants comprised of 45 laboratory confirmed COVID-19 patients at admission followed up till discharge. The study was conducted between 6th May, 2020 and 19th July, 2020. None of the patients was in severe or critical condition and were able to give consent.The duration between admission and discharge of COVID-19 patients was 3–10 days. The participants did not have hypertension, diabetes mellitus, cardiovascular disease, cerebrovascular disease, cancer, chronic renal disease or inflammatory conditions. Serum was removed from clotted whole blood collected in a test tube without anticoagulant by centrifuging at 1500×g for 10 minutes. Interferon-gamma and IL-4 levels in the serum were determined using ELISA as previously described using manufacturer's instruction.[11] The values of IFN-γ and IL-4 obtained were compared with the reference value of 0.1–2.8 pg/L for serum IL-4 and reference value of 0.16-7.42 pg/L for serum IFN-γ to determine the proportion of patients having IFN-γ or IL-4 values above reference ranges.[20] Data from this comparison were represented as frequencies and percentages. Chi-square test was used to determine the differences between the frequencies while the differences in the mean of variables of COVID-19 patients at admission and discharge were compared using Student t-test. $p \leq 0.05$ was considered as statistically significant. A treatment protocol designed by the Case Management Team of the Oyo State COVID-19 Task Force was adopted.[13] The protocol included treating with a cocktail of chloroquine or hydroxychloroquine, zinc, vitamins C and D and or antibiotic(s) as indicated. Physiotherapy and nutritional support for these patients were also considered as priority.

Results

Vitamin C, vitamin D and Zn were given to COVID-19 patients from day of admission for 3 weeks while azithromycin and chloroquine or hydroxylchloroquine were given for 3 days (Table 1). The mean values of IFN-γ and IL-4 were significantly higher in COVID-19 patients at admission compared with COVID-19 patients at discharge whereas the mean value of IFN-γ:IL-4 ratio was significantly higher in COVID-19 patients at discharge compared with COVID-19 patients at admission (Table 2). Significantly higher proportion of COVID-19 patients at discharge had IFN-γ within the normal ref-

erence ranges compared with COVID-19 patients at admission whereas the proportions of COVID-19 patients at discharge and COVID-19 patients at admission having IL-4 within the normal reference ranges were the same (Table 3).

Table 1. Medication used among COVID-19 patients at IDC, Ibadan, Nigeria

Medications	COVID-19 Patients
Vitamin D (1,000 IU)	1,000 iu twice daily for 3 weeks
Vitamin C (1,000 mg)	1,000 mg twice daily for 3 weeks
Zn (20 mg)	100 mg daily for 3 weeks
Azithromycin	500 mg daily for 3 days
Hydroxychloroquine	400 mg on day 1 and 200 mg daily for 3 more days
Chloroquine (as an alternative to hydroxychloroquine)	500 mg on day 1 and 250 mg daily for 3 more days

Table 2. Mean (±SD) values of IFN-γ and IL-4 in COVID-19 patients at admission compared with COVID-19 patients at discharge

Variable	COVID-19 patients at admission (n=45)	COVID-19 patients at discharge (n=45)	p
IFN-γ (pg/L)	0.162±0.96	0.149±0.099	<0.05
IL-4 (pg/L)	0.3±0.1	0.1±0.1	<0.05
IFN-γ/IL-4 ratio	0.51±0.03	1.23±0.98	<0.05

Table 3. Frequency (percentage) of COVID-19 patients at admission compared with COVID-19 patients at discharge having of IFN-γ and IL-4 within and outside reference ranges*

Variable	Category	COVID-19 patients at admission (n, %)	COVID-19 patients at discharge (n, %)	p
IFN-γ	Within RR	31 (70.45)	33 (75)	<0.05
	Outside RR	13 (29.55)	11 (25)	
IL-4	Within RR	43 (100)	43 (100)	>0.999
	Outside RR	0 (0)	0 (0)	

* RR – reference range

Discussion

COVID-19 patients develop clinical signs ranging from mild to life-threating symptoms as a result of SARS-CoV-2 invasion of epithelial cells of the mucosal surfaces through attachment to angiotensin-converting enzyme 2 (ACE2) receptor.[18] In the cytosol of lung cell, double stranded RNA replication stimulates hyperinflammation and the presence of ACE 2 receptors on several organs in humans apart from lungs, causes multi-organ damages by SARS-CoV-2.[12] However, the present study proposed that excessive stimulation of cytokine production might be responsible for organ damage in COVID-19 patients, which was curtailed by repurposed drugs. Understanding the precise drivers of immune dysfunction is crucial to guide the application of appropriate immunomodulatory treatments since management strategies of COVID-19 are not specific. Previous management strategy of COVID-19 patients involved the use of immunosuppressive therapies, anti-inflammatory and immunomodulatory agents.[13-16] Due to variations in the management strategies of COVID-19, the mechanism of individual repurposed treatment might vary. The present study suggests that reduction of cytokine storm might be one of the actions mitigated by repurposed management strategies applied in our IDC.

There have been several studies comparing cytokine levels in COVID-19 patients and control but a longitudinal study (comparing admitted versus discharged patients) or comparing data with normal reference ranges will give better clinical relevant information.[17-20] Moreover, inconsistent results were obtained for cytokine levels in COVID-19 cases versus unifected control studies. No study till date, have reported prevalence of COVID-19 patients having biological markers within and outside normal reference ranges at admission or during treatment. IFN-γ is an antimicrobial protein upregulated in the COVID-19 patients.[17,18] Our result of raised mean IFN-γ level in newly admitted COVID-19 patients is supported in a study by Zhou et al., which reported a significantly increased peripheral blood monocytic cells frequency of polyclonal GM-CSF+CD4 T cells capable of prodigious ex-vivo IFN-γ production only in critically ill COVID-19 patients.[21] IFN-γ-mediated Th1 plays a critical role in antigen-specific defense mechanisms at the epithelial surface associated with multiple inflammatory airway diseases.[17] Other reports stated that IFN-γ acts as a regulator of efficient antigen presentation, and lowering the IFN-γ level or deviation in the IFN-γ-IFN-γR system severely impedes host immune responses to infections.[22] A study also showed that IFN-γ level was associated with respiratory distress and mortality in patients with COVID-19.[23] Other studies showed that IFN-γ induces PANoptosis and a deadly cytokine storm, causing acute lung damage and mortality among patients.[24] It is likely that the natural defence phenomenon of the host was to stimulate concurrent rise in IL-4 level to conteract this negative effect of raised IFN-γ level during SARS-CoV-2 infection.

Limited data existed for IL-4 level in COVID-19 patients, though IL-4 was found to be associated with impaired lung lesions and its potential mediator effect was emphasised in COVID-19 patients.[25] A data reported that IL-4 level was significantly increased, whereas the level of IFN-γ was significantly reduced in patients with severe COVID-19 compared with those in patients with mild and/or moderate COVID-19.[17] Increase in IL-4 level, but no reduction in the IFN-γ level has also been reported by other study.[26] High levels of IFN-γ and IL-4 observed in newly admitted COVID-19 patients

compared with discharged COVID-19 implied cytokine storm in COVID-19 patients and this was reported contributing to tissue damage in the respiratory tract and in other organs.[7,12,18,19] Also, reduced IFN-γ and IL-4 in discharged COVID-19 compared with COVID-19 at admission could also be linked with the fact that adaptive immunity (both humoral- and cellular mediated responses but more especially cellular mediated response) is needed for recovery of COVID-19 patients. In addition, our data suggest that the systemic inflammation in COVID-19 patients at admission was due to ongoing infection and viral replication as confirmed by absence of SARS-CoV-2 by PCR analysis in all discharged patients after repurposed therapy. The implication of our finding is that repurposed treatment suppressed systemic inflammation.

The cytokine environment determines the subsets of predominant T helper cells by influencing their differentiation. IFN-γ is critical for determining Th1 development, whereas IL-4 is necessary for the development of Th2 proliferation, thus Th1-Th2 balance determines the disease outcomes through regulation of cellular immune responses and inflammation.[27] It has also been reported that higher IFN-γ:IL-4 ratio (higher Th1/Th2) mediate diminished serum antibodies and increased production of cell-mediated immunity.[27] Among intriguing finding of our study is that discharged COVID-19 patients have a higher IFN-γ:IL-4 ratio than in newly admitted COVID-19 patients. This implied an enhanced cell-mediated immunity in discharged COVID-19 patients. This supported previous findings of increased lymphocyte numbers in recovered patients but decreased number in non-surviving patients.[18-20] Our present finding of higher IFN-γ:IL-4 ratio in discharged COVID-19 patients compared with newly admitted COVID-19 patients also supported the established fact that cell-mediated immunity is needed for the control of viral infection and that lethal outcomes in virus infection correlated with elevated Th2 cell serum cytokines including IL-4.[22-25,28]

Vitamin intake lowers oxidative stress markers, alleviates cytokine storm, reduces disease severity, lowers-proinflammatory cytokine, -hyperinflammation and -organ failure.[29] Vitamin C as a basic exogenous vitamin is known for its strong antioxidant and anti-inflammatory properties because it scavengers free radical, activates and stimulates biosynthesis of antioxidants. Other involvement of vitamin C against inflammation encompasses modulation of nuclear transcription factor kappa B (NF-kB) and lowering of pro-inflammatory cytokines production.[30] An observation corroborated the anti-inflammatory and immune supporting properties of vitamin D which usually result in dampening of hyperinflammation.[31] Zinc supplementation decreases -oxidative stress biomarkers, -inflammatory cytokines, -generation of IL-1β and its mRNA but upregulates A20 (a zinc transcription factor).[32] Taken together, these previous reports thus explain our present observation of suppressed pro-inflammation cytokine (IFN-γ) in most discharged COVID-19 patients after treatment with repurposed drugs containing antioxidant micronutrients (vitamin D, vitamin C and Zn).

Conclusion

Taken together, higher IL-4 and IFN-γ levels in admitted COVID-19 patients compared with discharged COVID-19 patients observed in our study might indicate reduced cytokine storm in discharged patients. However, to prove it broadly, larger panel of cytokine in a larger population of COVID-19 patients is needed to be examined.

Declarations

Funding

The project was self-funded.

Author contributions

Conceptualization, G.O.A., A.A.O., K.A. and M.B.O.; Methodology, G.O.A., A.A.O., K.A. and M.B.O.; Formal Analysis, G.O.A., A.A.O., K.A. and M.B.O.; Data Curation, G.O.A. and A.A.O.; Writing – Original Draft Preparation, G.O.A., A.A.O., K.A. and M.B.O.; Writing – Review & Editing, G.O.A., A.A.O., K.A. and M.B.O.; Project Administration, G.O.A. and A.A.O.

Conflict of interests

The authors declare no competing interests.

Data availability

For ethical reasons, the datasets generated during and/or analysed during the study are confidential. However, they would be available by the corresponding author on genuine request.

Ethics approval

Ethical approval was granted by the Institutional Ethical Committee with approval number UI/EC/20/0283.

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ORIGINAL PAPER

Satisfaction levels of patients attending the outpatient department of a tertiary care center of India

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ABSTRACT

Introduction and aim. Any hospital's outpatient department (OPD) is regarded as the storefront of the facility, and patient satisfaction is an important measure of healthcare quality. Hence, this cross-sectional study was conducted in different OPDs at a tertiary care center.

Material and methods. Two hundred patients were recruited, and structured personal interviews were conducted with questions based on the Patient Satisfaction Questionnaire Short Form (PSQ-18). T-tests and analysis of variance (ANOVA) were used to compare satisfaction scores between variables.

Results. Upon analysis, 86% of the patients rated their overall experience as either 'very good' or 'good'. Interpersonal manners (mean score±SD; 4.60±0.55), communication (4.39±0.66), general satisfaction (4.03±0.79), and technical qualities (3.86±0.57) were the domains in which the patients were most satisfied, while time spent with the doctor (3.77±0.89), accessibility and convenience (3.77±0.67), and financial aspects (3.37±0.83) were the areas that lagged.

Conclusion. Satisfaction scores were found to vary significantly with gender, age, waiting times, and the number of visits per day. Regular patient satisfaction surveys should be conducted in all hospitals for devising interventions to provide patients with the best possible care.

Keywords. health care quality, patient care, patient satisfaction, PSQ-18, quality improvement research

Introduction

Any hospital's outpatient department (OPD) is regarded as the facility's storefront. Our institute is one of the oldest medical colleges in India, catering to the medical needs of three states as well as the neighboring country of Nepal. The OPDs of the institute are jam-packed with an average footfall of 2500 patients per day, a number that is increasing day by day.

Patient outcomes are dependent on the interplay of many different factors, including not just the medical or surgical management but also their mental states and perceptions. Alas, the latter aspect is often overlooked, especially in developing nations where resources are limited, and a relatively large population needs care.[1]

Patient satisfaction or dissatisfaction is a complicated phenomenon that is linked to patients' expectations, health status, personal characteristics, and health system.[2] Longer contact periods, appropriate privacy, confidentiality, and professional etiquette have all been linked to higher patient satisfaction rates, which ultimately enable a trustworthy, frank, and open connection with the doctor, improving patient care.[3–5] Studies from various Indian institutes report quite different levels of satisfaction, thus necessitating individual assessments.[6–8] Measuring patients' satisfaction has many purposes, in-

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cluding helping to evaluate healthcare services from the patient's point of view, facilitating the identification of problem areas, and helping in the generation of ideas towards resolving those problems.[9]

Aim

Hence, this study was carried out to evaluate the level of patients' satisfaction in terms of general satisfaction, time spent with the doctor, interpersonal manner, communication, technical quality, financial aspects, and accessibility and convenience among those attending various OPDs of our institute, to identify the problems of the patients and suggest measures for enhancement of service quality.

Material and methods

Study duration and setting

This was a cross-sectional observational study conducted over a period of three months (March to May 2022) at a tertiary-care hospital. Approval was taken from the institute's ethics committee of Patna Medical College (IEC/PMC/624/2022). The study was conducted in accordance with the Declaration of Helsinki after taking consent from all the participants.

Sample size and sampling technique

Considering a confidence level of 95%, a margin of error of 10%, and assuming maximum variability (i.e., taking the population proportion as 50%), the minimum sample size was calculated as 97 with the help of an online calculator. A total of 200 patients were recruited through convenient sampling.

Study population

Patients attending various OPDs (medicine, surgery, obstetrics & gynecology, orthopedics, ophthalmology, and otorhinolaryngology) who were willing to participate in the study were included in the study after taking verbal consent. Inclusion criteria: adult patients of either sex, aged 18 years or more. Exclusion criteria: patients not giving consent.

Study tool

A standard, pre-validated questionnaire, the Patient Satisfaction Questionnaire Short Form (PSQ-18), was administered to the subjects as structured personal interviews along with demographic and basic details (including sex, age, department visited, waiting time before consultation, and number of visits per day). The patients were also asked to rate their overall experience on a four-point Likert scale from 'very good' to 'bad'. The PSQ-18 is an 18-item tool with good internal consistency and reliability and is divided into seven subscales: general satisfaction, technical quality, interpersonal manner, communication, financial aspects, time spent with the doctor, and accessibility and convenience.[10]

The PSQ-18 gives scores separately for each of the seven subscales: general satisfaction (items 3 and 17); technical quality (items 2, 4, 6, and 14); interpersonal manner (items 10 and 11); communication (items 1 and 13); financial aspects (items 5 and 7); time spent with the doctor (items 12 and 15); accessibility and convenience (items 8, 9, 16, and 18).[11] All items were rated by the subjects on a five-point Likert scale ranging from 'strongly agree' to 'strongly disagree'. Certain PSQ-18 items are formulated to indicate agreement when expressing satisfaction with medical care, while others are structured to indicate agreement when expressing dissatisfaction with medical care, but the scores were assigned for each item from one to five in such a manner that higher scores meant better satisfaction, as recommended. The score for each individual subscale was then calculated by averaging all the item scores in that subscale.[11]

Care was taken to conduct the interviews in a structured and objective manner as much as possible, so as to minimize the risk of confirmation bias, interviewer bias, bias due to leading questions, and the question order effect.

Statistical analysis

The data was cleaned and coded in Microsoft Excel and then analyzed using IBM SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Various parameters of descriptive statistics, such as proportion, mean, and standard deviation (SD), were calculated. After checking for normality, t-tests were used to compare satisfaction scores between variables with two groups (like gender and number of visits per day), and analysis of variance (ANOVA) tests were used when comparing scores between variables with three or more groups (like age group, waiting time, and overall experience). A p-value of less than 0.05 was considered significant.

Results

A total of 200 patients participated in the study. 142 (71%) of them were male, and 46 (23%) were older than 60 years. 52 patients (26%) came to the medicine OPD and 38 patients (19%) came to the surgery OPD. 148 (74%) patients reported that they had to wait less than an hour before their consultations. When asked to rate their overall experience, 116 (58%) patients rated it as 'good' while only 6 (3%) rated their experience 'bad'. 110 (55%) patients visited the OPD twice in one day, and the rest visited only once a day (Table 1).

The (mean±SD) satisfaction scores for different subscales (arranged from highest to lowest scores) were: interpersonal manner (4.60±0.55), communication (4.39±0.66), general satisfaction (4.03±0.79), technical quality (3.86±0.57), time spent with the doctor (3.77±0.89), accessibility and convenience (3.77±0.67), and financial aspects (3.37±0.83) (Fig. 1).

Table 1. Demographic characteristics of the study subjects (n=200)

Variable	Categories	n (%)
Gender	Male	142 (71%)
	Female	58 (29%)
Age (in years)	18-30	40 (20%)
	31-40	28 (14%)
	41-50	46 (23%)
	51-60	40 (20%)
	>60	46 (23%)
Department	Medicine	52 (26%)
	Surgery	38 (19%)
	Gynecology & Obstetrics	34 (17%)
	Orthopedics	28 (14%)
	Ophthalmology	25 (12.5%)
	Otorhinolaryngology	23 (11.5%)
Waiting time	<1 hour	148 (74%)
	1-2 hours	32 (16%)
	>2 hours	20 (10%)
Number of visits per day	Once a day	90 (45%)
	Twice in one day	110 (55%)
Overall experience	Very Good	56 (28%)
	Good	116 (58%)
	Fair	22 (11%)
	Bad	6 (3%)

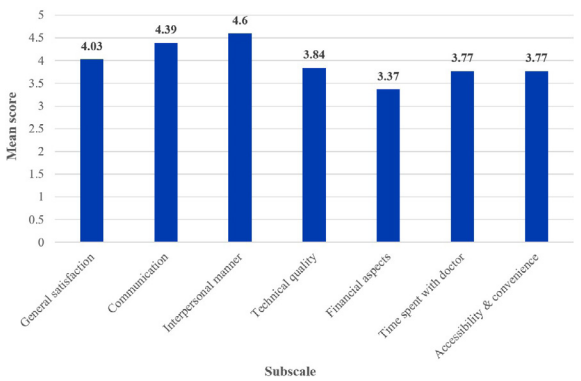

Fig. 1. Mean satisfaction scores for different subscales (n=200)

When the subscale scores were compared between the two genders, it was observed that the scores (mean±SD) for only one subscale, i.e., general satisfaction, were significantly higher in females (4.22±0.65) than males (3.96±0.83) with a p of 0.02.

When comparison was done considering the different age groups, it was seen that the mean (±SD) subscale scores for interpersonal manner were significantly different among various age groups, ranging from 4.78 (±0.41) in the 31–40 years group to 4.40 (±0.70) in the 18–30 years age group (p=0.04). The subscale scores for time spent with doctor also differed significantly from 4.11 (±0.75) among 18- to 30-year-olds to 3.58 (±0.93) among 51- to 60-year-olds (p=0.02) (Table 2).

Table 2. Distribution of patient satisfaction scores by gender and age group (n=200)

Subscale	Sex (Mean score±SD)			Age groups (in years) (Mean score±SD)					
	Male (n=142)	Female (n=58)	p	18-30 (n=40)	31-40 (n=28)	41-50 (n=46)	51-60 (n=40)	>60 (n=46)	p
General satisfaction	3.96 ±0.83	4.22 ±0.65	0.02	3.89 ±0.77	4.19 ±0.51	4.13 ±0.71	3.98 ±0.85	4.03 ±0.96	0.5
Communication	4.36 ±0.68	4.48 ±0.58	0.23	4.40 ±0.64	4.41 ±0.52	4.44 ±0.68	4.23 ±0.78	4.48 ±0.64	0.46
Interpersonal manner	4.56 ±0.57	4.68 ±0.48	0.16	4.40 ±0.7	4.78 ±0.41	4.69 ±0.41	4.59 ±0.58	4.58 ±0.56	0.04
Technical quality	3.82 ±0.54	3.89 ±0.63	0.7	3.79 ±0.54	3.76 ±0.47	3.89 ±0.56	3.79 ±0.64	3.92 ±0.59	0.64
Financial aspects	3.41 ±0.74	3.27 ±0.01	0.32	3.40 ±0.69	3.50 ±0.78	3.42 ±0.87	3.38 ±0.88	3.24 ±0.91	0.73
Time spent with doctor	3.79 ±0.91	3.71 ±0.85	0.53	4.11 ±0.75	3.97 ±0.72	3.71 ±0.86	3.58 ±0.93	3.59 ±1.02	0.02
Accessibility and convenience	3.78 ±0.67	3.74 ±0.65	0.76	3.82 ±0.65	3.71 ±0.41	3.83 ±0.77	3.71 ±0.69	3.79 ±0.71	0.88

When the scores were compared between the waiting times, it was observed that the mean (±SD) subscale scores for interpersonal manner varied significantly with different waiting times, ranging from 4.65 (±0.45) in patients who had to wait less than an hour before their consultations to 4.42 (±0.76) in patients who had to wait more than 2 hours (p=0.04). However, it was observed that there was a statistically significant difference for financial aspects as well, with the scores ranging from 3.50 (±1.01) in patients with wait times of more than 2 hours to 3.43 (±0.78) in patients with wait times less than an hour (p=0.02).

On comparison of scores among the number of visits per day, the subscale score for time spent with the doctor was found to be significantly higher among patients who visited the OPD only once in a day (mean±SD; 3.96±0.87) compared to patients who visited twice in a day (3.61±0.88) with a p=0.01 (Table 3).

Table 3. Distribution of patient satisfaction scores by waiting time and number of visits per day (n=200)

Subscale	Waiting Time (Mean score±SD)				Number of visits per day (Mean score±SD)		
	<1 hour (n=151)	1-2 hours (n=31)	>2 hours (n=18)	p	Once a day (n=90)	Twice in one day (n=110)	p
General satisfaction	4.03±0.78	3.86±0.84	4.27±0.71	0.18	3.92±0.78	4.12±0.78	0.08
Communication	4.41±0.64	4.39±0.64	4.27±0.8	0.69	4.35±0.72	4.42±0.6	0.48
Interpersonal manner	4.65±0.45	4.43±0.75	4.42±0.76	0.04	4.53±0.58	4.65±0.52	0.15
Technical quality	3.86±0.68	3.85±0.75	3.85±0.52	0.1	3.82±0.77	3.88±0.59	0.51
Financial aspects	3.43±0.78	3.01±0.87	3.50±1.01	0.02	3.33±0.69	3.40±0.93	0.54
Time spent with doctor	3.77±0.89	3.65±0.96	4.00±0.77	0.39	3.96±0.87	3.61±0.88	0.01
Accessibility and convenience	3.77±0.66	3.73±0.64	3.82±0.77	0.89	3.75±0.63	3.79±0.70	0.64

It was observed that the mean (±SD) subscale scores for general satisfaction were significantly different among the categories of the overall experience ratings, ranging from 4.24 (±0.81) in patients who rated their overall experience as 'very good' to 3.12 (±0.94) in those who categorized their experience as 'bad' (p<0.01). Similarly, the scores for communication varied significantly from 4.44 (±0.56) in patients whose overall opinion was 'good' to 3.25 (±1.50) in patients with a 'bad' opinion about the provided care (p=0.01) (Table 4).

Table 4. Distribution of patient satisfaction scores by overall experience rating (n=200)

Subscale	Overall experience (Mean±SD)				
	Very good (n=56)	good (n=116)	Fair (n=22)	Bad (n=6)	p
General satisfaction	4.24±0.81	4.05±0.71	3.60±0.86	3.12±0.94	0.01
Communication	4.36±0.75	4.44±0.56	4.41±0.59	3.25±1.50	0.01
Interpersonal manner	4.54±0.7	4.62±0.44	4.65±0.61	4.12±0.75	0.34
Technical quality	3.87±0.63	3.90±0.70	3.64±0.62	3.56±0.65	0.45
Financial aspects	3.34±0.98	3.40±0.79	3.28±0.59	3.25±1.04	0.88
Time spent with doctor	3.73±0.98	3.89±0.82	3.30±0.77	3.50±1.47	0.06
Accessibility and convenience	3.86±0.76	3.77±0.56	3.60±0.82	3.18±0.94	0.15

Discussion

The level of satisfaction with the care received by two hundred OPD patients at a tertiary care center was measured, and it was observed that 86% of the patients rated their overall experience as either 'very good' or 'good'. In terms of the PSQ-18 scores, we found that out of a maximum score of 5, the mean subscale scores for interpersonal manners, communication, and general satisfaction were quite high (4.60, 4.39, and 4.03, respectively), followed by technical qualities with a mean score of 3.84. The areas that lagged were time spent with the doctor, accessibility and convenience, and financial aspects (3.77, 3.77, and 3.37, respectively). In line with findings of many previous studies, this study corroborated that interpersonal manners and communication were consistently rated highly by the patients, while the financial aspect was often the most unsatisfactory domain for many patients.[12-16]

Ours is a tertiary care specialty center where most patients come via referrals from faraway places after having exhausted all other local options, with many requiring advanced (and thus generally expensive) investigations or procedures. It caters to a large population with an ever-growing daily patient footfall that far exceeds the rate of increase in the number of healthcare providers available to treat them. Furthermore, our hospital (a government-funded public hospital where the cost of treatment is much lower than in private settings) attracts many patients who belong to the economically weakest sections of society and who often find it extremely difficult to pay their medical bills. In their study conducted at a similar tertiary care center in the neighboring state of Odisha, Kshatri et al. also found that 80% of the patients thought that the amount paid by them was unreasonable.[15] These may be the reasons behind the relatively lower scores in the areas of time spent with the doctor, accessibility and convenience, and financial aspects observed in our study.

It was observed that the general satisfaction scores were significantly higher among women than men. We also observed that, even though all age groups rated the interpersonal manner subscale quite highly, the 31- to 40-year-olds seemed to be the most satisfied with interpersonal manner. It was also interesting to see that the younger age groups were considerably more satisfied with the time they spent with the doctor as compared to the older patients. It may be possible that the younger people (who often have limited time themselves due to studies, work, and other social engagements) may value the limited amount of time they spent with the doctor more than the older patients (who generally have relatively more time and often have multiple co-morbidities that require lengthier discussions).

For patients who had to wait less than an hour before their consultations, the scores of interpersonal manners were higher compared to those who had to wait more. But surprisingly, patients were more satisfied with the financial aspects of their care when they had to wait for more than two hours before their consultations. It could point to the possibility that doctors, when informed about a patient's long wait time, may handle that patient more efficiently, leading to an overall more satisfying consultation and ultimately giving the sense of money well spent. This finding is similar to that of Chandra et al., who reported that patients considered satisfying consultations worth the wait.[17]

The patients who visited the OPD only once a day appreciated the time spent with the doctor significantly more compared to the patients who visited twice in one day (i.e., once for the initial consultation and the subsequent visit for showing the investigation reports). The reason behind this may be that when doctors call the patients for a subsequent visit along with the investigation reports without having in-depth conversations first, it may be perceived as dismissive.

Based on the findings of this study, we recommend that the following measures be taken to increase patient satisfaction levels: efforts should be made to reduce the patient load at referral-level facilities so that the healthcare providers can devote more of their time and attention to each patient; such improvements should be made that aim to reduce the wait times, like the implementation of an appointment-based system for OPDs in which the patients are only required to arrive for their consultations just before their assigned times; the overall quality of care should be improved; all healthcare workers should undergo regular training courses on attitude, be-

havior, and communication skills required during patient care; reducing the financial burden on patients; and spreading awareness about the various health schemes launched by the government.

There are certain limitations to the study. Since this was a single-center study conducted at a tertiary care hospital in a limited number of OPDs, the findings of the study are difficult to generalize. The convenient sampling technique could have led to selection bias. OPD samples were collected only in the morning hours, which could have influenced the selection of the patients as well as the care provided.

Conclusion

Four out of five patients described their overall experience as 'very good' or 'good'. Interpersonal manners, communication, general satisfaction, and technical qualities were the domains in which the patients were most satisfied, while time spent with the doctor, accessibility and convenience, and financial aspects were the areas that lagged. Satisfaction scores were found to vary significantly with gender, age, waiting times, and the number of visits per day.

There is always scope for further improvement, and proper steps should be taken to increase patient satisfaction levels, especially by focusing on the domains that lag. Such patient satisfaction surveys should be conducted periodically in all hospitals for continuous monitoring and identification of issues, which will help in the formulation of policies and interventions with the goal of providing patients with the best possible care.

Declarations

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Author contributions

Conceptualization, V.K. and K.K.; Methodology, V.K.; Software, V.K. and R.B.; Validation, V.K., R.B., V.S.O. and K.K.; Formal Analysis, V.K.; Investigation, V.K., R.B., V.S.O. and K.K.; Resources, K.K.; Data Curation, V.K. and K.K.; Writing – Original Draft Preparation, V.K., R.B. and V.S.O.; Writing – Review & Editing, V.K. and K.K.; Visualization, R.B.; Supervision, K.K.; Project Administration, V.K.

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Approval was taken from the institute's ethics committee of Patna Medical College (IEC/PMC/624/2022). The study was conducted in accordance with the Declaration of Helsinki after taking consent from all the participants.

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ORIGINAL PAPER

The use of the "Talk To Me" application in the therapy of speech development delays

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ABSTRACT

Introduction and aim. Computer techniques are increasingly used in speech therapy. The aim of the work is to present the results of the preliminary evaluation of the new "Talk To Me" application and its functionality in the treatment of speech development delays.

Material and methods. The study was conducted in 3 groups of children: the study group with the use of the "Talk To Me" application, the conventional therapy group and the control group - in the case of both groups with the intervention, additional reinforcements were used. All children included in the project showed delays in speech development. The recorded age of children in all analyzed groups ranged from 2 to 6 years. In order to verify language progress, the Scale of Language Skills Acquisition in the Field of Communication Competence was used.

Results. Acquisition of language skills varies depending on the group affiliation. The analysis of simple main effects for the time of measurement showed that in each group the differences between successive measurements turned out to be significant. In the study group, the increase in language skills differs from the other two groups ($p< 0.001$). However, there is no difference between the groups with conventional and control therapy ($p=1.00$).

Conclusion. Analysis of the research results allows us to conclude that the "Talk To Me" application is a tool that significantly affects the speed of therapy progress in the case of speech development delays concerning communicative competence.

Keywords. "Talk To Me" application, speech development delay, therapy

Introduction

Speech is an essential function enabling linguistic expression through which conceptualization, reasoning and understanding are possible.[1] One of the most frequent developmental problems that pre-school children face is delayed language acquisition.[2] Issues related to acquiring communicative competence at this age may result from a number of underlying causes, both environmental and biological. Environmental impact on acquiring communicative competence may commonly be connected with unintentional mistakes made by the caregivers, such as, fulfilling child's non-verbal requests or giving verbal explanation to what is expressed non-verbally by the child. Biological factors may be associated with autism, hearing impairment, intellectual disability or can be an effect of a combination of developmental delays, neurological dis-

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orders and genetic diseases. Unless there is a significant clinical factor involved in acquiring language the aforementioned problems are often attributed to developmental language disorder (DLD).[2] Any difficulties in the matter may significantly hinder child's social competence leading to frustration, relationships withdrawal and a variety of limitations in social functioning or even isolation. Additionally, it may have a negative impact on other areas such as academic achievements, mental health or quality of life.[3] The incidence of this developmental abnormality, depending on the source, may range from 5% to 16% for the age group 0-7 years. For half of these children, the difficulties are persistent.[4-7] For example, on a European scale, it is estimated that the problem of DLD affects about 5.8 million children.[8] Furthermore, language emergence in human growth plays crucial role in diagnosing delays in children with various impairments.[9]

In terms of language problems, prophylaxis is critical, because early diagnosis and intervention in many cases will prevent the occurrence of derivative problems, e.g., low level of school achievement or behavioral adjustment difficulties.[10]

Important factors accelerating the process of acquiring new skills by children, including language, are positive atmosphere and an appropriate level of motivation. Accordingly, in speech development delay therapy, one of the key factors leading to an optimal therapeutic result is the introduction of solutions that make exercising more attractive. Therefore, speech therapy solutions increasingly use modern technologies in the form of specially created games or programs. use modern technologies in the form of specially created games or programs.

Aim

Therefore, the purpose of this paper is to present the results of the preliminary evaluation of the new "Talk To Me" application and its functionality in the treatment of speech development delays.

Material and methods

The study was approved by the Bioethics Committee of the College of Medical Sciences of the University of Rzeszow No. 4/11/2020. It was conducted in 3 groups: the study group with the use of the "Talk To Me" application, the conventional therapy group and the control group - in the case of both groups with the intervention, additional reinforcements were used. All children included in the project showed delays in speech development. The recorded age of children in all analyzed groups ranged from 2 to 6 years. The size of each group was 15 participants. Approximately 64% of the children were boys and 36% were girls.

Therapy was conducted by a specialist in the presence of a parent. The whole therapeutic idea has been based on provoking the child to verbally realize speech sounds, syllables, in a strictly communicative situation. Therefore, before starting the therapy, an analysis of language capabilities was carried out – it was checked what syllables the child implements in spontaneous situations, which constituted the foundation for further proceedings. The application was complementary to therapeutic activities provoking the child to produce syllables – so that it would not be challenging for the child in order to avoid any kind of traumatization. The entire therapeutic process is to be regarded by the child as fun and pleasant, but above all it is to convince the child that by using speech (in this case syllables) and no other non-verbal/verbal behavior (moaning, grunting), the desired goal – the continuation of watching favorite cartoon etc. – can be achieved.

In the study, during therapy the child answered the therapist's questions, such as: „Do you want more?", „Do you want to continue watching the cartoon?" What was expected was a positive, verbal response, at this stage with any syllable (ta/da/na being most desirable). On creating the conditions facilitating verbal reaction to the specialist's commands, the child began sessions, during which the application was used. Similarly, the application automatically stopped displaying whatever the child was watching at the time eliciting functional syllables. Ultimately, the goal was to shorten the verbal reaction time to a minimum and to automate the ability to use speech for communication.

The duration of the study was 12 weeks. The examined functions were measured three times: 1 - before the start of therapy, 2 - after 6 weeks and 3 - after 12 weeks. The therapeutic tool in the main study group was the newly created application for speech therapy („Talk To Me"), an easy-to-use program aimed primarily at being used in therapy of speech delays and at accelerating the acquisition of communication and language competences. The therapy consists of controlling the application through voice commands in order to start and continue watching a selected film from various websites. At the research stage, it was a command in the form of functional syllable or syllables. The application aims to provoke the child to verbalize and convincing them at the same time that verbalization has a significant causative meaning (Fig. 1-3).

Therapy in "the conventional therapy group" consisted of conventional treatment based mainly on repetition and naming. Children imitated animal sounds, and attempted to name the presented pictures with nouns, answering the question: where is it? what is it? Material was adapted to the age and abilities of children. The control group consisted of children who, for various reasons, were not subject to therapy in the study period, despite the diagnosed delay in speech development.

In both cases, therapy sessions lasted 30 minutes once a week.

In order to verify language progress, the Scale of Language Skills Acquisition in the Field of Communica-

tion Competence (LSAFCC) was used. It is a quick and simple tool for verifying language skills used for communication, and assessing a given competence on a scale of 0–1 or 0–2. Whereas, depending on the competence, 0 means a correct presentation of a given language competence, 1 – a correct result or an intermediate grade between a correct and incorrect result, 2 – a incorrect result for the selected competences. Assessment issues concern the way of communicating needs, helping the child with verbalization or stimulating verbalization, the way of nodding/denying (verbally, non-verbally), spontaneously uttering syllables and repeating them.

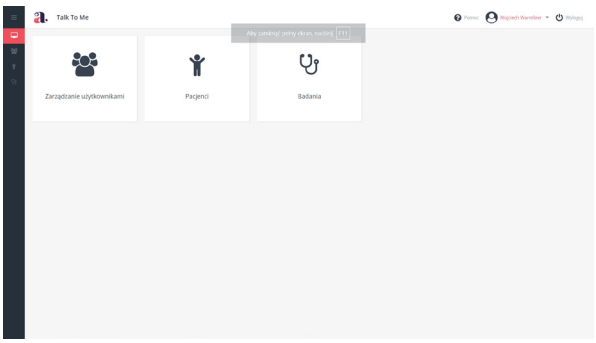

Fig. 1. The main menu of the application presenting buttons corresponding to individual functionalities: user management, patients, examinations

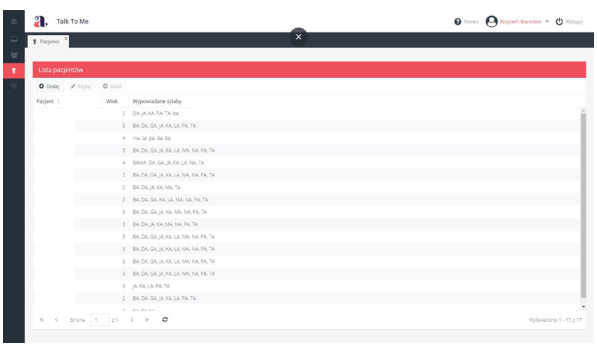

Fig. 2. Submenu of the application that allows to select the syllables to practice

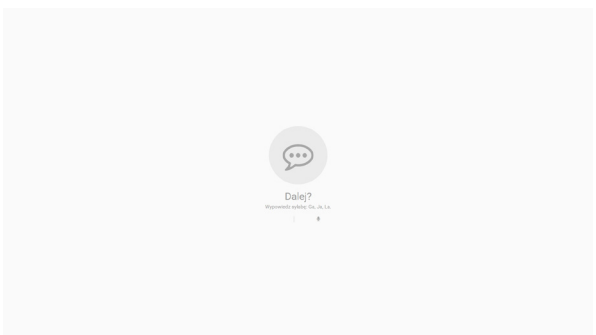

Fig. 3. The application window for the moment when syllables should be spoken in order to continue displaying the footage

The statistical analysis of the collected data was performed with the SPSS Statistics 23.0 (IBM, Armonk, NY, USA). In order to test the hypothesis regarding the impact of the type of therapy applied on the acquisition of language skills, a two-factor analysis of variance was performed in a mixed 3x3 scheme, where the intra-subject variable is the time of measurement (in weeks 1, 6 and 12), and the between-subject factor – group affiliation (group with the application conventional therapy group or control group).

Due to the fact that the assumption about the sphericity of the variables was not met, the Greenhouse-Geisser epsilon correction was used to calculate the significance level of the F test. A statistical significance level of $p<0.05$ was assumed. The "Talk To Me" application was created as part of a project implemented by BD Center sp. z o.o. entitled: "R&D works leading to the development of an innovative method of early diagnosis and speech therapy of children using a mobile application stimulating speech development", No. RPPK.01.02.00-18-0033/19-00, co-financed by the European Regional Development Fund under Priority Axis 1 "Competitive and innovative economy" of the Regional Operational Program of the Podkarpackie Voivodeship 2014-2020.

Results

The results of the analysis indicate a statistically significant main effect of the time of measurement/therapy ($F(2.84)=293.10$, $p<0.001$; $\eta^2 = 0.87$), the main effect of group membership ($F(2.42)=56, 24$, $p<0.001$; $\eta^2=0.72$) and the interaction effect ($F(4.84)=134.79$, $p<0.001$; $\eta^2=0.63$).

Post-hoc analysis for the main effect of the measurement time showed that, regardless of group affiliation, the Language Skills Scale score decreases over time (Fig. 4) – significant differences occur both between week 1 and 6, as well as between week 6 and 12 ($p<0.001$).

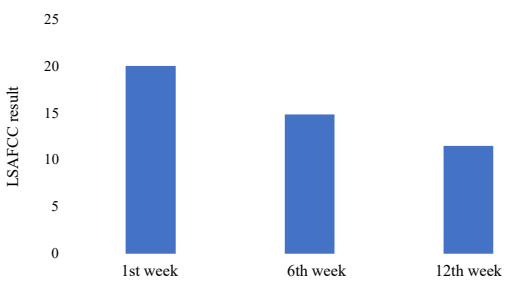

Fig. 4. The average level of language skills depending on the measurement time (weeks) in 1st, 6th and 12th

In the case of the main effect of belonging to a group, the post-hoc analysis showed that the average score of the Language Skills Scale in the group with the application is significantly lower than in the case of both other

groups (p<0.001) (Fig. 5). However, there is no significant difference between the conventional therapy group and the control group (p=1.00).

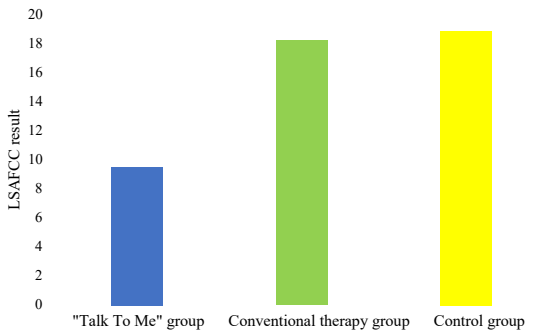

Fig. 5. Average level of language skills depending on group affiliation: blue – „Talk To Me group", green – conventional therapy group, yellow – control group

The statistically significant interaction effect means that the acquisition of language skills varies according to group affiliation. The analysis of simple main effects for the time of measurement showed that in each of the groups the differences between successive measurements turned out to be significant - the score on the Language Skills Acquisition Scale at week 6 is significantly lower than the score at week 1, and the score after 12 weeks is significantly lower than at week 6 in both the application and conventional therapy groups and the control group. This means that over time, even without therapy, language skills improve. The results of these analyzes are presented in Table 1 and Fig. 6. In the application group, however, the increase in language skills differs from the other two groups (p<0.001). However, there is no difference between the conventional and control treatment groups (p=1.00).

Table 1. Differences in the acquisition of language skills at 1, 6 and 12 weeks in the group with the application, in the conventional therapy and in the control group[a]

					F	η^2	
Group	n	1 week *M (SD)*	6 weeks *M (SD)*	12 weeks *M (SD)*			
Study group	15	17.47 (4.50)	7.60 (3.83)	3.47 (4.55)	194.33***	0.93	T1>T2>T3
Conventional therapy group	15	21.80 (1.47)	17.93 (2.76)	14.93 (2.66)	48.25***	0.77	T1>T2>T3
Control group	15	21.13 (1.88)	19.27 (1.71)	16.27 (2.46)	98.05***	0.87	T1>T2>T3

[a] F based on the Greenhouse-Geisser test; *p<0.05; **p<0.01; ***p<0.001

Discussion

It is natural to acquire passive language skills in the process of development through auditory stimulation, observation of articulation and repetition. The acquisition of the ability to transmit a language is inevitably connected with its purposeful use, the intention of using it in a communicative situation. Communication difficulties can be the cause of frustration, subsequent difficult behaviors, social isolation or changes in social relationships. Undoubtedly, this affects the reduction of the quality of life and the lack of optimal achievements in many spheres of functioning.[8] Usually, abnormalities in this area are detected early and thus early, most often in the age range of 2-3 years, children are referred for speech therapy. At the same time, the results of research in this area indicate that this problem affects boys more often (approximately 70% of cases) than girls.[5,11] A similar percentage was represented by boys in our study - 64%.

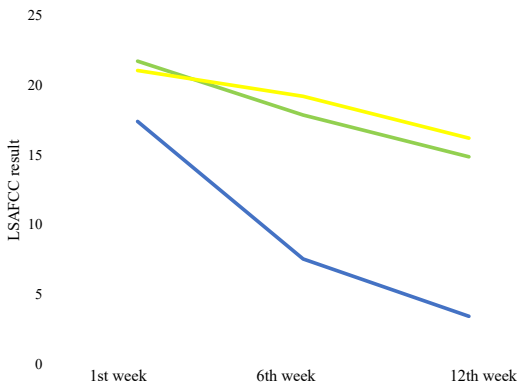

Fig. 6. Acquisition of language skills depending on belonging to a group in 1st, 6th and 12th: blue – „Talk To Me group", green – conventional therapy group, yellow – control group

The results of the research indicate that generally a positive measurable effect of speech therapy is achieved, which of course varies depending on many factors related to the disorder, attributable to the patient or external factors.[12] Therapeutic treatment of children with speech development delay is very diverse, because there are no universal guidelines for its unification. There are also no obvious reasons for undertaking a given therapeutic path, hence the decision depends on the therapist and the availability of services in this area.[12]

Therapeutic treatment can be carried out at home, in an educational unit, or in a specialist center. Differences can be observed in the intensity of therapy, its duration, availability of methods, forms and therapeutic agents, individual needs of the child, as well as regulations in the field of functioning of speech and language therapy services. The form of direct proceedings may also vary. It can be individual or group, depending on the age and needs of the child, as well as the facilities available. On the other hand, indirect interventions are based on the reorganization of the functioning environment in such a way that it facilitates and encourages communication development. Such a

naturalistic approach is progressively being used to maximally support/stimulate the child to communicate with its environment, while boost positive child/adult and child/parent (caregiver) interactions. The indirect approach is increasingly used. The therapist plays a crucial role in training those who work and care for children, providing them with programs and advice on creating an environment that stimulates the child's speech development.[12]

In speech therapy, new technological solutions are increasingly used, e.g., in the form of games aimed at children with speech and communication problems. For instance, what is often used to motivate the child to appropriate articulation are schemes introducing point systems or simply a possibility to advance to the next game level.[13] In the case of the analyzed application, the motivation factor is the willingness to continue the video material that children select independently according to their own preferences from a publicly available website (e.g., YouTube). The assumption of the therapy is to activate the film/cartoon by the child saying a pre-programmed functional syllable or syllables.

It is emphasized that therapy based on fun and modern electronic interactive solutions is imperative for the regularity of the therapeutic process and better prognosis. It is also a meaningful factor in involving parents in therapy and raising their awareness of its importance.[14-17]

Preliminary research on the effectiveness of the application showed differences in the process of acquiring language skills depending on group affiliation. The result was obtained both in the time interval of 1-6 weeks and after 12 weeks of the intervention – the relationship concerned all analyzed groups. In the study group, the increase in language skills was significantly different from the results obtained for the conventional therapy group and the control group, thus confirming the potency of the new tool in the therapeutic process ($p<0.001$). Similar results in terms of the effectiveness of speech therapy were obtained by Matej et al. conducting research on tablet game-supported speech therapy in environments with children. What's more, the authors emphasize the fact of increasing motivation and time spent on exercises thanks to the use of modern technological solutions.[18] Another team of researchers verified the effectiveness of SS4Kids – online music-based speech and language learning game as part of early intervention. This tool was also shown to be effective in supporting targeted word formation over the two-week intervention period. Likewise, researchers verified the effectiveness of the tool depending on the circumstances of its use. The results showed that the group conditions did not improve the effectiveness, speaking in favor of therapy in a clinical or home setting.[19]

Moreover, Saeedi et al. reviewing the literature in this field, based on 69 publications, confirmed the fact that the use of games in speech therapy was an important motivating factor and they can successfully be a component of the therapeutic process. However, the authors emphasize the fact that the initial process of creating a tool is very important, which should take into account the purposefulness of its operation as well as obstacles and challenges.[20]

To sum up, the conducted research showed the high effectiveness of the newly developed speech therapy tool in relation to the conventional method. A significantly higher effect was recorded in the form of activating the communicative competence of children from the study group. Increasingly, the literature on the subject emphasizes the importance of using modern technologies, including gamification, applications and online services as important components of speech therapy in children, which bring a positive therapeutic effect. Therefore, also in the case of the pronated therapeutic tool as well as other available solutions, due to their proven effectiveness, continuation of research is seen in order to optimize their operation, which is a factor that significantly encourages children to engage in therapy.[21]

It is true that the "Talk To Me" application still requires further work to improve its operation, making the graphic design more attractive and expanding its functionality. Nonetheless, as a therapeutic tool it is in line with the modern trend (the use of modern technological solutions, therapy in any environment in which the child functions, the involvement of people working/caring for the child in the therapeutic process and the possibility of constant observation of therapeutic progress) and already at the test level it has given promising therapeutic results.

Conclusion

The analysis of the research results allows us to conclude that the "Talk To Me" application is a tool significantly affecting the speed of therapy progress in the case of speech development delays. Further specific studies are needed to test the effectiveness and added functionalities of the application in subgroups of patients suffering from disorders presenting itself, among others, with speech development delay.

Declarations

Funding

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Author contributions

Conceptualization, W.A.W.; Methodology, W.A.W., L.P. and J.P-B.; Software, A. G-M. and J.P-B.; Validation, L.P. and J.P-B.; Formal Analysis, A.G-M. and J.P-B.; Investigation, W.A.W. and J.P-B.; Resources, L.P. and J.P-B.; Data Curation, A.G-M.; Writing – Original Draft Preparation, W.A.W., J.P-B. and L.P.; Writing – Review & Editing, W.A.W., L.P. and J.P-B.; Visualization, A.G-M. and J.P-B.; Supervision, L.P.; Project Administration, L.P.; Funding Acquisition, L.P. and J.P-B.

Conflicts of interest

The authors declare no conflicts of interest in the research reported in this paper.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Bioethics Committee of the College of Medical Sciences of the University of Rzeszów No. 4/11/2020.

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ORIGINAL PAPER

Oxidative stress and antioxidants markers in individuals with thyroid hormones dysfunction

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ABSTRACT

Introduction and aim. Thyroid hormone abnormalities have been associated with oxidative changes in human beings. The aim of the study was to evaluate the oxidative stress marker and antioxidants status in individuals with thyroid hormone dysfunction in Ekiti State.

Material and methods. A total of eighty samples were recruited in this study comprising forty subjects with thyroid hormones dysfunction and forty apparently healthy controls. Malondialdehyde (MDA), reduced glutathione (GSH) and catalase were determined spectrophotometerically.

Results. MDA was non-significantly higher ($p>0.05$) in subjects (4.33±0.84 nmol/mL) compared with control (4.12±0.63 nmol/mL), catalase was non-significantly higher ($p>0.05$) in subjects (199.36±20.21 µm/mL) compared with control (181.55±16.61 µm/mL), while GSH was significantly lower ($p<0.05$) in subjects (79.31±10.12 µmol/mL) compared with control (127.21±7.29 µmol/mL).

Conclusion. It can be concluded that the increase in the reactive oxygen species accompanied with impairment of the antioxidant system occurs in patients with thyroid hormone dysfunction. Hypothyroidism and hyperthyroidism induces disequilibrium of the oxidative/anti-oxidative balance that can lead to subsequent development of inflammation and associated diseases.

Keywords. antioxidants, dysfunction, malondialdehyde, oxidative stress, thyroid hormone

Introduction

The thyroid gland produces thyroid hormones that are essential for the healthy development of body organs. The thyroid is a significant endocrine gland since it essentially controls how quickly metabolism occurs in the cells.[1] They are crucial for the mental and psychological growth of infants and young children. Thyroxine and L-triiodothyronine, which are referred to as T4 and T3 respectively, are two distinctive hormones secreted by the thyroid gland.[2] Hypothyroidism is referred to a deficiency in thyroid hormone secretion and action. Between 2% and 15% of the population suffers from this disorder, which can be minor or severe.[3] Hyperthyroidism arise from over secretion of thyroid hormone from the thyroid gland or extra thyroidal tissues which can be generally divided into primary and secondary variants.[4] The incidence of hyperthyroidism is about 3 per thousand and women are 8 times more likely than males to have it. Despite the availability of free T4 and free T3, hyperthyroidism is characterized by a reduction in blood thyrotropin levels, which leads to metabolic formation and an acceleration of free radical production, changing the activity of antioxidant enzymes.[5] The main components of oxidative metabolism are thyroid hormones.[6]

Oxidative stress, which is defined as a free radical/antioxidant imbalance favouring radicals, plays a role

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in the pathophysiology of numerous diseases and their consequences.[7] Reactive oxygen species (ROS) comprising of superoxide, hydrogen peroxide and hydroxyl radicals, have traditionally been thought to have the ability to cause cancer and enhance invasiveness. Under physiological conditions, production of ROS is controlled by a large quantity of antioxidant systems which proceed as protective mechanisms. These systems comprise of antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase.[8]

Malondialdehyde (MDA)is one of by-products of lipid peroxidation and the most extensively researched marker of oxidative stress having a three-carbon molecular weight aldehyde.[9] Monitoring MDA levels in many biological systems can serve as a crucial marker of lipid peroxidation both in-vitro and in vivo for a variety of medical conditions.[10] Catalase is a typical enzyme present in all most all living things that are exposed to oxygen. Catalase is an essential antioxidant enzyme in charge of degradation of the reactive oxygen species and hydrogen peroxide to water and oxygen.[11] Glutathione (GSH) is a unique molecule that takes part in a wide range of metabolic, transport and detoxifying functions.[12] In addition to being a significant antioxidant, the cysteine residue of glutathione makes it the most prevalent low-molecular-weight thiol-containing peptide in most live cells. Reduced GSH and oxidized glutathione (GSSG) are the two distinct forms of intracellular glutathione.[13]

Fundamentally, oxidative stress is defined as an imbalance between the generation of oxidants and the protective actions of antioxidants, which may be brought on by normal metabolic processes or pathological situations.[14] ROS are produced as a byproduct during the synthesis of thyroid hormones. However, the body's antioxidant systems eliminate the ROS when the redox balance is normal, reducing oxidative damage.[15] On the other hand, some situations, such as thyroid gland inflammation and tumour cell growth, may change the equilibrium between ROS and antioxidant levels in favour of the former, which would then result in oxidative damage.[16] To stop or even manage a wide range of disease problems, appropriate antioxidant levels must be maintained. The various biochemical parameters associated with thyroid disease have been extensively studied, but little is known about the role of antioxidants and oxidative stress markers in thyroid disease. Furthermore, studies examining the state of antioxidants and markers of oxidative stress in individuals with thyroid hormone abnormalities are lacking in our study area.

Aim

This study was carried out to determine the oxidative stress marker and antioxidants status in individuals with thyroid hormone dysfunction in Ekiti State.

Material and methods

Study area

This study was carried out in Ikere. Ikere is the second most populous and principal city of Ekiti State, Nigeria. The area lies between latitudes 70 30' North of the equator and longitudes 50 14' East of the Greenwich meridian. The city has an area of 262 km^2 and population density of 778.3/km^2.

Study design

A cross sectional study design was employed in this study. Male and female individuals with thyroid hormone dysfunction between the ages of 15 and 45 years were recruited for this study.

Ethical approval for this study was obtained from the Ethics and Research Committee, Bamidele Olumilua University of Education, Science and Technology Ikere (BOUESTI), Ekiti State, Nigeria (BOUESTI/HREC/23/02/0112). Institutional permission/approval was also equally obtained. Informed consent was sought from each subject who participated in the study before the collection of sample.

Sample size

Sample size was determined using the formula:

$n = z^2pq / d^2$

Where; n – the desired sample size; z – is a constant given as 1.96; p – prevalence (2.6%); q – 1.0 – p; d – acceptable error (5%)

$n = (1.96^2 \times 0.026 \times (1 - 0.026)) / 0.05^2$

$n = 38.9 \approx 39$

Study population

The study population was individuals with thyroid hormone dysfunction visiting University Health Center, BOUESTI, Ekiti State. A total of eighty (80) individuals comprising of forty (40)subjects (individuals with thyroid hormone dysfunction) and forty (40) apparently healthy individuals (controls) were recruited for this study.

Inclusion criteria

Individuals with thyroid hormones dysfunction (hyperthyroidism and hypothyroidism) in the study area who gave their consent were included in this study.

Exclusion criteria

Individuals currently undergoing medications, tobacco smokers, alcohol drinkers, pregnant and lactating women, those using imunosuppressive drugs, those with underlying health conditions such as diabetics, HIV/AIDs and cardiovascular diseases etc. and those who did not give their consent were excluded from the study.

Sample collection

For each participant, about five millimeters (5 mL) of blood was collected via vein puncture and dispensed into plain bottle and allowed to clot. The serum was separated by centrifugation and carefully withdrawn into a pre-labeled tube for the determination of reduced glutathione, malondialdehyde and catalase activity. Specimens not tested immediately were stored at -20^{0}C.

Analytical methods

Malondialdehyde, glutathione and catalase activities were determined spectrophotometrically using UV Visible spectrophotometer (PXUV-2601 model) manufactured by Panomex Inc., New Delhi, India.[11-13]

MDA is a product of lipid peroxidation. When heated with 2-thiobarbituric acid (TBA) under alkaline condition, it forms a pink coloured product, which has absorption maximum at 532 nm. The intensity of colour generated is directly proportional to the concentration of MDA in the sample.[11]

GSSG in the samples treated with trichloroacetic acid (TCA) solution was reduced with reagents including Sodium borohydride ($NaBH_4$)and sodium hydroxide (NaOH) to form GSH. NaOH was used to increase the pH, which was low due to the utilization of TCA solution, to enable the reduction of the GSSG molecules. $NaBH_4$ was used as a reductant. After the reduction was completed, hydrochloric acid (HCl) solution was added to remove the remnant $NaBH_4$ in order to prevent extra-reduction of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) molecules and re-oxidation of GSH molecules. GSH levels were measured using 500 mM Tris solution (pH=8.2). The thiol residues of GSH reduced the DTNB molecules to 2-nitro-5-benzoic acid which has an absorbance at 412nm spectrophotometrically. GSH was measured before and after the reduction process.[13]

The catalase activity in a sample is determined by measuring the decrease in hydrogen peroxide (H_2O_2) concentration observed following an incubation of the analyte sample with an H_2O_2 standard solution. Catalase reacts with a known quantity of H_2O_2. The reaction is stopped after exactly one minute with catalase inhibitor.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

In the presence of peroxidase (HRP), the remaining H_2O_2 reacts with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample.[12]

$$2\,H_2O_2 + DHBS + AAP \xrightarrow{\text{HRP}} \text{Quinoneimine Dye} + 4\,H_2O$$

Statistical analysis

All results were presented in tables and chat as mean ± standard deviation. Statistical analysis was done using one way analysis of variance (ANOVA) and Student's t-test using Statistical Package for Social Sciences (SPSS) version 25 (IBM, Armonk, NY, USA). Additionally, nonparametric Kruskal-Wallis test was used to verify the ANOVA-test results. A p-values <0.05 was considered significant.

Results

Table 1 showed the socio-demographic variable of the subjects and control group. The age (years) in mean ± SD of the subjects and control group was 35.36±5.61 and 36.10±4.64, while the body mass index (BMI) (kg/m^2) was 25.22±2.32 and 22.14±1.12 respectively. BMI was significantly higher ($p<0.05$) in subjects compared with control, but BMI did not show any significant difference ($p>0.05$). With respect to classification of thyroid dysfunction, 25 (62.5%) had hyperthyroidism, while 15 (37.5%) had hypothyroidism.

Figure 1 shows the mean values of thyroid stimulating hormone (TSH)andT3 in hypothyroidism, hyperthyroidism and control subjects. TSH was significantly higher ($p<0.05$) in hypothyroidism (10.86±1.59 mU/L) compared with control group (3.36±0.55 mU/L) and hyperthyroidism (1.12±0.15 mU/L), and in control group compared with hyperthyroid group. T3 was significantly higher ($p<0.05$) in hyperthyroid subjects (2.91±0.34 nmol/L) compared with hypothyroid subjects (1.46±0.22 nmol/L) and control subjects (1.51±0.26 nmol/L) respectively.

Figure 2 shows the mean values of thyroxine (T4) in hypothyroidism, hyperthyroidism and control subjects. T4 was significantly higher ($p<0.05$) in hyperthyroidism (155.12±22.31 nmol/L) compared with hypothyroidism (89.33±12.21 nmol/L) and control subjects (96.58±10.34 nmol/L) respectively.

Table 2 presents the antioxidants and oxidative stress parameter of subjects and control. The mean value of MDA (nmol/mL) in subjects and control was 4.33±0.84 and 4.12±0.63, Catalase (μm/mL) was 199.36±20.21 and 181.55±16.61, while reduced glutathione (μmol/mL) was 79.31±10.12 and 127.21±7.29 respectively. MDA and catalase were non-significantly higher ($p>0.05$) in subjects compared with control, while reduced glutathione was significantly lower ($p<0.05$) in subjects compared with control.

Table 3 shows the antioxidants and oxidant stress parameters of male and female subjects. The mean MDA (nmol/mL) levels of male (4.76±0.86) was non-significantly higher ($p>0.05$) compared with female subjects (4.13±0.79). Catalase (μm/mL) activity was non-significantly higher in male subjects (221.41±31.56) in comparison with female subjects (186.17±28.50). On the other

hand, reduced glutathione (μmol/mL) level was non-significantly higher in female subjects (80.21±10.61) compared with male subjects (73.44±15.24).

Table 4 presents the antioxidants and oxidative stress parameter of subjects with hyperthyroidism and hypothyroidism. The mean values of MDA (nmol/mL) was significantly higher ($p<0.05$) in subjects with hypothyroidism (6.34±1.39) compared with subjects with hyperthyroidism (4.89±1.12). Catalase (μm/mL) was significantly higher ($p<0.05$) in subjects with hyperthyroidism (248.33±26.48) compared with hypothyroidism (126.24±11.72). GSH (μmol/mL) was significantly higher ($p<0.05$) in subjects with hyperthyroidism (93.26±9.69) in comparison with hypothyroidism (56.77±8.36).

Table 5 shows the correlation between T3, T4 and TSH hormone levels and oxidative stress parameters in subjects. The results obtained showed that there was a significant positive correlation between T3 and MDA ($r=0.802$, $p=0.000$), T3 and catalase ($r=0.760$, $p=0.001$), T3 and reduced glutathione ($r=0.786$, $p=0.001$), and T4 and catalase ($r=0.727$, $p=0.026$) respectively.

Table 1. Socio-demographic variable of the subjects and control group*

Variable	Subjects (%) (n=40)	Control (%) (n=40)	p
Male	15 (37.5%)	14 (35%)	
Female	25 (62.5%)	26 (65%)	
Age (Mean ± SD) (years)	35.36±5.61	36.10±4.64	0.612
BMI (Mean ± SD) (kg/m²)	25.22±2.32	22.14±1.12	0.001*
Marital status			
Single	12 (30%)	25 (62.5%)	
Married	28 (60%)	15 (37.5%)	
Educational status			
Primary	2 (5%)	1 (2.5%)	
Secondary	18 (45%)	22 (55%)	
Tertiary	20 (50%)	19 (47.5%)	
Occupation			
Students	8 (20%)	15 (37.5%)	
Self-employed	18 (45%)	12 (30%)	
Civil servants	9 (22.5%)	10 (25%)	
Unemployed	5 (12.5%)	3 (7.5%)	
Classification of thyroid dysfunction			
Hyperthyroidism	25 (62.5%)	–	
Hypothyroidism	15 (37.5%)	–	

*BMI – body mass index, % – percentage, SD – standard deviation

Table 2. Antioxidant and oxidative stress parameter of subjects and control

Parameters	Subjects Mean±SD	Control Mean±SD	t	p
MDA (nmol/mL)	4.33± 0.84	4.12 ± 0.63	0.675	0.369
Catalase (μm/mL)	199.36 ± 20.21	181.55 ± 16.61	1.019	0.115
GSH (μmol/mL)	79.31± 10.12	127.21 ± 7.29	4.133	<0.0001

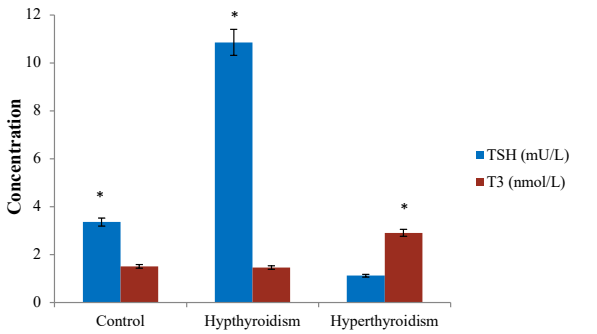

Fig 1. TSH and T3 in hypothyroidism, hyperthyroidism and control subjects (values with * significantly differ from other groups at $p<0.05$)

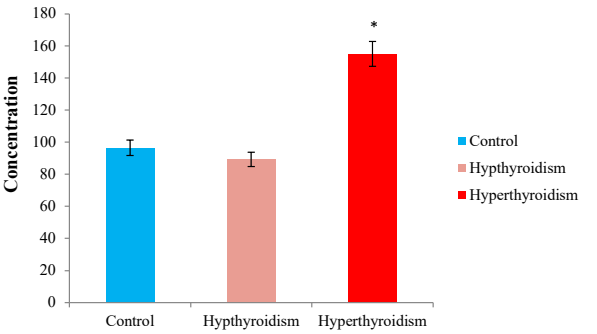

Fig 2. T4 in hypothyroidism, hyperthyroidism and control subjects (values with * significantly differ from other groups at $p<0.05$)

Table 3. Antioxidants and oxidative stress parameter of male and female subjects

Parameters	Male Mean±SD	Female Mean±SD	p
MDA (nmol/mL)	4.76±0.86	4.13±0.79	0.512
Catalase (μm/mL)	221.41±31.56	186.17±28.50	0.072
GSH (μmol/mL)	73.44±15.24	80.21±10.61	0.068

Table 4. Antioxidant parameters and oxidative stress parameter of Subjects with hyperthyroidism and hypothyroidism*

Parameters	Control Mean±SD	Hyperthyroidism Mean±SD	Hypothyroidism Mean±SD	p
MDA (nmol/mL)	4.12±0.63^{a}	4.89±1.12^{b}	6.34±1.39^{c}	0.412^{a+b} 0.003^{a+c} 0.002^{b+c}
Catalase (μm/mL)	181.55±16.61^{a}	248.33±26.48^{b}	126.24±11.72^{c}	0.002^{a+b} 0.001^{a+c} <0.000^{b+c}
GSH (μmol/mL)	127.21±7.29^{a}	93.26±9.69^{b}	56.77±8.36^{c}	<0.000^{a+b} <0.000^{a+c} 0.001^{b+c}

* values are statistically significant at $p<0.05$; $^{a+b}$ – represent control group vs hyperthyroid group, $^{a+c}$ – represent control vs hypothyroid group; $^{b+c}$ – represents hyperthyroid vs hypothyroid group

Table 5. Correlation between T3, T4 and TSH hormone levels and oxidative stress parameters in subject[a]

Variables	T3 r (p)	T4 r (p)	TSH r (p)
MDA	0.802 (<0.001)**	0.005 (0.990)	0.062 (0.874)
Catalase	0.760 (0.001)**	0.727 (0.026)*	-0.176 (0.652)
GSH	0.786 (0.001)**	0.177 (0.649)	-0.099 (0.799)

[a]** – correlation is significant at the level 0.01 level (2-tailed); * – correlation is significant at the level 0.05 level (2-tailed), r – Pearson's correlation

Discussion

This study was carried out to assess the oxidative stress marker and antioxidants status of individuals with thyroid hormone dysfunction in Ekiti State. In this study, MDA was non-significantly higher ($p>0.05$) in subjects compared with control. This increase could be the result of altered metabolic rates that produce too much H_2O_2 and nitric oxide.[17] Additionally, increased lipid peroxidation and the depletion of the body's antioxidant defense system may contribute to the rise in free radicals seen in thyroid dysfunction.[18] Elevated levels are observed in ROS-damaged tissues, as the final product of peroxidation, making them markers of oxidative stress in the body.[19] The increase in MDA found in this study is a sign of oxidative stress and lipid peroxidation. It's possible that the test subjects' oxidative stress was reduced by the action of catalase which was slightly elevated. This finding is agreement with previous research.[6,20-21] According to Basant et al., serum enzyme activities of MDA levels are good indicators for the systemic oxidant/antioxidant status, but they do not always correspond to changes that really occur in the thyroid directly.[6] This is because many different factors might affect the serum result.[6] In comparison to samples from normal thyroid tissue, the specimens from papillary carcinoma had significantly greater lipid peroxide concentrations expressed as MDA concentration.[20] In their investigation, Terziogluet al. observed that MDA levels before thyroidectomy were higher in thyroid dysfunction patients than in controls of same age, indicating enhanced free radical generation.[21]

The result of this study showed that catalase activity was non-significantly higher ($p>0.05$) in subjects compared with control. This research suggests an imbalance between antioxidants and oxidants in relation to aberrant thyroid function. ROS are produced more frequently as a result of changes in thyroid secretions. In the thyroid gland, free radicals and ROS play a role in both normal and pathological processes. ROS or free radicals are used by the endocrine system in the production of hormones. Since thyroid cells release enzymes that catalyse ROS production, the body's defence mechanisms and non-enzymatic antioxidants play a crucial role in neutralising excess ROS that isn't needed to produce thyroid hormones thereby maintaining overall homeostasis.[19] The thyroid hormone can control levels of enzymatic antioxidants including *superoxide dismutase*, glutathione peroxidase, catalase, and glutathione reductase as well as nonenzymatic antioxidants like vitamin E and C, glutathione, and uric acid, which can also influence the oxidative metabolism.[22]This finding is in agreement with previous studies which reported an increase in catalase activity in patients with thyroid dysfunction.[19,22-23] However dissimilar findings, showing markedly increased catalase in patients with thyroid dysfunction has also been described in literature when compared to controls.[24] Variations in ethnicity and dietary or ecological differences may be responsible for the discrepancy in findings. Additionally, the variation in antioxidant activity is tissue-specific and the reactions of antioxidant enzymes are not always the same.[25]

In this study, reduced GSH level was significantly lower ($p<0.05$) in subjects compared with control. Antioxidants can combat free radicals and neutralize oxidants. The lower levels of reduced glutathione as seen in this study may be due to its involvement in neutralizing and counteracting the effect of free radicals and oxidative stress occasioned by thyroid hormone dysfunction in our subjects. Reduced glutathione, an essential intracellular antioxidant, works as both a co-factor for glutathione peroxidase and a direct active scavenger to eliminate reactive species like the hydroxyl radical, carbon-centered radicals, peroxynitrite and singlet oxygen .[18]The protection of erythrocytes from oxidative damage is another highly important function of reduced GSH. It is a crucial antioxidant enzyme needed to neutralize ROS in different cell compartments and to respond to demanding circumstances. This finding is in consonant with previous studies which reported significant lower GSH levels in patients with thyroid gland dysfunction compared with healthy control.[17,19,22,24]

With respect to gender, MDA and catalase was non-significantly higher in males compared with female subjects, while reduced GSH level was non-significantly higher in female subjects compared with male subjects. According to a study, young men exhibit higher levels of in vivo oxidative stress indicators than women of the same age.[26] The generation of ROS was also shown to be higher in male vascular cells than female vascular cells.[27] Furthermore, research from both clinical and experimental settings revealed that women have a higher antioxidant potential than men.[28] According to these studies, there is a possible link between oxidative stress and gender, with women appearing to be less vulnerable.[26] ROS generation and the antioxidant defense system must be out of balance for oxidative stress to occur. The expression and/or activity of antioxidant enzymes appear to differ between males and females.[29]

In this study, MDA and catalase was significantly higher ($p<0.05$) in subjects with hypothyroidism com-

pared with hyperthyroidism, while reduced GSH was significantly higher in subjects with hyperthyroidism when compared with hypothyroid subjects. Both hyperthyroidism and hypothyroidism have been demonstrated to be related to oxidative stress. However, these two clinical diseases have different ways of producing oxidative stress: In hyperthyroidism, ROS generation is elevated, and in hypothyroidism, antioxidant availability is reduced.[5] This finding is in tandem with previous studies.[30-33] GSH is responsible for preserving the redox balance of cells. Reducing ROS levels and combating oxidative stress are possible effects of raised GSH levels.[32] Long-term oxidative stress can be associated with cell damage because it exceeds the capacity of antioxidant synthesis by the target organs or extracellular.[33] Ramli et al. reported that the activity of antioxidant enzymes was lowered and the quantity of TBA-active lipid peroxidation products (MDA) was much higher in hyperthyroid tissue.[30] Joshi et al.also shown that patients with hypothyroidism had lower MDA levels than patients with hyperthyroidism.[31] They conclude that lipid peroxidation was higher patients with hyperthyroidism compared with hypothyroid patients.[31]

Increased T4 and T3 thyroid hormone concentrations in hyperthyroidism lead to a rise in baseline metabolic rate, increased oxygen consumption and the production of significant amounts of reactive oxygen species, which heighten oxidative stress.[34] The elevated plasma TSH content in hypothyroidism is what causes the rise in lipid peroxidation. H_2O_2 generation is enhanced by high plasma TSH concentrations. H_2O_2 is an essential component in the production of thyroid hormones. It serves as an acceptor of electrons produced during oxidative hormone production processes.[35] Nicotinamide adenine dinucleotide phosphate oxidase system located in the apical membrane of the thyroid cell is responsible for producing H_2O_2 in the thyroid gland. The free radicals that are present in hypothyroidism are enhanced by H_2O_2 levels. Increased levels of TSH, H_2O_2, free radicals, and antioxidants as well as excessive vascularization, free radicals, and antioxidant levels all play a role in the development of thyroid conditions, which can result in oxidative stress.[36]

In this study, there was a significant positive correlation between T3 and MDA (r=0.802, p=0.000), T3 and catalase (r=0.760, p=0.001), T3 and GSH (r=0.786, p=0.001), and T4 and catalase (r=0.727, p=0.026) respectively. Sultana et al. in their study reported that in patients with hyperthyroidism, oxidative stress markers and thyroid hormone levels significantly correlate, and these markers can be useful in the assessment of the disease's prognosis.[37] The thyroid glands inflammatory process produces an excessive amount of ROS and free radicals, which suppress the antioxidants and cause an imbalance of oxidants and antioxidants.[19] Enhancing antioxidant defences to restore the equilibrium through therapeutic interventions may be helpful in the management of patients with hyperthyroidism since the oxidant-antioxidant balance is crucial for the regular function of the thyroids.[19] In order to improve thyroid function and restore oxidant-antioxidant equilibrium in hyperthyroid individuals, antioxidant supplementation with antithyroid medications may be used.[37]

Conclusion

It can be concluded that the increase in the reactive oxygen species accompanied with impairment of the antioxidant system may occur in patients with thyroid hormone dysfunction. Our results suggest that thyroid hormones in excess may be accompanied by increase in oxidative stress and impairment of the antioxidant system especially in females. Further study with larger sample size is required. Hypothyroidism and hyperthyroidism induces disequilibrium of the oxidative/anti-oxidative balance that can lead to subsequent development of inflammation and many associated diseases. This can be prevented by the adequate management of the disease and supplementation with anti-oxidative dietetics. Thus, reduction of oxidative stress may be beneficial in patients with subclinical hypothyroidism.

Declarations

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Author contributions

Conceptualization, E.A.O.; Methodology, E.A.O. and O.D.A.; Validation, E.A.O. and O.D.A.; Formal Analysis, E.A.O. and O.D.A.; Resources, E.A.O. and O.D.A.; Writing – Original Draft Preparation, E.A.O.; Writing – Review & Editing, E.A.O. and O.D.A.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data available on request from the authors.

Ethics approval

Ethical approval for this study was obtained from the Ethics and Research Committee, Bamidele Olumilua University of Education, Science and Technology Ikere (BOUESTI), Ekiti State, Nigeria.

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ORIGINAL PAPER

Manifestations of post-traumatic stress in military personnel after participating in hostilities in the Russian-Ukrainian war

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ABSTRACT

Introduction and aim. Since the beginning of the Russian-Ukrainian war, many Ukrainian military personnel began to show post-traumatic stress (PTS). The purpose of the article is to identify the features of PTS exhibited in different age groups in trauma-exposed military personnel during their participation in the psychological recovery program ("Invincibility Program").
Material and methods. Ukrainian Defense Forces military personnel (n=546 males, between 20 and 60 years of age) participated in this study. All participants were divided into three age groups. Determination of the features of PTS in military personnel was carried out using psychodiagnostic tests.
Results. In each age group, more than half of the participants in the "Invincibility Program" had PTS characteristics such as the sub-threshold or clinical manifestations of PTSD, adjustment disorders, low resilience to combat mental trauma, and various sleep disorders combined with somatic complaints. The data showed higher features of PTS in the younger participants and they decreased in both the 2 older sets of participants.
Conclusion. The age-related features of the manifestation of PTS in military personnel must be taken into account when developing psychological recovery programs: for younger participants, such events should be carried out longer and more intensively.
Keywords. hostilities, military personnel, post-traumatic stress

Introduction

On February 24, 2022, the armed forces of the Russian Federation invaded Ukraine and large-scale hostilities began, in which hundreds of thousands of military personnel from both sides took part. Combat injuries received by Ukrainian military personnel were accompanied by particular severity, multiplicity, and combined defeat.[1] They were caused by the use of many types of modern weapons by Russian military personnel: ballistic missiles, unmanned aerial vehicles, artillery shells, mines, multiple-launch rocket systems, and firearms. Also, almost all military personnel participated in hostilities combat stress manifested in the form of acute stress reactions, affective and anxiety disorders, addictive and delinquent behavior, adjustment disorders, and suicidal manifestations.[2-5] These negative consequences of post-traumatic stress (PTS) in combat conditions were combat stress reactions (CSRs), requiring the provision of psychological first aid to military personnel and recovery of mental resources.[6,7]

PTS consisted of immediate, long-term, and delayed mental consequences. At first, the PTS symptoms

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were not subjectively felt as a disease, without disturbing the adaptation and resilience of military personnel to combat conditions.[8] While most soldiers were able to adequately cope with these stressors, others become so overwhelmed that their psychological defenses become exhausted, causing a psychological breakdown.[9] They struggled with loneliness, isolation, forced separation from their loved ones, and lack of their physical needs being met regarding food, drink, and sleep. Combatants during the war faced constant danger, not only in the line of duty. They also witnessed the injury and death of their fellow soldiers, commanders, and enemies as well as innocent civilians. Added to the enormous stress was the unpredictability of modern warfare, including the risk of weapons of mass destruction and asymmetric combat operations, making it difficult to predict where and when the next attack might occur.[10] Subsequently, these changes in some military personnel were transformed into a delayed response to a stressful event of a threatening or catastrophic nature, which could later cause more pronounced stress or distress.[11-14]

Data reporting the prevalence of PTS in military personnel vary considerably. This was due to many factors: the intensity and duration of hostilities, the experience of the combatants, their level of professional and psychological readiness, motivation, as well as on gender, marital status, age, personal participation in combat, and other factors. For example, at the beginning of the Russian-Ukrainian war (March 2022), PTS in Ukrainian combatants reached threshold levels of clinical symptoms of anxiety (44.4%), depression (43.3%), and insomnia (12.4%).[15] The researchers found that although protective and mediating factors were in place, 11% to 17% of combat veterans were at risk for mental disorders in 3 to 4 months after return from combat duty.[16] It was revealed that out of 103,788 US veterans, 25,658 (25%) received mental health diagnosis(es); 56% of whom had two or more distinct mental health diagnoses.[17]

The long-term pathogenic effects of PTS may be reflected not only in higher rates of PTSD but also in its severity. During 20 years of follow-up, it was found that Israeli veterans who had PTS in the form of CSRs also suffered from more severe PTSD than combat veterans without CSRs.[9] Thus, for many PTS victims, the initial mental breakdown at the line of contact marked the beginning of a lifelong struggle with the psychopathological consequences of the war.

Post-conflict studies have shown that combat participation was associated with a significant risk of mental health problems, including post-traumatic stress disorder (PTSD), major depression, suicides, substance abuse, impairment in social functioning and in the ability to work, and the increased use of healthcare services.[18-20] Therefore, mental health issues must be addressed before and during deployment to ensure optimum individual and unit functioning, as well as to prevent chronic mental illness and disability in the future.[16,17,21] The US Department of Defence guidelines on PTSD recommend outpatient trauma-focused psychological therapies (TFPT) before pharmacological interventions or other forms of therapy due to the benefits of TFPT being longer-lived.[22]

We did not find scientific papers that studied the features of the manifestation of PTS in military personnel of different age groups after participating in long-term hostilities. In this regard, we suggested that PTS in military personnel of different age groups may manifest itself in different ways. It was found that no generally accepted international definition of human age groups. Therefore, we used the classification age groups with the recommendations of the UN and WHO, in which the crown of youth age is from 18 to 24; the young age is 25 to 44; the middle age is 45–60; the elderly age is 60–75; the senile age is 75–90, and the long-livers are after 90.[23] In this regard, the age groups used in this study are uneven. Age-related periodization of mental development was based on determining a person's ability to interact with society, taking into account bodily changes due to the laws of the biological development of the human body. This classification also considered the social situation of human development, leading professional activities, critical and sensitive periods of development (age crises), and other factors. It should be noted that in Ukraine, citizens who are 20 years old are called up for military service (therefore, in Group 1, the age of the study participants was 20–24 years old).

Aim

The purpose of the article was to identify the features of PTS exhibited in different age groups in trauma-exposed military personnel during their participation in the psychological recovery program.

Material and methods

Study design and participants

All participants gave their informed consent for inclusion before they participated in the study. The approval of the ethics committee was obtained before the initiation of the study (meeting date; 11/01/2023, decision number; 2023/14). All procedures performed in this study involving human participants were in accordance with the ethical standards specified by the institutional and national research committee and with the Helsinki Declaration and its later amendments or comparable ethical standards.

This study was a cross-sectional, descriptive study. After the start of the war and three months of intense hostilities, Ukrainian military personnel began to experience acute stress reactions, more often showing signs of distress and adjustment disorders, depression, demoral-

ization, PTS, and suicidal behavior. Therefore, the Command of the Operational Group of Troops "Kharkiv" created the rehabilitation center. The rehabilitation center was located 30–40 km from the combat zone. The psychological recovery project for military personnel after being in combat conditions began in June 2022 and continues to this day based on the clinical sanatorium in the Kharkiv region. The psychological recovery program for military personnel was specially developed for its practical implementation: we named it the "Invincibility Program." The purpose of this program was to reduce the impact of PTS on combatants, strengthen mental health and mobilize their psychological resources, improve adaptation and resilience, and promptly return to combat activities. The duration of stay of the program participants in the rehabilitation center was seven days. In this program eight military psychologists were involved, working with 60–80 military personnel. All participants were divided into 4–5 groups for group psychotherapy and psycho-correction (10–15 people in a group with 1–2 psychologists). Group activities were held in the morning, and individual consultations were held in the afternoon. Psychologists were constantly with their participants for the entire duration of the program. Participants of the "Invincibility Program" were allowed to live with their families and family members (wives, children) could participate in all activities of the program. Only about 30% of participants took advantage of this option: most of the families became forced refugees and left Ukraine or lived far from the rehabilitation center. The total number of military personnel involved since the beginning of the "Invincibility Program" has amounted to more than 3 500 people.

Table 1. Distribution of study participants by age groups

Age groups	Participants	
	n	%
Group 1 (20–24 years old)	73	13.37
Group 2 (25–44 years old)	278	50.92
Group 3 (45–60 years old)	195	35.71
Total (20–60 years old)	546	100.00

Ukrainian Defense Forces military personnel (n=546 males, between 20 and 60 years of age, 35.84±6.49 years) participated in this study. All combatants took part in the Russian-Ukrainian war and had combat experience after February 24, 2022 (6–10 months), (8.75±2.58 months). Combatants were sent to the rehabilitation center from combat positions based on a list of indications for psychological recovery. According to military specialties, there were infantrymen, attack aircraft, scouts, snipers, tankers, artillerymen, and other military specialists. Female military personnel were not included in this study because, over the entire period of the program, less than 0.5% of female combatants participated. Participants were randomly selected for the study. All participants were divided into three age groups (Table 1).

Instruments

Determination of the PTS manifestations in military personnel was carried out after arrival at the rehabilitation center using psychological tests.

The "Mississippi Scale for Combat-Related Posttraumatic Stress Disorder" (MSCRPTSD) was used to diagnose PTSD in military personnel on missions in the war zone, translated into Ukrainian.[24,25] The scale consists of 35 statements (4 subsets), the answers to which were given on a 5-point Likert scale (Cronbach's α=0.887). Subset 1 (11 statements) describes the symptoms of the "intrusion" group when the traumatic event is constantly repeated in the experience in one (or more) ways. Subset 2 (11 statements) relates the symptoms of the "avoidance" group when there is a constant avoidance of stimuli associated with trauma, blocking of emotional reactions, and numbness, which was not observed before the trauma. Subset 3 (8 statements) describes the symptoms of "excitability" when persistent symptoms of arousal increase that were not observed before the injury. Subset 4 (5 statements) describe symptoms associated with guilt and suicidal tendencies. Despite the grouping of statements into four subsets, one general indicator was calculated taking into account the conversion of the answer into a score for direct and inverse statements, reflecting the severity of PTSD symptoms (range from 35 to 175 points, where 35–80 points is a variant of the norm; 81–114 points – separate symptoms of PTSD; 115–175 points – clinical manifestations of PTSD, a psychiatric examination and inpatient examination were recommended).

The "Disadaptation Express Questionnaire" (DEQ) is an abbreviated modified version of the Multilevel Personality Questionnaire "Adaptation".[25] The DEQ made it possible to identify signs of a violation of the adaptability of the soldier's personality: violation of the regulatory function of the emotional-volitional sphere and self-esteem; lack of prospects for continuing life and the ability to overcome life's difficulties (probability of committing suicidal attempts); loss of moral convictions, the likelihood of committing addictive and delinquent acts; loss of communicative potential (comradely support, reduced ability to accept the help of one's team). The DEQ consists of 45 statements included in 5 subscales (Cronbach's α=0.848): "Sincerity of answers," "Violation of behavioral regulation," "Probability of committing suicide attempts," "Violation of moral normativity," and "Loss of communicative potential." Each positive response was worth 1 point, and negative 0 points. The overall DEQ scale was calculated as the sum of scores on 4 scales (values on the "Sincerity of answers" scale were not included). The results of the overall DEQ scale were

evaluated as follows: 1–10 points – high adaptation to combat operations, sufficient tolerance to adverse mental and physical stress, including under conditions of severe combat stress; 11–14 points – average adaptation, unstable level of performance, especially in combat conditions; 15 points or more – low adaptation (distress and adjustment disorders) that does not meet the requirements for soldiers in combat conditions.

The "Resilience to Combat Mental Trauma Questionnaire" (RCMTQ) was used for psychodiagnostic participants upon completion of the program. RCMTQ is the modified Combat Experiences Scale (CES).[25,26] CES is a 33-item measure that assesses deployment-related experiences. RCMTQ is a 45-item measure combined into 3 scales, answered on a 6-point Likert scale that assesses resilience to combat mental trauma based on combat experience gained (Cronbach's α=0.887). "The expectation from participating in hostilities scale" made it possible to assess the professional potential of military personnel in possible combat situations: their assessment as potentially (non)removable; expected mental (feeling of safety), psycho-physiological (somatic well-being) and social consequences of participation in hostilities; the ability to use the experience of their professional group and own experience. "The overcoming a stressful situation scale" made it possible to assess the mechanisms for overcoming stressful (combat) situations: to assess the role of anxiety, search activity, defense mechanisms (definition of the priority of problem-oriented and emotionally-oriented coping), behavior (hierarchies of the relations system, purposefulness and awareness of professional actions, responsibility), the role and importance of supporting the military team. The scale "Realization of the acquired combat experience" made it possible to assess the ability to process the acquired combat experience: to realize, accept, and determine its place in one's life path and the ability to apply it in the future adequately. The overall indicator of resilience to combat mental trauma (RCMT) was calculated as the sum of points on 3 scales, taking into account the conversion of the answer into a score for direct and inverse statements. The obtained results of the RCMT indicator were evaluated as follows: 193–225 points – a high level of RCMT, even with a significant complication of the combat situation, such military personnel are able to cooperate and provide assistance to colleagues; to process, assimilate one's own experience and adopt the experience of comrades; will be able to maintain focus, efficiency, and control over their mental state; 144–192 points – the average level of RCMT reflected a reduced ability to provide support to colleagues; do not always maintain the effectiveness of their activities and control over their mental state; 0–143 points – low level of RCMT reflected psychological unpreparedness to participate in hostilities; rapid exhaustion, irritability, inability to interact, and to assist colleagues.

For the data presented basic descriptive statistics were used (arithmetical mean M, standard deviation SD). The reliability of differences in the results of the mean values in three interrelated samples was determined using Student's t-test and Fisher's φ-test. For the assessment of the statistical significance of differences, we used the level of significance from $p<0.1$ to $p<0.001$. The statistical analysis of the study results was carried out using the program SPSS 20.0 (IBM, Armonk, NY, USA).

Results

The results of the study showed that in each of the 3 groups, approximately half of the participants had PTS characteristics such as the sub-threshold or clinical manifestations of PTSD according to the MSCRPTSD (Table 2).

Table 2. PTS manifestations in participants of different age groups[a]

PTS indicators	Groups of participants (%)			Differences between groups		
	Group 1	Group 2	Group 3	φ_{1-2}	φ_{1-3}	φ_{2-3}
Norm	46.58	47.12	51.28	0.08	0.69	0.90
Sub-threshold PTSD	20.55	31.65	34.87	1.93*	2.35**	0.73
Clinical manifestations of PTSD	32.88	21.22	13.85	2.02*	3.34***	2.07*

[a] *p<0.05; **p<0.01; ***p<0.001

Participants with normal PTS scores (<80 points) were not included in further analysis of the results. Further, of all the participants whose PTS indicators (sub-threshold and clinical manifestations of formed PTSD) exceeded the normative ones (>80 points), three age groups were made according to the above criteria (n=281): Group 1 consisted of 39 participants (13.88%); Group 2 consisted of 147 participants (52.31%); Group 3 consisted of 95 participants (33.81%). In Group 1, the intensity of PTS manifestations was the highest and decreases with age; the differences between groups of participants in these PTS indicators reach the level of statistical significance (Table 3).

Table 3. PTS manifestations in participants of different age groups (MSCRPTSD points)[a]

PTS indicators	Groups of participants			Differences between groups		
	Group 1 (n_1=39)	Group 2 (n_2=147)	Group 3 (n_3=95)	t_{1-2}	t_{1-3}	t_{2-3}
Sub-threshold and clinical manifestations of formed PTSD	102.15 ±14.41	96.94 ±14.85	92.77 ±11.84	2.00*	3.60**	2.42*

[a] *p<0.05, **p<0.001

Table 4 presents the results of the adjustment disorders in participants of different age groups.

Table 4. Adjustment disorders in participants of different age groups (DEQ points)[a]

Adjustment disorder indicators	Groups of participants			Differences between groups		
	Group 1 (n_1=39)	Group 2 (n_2=147)	Group 3 (n_3=95)	t_{1-2}	t_{1-3}	t_{2-3}
Sincerity of answers	3.87 ±1.13	3.42 ±1.24	3.28 ±1.23	2.17*	2.67**	0.85
Violation of behavioral regulation	5.90 ±2.40	4.64 ±2.36	4.22 ±2.06	2.92**	3.82***	1.46
Probability of committing suicide attempts	3.36 ±2.36	3.12 ±2.46	2.80 ±2.43	0.57	1.24	0.98
Violation of moral normativity	4.49 ±1.97	4.07 ±2.18	3.28 ±1.74	1.15	3.32**	3.09**
Loss of communicative potential	4.85 ±1.80	3.99 ±2.35	3.94 ±2.19	2.46*	2.49*	0.19
Total indicator of adjustment disorders	18.59 ±6.19	15.82 ±7.45	14.24 ±6.10	2.38*	3.71***	1.80^0

[a] ^{0}p<0.1; *p <0.05, **p<0.01; ***p<0.001

Table 5 presents the results of resilience to combat mental trauma in participants of different age groups.

Table 5. Resilience to combat mental trauma in participants of different age groups (RCMTQ points)[a]

Resilience to combat mental trauma indicators	Groups of participants			Differences between groups		
	Group 1 (n_1=39)	Group 2 (n_2=147)	Group 3 (n_3=95)	t_{1-2}	t_{1-3}	t_{2-3}
Expectation from participating in hostilities	40.91 ±11.62	42.74 ±10.73	46.29 ±11.01	0.89	2.47*	2.47*
Overcoming a stressful situation	47.44 ±11.30	49.40 ±10.29	51.03 ±10.76	0.98	1.69^0	1.17
Realization of the acquired combat experience	40.82 ±11.63	41.79 ±10.05	43.90 ±9.95	0.48	1.45	1.60
Total indicator of resilience to combat mental trauma	129.18 ±31.43	133.93 ±26.49	141.22 ±27.73	0.87	2.08*	2.03*

[a] ^{0}p<0.1; *p <0.05

Table 6 shows data on participants with various sleep disorders and somatic complaints requiring consultations and treatment of doctors (therapist, cardiologist, traumatologist, neuropathologist, otorhinolaryngologist).

Table 6. The presence of various sleep problems and somatic complaints in participants of different age groups with PTS manifestations[a]

Sleep and health problems	Groups of participants (%)			Differences between groups		
	Group 1 (n_1=39)	Group 2 (n_2=147)	Group 3 (n_3=95)	φ_{1-2}	φ_{1-3}	φ_{2-3}
Sleep problems	61.54	54.42	55.79	0.80	0.61	0.21
Somatic complaints	56.41	85.03	84.21	3.59***	3.29***	0.17

[a] ***p<0.001

Discussion

There are no studies that have examined the relationship between age and post-traumatic stress after the participation of military personnel in prolonged combat operations. As a result of the study, it was revealed that PTS in military personnel of different age groups manifested itself in different ways. It was found that in each age group, about half of the participants in the "Invincibility Program" had PTS characteristics such as the sub-threshold or clinical manifestations of PTSD. However, the intensity of symptoms in different groups is not the same. In Group 1, the largest number of participants were identified who had clinical manifestations of PTSD – 32.88%, in Group 3 this percentage is the lowest – 13.85%. According to the intensity gradation of PTS manifestations, the discrepancies between the age groups reach the level of statistical significance. In Group 1, the intensity of PTS manifestations was the highest and decreased with age.

A similar trend was also found in participants with adjustment disorders: in the direction from Group 1 (adjustment disorders are most pronounced) to Group 3, in which the indicators slightly exceed the norm. Differences between groups of participants in almost all indicators of adjustment disorders reach the level of statistical significance. The exception is the indicator "Probability of committing suicide attempts", which in all groups was approximately the same and relatively low.

In all groups with PTS manifestations, an unsatisfactory indicator of resilience to combat mental trauma was revealed (<143 points). However, Group 3 participants had the most realistic expectations about taking part in combat operations, which allows them to better tune in to the action of combat stress factors; according to this indicator, they significantly differ from Group 1 and Group 2. The revealed differences in resilience to combat mental trauma were not very pronounced in order to conclude that the personal life experience of Group 3 participants helps them in overcoming combat stress factors. Perhaps the challenges of war were such stress factors that even with good professional and psychological training it is impossible to be fully prepared for real combat activities, which predetermines the development of PTS in military personnel. However, it is easier for Group 3 participants to compare their own experiences of overcoming life's problems with the techniques that they were taught in preparation for participating in hostilities. This was a more reliable way to develop skills for coping with stressful situations.

More than half of the participants with PTS symptoms in each group had some type of sleep disorder. For Groups 2 and 3, 85% had somatic complaints: sleep problems superimposed on existing health problems and the consequences of injuries and concussions, worsened physical well-being, or, due to the mechanisms of reflection, the hypochondriacal component increased. Group 1 participants had more than 56% somatic complaints; according to this indicator, partici-

pants in Group 1 differ from the other two groups at a statistically significant level.

Thus, it can be argued that the impact of combat stress on mental and somatic health manifests itself differently in different age groups of military personnel. Perhaps this was due to life experience of coping with stress, and productive coping strategies developed by more mature military personnel. This trend can be explained by two paradoxes of stress and aging.[27] Although older adults are thought to experience more stress and to be more vulnerable to its adverse effects, they report less stress.[28] Older adults learn to appraise and cope with stress differently and that protects them despite the increased physiological vulnerability. The second paradox is related to the positive aspects of stress in that under certain conditions stress can have positive or 'toughening' effects that can be construed as building resilience.[27]

We found that in all three groups of participants, at the beginning of the "Invincibility Program", there is still no reflection and integration of the experience of participating in hostilities into their "lifeline". This can be explained by the fact that all participants had not yet completed the combat mission (after the program, all military personnel returned to the combat zone). Also, they had not yet realized the latent period (a month after the trauma event), the results of which may be post-traumatic growth or clinical manifestations of formed PTSD. It should be noted that the study participants had not yet gone through a full cycle of combat readiness recovery and combat stress resilience formation, which includes professional and psychological training, development of productive coping strategies, and awareness of the acquired experience to counter combat stress factors. However, at this stage of its formation, more mature military personnel had higher resilience to combat stress factors. It can also be assumed that this group included the most experienced military personnel. However, the data obtained show that their experience was not related to coping with the combat stressors that were characteristic of large-scale hostilities (in this indicator they do not differ from other age groups).

Combat-induced mental disorders were known to be often recognized either at the beginning or immediately after the end of the war, often in the absence of continuous systematic long-term follow-up of the victims.[20,29] It was found that military personnel who experienced PTS during combat in the form of CSRs had a higher likelihood of developing PTSD.[30] The results of our study may indicate that in the future many veterans may develop mental health problems after the end of hostilities. It can be assumed that intense and prolonged exposure to post-traumatic stress in adolescence, which, due to the maturation of brain structures and changes in social status, is sensitive to the formation of self-regulation, will not only be a temporary mental disorder, but a significant and lifelong vulnerability. This process can become the basis for the formation of lifelong PTSD.

PTS may not be the only negative outcome identified in military personnel after participating in hostilities. Israeli veterans experienced significantly more psychiatric disorders, distress, social functioning difficulties, health problems, accelerated aging, and earlier all-cause mortality than those who survived PTS.[31] The PTS experience is a typical moment when the combatant begins to feel vulnerable and helpless as he loses his sense of safety.[9,32] The results of previous studies and the new data we obtained confirm that combatants who experienced PTS may be at greater pre-combat risk and intensity than those who were not found PTS.

The presence of sleep disorders and somatic complaints in the participants significantly complicated the overcoming of PTS and reduced the effectiveness of the "Invincibility Program". The large difference between 20-24-year-olds and the other two groups for somatization (~50 vs. 85%) shown in Table 6 can be explained by some subjective and objective factors. Firstly, military personnel stayed in the combat zone for more than 6-8 months without rotation. Secondly, in addition to a direct threat to life, physical and mental health, a negative impact was exerted by: the lack of satisfaction of elementary physiological needs (safety, accommodation in trenches and dugouts, insufficient sleep, food, prolonged separation from the family, etc.), climatic effects of heat, cold, rain, snow, frost, etc. Thirdly, as the results of this study showed, mental traumatization by participation in combat actions was perceived more acutely in youth than in other age categories. This triggered the mechanisms of reflection, which were characterized by special attention to internal sensations, as a result of which somatic complaints of the hypochondriacal type could arise. Fourthly, young military personnel had less experience in taking care of their health. This was especially true for young people who had a "protracted childhood" and until recently their parents took care of them. Left alone with the onset of the disease, these young people were more likely to engage in more impulsive behavior, ranging from significant exaggeration to ignoring obvious problems, as well as greater dramatization of attitudes towards a possible disease. This could also be related to the purpose of a certain "manipulation": drawing attention to oneself and one's problems, obtaining help from outsiders, and reducing responsibility for erroneous actions.

Sleep problems were a core presenting symptom of many mental health disorders, including PTSD, depression, and anxiety.[33] Sleep disturbances, pain, and somatic problems were debilitating, significantly reducing the basis for recovery and opportunities for post-traumatic growth.

That is why it was advisable to carry out recovery programs at the sanatorium clinical base which has the necessary equipment and specialists to work with various sleep disturbances and diseases which has a psychosomatic, neurological component. In addition, sanatorium treatment should be aimed at general strengthening of the body, reducing anxiety, and tension, relieving muscle spasms by means of massage and exercise therapy, restoring the respiratory system, and anti-stress nutrition.

The results of the study allow us to offer practical recommendations for the formation of military units. It is expedient to limit (even if there is consent and motivation) the entry of youth military personnel who have not undergone purposeful military training into units participating in intense and prolonged combat clashes. Consider the possibility of expanding the involvement of military personnel of mature age in combat operations while maintaining sufficient physical health, motivation to participate in hostilities, and experience in professional military activity. Mature military personnel, while maintaining good physical shape and having motivation were more resistant to the action of combat stress factors, less than other age groups are prone to the formation of PTSD. If they had an adequate attitude to the peculiarities of their physical status (awareness of the consequences of aging and the presence of diseases), they would be able to quite effectively compensate for a number of physical shortcomings due to experience, a high-quality choice of military equipment, etc.

Study limitations

This study certainly had limitations. First, female military personnel were not included in this study because, over the entire period of the "Invincibility Program", less than 0.5% of female combatants participated. Secondly, it was impossible to study the further fate of program participants with symptoms of PTS, a high level of adjustment disorders, and low rates of resilience to combat mental trauma, which needed longer psychological recovery programs or additional examinations. Finally, the current study was limited by not having an active comparison condition and by not having a longitudinal follow-up.

Conclusion

As a result of the study, it was found that half of the military personnel after participating in hostilities had PTS characteristics such as the sub-threshold or clinical manifestations of PTSD, adjustment disorders, and low resilience to combat mental trauma. However, the manifestation of PTS in each age group of participants has its own characteristics. The data showed higher features of PTS in the younger participants and they decreased in both the 2 older sets of participants. Group 3 participants (age 45–60 years old) had higher rates of adaptation and resilience to combat mental trauma. They were aware of their age characteristics and did not expect significant changes in their mental state. Group 2 participants (age 25–44 years old) occupied an intermediate position in terms of PTS manifestations. Therefore, for this group, it was necessary to carry out additional activities to implement positive changes in their mental state. Group 1 participants (age 20–24 years old) showed the greatest manifestations of PTS. However, they also had the greatest mental and physical resources to overcome stressful events. But this required more time to move into a more harmonious mental state and consolidate the results achieved. The age-related features of the manifestation of PTS in military personnel must be taken into account when developing psychological recovery programs: for younger people, such events should be carried out longer and more intensively. The obtained results of the PTS manifestation made it possible to develop individual psycho-correctional and psychotherapeutic measures for the recovery of military personnel after participating in hostilities.

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Declarations

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Author contributions

Conceptualization, I.P. and Y.M.; Methodology, I.P.; Software, K.H.; Validation, Y.M., I.P. and N.K.; Formal Analysis, L.F.; Investigation, O.K.; Resources, N.A.; Data Curation, Y.M. and I.P.; Writing – Original Draft Preparation, Y.M.; Writing – Review & Editing, I.P.; Visualization, K.H.; Supervision, N.K.; Project Administration, I.P.; Funding Acquisition, I.P.

Conflicts of interest

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethical approval

The approval of the ethics committee was obtained before the initiation of the study (meeting date; 11/01/2023, decision number; 2023/14).

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ORIGINAL PAPER

Prevalence, prescription patterns, and quality of life of anaemia in adults with chronic renal disease

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ABSTRACT

Introduction and aim. Anaemia is a chronic kidney disease (CKD) condition characterised by a decline in glomerular filtration rate (GFR) and impaired kidney function. The aim of this study was to assess the prevalence, prescribing patterns, and quality of life of anaemia in patients with chronic renal disease who were adults (>18 years of age) at a tertiary care hospital.

Material and methods. Data on demographic characteristics, laboratory results, medication prescriptions, and quality of life assessments were collected. Statistical tests were performed to determine associations between anaemia prevalence and factors like age, gender, and CKD stage. The study included 132 patients, with a gender distribution of 89 men and 43 women.

Results. The most frequently prescribed drugs are epoetin (15.06%), multivitamins (14.82%), iron (10.65%), folic acid (10.22%), calcium carbonate (8.17%), calcitriol (5.6%), and omeprazole (4.22%). The cardiovascular system, blood disorders, and blood-producing organs come after the gastrointestinal tract and metabolism in the first anatomical level of the ATC classification.

Conclusion. It suggests hospital audits and recommendations for improved prescription practices. Further investigation into anaemia causes and drug class appropriateness is needed, and implementing improvements could potentially improve health outcomes.

Keywords. anaemia, chronic kidney disease, prescribing pattern, quality of life

Introduction

Chronic kidney disease (CKD) is a condition characterised by a decline in glomerular filtration rate (GFR) and impaired kidney function. The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative divides CKD into five stages: stage 1, stage 2, stage 3, and stage 4. Anaemia is a common complication of CKD, affecting a significant number of individuals.[1,2] Management of anaemia in CKD often involves drug classes like erythropoiesis-stimulating agents and iron supplements. However, further investigation into the underlying causes of anaemia in CKD and the appropriateness of prescribed drug classes is needed. Anaemia is often associated with poor quality of life, decreased physical activity, and cognitive impairment in CKD patients.[3] It is also closely related to the development of heart failure

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and stroke, as well as the progression of CKD. Normocytic, normochromic, and hyperproliferative anaemias are frequently associated with CKD. A decline in kidney function is the cause of anaemia, as the kidneys can create erythropoietin, a signaling protein that increases red blood cell production in response to a drop in blood oxygen levels.[4] When kidney function declines, the production of erythropoietin is reduced, leading to a decrease in red blood cell production. This can cause fatigue and other symptoms associated with anaemia and further worsen kidney function by reducing oxygen supply to the kidneys and promoting tissue damage.[5] Since recombinant human erythropoietin was made, erythropoiesis-stimulating agents (ESA) have been the main treatment for CKD anaemia. They improve the quality of life for patients and reduce morbidity, mortality, and left ventricular hypertrophy. International recommendations recommend considering ESA treatment for patients with a hemoglobin (Hb) level of 11 g/dL in pre-dialysis and 10 g/dL in dialysis patients.[6] However, it is crucial to carefully monitor Hb levels and adjust ESA dosage to avoid potential risks such as cardiovascular events or thromboembolic complications.

Aim

The current study was to evaluate the prevalence, prescription trends, and quality of life of anaemia in adult (patients older than 18 years of age) chronic renal disease patients at a tertiary care hospital.

Material and methods

A study of 132 adults aged 18 and older with chronic renal disease and confirmed anaemia participated. Data were collected from their medical records, including Hb levels, ESA prescriptions, and cardiovascular events or complications. A quality-of-life questionnaire was completed to assess anaemia's impact on daily functioning and well-being. The data was analysed to determine anaemia prevalence, ESA prescription patterns, and overall patient quality of life.

Study design, population, and approval

The Raghavendra Institute of Pharmaceutical Education and Research Institution Review Board approved a cross-sectional observational study involving 132 prescriptions from CKD patients aged over 18 (October 22, 2022, RIPER/IRB/2022/045). The study collected demographic and clinical data from medical records, including patient age, comorbidities, and laboratory results. Patient quality of life was assessed using validated questionnaires like the EQ-5D and SF-36.[7,8] The findings will provide valuable insights into anaemia management in CKD patients and contribute to improving patient care and outcomes. The study's demographic and clinical data will help establish a comprehensive understanding of the characteristics and health status of CKD patients. By examining factors such as age, comorbidities, and laboratory results, researchers can identify potential risk factors and determine the most effective strategies for anaemia management in this population. The assessment of patient quality of life using validated questionnaires will provide important information on the impact of anaemia on overall well-being and functioning.[9,10] These insights will be crucial in developing targeted interventions and improving care and outcomes for patients with anaemia in this population. If older patients with comorbidities and low Hb levels are at higher risk for anaemia-related complications, healthcare providers can prioritise regular monitoring and early intervention strategies. However, some counterarguments may arise, such as the potential for regular monitoring and early intervention strategies to not be feasible or cost-effective for all individuals at higher risk for anaemia-related complications.

Inclusion criteria

The CKD patients in stages 3a–5 who are not on dialysis and have an eGFR of 60 mL/min/1.73 m^2 and an Hgb level of 10 g/dL can get ESA therapy. However, these interventions may be challenging due to diverse needs and comorbidities, and their efficacy may vary depending on the severity and underlying cause of the anaemia.[11] Some potential interventions for individuals with anaemia include dietary modifications, iron supplementation, blood transfusions, and medication management. Dietary modifications may involve increasing the intake of iron-rich foods, while iron supplementation may be prescribed to replenish iron stores. Blood transfusions may be necessary in severe cases or when other interventions have been ineffective. Medication management may involve treating underlying conditions that contribute to anaemia, such as chronic kidney disease or certain types of cancer. If these interventions prove ineffective, blood transfusions may be required to replenish red blood cells and improve overall health.[12] If an underlying condition like chronic kidney disease or cancer is the cause of anaemia, managing the medication may involve first treating these conditions rather than concentrating solely on iron intake. Additionally, certain individuals may have a genetic condition called hemochromatosis, where their body absorbs too much iron, which can be harmful and exacerbate the condition.[13-16]

Exclusion criteria

The study aimed to evaluate the safety and appropriateness of blood transfusions for patients with chronic renal replacement therapy, active cancer, bleeding episodes, and documented iron-deficiency anaemia. Patients with a history of severe allergic reactions to blood products, infectious diseases like HIV or hepatitis, or

heart or lung conditions that may worsen the procedure are excluded.[17] Healthcare professionals must evaluate each individual's medical history and overall health status before determining if a blood transfusion is necessary and appropriate. Informed consent from the patient or their legal guardian is typically required before proceeding with the procedure. Blood transfusions are not without risks and potential complications, including transfusion reactions ranging from mild allergic reactions to more severe immune reactions.[18] These reactions can occur due to incompatibility between the donor's blood and the recipient's blood type or the presence of antibodies in the recipient's blood. Healthcare professionals must carefully match the blood type and perform compatibility tests to minimise these risks.

Data collection

The researcher collected information using a questionnaire after obtaining written informed consent from participants. Patients were interviewed to collect demographic data and medical histories to establish a context for current symptoms. Laboratory results, such as Hb, serum creatinine, eGFR, BUN, blood urine, and pharmaceutical information, were collected using the Anatomy Therapeutic Chemical Classification System (ATC).[19] The Form 12 Health Survey (SF-12) was used to measure well-being. The SF-12 questionnaire is a widely used instrument for evaluating self-reported health and well-being. The questionnaire evaluates physical functioning, roles incongruous due to physical and emotional health issues, physical discomfort, general health, vitality, social functioning, and mental health. The SF-12 questionnaire uses a scoring algorithm that takes an average of 50 and uses it to figure out an eight-dimensional summary of physical and mental health. This score is inversely related to the subjective health functions that were reported.[20] The SF-12 questionnaire is widely used in research and clinical settings to assess an individual's subjective perception of their overall health and well-being, providing a comprehensive evaluation of various aspects of physical and mental health. The scoring algorithm takes into account the responses provided by the individual across different dimensions, providing a reliable summary score that reflects their subjective health functioning.

Statistical analysis

The study used the student's T-test to compare the average of independent category variables and dependent continuous variables. Using Graph Pad software version 8.0, GraphPad Software (Boston, MA 02110, USA). Statistical analysis was conducted on the SF-12 questionnaire data to determine correlations and patterns between subjective health functioning and other variables.[21] This analysis helps researchers and clinicians gain insights into factors influencing an individual's overall health and well-being. Statistical analysis can also be used to compare the subjective health functioning of different populations or track changes in an individual's health over time. Overall, statistical analysis is crucial for interpreting the data from the SF-12 questionnaire and drawing meaningful conclusions about an individual's health status.[18]

Results

The study included 132 prescriptions written by CKD patients aged 40 to 50, with a gender split of 89 men (67.34%) and 43 women (32.57%), and Hb levels ranging from 7-9.9 g/dL. When questioned, every patient admitted to strictly adhering to the dietary changes advised by nephrologists. Overall, 765 different drugs were prescribed. Statistical analysis can provide valuable information about an individual's health status. By examining the data, researchers can identify patterns, trends, and correlations that may be indicative of certain health conditions or risk factors. This information can then be used to develop targeted interventions or treatments to improve the individual's overall well-being. Additionally, statistical analysis allows for the identification of potential confounding factors that may influence the relationship between health functioning and other variables. This helps ensure that any conclusions drawn are accurate and reliable. Out of the 132 prescriptions that were looked at, the average number of medications per prescription was 7.21. According to the first anatomical level of the ATC classification, drugs for the gastrointestinal tract and metabolism were most frequently prescribed (44.86%), followed by drugs for the cardiovascular system (21.85%), drugs for blood disorders and drugs for blood-forming organs (20.12%), and vitamin and mineral supplements (18.42%) (Table 1). The seven drugs that were most frequently prescribed were epoetin (15.06%), multivitamins (14.82%), iron (10.65%), folic acid (10.22%), calcium carbonate (8.17%), calcitriol (5.60%), and omeprazole (4.22%). According to the stages of chronic renal illness listed in Table 2, the patients were classified based on their eGFR values. Chronic renal disease was diagnosed in 61 cases (46.2%) at stage 1, 37 cases (28.1%) at stage 2, 26 cases (19.6%) at stage 3, and 8 cases (6.1%) at stage 4. Eight patients in stage 1 CKD had Hb values as high as 10–10.9 g/dL, whereas a maximum of 21 patients had values as low as 7–7.9 g/dL. The Hb levels in the 15 patients with stage 2 CKD were as low as 7–7.9 g/dL, while in 5 patients, they were as high as 10–10.9 g/dL. Hb levels in 12 individuals with stage 3 illness ranged from 7 to 7.9 g/dL to 10 to 10.9 g/dL in 2 cases. Finally, stage 4 patients have extremely low Hb levels of 7–7.9 g/dL. No patients exhibited Hb levels of 10–10.9 g/dL (Table 3).

Table 1. Analysis of drugs class based on ATC classification*

Class No.	Drugs class (Abbreviation of ATC class)	Total no. of drugs prescribed (%)
A	alimentary tract and metabolism	452 (43.67)
B	blood disorders and blood forming organs	218 (20.12)
C	cardiovascular system	226 (21.85)
D	dermatological drugs	3 (0.21)
G	genitourinary system and sex hormones.	2 (0.14)
J	ant infectious drugs for systemic use	9 (1.02)
L	immunomodulating agents	1 (0.09)
M	drugs for musculoskeletal system	10 (1.09)
N	drugs acting on nervous system	5 (0.56)
P	drugs against parasites and insecticides	0
R	drugs for respiratory system	2 (0.26)
S	drugs for eye, ear	0
V	vitamin and mineral supplements	209 (18.42)

* ATC – Anatomic therapeutic chemical

Table 2. CKD stage wise distribution based on eGFR values

S. No.	Stages	n=132	%
1.	1 (90 mL/min)	61	46.2
2.	2 (60–89 mL/min)	37	28.1
3.	3 (30–59 mL/min)	26	19.6
4.	4 (15–29 mL/min)	8	6.1
5.	5 (15 mL/min)	0	0

Table 3 Association between hemoglobin and stages of CKD (n=132)

S. No.	Hb levels (g/dL)	1	2	3	4
1.	7–7.9	21	15	12	4
2.	8–8.9	17	11	7	3
3.	9–9.9	15	6	3	1
4.	10–10.9	8	5	2	0

Hypertension was the most common risk factor, occurring in 41.6% of men and 37.2% of women. Following this was age: 20.2% of men and 32.6% of women; diabetes: 10.1% of men and 4.6% of women; a family history of the condition: 11.3% of men and 9.3% of women; drug abuse: 14.6 of men and 16.4% of women; and trauma: 2.2 and 0% of women. The mean and standard deviation for all patients in the study are shown in Table 4, which illustrates how the SF-12 health survey correlates with patients' Hb levels. The treatment period for darbepoetin alfa (40 mcg per kg) and epoetin beta-methoxy polyethylene glycol (0.6 mcg per kg) is used to treat anaemia. The study found that mental health (MCS) was significantly better than physical health (PCS) in both groups. Women had higher mean scores on the SF-12 mental health survey compared to men, suggesting they may have better overall mental well-being despite higher drug abuse rates. Men showed slightly higher mean scores on the SF-12 physical health survey, suggesting they may have better physical health outcomes. These findings highlight the complex relationship between gender, health, and risk factors associated with the condition. However, some cultural contexts, where gender roles and expectations heavily influence health outcomes, may result in lower scores for women compared to men. Individual choices and behaviours, as well as genetics and socioeconomic status, also play a significant role in health outcomes.

Prevalence

A study of 150 patients with CKD found that 88% had anaemia, while 12% did not. The study included 89 males and 43 females, with 128 individuals with Hb values between 7 and 9.9 g/dL. Age-related loss of renal function increases the number of CKD patients experiencing anaemia. Men have higher prevalence rates of the condition, possibly due to occupational hazards, lifestyle choices, and biological differences. Cultural norms and expectations may also contribute to underreporting or delayed diagnosis among women. Understanding the prevalence of the condition across different genders is crucial for addressing the specific needs and challenges faced by each group.

Health-related quality of life (HRQoL)

The mean and standard deviation (SD) were calculated using the SF-12 Questionnaire form. In contrast to feeling depressed, which had a high mean of 4.588 and a low SD of 0.8314, performing many tasks (such as moving a table or lifting any weight) had low mean and SD values of 1.039 and 0.2260, respectively, and had a significant impact on anaemia in CKD patients (Tables 5 and 6). Anaemia in CKD patients, particularly among different genders, leads to decreased energy levels, fatigue, and poor well-being, affecting daily activities and overall health. Addressing anaemia is crucial for improving HRQoL and overall health outcomes.

Table 4. Distribution based on risk factors among patients

S. No.	Risk factors	Male (n=89)	%	Female (n=43)	%
1.	Hypertension	37	41.6%	16	37.2
2.	Age	18	20.2%	14	32.6
3.	Diabetes	9	10.1%	02	4.6
4.	Family history	10	11.3%	04	9.3
5.	Drug abuse	13	14.6%	07	16.3
6.	Trauma/Accident	2	2.2%	0	0

Table 5. Health-related quality of life by SF-12 survey

S. No	Characteristics	Mean	Standard deviation
1.	Physical functioning	2.83	0.4102
2.	Role physical	1.516	0.6188
3.	Role emotional	1.039	0.226
4.	Mental health	1.052	0.4102
5.	Bodily pain	1.156	0.5515
6.	General health	1.13	0.4959
7.	Vitality	1.196	0.3983
8.	Social functioning	2.869	0.6948

Table 6. The association of SF-12 health survey with Hb levels

Overall Survey Questions	Hb 7–9.9 g/dL	Hb 10–10.9g/dL	p
Physical functioning	49.07±37.92	51.89±37.08	0.603
Role physical	47.22±29.5	47.35±32.31	0.977
Role emotional	51.62±30.52	53.01±33.32	0.772
Mental health	65.74±25.14	57.72±26.97	0.04
Bodily pain	50.46±34.16	58.46±37.24	0.137
General health	37.50±26.93	29.12±25.6	0.026
Vitality	53.70±30.48	50.06±34.5	0.464
Social functioning	44.90±31.98	45.79±37.71	0.869
PCS score	36.79±10.72	38.05±10.49	0.412
MCS score	40.25±11.06	39.53±10.45	0.213

* PCS – physical component summary; MCS – mental component summary

Discussion

The study analysed the gender distribution and mean age of CKD patients, who received an average of 7.21 prescriptions. Anaemia in CKD patients significantly impacts their daily activities and overall health, leading to decreased energy levels and fatigue.[19-21] This condition negatively affects well-being, emphasising the need for effective management and treatment to improve HRQoL and overall health outcomes. Darbepoetin Alfa, administered weekly, has been found to effectively raise blood sugar levels in CKD patients with anaemia.[22] This treatment leads to improved energy levels, reduced fatigue, and overall well-being. It also reduces the need for costly blood transfusions, which can be risky and costly. Darbepoetin Alfa not only increases Hb levels but also improves the quality of life for CKD patients, reducing symptoms like shortness of breath, dizziness, and difficulty concentrating. Additionally, studies have shown that Darbepoetin Alfa can help improve cardiovascular health in CKD patients by reducing the risk of heart failure and improving exercise capacity.[23] Furthermore, this treatment has been found to be well tolerated with minimal side effects, making it a viable option for long-term management of anaemia in CKD patients. This improvement in symptom management enhances daily functioning and productivity while minimising complications like infections and transfusion reactions, improving patient safety and healthcare costs.[24]

Epoetin beta-methoxy polyethylene glycol is a medication that has been shown to increase blood sugar levels in CKD patients with anaemia. Studies show that it stimulates red blood cell production, leading to improved blood sugar levels and alleviating anaemia symptoms. This medication is well-tolerated and safe, making it a viable treatment option for CKD patients.[25] It also improves exercise capacity and reduces the need for blood transfusions in CKD patients. Epoetin beta-methoxy polyethylene glycol mimics the action of erythropoietin, a hormone responsible for regulating red blood cell production. This leads to improved oxygen delivery to tissues and organs, improved energy levels, and reduced fatigue.[26] Polypharmacy is defined as the simultaneous use of five or more prescriptions for the same patient. The study found a significant correlation between the severity of anaemia and the degree of physical limitations experienced by CKD patients. Patients with more severe anaemia reported greater difficulty performing daily tasks, such as climbing stairs or walking short distances.[27] This underscores the need for effective management and treatment of anaemia to enhance the quality of life and overall functioning of these patients. Interventions aimed at improving haemoglobin levels and addressing anaemia-related symptoms may have a profound impact on the well-being and functional abilities of CKD patients.[28] The study included only prescribed drugs, but over-the-counter pharmaceutical use is common in the country, resulting in drug interactions and negative drug responses. Encouraging the use of generic medications can help reduce healthcare costs and promote better patient outcomes.[29] Healthcare providers should educate CKD patients about potential drug interactions and the importance of discussing all medications, including over-the-counter drugs, with their healthcare team.[30] Additionally, healthcare providers should also emphasise the importance of regular medication reviews to identify and address any potential drug interactions or adverse reactions. This proactive approach can further enhance patient safety and optimise treatment outcomes.[31] The study discovered that hypertension was the most frequent risk factor, followed by age, diabetes, family history, drug use, and trauma.[32] This finding is comparable to a study by Lori et al., which found that coronary artery disease, diabetes, and anaemia were the most common risk factors. However, 84% of CKD patients had hypertension, compared to 80% who had anaemia in the Strauss et al. study.[33] The study showed a correlation between Hb levels and the SF-12 health survey, with those with levels between 7 and 9.9 g/dL and 10 to 10.9 g/dL having significantly higher MCS scores than PCS scores. The majority of studies demonstrate that chronic illnesses – not just renal disease – hurt poor physical and mental health. Nephrologists should pay special attention to reducing the disease's progression, as the condition has a major impact on HRQoL.[34] The study highlights the need for comprehensive interventions that address both physical and mental well-being to improve overall HRQoL in CKD patients. These interventions could include a combination of medical treatments, lifestyle modifications, and psychological support. By addressing both the physical and mental aspects of CKD, patients may experience improved quality of life and better overall health outcomes.[35] Healthcare providers should consider implementing multidisciplinary care teams to

provide holistic care for CKD patients. These care teams could consist of nephrologists, dietitians, mental health professionals, and social workers who work together to address the various needs of CKD patients. By taking a multidisciplinary approach, healthcare providers can ensure that patients receive comprehensive and coordinated care, leading to better management of their condition and improved HRQoL.[36] Furthermore, patient education and empowerment are crucial in promoting self-management and adherence to treatment plans, which can positively impact both physical and mental well-being in CKD patients. Collaboration between healthcare professionals and social workers plays a vital role in supporting CKD patients.[37] They provide essential resources and support services to help patients navigate the challenges of living with a chronic illness. Social workers can assist with financial counselling, connecting patients with community resources, and addressing any psychosocial issues that may arise.[38] Their involvement in the multidisciplinary team ensures that patients receive holistic care that addresses all aspects of their well-being. Moreover, patient education and empowerment are key components of improving the overall quality of life for CKD patients. By providing patients with knowledge about their condition, treatment.[39]

Study limitations

The study's cross-sectional design, lack of follow-up, and quantitative data may have hindered the identification of patients' poor quality of life and its underlying causes. In-depth interviews or focus groups could have better highlighted the causes of poor quality of life. The study's small sample size and self-reported HRQoL measures may introduce bias, and it did not consider potential confounding factors like socioeconomic status or comorbidities. Further research with larger sample sizes and more comprehensive assessments is needed to fully understand the impact of CKD on HRQoL.

Conclusion

In conclusion, while this study provided valuable insights into the relationship between chronic kidney disease and quality of life, there are limitations that need to be addressed in future research. By conducting in-depth interviews or focus groups, researchers can gain a more nuanced understanding of the underlying causes of the poor quality of life associated with CKD. Epoetin beta-methoxy polyethylene glycol and Darbepoetin Alfa are the drugs of choice for the treatment of anaemia in CKD patients. Additionally, it is crucial to consider potential confounding factors such as socioeconomic status and comorbidities to obtain a more accurate picture of the impact of CKD on HRQoL. To ensure a comprehensive understanding, future studies should aim for larger sample sizes and utilise more comprehensive assessments to provide a clearer understanding of the complex relationship between CKD and quality of life.

Declarations

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Author contributions

Conceptualization, A.S. and K.E.; Methodology, H.W.; Software, S.S.; Validation, S.A., S.S. and A.S.; Formal Analysis, H.W.; Investigation, A.S.; Resources, S.A.; Data Curation, S.S.; Writing – Original Draft Preparation, S.S.; Writing – Review & Editing, S.A.; Visualization, K.E.; Supervision, H.W.; Project Administration, K.E.; Funding Acquisition, S.A.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data available on request from the authors.

Ethical approval

The Raghavendra Institute of Pharmaceutical Education and Research Institution Review Board gave its approval for this study, and it also obtained data usage permission. The study period was November 2022 to April 2023 (date of approval: October 22, 2022, RIPER/IRB/2022/045).

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ORIGINAL PAPER

Fall risk and frailty level in older adults admitted to the emergency department with a complaint of falling

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ABSTRACT

Introduction and aim. Falls are the second leading cause of unintentional death in the world. The study was conducted to examine the risk of falls and levels of frailty in older adult patients admitted to the emergency department due to fractures, as well as to identify the factors that influence fall risk and frailty levels.

Material and methods. This cross-sectional and correlational study conducted with 155 older patients. Data collected by the patient information form, Itaki Fall Risk Scale and Edmonton Frail Scale.

Results. Patients diagnosed with fracture in the emergency department had a high risk of falling with a mean score of 9.55±3.84.70.3% of the patients were frail. The one-third (30.3%) had severe frailty. There was a moderate positive correlation between the risk of falling and the mean frailty score of the older adult patients (p<0.001).

Conclusion. The study showed that older adults admitted to the emergency department due to falls are at high risk of falling and the majority of them are frail. Early determination of fall risk and frailty levels in the older adults with a history of falling, prevention of falls and fractures due to falls will be beneficial in increasing the quality of life of the older adults.

Keywords. emergency department, fracture, frailty, older adults, risk of falling

Introduction

A fall is defined as an event that results in undesirable movement of a person on the ground or other lower level. Falls are the second leading cause of unintentional death in the world. The age range with the highest number of fatal falls is adults aged 60 and over. The World Health Organization states that the number and proportion of individuals aged 60 and over in the population has increased.[1] According to statistical data, one in six people in the world is expected to be 60 years or older by 2030. It is estimated that the world population aged 60 and over will double by 2050 (2.1 billion). The number of people aged 80 and over is expected to reach 426 million between 2020 and 2050.[2] In Turkey, according to the data of the Turkish Statistical Institute, the proportion of the older population in the total population has increased to 9.5% in 2020. In 2020, 44.2% of the older population is male and 55.8% is female. According to population data, it is estimated that the proportion of the older population will reach 11% in 2025, 12.9% in 2030, and 16.3% in 2040.[3]

Prolonged life expectancy is increasing significantly from year to year due to falls.[4] In a meta-analysis study, it reported as 26.5% the prevalence of fall among the older adults in the world.[5] Falls in the older adults constitute approximately 10% of admissions to emergency department and 6% of hospitalizations from emergency department.[6] Centers for Diseases Control and Prevention reported that every year 3 million older adults treated in emergency rooms due to fall injuries.[7] Erdem

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and Atay stated that 95.5% of older patients in Turkey have a high risk of falling.[8] In a different study conducted in Turkey, it was determined that 19.5% of older patients applied to the emergency department due to falls, and 31.7% of them had a fall history.[9]

It is emphasized that the risk of falling is high in the older population with low education level, unemployed, using multiple drugs, malnourished, living in urban areas, consuming cigarettes and alcohol in developed countries. In addition, comorbidities such as heart disease, hypertension, diabetes, stroke, frailty, previous history of falls, depression, Parkinson's disease and pain have been shown to increase the risk of falling.[10-16] It has been determined that the older adults in Turkey also experience falls for similar reasons.[17-21]

All studies conducted in previous year show that falls will continue as an important problem among the older adults in our country and in the world. Therefore, it is important to identify of falling risk with sensitive, specific and reproducible tools on the older adults.[22] In a study conducted in the emergency department, it emphasized that the need to determine fall risk and determine appropriate fall risk assessment tools continues.[23] In addition, a limited number of studies evaluating the fall risk and frailty of patients who applied to the emergency department with a fall found.

Aim

The study was conducted to examine the risk of falls and levels of frailty in older adult patients admitted to the emergency department due to fractures, as well as to identify the factors that influence fall risk and frailty levels.

Research questions

- What is the risk of falling in older adult patients admitted to the emergency department and diagnosed with a fracture?
- What is the frailty levels in older adult patients admitted to the emergency department and diagnosed with a fracture?
- What are the factors affecting the risk of falling and frailty levels in older adult patients admitted to the emergency department and diagnosed with a fracture?
- Is there a relationship between the risk of falling and frailty levels of older adult patients admitted to the emergency department and diagnosed with a fracture?

Material and methods

Ethical approval

Firstly, ethical approval was obtained from the Social and Human Sciences Ethics Committee of the University (decision date: 07.01.2022, approval number: 21). Subsequently, written permission was obtained from the health directorate. Informed consent was obtained from the patients. Throughout the research, adherence to the Helsinki Declaration was maintained.

Design and sample

The population for this cross-sectional and correlational study comprised 258 patients aged 60 and over who were admitted to the orthopedic service and emergency department with fractures in a state hospital in Turkey between January 1 and December 31, 2020. The sample size was determined as 155 patients using the known population sample formula (n=258, t=1.96, p=0.5, q=0.5, d=0.05).

The inclusion criteria for the study were as follows: i) willingness to participate, ii) age over 60 years, iii) presentation to the emergency department with a complaint of falling and subsequently being admitted to the orthopedic service due to fracture, iv) ability to speak and understand Turkish, and v) conscious state. Patients who were under the age of 60 (n=3179), who did not volunteer to participate (n=6), who did not experience fractures after falling (n=1880), and who were not admitted to the orthopedic service (n=42) were excluded from the study.

Data collection

Data for the study were collected between March 2022 and January 2023 using three instruments: the patient information form, the Itaki Fall Risk Scale, and the Edmonton Frail Scale. The data collection process involved face-to-face interviews with the participants.

Patient Information Form

This form consists of 23 questions evaluating the sociodemographic characteristics and clinical status of the older adults.

Itaki Fall Risk Scale

It is a scale developed by the Republic of Turkiye Ministry of Health to evaluate the fall risks of adult patients in health institutions in our country. The Itaki Fall Risk Scale used to question 19 risk factors (8 items major risk factors, 11 items minor risk factors) that cause patient falls. Minor risk factors scored with 1 point and major risk factors with 5 points. A score of less than 5 points considered low risk and 5 points or over considered high risk.[24]

Edmonton Frail Scale

The scale developed by Rolfson et al. in 2006. Turkish reliability and validity were done by Aygör et al.[25] The scale consists of 11 items. A minimum of 0 and a maximum of 17 points can be obtained from the scale. There are areas of vulnerability, item section consisting

of questions and scoring areas according to the answers to be given between 0 and 2 points. According to the answers received, 0-4 is not fragile, 5-6 is apparently vulnerable, 7-8 is mildly fragile, 9-10 is moderately fragile, 11 and above is severely fragile. The Cronbach alpha value of the Turkish version of the scale is 0.75.[25] The cronbach's alpha was determined as 0.78 in this study.

Data analysis

Data evaluated by entering the SPSS 22.0 package program (IBM, Armonk, NY, USA). The normally distribution analyzed with the Shapiro Wilk test. Data evaluated with chi-square test, Mann Whitney U test, Kruskal Wallis test and Spearman Correlation analysis as well as descriptive statistical analysis methods. The significance was evaluated at the $p<0.05$ level.

Table 1. Profile of older adult patients (n=155)[a]

		Mean (SD)
Age		75.85 (9.22)
		n (%)
Gender	Female	109 (70.3)
	Male	46 (29.7)
Employment status	Working	2 (1.3)
	Not working	153 (98.7)
Residential location	Village	82 (52.9)
	Town	35 (22.6)
	City	38 (24.5)
People they live with	Alone	19 (12.3)
	Family	134 (86.5)
	Nursing home	1 (0.6)
	Caregiver	1 (0.6)
Assistive device use	Yes	77 (49.7)
	No	78 (50.3)
Chronic disease*	Absent	29 (18.7)
	Diabetes mellitus	62 (40)
	Hypertension	93 (60)
	Heart disease	46 (29.7)
	COPD	19 (12.3)
	Osteoporosis	14 (9.0)
Co-morbidity*	Absent	95 (61.3)
	Cerebrovascular disease	17 (11)
	Alzheimer's	20 (12.9)
	Parkinson's	4 (2.6)
	Loss of physical activity	26 (16.8)
	Psychiatric illness	14 (9)
Continuous drug use	No	24 (15.5)
	One drug	16 (10.3)
	Two drug	27 (17.4)
	Three and more	88 (56.8)
History of fall	Yes	116 (74.8)
	No	39 (25.2)
History of falling in the last 6 months	Yes	51 (32.9)
	No	104 (67.1)
Fracture after falling in the last 6 months	Yes	31 (26.7)
	No	85 (73.3)

[a] COPD – chronic obstructive pulmonary disease; SD – standard deviation; * – more than one answer was given, the percentages were taken according to the number n (155)

Results

Table 1 presents the findings related to the demographic characteristics and previous health history of the patients. According to the table, the average age of the patients was 75.85 (SD=9.22) years, with 70.3% being female and 98.7% being non-working. Among the patients, 52.9% resided in rural areas, and 86.5% lived with their families. Around 81.7% of the patients had chronic diseases, with 49.7% of them using assistive device. Approximately 85.5% of the patients were on continuous medication, and 56.8% of them were taking three or more drugs. Furthermore, 74.8% of the patients had a history of falling, with 32.9% experiencing a fall in the last 6 months, and 26.7% of those falls resulting in fractures (Table 1).

Among the older adult patients admitted to the emergency department, 80% of them had femur fractures and 9.7% had arm fractures. Additionally, it was found that 3.6% of the patients experienced multiple fractures. In Table 2, it is shown that 59.4% of the patients fell at home, 32.3% while walking, and 40.6% of the falls occurred at noon (Table 2).

Table 2. Fracture types and characteristics of falls on the older adult patients (n=155)

	n (%)
Fracture types	
Femur	124 (80)
Femur and pelvis	1 (0.6)
Spine and arm	1 (0.6)
Pelvis and spine	1 (0.6)
Pelvis	3 (1.9)
Arm	15 (9.7)
Ankle	6 (3.9)
Wrist	1 (0.6)
Wrist and femur	1 (0.6)
Arm and wrist	1 (0.6)
Spine and ankle	1 (0.6)
Place of fall	
Indoor	92 (59.4)
Outside of home	63 (40.6)
Circumstances for falling	
While turning in bed	16 (10.3)
Getting up from the toilet	25 (16.1)
While performing ablution	7 (4.5)
Getting up	15 (9.7)
Working in the garden	23 (14.8)
While bathing	8 (5.2)
When using stairs	11 (7.1)
While walking	50 (32.3)
Falling Time	
Morning	39 (25.2)
Noon	63 (40.6)
Evening	37 (23.9)
Night	16 (10.3)

The older adults patients in the study exhibited a high risk of falling, with a mean score of 9.55 (SD=3.84). This high risk was observed in 91.6% of the older adults. Furthermore, the older patients had mild frailty, with an average score of 8.51 (SD=3.39). However, 70.3% of

the older adults patients were classified as frail, and one-third (30.3%) had severe frailty. There was a moderate positive correlation between the risk of falling and the average frailty score of the older patients (p<0.001) (Table 3).

Table 3. Fall risk and frailty level in the older adult patients (n=155)[a]

		Mean (SD)	Min–Max
İtaki Fall Risk Scale		9.55 (3.84)	1–20
Edmonton Frail Scale		8.51 (3.39)	1–16
rho p		0.468 <0.001*	
		n	%
Fall Risk	Low	13	8.4
	High	142	91.6
Frailty level	Not fragile	25	16.1
	Seemingly defenseless	21	13.5
	Slightly fragile	26	16.8
	Medium fragile	36	23.2
	Severely fragile	47	30.3

[a] SD – standard deviation; Min – minimum; Max – maximum; * – p<0.001

The mean scores of the Itaki Fall Risk Scale and Edmonton Frail Scale were found to be significantly higher (p<0.05) in patients who used assistive devices, had a chronic disease, had a concomitant disease, and were consistently taking medication. Furthermore, the mean scores of the Edmonton Frail Scale were significantly higher (p<0.05) in patients who were not working and had a history of falling in the last 6 months (Table 4).

In Table 4, it was observed that there was a weak negative correlation between the mean fall risk score of the patients and hemoglobin levels, as well as a weak positive correlation between the mean frailty score and the INR value (p<0.05). Additionally, a moderate negative correlation was found between T3 levels and the mean scores of fall risk and frailty (p<0.05) (Table 4).

Table 4. Comparison of the profile of the older adult patients and their fall risk and frailty level (n=155)[a]

		Itaki Fall Risk Scale Mean (SD)	Edmonton Frail Scale Mean (SD)
Gender	Female	9.83 (3.81)	8.49 (3.15)
	Male	8.87 (3.87)	8.57 (3.93)
		Z=-1.494 p=0.135	Z=-0.112 p=0.911
Employment status	Working	7 (0.00)	3.50 (0.70)
	Not working	9.58 (3.38)	8.58 (3.36)
		Z=0.161 p=0.188	Z=-2.037 p=0.03*
Residential location	Village	9.29 (4.06)	8.48 (3.28)
	Town	8.97 (3.49)	7.86 (3.69)
	City	10.63 (3.53)	9.18 (3.31)
		c^2=4.419 p=0.110	c^2=3.010 p=0.222
Assistive device use	Yes	10.44 (3.61)	9.73 (2.75)
	No	8.67 (3.88)	7.31 (3.55)
		Z=-3.045 p=0.002*	Z=-4.471 p<0.001*
Chronic disease	Yes	10.50 (3.12)	8.80 (3.32)
	No	5.41 (4.00)	7.24 (3.47)
		Z=-6.364 p<0.001*	Z=-2.151 p=0.031*
Co-morbidity	Yes	10.58 (3.67)	10.58 (2.54)
	No	8.89 (3.82)	7.20 (3.21)
		Z=-2.808 p=0.005*	Z=-6.228 p<0.001*
Continuous drug use	Yes	10.51 (3.21)	8.87 (3.32)
	No	4.29 (2.59)	6.54 (3.17)
		Z=-6.961 p<0.001*	Z=-2.984 p=0.003*
History of falls	Yes	9.77 (3.67)	8.82 (3.34)
	No	8.90 (4.30)	7.59 (3.41)
		Z=-1.571 p=0.116	Z=-1.798 p=0.072
History of falling in the last 6 months	Yes	10.20 (3.53)	9.82 (3.01)
	No	9.23 (3.96)	7.87 (3.39)
		Z=-1.474 p=0.141	Z=-3.347 p=0.001*
Fracture after falling in the last 6 months	Yes	10.06 (3.62)	8.90 (3.03)
	No	9.66 (3.71)	8.79 (3.46)
		Z=-0.888 p=0.375	Z=-0.003 p=0.998
		rho p	rho p
Laboratory results in the emergency department	Hemoglobin	-1.179 0.026*	-0.184 0.022
	Glucose	0.093 0.249	-0.023 0.775
	Sodium	0.006 0.945	-0.001 0.988
	Potassium	-0.056 0.49	-0.052 0.519
	INR	0.084 0.297	0.230 0.004*
	T3	-0.334 <0.0001*	-0.237 0.003*
	T4	0.016 0.844	0.130 0.106
	TSH	-0.059 0.467	-0.040 0.625

[a] SD – standard deviation; INR – international normalized ratio; T3 – triiodothyronine; T4 – thyroxine
Z – Mann Whitney U test; rho – Spearman correlation; * – p<005

Discussion

In this study, we investigated the risk of falls and levels of frailty in older adult patients who were admitted to the emergency department due to fractures. Additionally, we examined to identify the factors that influence fall risk and frailty levels in this population. The results revealed that the majority of the patients experienced a femoral fracture as a result of a fall, followed by fractures of the arm. Furthermore, it was observed that some older adult patients had multiple fractures. Falls were more frequent in the home environment, during walking, and at noon. Our study findings regarding fracture type and fall environment were consistent with previous research, except for the timing of falls. In a report published by the Australian Institute of Health and Welfare, it was stated that femur fractures (73%) are the most common type of injury resulting from falls in older adults. In the same report, it was highlighted that the most common causes of falls in older adults are slipping, tripping, and stumbling while walking.[26] Another study found that the most common cause of injury in older adults was falling from a level (55.2%), with the pelvis and lower extremities being the most frequently affected areas (43.7%).[27] Pierre et al. also reported that the leading cause of fractures among all older patients presenting with a complaint of falling was falling during movement (82.8%).[28]

It has been demonstrated that hip and femur fractures are the predominant causes of hospitalization due

to fall-related injuries in Sweden.[29] However, there are varying results in the literature regarding the location and timing of falls. One study reported that over half of the falls took place at home and during daytime hours.[30] In another study, it was reported that falls occurred at night and the reasons behind them were unknown.[31] Choi et al. also found that more than half of the falls occurred within the individual's home, with 60% of them being attributed to slipping or tripping over objects. They further identified a significant association between admission to the emergency department and the diagnosis of fracture in these patients (RR = 3.59, 95%CI = 2.58–5).[32]

In this study, it was found that older adult patients diagnosed with fractures in the emergency department had a high risk of falling. Furthermore, there was a significant association between an increased risk of falling and higher levels of frailty (p<0.001). In the literature, various fall risk assessment tools such as the Morse Fall Risk Scale, Hendrich II Fall Risk Scale, and STRATIFY Fall Risk Scale have been utilized to assess the fall risk in older adult patients. In our country, the Itaki Fall Risk Scale, developed by the Ministry of Health, is extensively employed in health institutions to assess the fall risks of older adult patients.[24] Several studies conducted in our country utilizing the Itaki Fall Risk Scale have consistently demonstrated that older adults are at a high risk of falling.[33-37] The results of our study are in line with these previous studies conducted in our country, further supporting the notion of a high fall risk among older adults.

Frailty is indeed a crucial aspect of older adult care and has significant implications for hospitalization and health outcomes. Numerous studies have demonstrated the association between frailty and adverse health outcomes.[14,38,39] It underscores the importance of recognizing and addressing frailty as a key consideration in the healthcare management of older adults. In our study, the patients exhibited mild frailty based on the total score of the Edmonton Frail Scale. However, it is noteworthy that the majority of the patients were classified as frail, and approximately one-third of them were identified as severely frail. These results highlight the significant prevalence and severity of frailty among the older adult population included in our study.

A study utilizing the Edmonton Frail Scale revealed that 51% of older patients who were admitted to the hospital with a femoral neck fracture exhibited a high frailty score.[40] Furthermore, another study reported that one-third of the patients included in their sample were identified as frail, highlighting the association between frailty and increased risk of falls and fractures in older adults.[41] Jankowska-Polańska et al. highlighted in their study that a significant proportion (41.9%) of individuals aged over 65 years exhibit frailty.[42] Moreover, a systematic review and meta-analysis of 29 prospective studies demonstrated that frailty serves as a significant risk factor for falls and the development of bone fractures.[43] These results underscore the need for early assessment of frailty in order to prevent falls and enhance the quality of life for older adults.[38]

In our study, it was observed that patients who used assistive devices, had a chronic disease, had a concomitant disease, and were on constant medication had higher levels of both fall risk and frailty (p<0.05). Furthermore, it was found that patients who were unemployed and had a history of falling in the last 6 months had significantly higher levels of frailty (p<0.05). In the literature, several studies have reported a statistically significant relationship between various factors and fall risk, including advanced age, chronic disease, need for physical support while standing/walking, urinary/fecal disorders, use of more than four drugs, high-risk drug use in the last week, and environmental factors.[32,35,43-47] A study reported that frail individuals had a higher risk of falling in older adults, and this association was statistically significant (p<0.05).[48] According to our results, 32.9% of the patients experienced a fall in the last 6 months. Moreover, our study revealed that patients with a history of falls in the last six months had significantly higher mean scores on the Edmonton Frail Scale. This suggests that the occurrence of recurrent falls in older adult patients should be carefully assessed and monitored. Indeed, recurrent falls in older adults can serve as an indicator of underlying frailty.[49] As supported by previous research, the likelihood of experiencing another fall is 33-51% higher in older adults exhibiting signs of frailty.[50] Sri-On conducted a follow-up study on 350 older patients who were initially admitted to the emergency department due to a fall. The study revealed that within the subsequent 6 months, a significant number of patients (43%) experienced another fall, leading to 31% of them seeking re-admission to the emergency department and 12% requiring hospitalization.[51] Similarly, Schaap et al. reported that out of 498 older patients, 26.1% experienced recurrent falls, with 12% of these falls resulting in fractures.[52] These findings highlight the concerning nature of recurrent falls among older adults and the potential for subsequent healthcare utilization and serious injuries.

In our present study, we observed that low hemoglobin levels in older adults were associated with an elevated risk of falling. It is worth noting that low hemoglobin levels are a prevalent health issue among older individuals and are linked to risk factors for fractures, including reduced physical function and decreased bone mass. A decline in hemoglobin levels can lead to symptoms such as fatigue, dizziness, and weakness. These symptoms can indeed contribute to an increased risk of falling and are associated with a significant elevation in fracture risk.[53,54]

A study conducted among the general patient population have demonstrated that low hemoglobin levels (p=0.003) and a history of falls within the previous year (p<0.001) are independent factors influencing the severity of falls and fall-related injuries.[55] It has been reported that approximately 40% of all hip fractures, including femoral neck, intertrochanteric, or subtrochanteric fractures, occur in patients with hemoglobin levels below 12 g/dL at the time of hospitalization. Additionally, a significant decrease in hemoglobin levels is observed in extracapsular fractures, particularly in the days preceding surgery.[56] Another study emphasized that older patients with a hemoglobin level below 11.55 g/dL, who presented to the emergency department after a fall, had a higher mortality rate. The risk of death was found to increase by 5.488 times within the first two months.[57]

Frailty is a prevalent biological syndrome observed in older adults, and it is known that thyroid functions are associated with the aging process. Thyroid hormones play a crucial role in various physiological processes, including skeletal muscle development, tissue formation, growth, differentiation, and metabolism. Triiodothyronine (T3), in particular, stimulates the myosin heavy chain of fast-twitch muscle fibers, leading to increased mitochondrial activity and an improved relaxation-contraction ratio.[58,59] These effects contribute to the overall functionality and performance of the musculoskeletal system. In our study, we observed a moderate negative correlation between T3 levels and the mean scores of fall risk and frailty, indicating that lower T3 levels were associated with higher levels of fall risk and frailty in the older adults (p<0.05). However, we did not find a significant correlation between T4 levels and fall risk or frailty levels (p>0.05). These findings differ from previous studies, suggesting that the relationship between thyroid hormone levels and fall risk/frailty may vary across different populations or study settings. Indeed, the study by Bano et al. reported that higher T4 levels in older patients were associated with an increased risk of becoming more fragile over time.[60] This suggests that T4 levels may play a role in the progression of frailty in older adults. Additionally, several other studies have found a significant relationship between T3 levels and frailty, further supporting the notion that thyroid hormone levels, particularly T3, may be associated with the development and progression of frailty in the older population.[59,61,62] These findings highlight the potential importance of thyroid function in relation to frailty and call for further investigations to better understand the underlying mechanisms and clinical implications of these associations.

In our study, a significant relationship was observed between the International Normalized Ratio (INR) values measured in the emergency department and the frailty levels of the patients. This finding suggests that as the INR level increases, the frailty levels also tend to increase in older adults. Previous studies have also indicated a link between high frailty levels and the use of anticoagulants in older adults. The uncontrolled use of anticoagulants can lead to an elevation in the INR level.[63-65] These findings are consistent with the existing literature, although it should be noted that there is a limited number of studies available on this topic. Further research is needed to explore the relationship between INR levels, anticoagulant use, and frailty in older adults, in order to better understand the underlying mechanisms and implications of these associations.

Conclusion

In conclusion, this study revealed that older adults presenting to the emergency department after falling are at a high risk of future falls and frailty. The majority of these patients were found to be frail, and femoral fractures were the most common type of injury resulting from falls. Several factors, including the use of assistive devices, chronic diseases, comorbidities, and continuous medication use, were identified as risk factors for frailty and fall risk in this population.

Based on these findings, it is recommended to assess fall risk and frailty levels in older adults at an early stage to prevent falls and fall-related fractures. This proactive approach can contribute to improving the independence and overall quality of life for older adults. Furthermore, it is crucial to provide education and training to older adults, healthcare professionals, family members, and caregivers on fall prevention strategies and frailty management. Increasing public awareness through public service announcements can also play a significant role in promoting the health and well-being of older adults.

Declarations

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Author contributions

Conceptualization, S.Ç.; Methodology, S.Ç.; Software, S.Ç. and E.K..; Validation, S.Ç; Formal Analysis, S.Ç. and E.K.; Investigation, N.U; Resources, S.Ç. and N.U; Writing – Original Draft Preparation, S.Ç., N.U.,E.K. and İ.D; Visualization, S.Ç., N.U. and İ.D; Supervision, S.Ç.; Project Administration, S.Ç. and N.U; Funding Acquisition, N.U

Conflicts of interest

The author declares no conflicts of interest.

Data availability

Data will be made available on request.

Ethics approval

Ethical approval was obtained from the Social and Human Sciences Ethics Committee of the University (decision date: 07.01.2022, approval number: 21).

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ORIGINAL PAPER

The investigation of the impact of education on sexual health/reproductive health knowledge levels of nurses and midwives in extraordinary situations – evidence from Turkey

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ABSTRACT

Introduction and aim. In extraordinary situations, sexual/reproductive health services are very important for the health of the whole society, especially women and children. The aim of this study is to assess the impact of training provided on sexual health/reproductive health during extraordinary situations on the knowledge levels of nurses and midwives.

Material and methods. The research was conducted using a single-group pre-test-post-test follow-up test measurement quasi-experimental design. It was completed between November 2021–June 2022 with 140 participants working in a province in Turkey. The data were collected with the "Descriptive Information Form" and the "Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations". Training was given with the Sexual Health/Reproductive Health Training Booklet for Extraordinary Situations.

Results. It was found that the participants scored 20.82±4.47 on the Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations in the pre-test, 27.63±2.67 in the post-test, and 27.07±3.46 in the follow-up test. As a result of the training, it was determined that the difference between the scores they got from the Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations was due to the pre-test ($p<0.05$).

Conclusion. In the study, the participants scored above the average in the pre-test, while they achieved significantly higher scores in the post-test and follow-up test. This shows that the training given to nurses and midwives is effective.

Keywords. education, extraordinary, midwife, nurse, reproductive health, sexual health

Introduction

Extraordinary situations are defined as "the serious disruption of public health and order due to natural disasters, dangerous pandemics, severe economic crises, or widespread violent events".[1] Given the ongoing trends of population growth, heavy migration to urban centers, illegal and unregulated construction, unplanned urbanization, and industrialization, the likelihood of facing greater damages and losses in the face of potential natural events continues to increase every day. Turkey frequently encounters natural disasters due to its geographical and climatic conditions.[2] Following disasters, there is a need to address the health needs of survivors.[3] In this context, in addition to meeting basic needs such as nutrition, shelter, and security, Sexual Health/Reproductive Health (SH/RH) services are also a critically important and prioritized issue.[4] Reproductive health has historically received low priority in the hierarchy of humanitarian interventions. Awareness of reproductive health needs in emergencies began in the mid-1990s and led to the establishment of the Inter-Agency Working Group (IAWG) for reproductive health. Subsequently, a set of guidelines known as the Minimum Initial Services Package (MISP) was developed to ensure the provision of reproductive health services in crisis situations.[5] Ensuring comprehensive access to SH/RH services for the

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entire population affected by an extraordinary situation is one of the key objectives of the healthcare sector.[6]

MISP was designed by the IAWG in 1996 as a coordinated reproductive health service aimed at addressing the needs of the affected population, particularly women and girls in vulnerable groups, at the onset of extraordinary situations, ideally within the first 48 hours.[7,8] Despite being often overlooked in the aftermath of emergencies, MISP serves essential and lifesaving objectives. The key goals of MISP for reproductive health services in extraordinary situations include reducing maternal and neonatal mortality and morbidity, reducing/preventing sexually transmitted infections (STIs) and HIV transmission, preventing gender-based violence, and preventing unwanted pregnancies.[5,6,9] In addition, the 2030 Sustainable Development Goals target healthy and quality living, reducing maternal and neonatal mortality and morbidity, reducing HIV and STIs, and achieving gender equality, peace, and justice.[10] In this context, it is emphasized that urgent and significant steps are required to achieve the Sustainable Development Goals and leave no one behind.[11]

Research indicates that in the aftermath of extraordinary situations, there is an increase in complications related to childbirth and the postpartum period, and the likelihood of women and girls encountering violence, harassment, and abuse also rises.[8,12] Interventions for Sexual and Reproductive Health (SRH) for populations affected by extraordinary situations require specialized knowledge and skills. Therefore, the effectiveness of SRH service coordination in such situations relies on the strength of existing emergency preparedness mechanisms developed before extraordinary events.[6] The management of extraordinary situations is based on disaster management.[13] The concept of disaster management includes measures taken before disasters, preparedness, education, the phase of preventing the development of disasters, emergency assistance after disasters, recovery, and rehabilitation phases. The most crucial aspect of disaster management is ensuring preparedness before disasters occur and reducing/preventing the damage caused by disasters. Taking measures, providing education to individuals, and facilitating technological advancements to navigate extraordinary situations with minimal harm are fundamental principles in disaster prevention.[2,10,14,15]

Starting with Florence Nightingale's work during the Crimean War, nurses have actively played a role in the care of the sick and wounded in all extraordinary situations throughout history.[16] In our country, nurses and midwives primarily work alongside other healthcare professionals to meet the needs of women, children, and infants, who are the groups most affected by extraordinary situations. The fact that nursing and midwifery are patient-centered professions and that they possess knowledge and skills in epidemiology, psychology, communication, collaboration, and problem-solving processes enhances their effectiveness in disaster management.[17-19] The roles of nurses and midwives extend beyond saving lives during the immediate phase of a disaster and preserving the health of disaster survivors. Nurses and midwives have significant roles and responsibilities in all stages of disaster management, including the preparation of disaster plans before disasters occur and the implementation, evaluation, and updating of these plans.[18,20,21] In the pre-disaster phase, nurses and midwives should provide routine or needs-based education to individuals in the community and to those involved in disaster response efforts, aiming to minimize damage and reduce potential adverse outcomes.[17,21,22]

To date, limited research has been conducted in the literature assessing the implementation and delivery of the MISP within the context of various extraordinary situations. It has been emphasized that there is a need to increase research related to MISP.[8] Research conducted thus far has indicated that there is still a lack of knowledge and awareness among healthcare workers regarding MISP, and deficiencies have been reported in staffing, logistics, and coordination during the delivery of MISP services.[4,5,8,23] As a follow-up to the United Nations Population Fund's 2010 Global Planning Meeting recommendation on "Improving humanitarian response and emergency preparedness systems," a decision was made to expand the implementation of MISP in Eastern and Central Europe as well as in Asia. As part of this initiative, seven countries, including Turkey, were selected for the first round of MISP implementation. A total of 27 participants attended the MISP regional trainer training held in Istanbul from March 28 to April 1, 2011, representing these seven countries.[12] In Turkey, there is a lack of ongoing training on MISP, and it is believed that the educational needs of healthcare workers at all levels in this regard are not adequately met. No research has been found on this topic in our country, making this study the first research endeavor representing our nation. Through this research, we aim to fill the existing gaps in both the international literature and in Turkey, providing evidence and guiding future research by focusing on priority areas to improve the scope, quality, and accessibility of Sexual and Reproductive Health (SRH) services.

Aim

The aim of this study is to assess the impact of training provided on sexual health/reproductive health during extraordinary situations on the knowledge levels of nurses and midwives.

Research hypotheses

H0 – The training on sexual health/reproductive health during extraordinary situations has no effect on the knowledge levels of nurses and midwives.

H1 – The training on sexual health/reproductive health during extraordinary situations increases the knowledge levels of nurses and midwives.

Material and methods

Design

The research was conducted using a single-group pre-test-post-test follow-up test measurement quasi-experimental design.

Population, sample, and data collection

Nursing and midwifery are internationally recognized professions with distinct education, practice, and legal regulations. However, in Turkey, both nurses and midwives have duties, authority, and responsibilities in identifying and meeting the healthcare needs of women of reproductive age, infants, and children aged 0–6, despite their different education, practice, and legal frameworks.[19] Therefore, nurses and midwives constitute the population of this research. According to the data from the Bartin Provincial Health Directorate, a total of 170 nurses and midwives are working in the city center of Bartin, a city in the northwest of Turkey. A power analysis was conducted to determine the sample size for the research. The power of the test was calculated using the G*Power 3.1 program. The effect size was set at 0.15, which is considered moderate according to multiple regression analysis by Cohen (1988).[24] To achieve a power of more than 95%, it was determined that 107 participants would be required at a 5% significance level and with an effect size of 0.15 (df=2; F=3.086). The study was completed with 140 participants who met the inclusion criteria.

Inclusion criteria

The participants included in the study were nurses or midwives who were employed in healthcare institutions within the city center of Bartin during the period from November 2021 to June 2022 and who willingly consented to take part in the research.

Instruments

To collect data, the researchers used the 'Descriptive Information Form' and a 'Sexual Health and Reproductive Health Knowledge Assessment Form' developed based on the literature.

Descriptive information form

This form was prepared by the researchers based on the literature and comprises a total of 18 questions aimed at revealing the socio-demographic characteristics of the participants (such as age, gender, educational background, workplace, years of experience, etc.) and their levels of knowledge and competence in sexual health/reproductive health (including whether they have received training on sexual health/reproductive health, how confident they feel in their knowledge and skills in sexual health/reproductive health in extraordinary situations, etc.).[25-27]

Sexual health and reproductive health knowledge assessment form for extraordinary situations (SHRHKAFES)

The form was developed in accordance with the content of the Sexual Health/Reproductive Health Education Booklet for Extraordinary Situations. Initially, research objectives related to knowledge were defined by the researchers. Subsequently, a 31-item Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations was created.[4–9,28,29] Each item in the form were evaluated with the options 'correct,' 'incorrect,' and 'I don't know.' Scoring was done based on the number of correct answers for each item. 1 point was awarded for correctly answered questions, and 0 points for incorrect or 'I don't know' responses. Accordingly, the minimum score that could be obtained from the questionnaire was 0, while the maximum score was set at 31. Expert opinions were sought from five faculty members who have specialized in the field for the Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations, and necessary revisions were made to finalize the form. Subsequently, a pilot study was conducted with 10 participants.

Sexual health/reproductive health training booklet for extraordinary situations

In order to provide training to nurses and midwives on sexual health and reproductive health in extraordinary situations, the researchers prepared the 'Sexual Health/Reproductive Health Training Booklet for Extraordinary Situations' in accordance with the literature. The preparation of the booklet was based on data from the Inter-Agency Working Group and relevant literature sources.[4–9,28,29] The topics covered in the booklet were aligned with the fundamental information and objectives of the MISP for Sexual and Reproductive Health services in extraordinary situations (IAWG, 2018), including basic information about MISP, prevention and management of sexual violence and abuse, reduction/prevention of HIV and STI transmission, prevention of maternal and neonatal mortality and morbidity, and prevention of unwanted pregnancies, as well as the supply of materials for implementing MISP. Prior to the research, expert opinions were obtained from five faculty members who specialize in the field to ensure the appropriateness of the educational content, and necessary adjustments were made.

Data collection process

The training on sexual health/reproductive health in extraordinary situations was provided to nurses and midwives in groups of 5-8 in two sessions, each lasting 45 minutes to 1 hour, on weekdays during office hours, at times convenient for the nurses and midwives.

Throughout the training process, an environment was created for nurses and midwives to ask questions and share their experiences. The participants were asked to fill out the Descriptive Information Form and the Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations before the training (pre-test). After providing the training on sexual health and reproductive health in extraordinary situations (post-test), the participants were asked to complete the Sexual Health and Reproductive Health Knowledge Assessment Form for Extraordinary Situations again (follow-up test) (Figure 1). It took approximately 10 minutes to complete the data collection form.

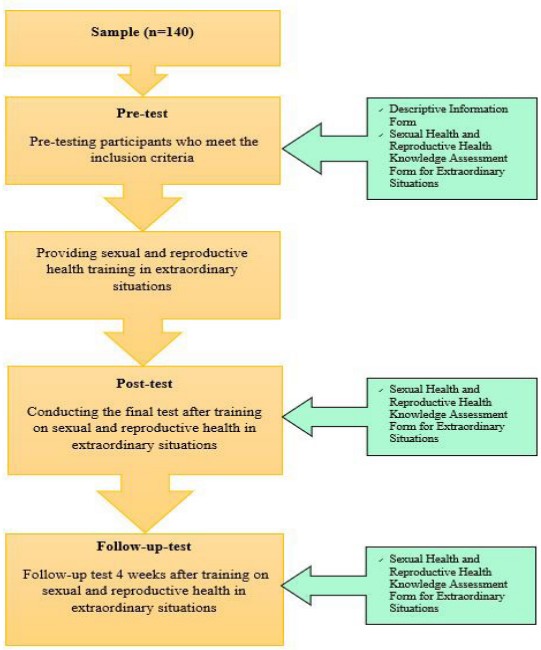

Fig. 1. Research flow chart

Statistical analysis

The Statistical Package for the Social Sciences version 25.0 package program (IBM Corp.; Armonk, NY, USA) was used to evaluate the data. Descriptive statistical methods (number, percentage, mean, standard deviation, minimum, median, and maximum) were employed for data analysis. The normal distribution of the data was tested using the Kolmogorov-Smirnov and Shapiro-Wilk tests. For measurements that did not follow a normal distribution, non-parametric tests were performed. The Mann-Whitney U test was used to compare the differences between the two groups for quantitative data when the measurements did not conform to a normal distribution. The reliability of the sexual health and reproductive health knowledge assessment form for extraordinary situations used in the study was determined through Cronbach's Alpha reliability analysis. The level of statistical significance was set at 0.05.

Ethical considerations

In order to collect the data, the researchers obtained institutional approval from the Ethics Committee of their affiliated institution (Date: 14/09/2021, Decision No: 2021-SBB-0332) and from the Bartin Provincial Health Directorate (Date: 13/10/2021, Reference No: E-12240456-600). The participants were informed about the purpose of the research, the scientific use of the data, and the voluntary nature of their participation, and their consent was obtained. The research was conducted in accordance with the principles of the Helsinki Declaration.

Results

Descriptive characteristics of the participants

The distribution of participants according to their demographic characteristics is presented in Table 1. The mean age of the participants was 34±8.25. 90.7% of the participants were female, and 77.9% had a bachelor's degree. 82.1% received education on sexual health and reproductive health, 52.1% received training on extraordinary situations, 89.3% did not hear about the MISP concept before, and 62.9% stated that they did not receive training on sexual health/reproductive health in extraordinary situations (Table 1).

Table 1. Characteristics of the participants[a]

Variables		n	%
Gender	Female	127	90.7
	Male	13	9.3
Educational status	High school	6	4.3
	Associate degree	22	15.7
	Licence	109	77.9
	Master's/PhD	3	2.1
Working status	I only work during the day	50	35.7
	I only work at night	0	0
	I work both day and night	90	64.3
Receiving education on sexual and reproductive health	Yes	115	82.1
	No	25	17.9
Source of education on sexual and reproductive health*	Mother and father	19	7.1
	Partner	8	3.0
	Friends. neighbors and relatives	10	3.8
	Health personnel (nurse, midwife, doctor)	49	18.4
	Press and broadcast media (television, radio, newspaper, etc.)	15	5.6
	Written resources such as books, magazines	29	10.9
	Internet	19	7.1
	Course	18	6.8
	In the lessons I took during my education	86	32.3
	Social media	13	4.9
Level of competence in sexual and reproductive health	Very enough	29	20.7
	Sufficient	83	59.3
	I'm undecided	24	17.1
	I'm not quite enough	4	2.9
	I'm not enough at all	0	0

Receive training on emergencies	Yes	73	52.1
	No	67	47.9
Source of education on emergencies*	Family members	3	2.5
	Health personnel (nurse, midwife, doctor)	26	21.3
	Press and broadcast media (television, radio, newspaper, etc.)	10	8.2
	Written resources such as books, magazines	12	9.8
	Internet	10	8.2
	Course	11	9.0
	In the lessons I took during my education	47	38.5
	Social media	3	2.5
Perceived importance of sexual and reproductive health	Very important	53	37.9
	Important	81	57.9
	I'm undecided	3	2.1
	low importance	2	1.4
	Does not matter	1	0.7
Earlier hearing about sexual/ reproductive health in emergency situations	Yes	62	44.3
	No	78	55.7
Perceived importance of sexual/reproductive health in emergency situations	Very important	36	25.7
	Important	73	52.1
	I'm undecided	24	17.1
	low importance	5	3.6
	Does not matter	2	1.4
The state of hearing the concept of MISP	Yes	15	10.7
	No	125	89.3
Obtaining information on sexual/reproductive health in extraordinary situations	Yes	52	37.1
	No	88	62.9
Resource of sexual/ reproductive education in emergencies*	Health personnel (nurse, midwife, doctor)	23	27.1
	Press and broadcast media (television, radio, newspaper, etc.)	7	8.2
	Written resources such as books, magazines	10	11.8
	Internet	11	12.9
	Course	11	12.9
	In the lessons I took during my education	23	27.1
Perceived proficiency in sexual/reproductive health in emergency situations	Very enough	19	13.6
	Sufficient	39	27.9
	I'm undecided	55	39.3
	I'm not quite enough	24	17.1
	I'm not enough at all	3	2.1
Age	X±SD	34±8.25	

[a] X – mean; SD – standard deviation; * – participants selected more than one option

Pre-test, post-test, and follow-up test evaluations for the SHRHKAFES

The Cronbach's Alpha reliability coefficient was calculated to reveal the internal consistency of the SHRHKAFES. It was calculated as 0.736 in the pre-test, 0.771 in the post-test, and 0.800 in the follow-up test (Table 2).

It was found that the participants scored 20.82±4.47 on the SHRHKAFES in the pre-test, 27.63±2.67 in the post-test, and 27.07±3.46 in the follow-up test (Table 3). A statistically significant difference was observed in the scores obtained by the participants from the SHRHKAFES in the pre-test, post-test, and follow-up test ($p<0.05$). According to the Bonferroni analysis conducted to determine between which two groups the difference existed, it was found that the difference stemmed from the scores obtained in the pre-test, and the scores obtained in the pre-test were lower than the scores obtained in the post-test and follow-up test (Table 4).

Table 2. SHRHKAFES reliability analysis

SHRHKAFES	Cronbach's Alpha
Pretest	0.736
Posttest	0.771
Follow-up test	0.800

Table 3. Descriptive statistics of the scores obtained during the pre-test, post-test and follow-up of the SHRHKAFES[a]

SHRHKAFES	n	Min	Max	X±SD
Pretest	140	8	27	20.82±4.47
Posttest	100	20	31	27.63±2.67
Follow-up test	99	15	31	27.07±3.46

[a] X – mean; SD – standard deviation

Table 4. Time comparison analysis results of SHRHKAFES[a]

SHRHKAFES	n	Min	Max	X±SD	Test statistic	p	Bonferroni	p
Pretest (1)	140	8.00	27.00	20.82±4.47	X^2= 88.813	<0.001*	1<3; 1<2	<0.001* <0.001*
Post test (2)	100	20.00	31.00	27.63±2.67				
Follow-up test (3)	99	15.00	31.00	27.07±3.46				

[a] X – mean; SD – standard deviation; * – $p<0.05$, X^2 – Friedman test statistic

Participants' descriptive characteristics and pre-test, post-test, and follow-up test evaluations for SHRHKAFES

The analysis of participants' descriptive characteristics and the scores obtained in the SHRHKAFES at the pre-test revealed a statistically significant difference in scores based on education level, work schedule, and having received training on SHRH ($p<0.05$). Specifically, participants with a high school or associate degree education level had higher scores on the SHRHKAFES compared to those with a bachelor's, master's, or doctoral degree. Additionally, participants who only worked during the day had lower scores on the SHRHKAFES compared to those who worked both day and night. The participants who received training on SHRH had higher scores on the SHRHKAFES compared to those who did not receive such training (Table 5). However, no statistically significant difference was found in the scores obtained at the post-test (Table 6) and follow-up test (Table 7) based on participants' descriptive characteristics ($p>0.05$).

Discussion

The research aimed to determine the impact of the training on sexual health/reproductive health in emergency situations on the knowledge levels of nurses and

Table 5. Comparison of the characteristics of the participants and the scores they got from the pre-test of SHRHKAFES[a]

Variables		n	Min	Max	X±SD	Z	*p*
Gender	Female	127	8	27	20.72±4.58	-0.588	0.556
	Male	13	15	26	21.85±3.08		
Educational status	High School-Associate Degree	28	16	27	22.46±3.29	-2.084	0.037*
	Bachelor-Master/PhD	112	8	27	20.41±4.64		
Working status	daytime only	50	8	27	19.36±5.70	-2.022	0.043*
	Both night and day	90	11	27	21.63±3.38		
Status of receiving education on SRH	Yes	115	8	27	21.19±4.32	-2.215	0.027*
	No	25	8	27	19.12±4.82		
Status of receiving training on emergency situations	Yes	73	8	27	21.07±4.31	-0.614	0.539
	No	67	8	27	20.55±4.65		
Prior hearing about sexual/reproductive health in extraordinary situations	Yes	62	8	27	20.82±4.69	-0.221	0.825
	No	78	8	27	20.82±4.32		
The state of hearing the concept of MISP	Yes	15	17	27	22.27±3.24	-1.263	0.207
	No	125	8	27	20.65±4.57		
Receiving information on sexual/reproductive health in extraordinary situations	Yes	52	13	27	21.69±3.11	-1.082	0.279
	No	88	8	27	20.31±5.05		

[a] Z – Mann Whitney U test statistic; * – p<0.05

Table 6. Comparison of the characteristics of the participants and the scores they got from the posttest of SHRHKAFES[a]

Variables		n	Min	Max	X±SD	Z	*p*
Gender	Female	91	20	31	27.76±2.57	-1.031	0.303
	Male	9	23	30	26.33±3.39		
Educational status	High School-Associate Degree	17	23	31	28.12±2.06	-0.421	0.674
	Bachelor-Master/PhD	83	20	31	27.53±2.77		
Working status	daytime only	33	23	30	27.82±2.54	-0.523	0.601
	Both night and day	67	20	31	27.54±2.74		
Status of receiving education on SRH	Yes	82	20	31	27.60±2.64	-0.462	0.644
	No	18	23	31	27.78±2.86		
Status of receiving training on emergency situations	Yes	55	23	31	27.84±2.33	-0.490	0.682
	No	45	20	31	27.38±3.03		
Prior hearing about sexual/reproductive health in extraordinary situations	Yes	44	20	31	27.73±2.57	-0.11	0.992
	No	56	21	31	27.55±2.76		
The state of hearing the concept of MISP	Yes	12	23	31	27.83±2.33	-0.016	0.987
	No	88	20	31	27.60±2.72		
Receiving information on sexual/reproductive health in extraordinary situations	Yes	40	23	31	28.05±2.32	-1.018	0.309
	No	60	20	31	27.35±2.86		

[a] Z – Mann Whitney U test statistic; * – p<0.05

Table 7. Comparison of the characteristics of the participants and the scores they got from the follow-up test of SHRHKAFES[a]

Variables		n	Min	Max	X±SD	Z	p
Gender	Female	90	15	31	27.18±3.47	-1.346	0.178
	Male	9	20	30	26.00±3.32		
Educational status	High School-Associate Degree	19	19	31	27.53±3.64	-1.050	0.294
	Bachelor-Master/PhD	80	15	31	26.96±3.43		
Working status	daytime only	35	17	31	27.34±3.76	-1.355	0.175
	Both night and day	64	15	31	26.92±3.30		
Status of receiving education on SRH	Yes	81	15	31	27.28±3.52	-1.937	0.053
	No	18	19	30	26.11±3.08		
Status of receiving training on emergency situations	Yes	54	15	31	27.13±3.35	-0.096	0.923
	No	45	17	31	27.00±3.62		
Prior hearing about sexual/reproductive health in extraordinary situations	Yes	43	20	31	27.28±2.86	-0.197	0.844
	No	56	15	31	26.91±3.87		
The state of hearing the concept of MISP	Yes	10	20	31	28.00±3.80	-1.390	0.165
	No	89	15	31	26.97±3.43		
Receiving information on sexual/reproductive health in extraordinary situations	Yes	39	20	31	27.10±3.03	-0.418	0.676
	No	60	15	31	27.05±3.73		

[a] Z – Mann Whitney U test statistic; * – p<0.05

midwives. To the best of our knowledge, no research has been conducted on this topic in Turkey. In addition to being the first study representing our country, this research is also significant in the international literature as it evaluates the effectiveness of the training on the subject. The participants in the study scored above average in the pre-test, while they obtained significantly high scores in the post-test and follow-up test conducted after the training. This indicates the effectiveness of the Sexual Health/Reproductive Health Training for Emergency Situations given to nurses and midwives. In this regard, the H1 hypothesis was accepted.

The majority of the participants (82.1%) reported having received education on sexual health and reproductive health, with school courses being the most common source of education (32.3%). More than half of the participants (57.9%) considered sexual health and reproductive health to be important, and the majority (59.3%) perceived themselves as competent in sexual health and reproductive health. However, various studies have indicated that nurses and midwives

do not have sufficient education in sexual health and do not consider themselves competent to provide education on sexual and reproductive health to individuals with specific needs, emphasizing the need for more attention to be given to this topic during their school education.[30-32] In the literature, it is recommended that nurses and midwives improve their knowledge and skills in sexual health and reproductive health.[33] Sexual health education provided during courses plays a crucial role in building students' fundamental knowledge. In addition, nurses and midwives should adopt a lifelong learning perspective, keeping up with current literature and refreshing their knowledge regularly.

In the study, more than half of the participants (52.1%) reported receiving training on emergencies, and over one-third (38.5%) acquired this knowledge during their coursework. Additionally, the majority of participants (62.9%) stated that they did not receive training on sexual health and reproductive health during emergencies, and among those who did receive training, over half (54.2%) obtained it from healthcare professionals such as nurses, midwives, or doctors, or during their educational courses. The literature contains studies both confirming that nurses receive education on disasters during their training[25] and indicating instances where they do not.[34,35] Another study conducted in Turkey found that a majority of nursing students (65.3%) did not receive education on disasters and the vast majority (85.4%) expressed a desire to take disaster-related courses during their education.[34] Taskiran and Baykal did not list sexual health and reproductive health in emergencies as a topic that nurses desired to receive education on during the disaster preparedness phase.[36] In a study involving nursing and midwifery students, over half of the participants (59.9%) reported not receiving any training on emergencies.[37] While there is limited information on whether midwives receive education on emergencies in the literature, a study conducted in the United Kingdom found that the training given to midwives on the management of childbirth during emergencies was effective.[22] This underscores the need to incorporate disaster-related courses into nursing and midwifery curricula.

More than one-third of the nurses and midwives in the study (39.3%) expressed uncertainty about feeling competent in sexual and reproductive health during emergencies, while over half of the participants (52.1%) emphasized the importance of sexual and reproductive health during emergencies. In line with these findings, a recent article highlights the significance of implementing MISP for nutrition, sanitation, access to healthcare services, sexual and reproductive health services, and protection from violence, harassment, and abuse for individuals affected by the Ukraine conflict.[38] Similarly, a study conducted after the Nepal earthquake emphasizes the need to prioritize sexual and reproductive health issues in disaster preparedness and emergency plans and to provide healthcare workers with relevant training.[39] Offering training to nurses and midwives on sexual and reproductive health during emergencies, conducting awareness sessions, and enhancing their knowledge and skills will contribute to their competence development.

It was found that almost all participants (89.3%) had never heard of the MISP concept before. In Indonesia, only half of healthcare workers were aware of the MISP concept and its implementation, and only one participant could correctly define MISP's objectives and priority activities.[40] Awareness of MISP among healthcare workers was found to be insufficient in Kenya, while it was found to be high in Haiti and Nepal.[4,5] Despite more than a decade since its establishment, it is emphasized that MISP is still unknown and not implemented by many government institutions and humanitarian health organizations worldwide.[5] The current research raised awareness of MISP among the participants, which is a positive outcome.

A statistically significant difference was observed in the scores obtained from the SHRHKAFES in the pre-test, post-test, and follow-up test ($p<0.05$). In the literature, a direct conclusion related to this finding cannot be reached, yet similar studies have shown parallel results to this outcome. In a study conducted in Chad, the Democratic Republic of the Congo, Mali, Djibouti, and Pakistan, aimed at improving the use of family planning services in humanitarian crisis settings, it was found that participants' knowledge and awareness of modern family planning methods increased between the pre-test and post-test.[41] After a psycho-education intervention provided by female healthcare workers following the 2012 Haiti earthquake, an increase in practices related to HIV/STI prevention was identified.[42] As in this study, positive results have been obtained in the literature in educational interventions aimed at improving the knowledge and skills that individuals want to acquire.

It was found that the scores obtained in the pre-test were significantly associated with some of the participants' demographic characteristics. In the literature, it has been noted that certain demographic variables of participants are related to their knowledge levels regarding emergencies.[25,34,36]

Study limitations

This research is expected to serve as a valuable resource for researchers working in the field of sexual and reproductive health. However, there are some limitations to this study. One limitation is that it was conducted in a single province in the northwest region of Turkey. The researchers considered this limitation as a pilot study in a selected province. Another limitation is that since the

number of nurses and midwives in the sample was not evenly distributed, no comparisons could be made between nurses and midwives. In future research, it may be beneficial to present this difference more clearly. Another limitation is the lack of a valid and reliable measurement tool that nurses and midwives can use to assess their knowledge of sexual and reproductive health in emergencies. Therefore, the researchers created a questionnaire based on the literature. The validity analysis conducted at the end of the study confirmed that the questionnaire is a reliable measurement tool. Finally, the scarcity of studies on this topic limited the discussion of the findings.

Conclusion

In the study, the participants scored above the average in the pre-test, while they achieved significantly higher scores in the post-test and follow-up test. This indicates the effectiveness of the training on sexual health/reproductive health during extraordinary situations. As a result of the training, the participants may feel more competent in their professional roles, which can positively impact the quality of care and treatment.

The SHRHKAFES, developed by the researchers based on the literature, was found to have a good level of internal consistency. It is recommended that this form be administered to healthcare professionals working in different regions of the world, including Turkey as further research in this field can enrich the literature. Furthermore, it is suggested to incorporate disaster management courses into the undergraduate curriculum for nurses and midwives. In addition, nurses and midwives should adopt a lifelong learning perspective by keeping up with current literature and cultivating a habit of refreshing their knowledge.

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Declarations

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Author contributions

Conceptualization, E.C.E., T.K., E.E. and N.D.; Methodology, E.C.E.; Software, E.C.E.; Validation, E.C.E., T.K., E.E. and N.D.; Formal Analysis, E.C.E., T.K., E.E. and N.D.; Investigation, E.C.E., T.K., E.E. and N.D.; Resources, E.C.E., T.K., E.E. and N.D.; Data Curation, E.C.E., T.K., E.E. and N.D.; Writing – Original Draft Preparation, E.C.E., T.K., E.E. and N.D.; Writing – Review & Editing, E.C.E., T.K., E.E. and N.D.; Visualization, E.C.E., T.K., E.E. and N.D.; Supervision, E.C.E.; Project Administration, E.C.E.; Funding Acquisition, E.C.E., T.K., E.E. and N.D.

Conflicts of interest

The authors declare that there is no conflict of interest regarding this article.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The protocol for the research project has been approved by Bartin University Social and Human Sciences Ethics Committee (Date: 14.09.2021, No: 2021-SBB-0332).

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ORIGINAL PAPER

Determination of attitudes and practices of nurses working in the psychiatry clinic towards physical health care of individuals with severe mental illness

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ABSTRACT

Introduction and aim. Individuals with severe mental illness experience more physical health problems than the general population and their life expectancy may be shorter. Therefore, the physical care provided to these patients is very important. This study aims to examine the attitudes, practices, and training needs of nurses working in psychiatry clinics towards physical health care of individuals with severe mental illness.

Material and methods. This descriptive, cross-sectional study collected data from 139 nurses in psychiatry clinics using the Personal Information Form and Physical Health Attitude Scale.

Results. This study found that nurses often provide physical healthcare as part of their routine care for individuals with severe mental illness. However, the study also revealed that nurses require additional education to better provide this type of care. Despite this, the nurses generally displayed a positive attitude towards physical health. The average total score on the physical health attitude scale was 80.33±10.14.

Conclusion. The study concluded that nurses have a positive attitude towards physical health in general. The role of nurses working in psychiatry clinics is crucial in evaluating and caring for physical health of individuals with severe mental illness, as it is an important aspect of holistic nursing care. It is necessary to make nurses aware of their shortcomings in providing physical health care and support them in improving in this area.

Keywords. attitude, nurse, physical health, severe mental illness

Introduction

Individuals with severe mental illness typically have a lifespan that is 25 years shorter than the average person, and they often suffer from physical health issues that can lead to social isolation and a lower quality of life.[1,2] Undiagnosed and untreated medical diseases are more common in individuals with mental illness.[3] Individuals with severe mental illness are more likely to suffer from various physical health problems, such as diabetes, cardiovascular diseases, respiratory diseases, and some types of cancer, compared to the general population.[4] This suggests that people with severe mental illness do not benefit from healthcare advances to the same extent as the general population.[4,5] In addition, it is stated that the evaluation of the physical health of the patients in primary and secondary health care services

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can be neglected.[6,7] Also, patients do not always receive the physical health care they need during their hospital stay.[8] The cause of this healthcare inequality is complex and multidimensional.[9] It has been reported that there are multifactorial barriers in the management, and diagnosis of physical diseases of individuals with mental illness, which can be caused by the patient and the disease, treatment, and health workers.[1,2,4,5] Individuals with severe mental illness cannot demand adequate physical health care, cannot recognize their physical problems, and may have difficulty in complying with health care recommendations due to symptoms such as cognitive impairment, social isolation, and skepticism.[2,10] In addition, the fact that healthcare professionals do not adequately inform individuals with severe mental illness about their physical health, have insufficient communication with patients, focus on mental illness rather than physical illness, and see physical complaints as psychosomatic complaints may adversely affect the physical health of patients.[2,10] In the qualitative study of Happel et al. with nurses, it was determined that stigma against patients prevents them from receiving physical health care.[11] Another study found that nurses performed routine physical health care with confidence but were less confident in interpreting physical health outcomes that required interpretation, such as lipid levels. In the same study, it was seen that the physical health needs of the patients were not adequately addressed.[7]

Individuals with severe mental illness need the support of nurses in recognizing, evaluating, and managing their physical health problems.[7,9,12] Therefore, psychiatric nurses have a key role in providing holistic care that includes mental and physical healthcare for individuals with mental illness.[8,12–14] The roles of nurses in the psychosocial care of individuals with mental illness are as important as their roles in the physical care of patients.[12,15] Psychiatric nurses can play a role in providing care for common physical problems in patients, encouraging a healthy lifestyle, and improving patients' self-care.[6,13,16] In this context, it is important to know the attitudes, behaviors, and thoughts of nurses working in psychiatry clinics towards the physical health of individuals with mental illness. However, there are limited studies in the literature on nurses' attitudes, behaviors, and thoughts about providing physical health care. It is seen that several quantitative and qualitative studies have been conducted in the last decade measuring nurses' attitudes, practices, and training needs regarding providing physical health care to individuals with severe mental illness.[7,11,12,15,17–20] When the studies are examined, it is seen that the attitudes of nurses towards physical health care and their physical health care practices, differ. It is seen that more studies are needed to reveal the attitudes and practices of nurses towards physical health care of individuals with mental illness.

Aim

This study analyzed the attitudes, practices, and training requirements of nurses working in psychiatry clinics regarding the physical health care of people with mental illness. The study aims to provide insights into the nursing care provided to individuals with mental illness and guide efforts to promote holistic care. This study aims to examine the attitudes, practices, and training needs of nurses working in psychiatry clinics towards physical health care of individuals with mental illness. This study sought answers to the following questions;

- What are the physical health attitudes of nurses working in psychiatry clinics in individuals with mental illness?
- What are the practices and training needs of nurses working in psychiatry clinics for physical health care for individuals with mental illness?
- How do sociodemographic characteristics and characteristics of physical health care affect the physical health attitudes of nurses working in psychiatry clinics with individuals with mental illness?

Material and methods

Study design

This study was conducted in a descriptive, cross-sectional design.

Setting

This study was conducted with nurses working in psychiatry clinics in a regional hospital in the Eastern Anatolia Region of Turkey between January 2022 and May 2022. The hospital is a large branch hospital with a capacity of 488 beds serving as a regional hospital in the field of mental health in Turkey. Alcohol and Drug Abuse Treatment and Research Center, Child and Adolescent Substance Addiction Treatment Centers, Community Mental Health Center is a hospital serving 18 provinces with specific units such as Protected Houses. There are physicians specialized in internal medicine, neurology, biochemistry and microbiology in the hospital to meet the physical care of the patients.

Participants

The population of this study consists of nurses working in psychiatry clinics in a regional hospital in the Eastern Anatolian Region of Turkey. The total number of nurses working in the hospital is 150. Nurses working in leprosy units, except psychiatry units, were not included in the sample. Since the researcher himself works as the nurse of this institution, he was not included in the sample. In this study, it was aimed to reach all other nurses working in psychiatry units, and sampling method was not used. Accordingly, 139 nurses (99.2%) who agreed to participate in the study and met the inclusion criteria were reached.

Sampling inclusion/exclusion criteria

The inclusion criteria were volunteering to participate, providing physical care to individuals with mental illness, and working as a nurse in psychiatry clinics for at least 6 months. Exclusion criteria from the study were not accepting to participate in the study and working outside of psychiatry clinics.

Data collection tools

In the study, "Personal Information Form" and "Physical Health Attitude Scale" were used as data collection tools in collecting the data of nurses working in psychiatry clinics. The data were collected face-to-face with the nurses working in the clinics of a regional hospital in the Eastern Anatolian Region of Turkey, who agreed to participate in the study, and by giving data forms to those who did not want this method, by asking them to fill in themselves. In addition, during the data collection phase, clinics were visited during working hours five days a week and data collection was carried out for a nurse in approximately 15-20 minutes.

Personal Information Form

This form, which was created by the researchers by scanning the literature, consists of 18 questions that evaluate the views, practices, and training needs of nurses regarding physical health care, as well as age, gender, professional education level, professional experience, and working style.[12,21]

Physical Health Attitude Scale (PHAS)

The original 28-item scale was developed by Robson and Haddad in 2012.[22] Turkish validity and reliability study was conducted by Özaslan et al. in 2020.[15] The scale is a 5-likert-type scale with 24 items and 4 sub-dimensions. The scale has four sub-dimensions. "nurses' attitudes towards their participation in physical health care sub-dimension", "nurses' confidence in providing physical health care sub-dimension", "perceived barriers to physical health service delivery sub-dimension" and "attitudes towards smoking and negative beliefs sub-dimension". Each item in the scale is scored between 1 and 5. Items 4, 6, 8, 11, 12, 13, 14, 16, 20, 23, 24 in the Turkish scale are calculated by reverse coding. The total score that can be obtained from the scale is between 24-120. A high score from the scale is an indicator of a positive attitude towards physical health. In the Turkish validity and reliability study, the scale total score Cronbach's alpha value was 0.83. Cronbach's alpha values for the sub-dimensions found in the validity and reliability study ranged from 0.64 to 0.88.[15] In this study, the total score cronbach's alpha value of the PHAS was found to be high as 0.79. As a result of this research, cronbach's alpha values for the sub-dimensions ranged from 0.60 to 0.84.

Data analysis

The data obtained in this study were evaluated through the SPSS 22.0 statistical program (IBM, Armonk, NY, USA). Frequency and percentage analyzes were used to determine the descriptive characteristics of the nurses participating in the research. Mean and standard deviation statistics were used to analyze the scale data. Kurtosis and Skewness values were examined to determine whether the research variables showed a normal distribution. In this direction, parametric tests were used in the analysis of normally distributed variables, and non-parametric tests were used in the analysis of non-normally distributed variables. Parametric tests such as t-test, one-way analysis of variance (ANOVA) and post hoc (LSD) analyzes were used to analyze the differences in scale levels according to nurses' descriptive characteristics, and non-parametric Man Whitney U test and Kruskal Wallis tests were used. The results were evaluated at the 95% confidence interval, at the $p<0.05$ level of significance.

Ethical consideration

Ethics committee approval (07.10.2021/88697) was obtained from the Human Research Ethics Committee of Zonguldak Bülent Ecevit University in order to conduct the study. In addition, written permission was obtained from the institution where the research was conducted. Nurses who agreed to participate in the study were informed about the purpose, duration and scope of the study, and verbal and written consent was obtained from the participants, explaining that participation in the study was voluntary.

Results

Findings obtained as a result of this research (1) sociodemographic characteristics; (2) physical health care characteristics of nurses; (3) nurses' training needs related to physical health care, and (4) nurses' PST scores are presented under four headings.

Sociodemographic characteristics

The mean age of the nurses is 32.97±8.12, and 29.5% of them are between the ages of 31-35. Of the nurses, 75.5% were women, 66.2% had a bachelor's degree, 30.9% had a working year of less than 5 years, and 54.0% had a working year of less than 5 years in psychiatry units. 31.7% of the nurses work in the male acute unit and 56.8% of them work in a shift for 24 hours. The mean number of patients per nurse was 11.53±5.88 (Table 1).

Characteristics of nurses regarding physical health care practices

50.4% of the nurses reported that obesity-weight gain is the most common physical health problem in individuals with severe mental illness. On the other hand,

85.6% of the nurses stated that smoking is the most common problem that can negatively affect physical health in individuals with severe mental illness (Table 2). It was determined that 93.5% of the nurses always evaluate the physical health status of the patient at the first admission. 51.8% of nurses think that the psychiatric care they provide to patients does not interfere with physical care. 58.3% of nurses stated that their working conditions were not sufficient to provide physical care, and 82.7% stated that "the high number of patients per nurse" prevented them from providing physical health care (Table 2).

Table 1. Sociodemographic characteristics of nurses working in psychiatry clinics (n=139)

	$\bar{x}$±SD (min–max)	
Age	32.97±8.12 (18–56)	
	n	**%**
<25	29	20.9
26-30	29	20.9
31-35	41	29.5
35>	40	28.8
Gender		
Female	105	75.5
Male	34	24.5
Educational status		
Health vocational high school	17	12.2
Associate degree	24	17.3
Undergraduate	92	66.2
Master degree	6	4.3
PhD	0	0
Years of work in nursing		
<5	43	30.9
5-9	32	23
10-14	35	25.2
≥15	29	20.9
Years of study in psychiatry clinics		
<5	75	54
5-9	30	21.6
≥10	34	24.5
Psychiatric unit		
Female acute	37	26.6
Female subacute	14	10.1
Male acute	44	31.7
Male subacute	24	17.3
Other (CMHC, AMATEM etc)	20	14.4
Working hours in one shift		
8	55	39.6
16	5	3.6
24	79	56.8
	$\bar{x}$±SD (min-max)	
Number of patients per nurse	11.53±5.88 (4–30)	
	n	**%**
1-5	20	14.4
6-10	61	43.9
11-15	34	24.5
>15	24	17.3
Total	139	100

Table 2. Characteristics of nurses working in psychiatry clinics regarding physical health care practices (n=139)[a]

	n	**%**
The most common physical health problems in individuals with severe mental illness*		
Obesity/Weight gain	70	50.4
Diabetes mellitus	67	48.2
Cardiovascular disorders	63	45.3
Thyroid disorders	36	25.9
Urinary infection	10	7.2
Cancer	2	1.4
Other	4	2.9
The most common problems in individuals with mental illness that may adversely affect physical health*		
Lack of hygiene	117	84.2
Smoking	119	85.6
Drinking alcohol	32	23
Being overweight	78	56.1
Not exercising or doing sports, sedentary life	83	59.7
Psychiatric symptoms in the patient	88	63.3
Psychotropic drug side effects	68	48.9
Evaluation of the physical health status of the patient at the first hospitalization		
Everytime	130	93.5
Sometimes	8	5.8
None	1	0.7
Psychiatric care interferes with physical care		
Yes	43	30.9
No	72	51.8
Undecided	24	17.3
Adequacy of working conditions for physical care		
Yes	27	19.4
No	81	58.3
Undecided	31	22.3
Barrier working condition		
Working shifts	3	3.7
High number of patients per nurse	67	82.7
Insufficient number of nurses	10	12.3
Other	1	1.2
Total	139	100

[a] * – more than one option has been marked

Nurses' practices regarding physical health care

81.3% of the nurses regularly monitor the patient's blood pressure, 82% always help the patient to pay attention to personal hygiene, 51.1% sometimes encourage patients to exercise regularly, 55.4% always encourage patients to eat healthy, It was determined that 78.4% of the patients always followed up the routine weight of the patients, and 81.3% of the patients at risk always followed up the blood glucose abnormalities regularly. It was determined that 51.8% of the nurses sometimes evaluate the oral and dental health of the patients, 46.8% sometimes make attempts to help the patients quit smoking, and 43.9% never inform the patients about birth control methods (Table 3).

Educational needs of nurses regarding physical health care

79.9% of the nurses have knowledge and skills related to physical health care, 78.4% of them received train-

ing on physical health care, 65.1% of those who received training received this training during their undergraduate education, 51.4% of them received training in physical health care. stated that it was sufficient in providing care (Table 4). It was determined that nurses stated that they mostly needed training on exercise (59.7%) and smoking cessation (50.4%) among the physical health care practices that could be given to patients (Table 4).

Table 3. Practices of nurses working in psychiatry clinics for physical health care of individuals with mental illness (n=139)

	Everytime n (%)	Sometimes n (%)	None n (%)
Blood pressure monitoring	113 (81.3)	24 (17.3)	2 (1.4)
Helping them take care of their personal hygiene	114 (82)	24 (17.3)	1 (0.7)
Encouraging patients to exercise regularly	61 (43.9)	71 (51.1)	7 (5)
Encouraging patients to eat healthy	77 (55.4)	54 (38.8)	8 (5.8)
Doing routine weight follow-up of patients	109 (78.4)	23 (16.5)	7 (5)
Monitoring blood glucose abnormalities of at-risk patients	113 (81.3)	22 (15.8)	4 (2.9)
Evaluation of patients' oral and dental health	37 (26.6)	72 (51.8)	30 (21.6)
Attempts for patients to quit smoking	36 (25.9)	65 (46.8)	38 (27.3)
Informing patients about birth control methods	25 (18)	53 (38.1)	61 (43.9)

Table 4. Training needs of nurses working in psychiatry clinics on physical health care practices that can be given to individuals with mental illness (n=139)[a]

	n	%
Having knowledge and skills		
Yes	111	79.9
No	28	20.1
Getting an Education status		
Yes I have been trained	109	78.4
No, I did not receive any training	30	21.6
Where did the training come from*		
In undergraduate education	71	65.1
In in-service training	38	34.9
Is the education received in caregiving sufficient?		
Yes	56	51.4
No	22	20.2
Sometimes	31	28.4
Which of the physical health care practices that can be given to patients are most needed for training**		
Exercise	83	59.7
Cardiovascular health management	64	46
Diabetes care	56	40.3
Healthy eating	56	40.3
Quit smoking	70	50.4
Weight management	44	31.7
Reproductive health	32	23
Oral and dental health problems	23	16.5
Practices to improve physical health	26	18.7
Total	139	100

[a] * – those who answered yes to the previous question answered; ** – more than one option is marked

Nurses' Physical Health Attitude Scale (PAHS) Scores

The mean total score of the nurses' PAHS was 80.33±10.14 (Table 5).

There is no statistically significant difference in the total score of the nurses' PAHS according to gender, unit of work, duration of work in a shift, number of patients per nurse, age, education level, working year as a nurse, and working year in psychiatry units (p>0.05) (Table 6).

Table 5. PAHS scores of nurse

	$\bar{x}$±SD (min–max)
Total score	80.33±10.14 (32–120)
Sub dimensions	
Attitudes towards participating in physical health care	25.20±6.08 (8–40)
Confidence in providing physical health care	18.85±3.1 (9–25)
Perceived barriers to physical health care	18.66±3.03 (6–25)
Attitudes and negative beliefs towards smoking	17.61±3.74 (6–30)

PAHS total scores show a statistically significant difference according to the nurses' knowledge and skills related to physical health care (U:1067.500; p=0.011). The total scores of PAHS show statistically significant differences according to the sufficiency of the education received by the nurses regarding physical health care (X2=9.483; p=0.009). It was determined that the total score of those who thought that education was sufficient in care was higher than those who thought that education was not sufficient in care (p<0.05) (Table 7).

It was found that there was no statistically significant difference in terms of the total mean scores of the PAHS of the nurses according to the psychiatric care's prevention of physical care, the adequacy of working conditions for physical care, and their educational status (p>0.05) (Table 7).

Discussion

The role of nurses working in psychiatry clinics in evaluating and caring for the physical health of individuals with mental illness is very important for holistic nursing care.[8] This study, it was aimed to determine the attitudes, practices, and training needs of nurses working in psychiatry clinics regarding the physical health care of individuals with mental illness.

In the study, nurses working in psychiatry clinics stated obesity and weight gain was the most common physical health problem in individuals with mental illness. This result aligns with the findings from previous studies in the literatüre.[4,23,24] In the study of Firth et al., it is stated that obesity is seen at a higher rate in individuals with severe mental illness compared to the general population.[4] Collins et al. stated in their study that deaths due to physical health problems caused by the increase in body weight of severe mental patients are

Table 6. Comparison of the total PAHS score according to the sociodemographic characteristics of nurses working in psychiatry clinics (n=139)[a]

Sociodemographic characteristics	n	PAHS total score $\bar{x}$±SD
Age		
<25	29	81.31±12.41
26–30	29	78.82±6.27
31–35	41	81.48±11.35
>35	40	79.55±9.40
		X^2= 3.431
		p=0.33
Gender		
Female	105	80.73±10.79
Male	34	79.11±7.8
		U= 1481.000
		p=0.136
Educational status		
Health vocational high school	17	79.58±6.08
Associate degree	24	80.08±12.94
Undergraduate	92	80.19±9.94
Master degree	6	85.66±10.63
		X^2= 1.761
		p= 0.624
Years of work in nursing		
<5	43	80.9±8.8
5–9	32	78.25±9.17
10–14	35	81.85±13.45
>15	29	79.96±8.33
		X^2= 4.522
		p=0.21
Years of study in psychiatric clinics		
<5	75	81.62±9.92
5-9	30	78.26±11.09
≥10	34	79.32±9.63
		X^2= 1.754
		p=0.416
Psychiatric unit		
Female acute	37	81.35±12.89
Female subacute	14	79.92±9.03
Male acute	44	80.22±10.48
Male subacute	24	80.50±7.71
Diğer (CMHC, AMATEM etc)	20	78.80±7.13
		X^2= 1.654
		p=0.799
Working hours in one shift		
8	55	82.56±7.1
16	5	85.8±19.7
24	79	78.44±10.86
		X^2= 5.68
		p=0.058
Number of patients per nurse		
1-5	20	81.75±10.62
6–10	61	81.98±10.1
11–15	34	78.58±7.85
>15	24	77.45±12.14
		X^2= 5.415
		p=0.144

[a] X^2 – Kruskal-Wallis Test; U – Mann Whitney U test; p<0.05

Table 7. Comparison of the PAHS total score and sub-dimension scores of nurses working in psychiatry clinics according to their physical health care characteristics (n=139)[a]

	n	PAHS total score $\bar{x}$± SD
Psychiatric care interferes with physical care		
Yes	43	79.27±6.05
No	72	80.93±12.75
Undecided	24	80.45±6.72
		X^2= 2.475
		p=0.29
Adequacy of working conditions for physical care		
Yes	27	81.29±10.05
No	81	80.03±11.39
Undecided	31	80.29±6.18
		X^2= 0.174
		p=0.916
Having knowledge and skills		
Yes	111	81.36±10.13
No	28	76.28±9.26
		U:1067.500
		p=0.011
Getting an education status		
Yes I have been trained	109	80.43±11.13
No, I did not receive any training	30	80.00±5.30
		U: 1526.000
		p=0.576
Where did the training come from		
In undergraduate education	71	81.08±10.97
In in-service training	38	79.21±11.46
		U:1228.500
		p= 0.443
Is the education received in caregiving sufficient?		
Yes	56	82.66±11.90
No	22	76.50±13.59
Sometimes	31	79.19±5.89
		X^2= 9.483
		p=0.009 PostHoc=1>2,1>3

[a] X^2 – Kruskal-Wallis Test; U – Mann Whitney U test; posthoc – Tukey and LSD

much higher than deaths due to accidents and suicide in these patients.[24] Kayar Erginer and Partlak Günüşen, in their research examining the physical health status and health lifestyle behaviors of individuals with severe mental illness, determined that most of the patients had hypertension, cardiovascular diseases, and diabetes mellitus.[13] It is stated that there are many factors affecting obesity and weight gain in individuals with severe mental illness. The presence of psychotropic drugs used by patients, insufficient exercise, unhealthy diet, and inability to cope with psychiatric symptoms can be shown among the most important causes of obesity.[10,12,25,26] While weight gain and obesity in patients cause many physical health problems, they increase the risk of mor-

tality in patients.[22,27] For this reason, it is very important for nurses working in psychiatry clinics to plan and implement interventions to prevent weight gain and obesity, and to monitor and evaluate patients in this respect.

As a result of this study, nurses stated that patients' smoking was the most common behavior that could negatively affect their physical health. This finding is similar to the studies in the literature. It is stated that smoking rates are higher in individuals with severe mental illness than in individuals without mental illness.[28–31] As a result of the study conducted by Dickerson et al., it was determined that the rate of smoking was 62% in patients with schizophrenia, 37% in patients with bipolar affective disorder, while this rate was 17% in patients without a psychiatric disorder. It is stated that individuals with severe mental illness have higher smoking rates as well as lower smoking cessation rates.[31] Although the barriers to quitting smoking are multifactorial, it is stated that the attempts of psychiatry services to quit smoking are limited.[32] For this reason, nurses related to smoking, which has an important contribution to the emergence of physical health problems in individuals with severe mental illness, should plan initiatives to encourage patients to quit smoking within the framework of multidisciplinary team cooperation. Nurses can teach patients effective coping methods instead of smoking.

In this study, the physical health care that nurses regularly give to their patients, evaluation of the physical health status of the patient at the first hospitalization, regular monitoring of the patient's blood pressure, helping the patient pay attention to personal hygiene, encouraging health nutrition, routine weight monitoring, regular blood pressure monitoring of risky patients. It has been determined that glucose abnormalities are followed. This finding in the study suggests that nurses provide physical health care, which is mostly included in routine care, in clinics. This result is similar to the results in the literatüre.[18,19] As a result of a similar study conducted with psychiatric nurses in Asian countries, it was found that the majority of nurses evaluate the patient's physical health while providing nursing care at the first hospitalization, and routinely apply physical health care such as monitoring blood pressure and helping personal hygiene.[33] As a result of a similar study conducted with nurses in Australia, it was determined that the majority of nurses saw it as a nursing responsibility to help patients manage their weight and to advise patients on nutrition and how to prevent heart disease.[17]

As a result of this study, an important result related to providing physical healthcare is that nurses rarely apply interventions to encourage patients to exercise regularly, evaluate oral and dental health, and quit smoking, and they never inform patients about birth control methods. These shortcomings in assessing patients' physical health and providing holistic physical health care can be explained by several factors. First; Another finding in this study is the perceived barriers of nurses in providing physical health care. The results of this study show that the psychiatric care given by the nurses to the patients, the working conditions and the high number of patients per nurse prevent them from providing physical health care. This result is similar to the literature.[17,21,33] In a similar study with psychiatric nurses in three Asian countries, nurses' workload, patients' lack of motivation to exercise, patients' lack of interest in improving their own physical health, and the presence of mental illness are shown as similar situations that prevent nurses from providing physical health care.[33] In a similar study, it was determined that nurses see workload as an obstacle in providing physical health care.[17] As the second factor that may cause deficiencies in providing physical health care, it is discussed in the literature that nurses do not trust themselves in providing holistic physical health care with all its dimensions, do not see physical health care as a priority, and do not have adequate training in assessing physical health and providing care.[8,21,27,34,35] In Gray and Brown's study, it was determined that psychiatric nurses do not find themselves sufficient to provide physical health care to individuals with mental illness, they think that patients tend to their own mental health, and that the physical health of patients is not among their priorities.[36] In their study with psychiatric nurses, Çelik İnce et al. determined that individuals with mental illness could not receive the physical health services they needed, that patients generally focused on their mental problems, and somatic interpretation of their physical complaints prevented these patients from receiving physical health care.[12] In this study, it was determined that the education of nurses on physical health care, especially in undergraduate education, was sufficient in providing physical health care. However, it has been determined that among the physical health care practices that can be given to the patients, the training needs are mostly about exercise and smoking cessation. Moreover, it is evident that nurses should possess adequate knowledge and confidence in assessing oral and dental health of individuals with mental illness, explaining contraceptive methods, and evaluating and managing physical health concerns. They should also receive appropriate training to fulfill the requirements of providing care and interventions to safeguard physical health.[27,33] It is important to provide training to nurses that caters to their specific needs, particularly in the area of patient care. Repetition of these trainings on an annual basis has been shown to increase nurses' confidence in administering physical health care to patients, and has a positive impact on the overall quality of care provided.[8]

In this study, when the scores of the nurses from the scale are evaluated, it can be said that they have a positive physical health attitude. In some of the studies in the literature, it was determined that psychiatric nurses have positive attitudes toward participation in physical health

care, it was determined that some of them had negative attitudes.[3,9,15,37] In addition, in the studies in the literature, psychiatric nurses have different views on the importance of physical health care and the role of nurses in this issue; It has been determined that the difference in the level of trust of nurses in providing physical health services is effective in the emergence of this situation.[18,19] The results of the study also show similarities with the studies of Ganniah et al. and Howard and Gamble.[3,7] However, Bradshaw and Pedley showed that mental health nurses generally have a low level of positive health promoting attitudes towards physical health care.[16] It has been noticed that nurses working in psychiatry clinics have varying attitudes towards providing physical health care to patients. Patients with severe mental illness require the assistance of nurses not only in managing their psychiatric illnesses but also in maintaining their physical health. Psychiatric nurses are crucial in providing comprehensive care to patients. Therefore, it is imperative to promote positive attitudes towards physical health care among nurses and identify their requirements in this area.

Clinical implications for nursing practice

Based on the research findings, it is advisable for nurses in psychiatric clinics to assess the overall physical health of their patients during regular care. Additionally, providing in-service training to nurses on topics that require training can help improve their ability to provide physical healthcare support. To address the issue at hand, it is important to increase nurses' awareness, knowledge, and skills in providing holistic care, reduce the nurse-to-patient ratio in psychiatry clinics, and implement evidence-based nutrition, exercise, and hygiene programs for patients. Incorporating activities like nutrition and sports into routine care in psychiatry clinics and developing infrastructure to support these efforts can also help. By doing so, we can reduce physical health problems in individuals with severe mental illness and improve nurses' knowledge and confidence levels on this topic.

Study limitations

Since the study was conducted only in a regional hospital located in the Eastern Anatolia Region of Turkey, the fact that it could not be generalized to all psychiatric nurses can be considered as the main limitation of the study.

Conclusion

As a result of this research, which was conducted to determine the attitudes, practices, and training needs of nurses working in psychiatry clinics toward physical health care of individuals with severe mental illness, it was determined that obesity and weight gain, diabetes, and cardiovascular diseases are the most common physical health problems of nurses in individuals with mental illness. It has been determined that lack of hygiene and smoking are among the problems that can negatively affect physical health. Almost all of the nurses evaluated the routine physical health status of the patients at the first hospitalization; It has been determined that the most frequently given physical health care practices are practices such as regular monitoring of blood pressure, helping patients to pay attention to their personal hygiene, and routine weight follow-up.

Overall, the research indicates that nurses working in psychiatry clinics have positive attitudes towards physical health. However, there is a need to improve their awareness and confidence in evaluating and caring for various aspects of physical health, such as oral and dental health assessments, explaining birth control methods, and interventions to protect physical health. It's important to determine and meet their training needs in order to address these areas of improvement.

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Author contributions

Conceptualization, H.Y.K. and S.Ç.İ.; Methodology, H.Y.K. and S.Ç.İ.; Software, H.Y.K.; Validation, H.Y.K. and S.Ç.İ.; Formal Analysis, H.Y.K. and S.Ç.İ., Expert Statistician; Investigation, H.Y.K. and S.Ç.İ.; Resources, H.Y.K. and S.Ç.İ.; Writing – Original Draft Preparation, H.Y.K. and S.Ç.İ.; Visualization, S.Ç.İ.; Supervision, S.Ç.İ.; Project Administration, H.Y.K. and S.Ç.İ.; Funding Acquisition, H.Y.K. and S.Ç.İ.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

Data available on request from the authors.

Ethics approval

Ethics committee approval (07.10.2021/88697) was obtained from the Human Research Ethics Committee of Zonguldak Bülent Ecevit University in order to conduct the study. In addition, written permission was obtained from the institution where the research was conducted. Nurses who agreed to participate in the study were informed about the purpose, duration and scope of the study, and verbal and written consent was obtained from the participants, explaining that participation in the study was voluntary.

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ORIGINAL PAPER

Nursing students' awareness of health-promoting lifestyle profile and sustainable development goals – a quasi-experimental study

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ABSTRACT

Introduction and aim. Health promotion is one of the common elements of sustainable development goals. The aim of this study was to identify the impact of "Healthy Life and Environment" course on nursing student's awareness of sustainable development and healthy life, and health-promoting lifestyle behaviours.

Material and methods. A pre-, and post-test control group quasi-experimental research design was used. One nursing department located in Nortwest Blacksea region, Turkey. A total of 160 nursing students pursuing bachelor nursing programs. Students from first and second years who enrolled in the "Healthy Life and Environment" course assigned to intervention group, those who did not attend the course assigned to control group.

Results. Female students had higher level of HPLP II and SDA than male. SDA had a weak correlation with HLA and a very week correlation with HPLP II at baseline. Intervention group had an improvement of .03 points in HPLP II, no change in HLA, and a slight improvement of .01 points in SDA, however these differences were not statistically important.

Conclusion. The results of our study highlighted that nursing curricula need to be modified to incorporate sustainable development methodology using student-centered learning.

Keywords. health promotion, nursing students, sustainable development goal

Introduction

Requirements of well-being is far more complex than focusing on diseases and organising the necessary care and treatment. Interaction with various intricate factors underlying the health problems and illnesses such as inadequate housing or poor air quality could cause health problems without the health care professionals being aware of it, despite their long-established and significant roles as health determinants.[1] The Sustainable Development Goals (SDGs), which were adopted by the United Nations in 2015, were defined as the overarching Global Goals to be achieved in the interrelationship between the social determinants of health and our environment, and address actions against climate that should be taken to contribute on individuals' health as well as global health.[2-5]

Improving individuals' well-being is at the centre of SDGs. A person's well-being is affected by various factors, for instance, physical and social environment, culture of the society in which his/her lives, and economic conditions.[6] An example of coronavirus disease is important in terms of evidencing that brought along social and economic problems as well as health issues.[7] Since individuals' well-being is not considered without all related determinants of health, achieving SDGs is an essential concept due to its general definition that meet the needs of the current generation without depleting the resources needed by the future generation.[8]

All the SDGs have one common components, which is health promotion due to its promoter and facilitator role in SDGs.[9,10] Today's health promotion

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programs generally include overall healthy lifestyle improvement, physical activity, and diet.[11] The concept of a healthy lifestyle comprises the last two components among them.[12,13] A healthy lifestyle allows a person to control all behaviours that could affect their health and to choose behaviours that fit their health status in regulating their daily activities. Healthy lifestyle behaviours, which are closely associated with diseases or mortality rates, is a part of a person's life helping her/him to stay healthy and improve her/his health status.[13]

The one of 17 the SDGs, which is "3. Good Health and Well-Being", focuses specifically on delivery healthy lives and promoting well-being for all at all ages.[3] This goal is core to the philosophy of Nightingale by not limited to caring for people during illness.[1] Although major progress was made in improving health, more efforts still are needed to reach better health conditions for all people in the World.[3] The health care professional, particularly nurses as a valued and trusted members of the health care team, play a critical role from providing care to reach wellness perspective to advocating for individuals' rights and contributing policy change.[2]

Environmental health, which is considered as one of the crucial drivers of individual well-being and global health, was first introduced in Nightingale's Environmental Theory (1863). Modern nursing have a global responsibility to apply her theory into current practice by optimizing the environment for individuals' wellness.[2] Nurses are a stronger position to be a role model in acquiring healthy lifestyle behaviours of society due to being healthcare professionals who closely and frequently serve people. Nurses assess society's healthy lifestyle behaviours using valid measurements and apply programs to improve their healthy lifestyle behaviours.[12] They also in position to be a health educator to explain society the potential risks of poor environmental quality.[14] Although expectation of being a role model for society, nurses themselves also have a high risk of unhealthy behaviours due to their stressful work environment and might contribute negatively to the environmental health through their actions without their knowledge.[2,15]

Contributions of nurses to healthy lifestyle of society and environmental health could be realized by integrating key issues and concepts of the philosophy of Nightingale into nursing education program. Courses including building and sustaining safety environment for individuals has been already included in the nursing curriculum.[4] Nurses are assumed to be knowledgeable regarding healthy lifestyle behaviours and improve environment for one's wellness based on their education.[2,15] Although building and sustaining safety environment for improving health and well-being in nursing curricula in Turkey and many other countries, evidence shows that nurses still need to have skills and competence in environmental sustainability as well as knowledge.[4,14]

Aim

Therefore, this study aimed to determine the effectiveness of the "Healthy Life and Environment" course on nursing students' awareness of sustainable development, healthy life, and health-promoting lifestyle behaviors.

Material and methods

Design and participants

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Bartin University (Reference number: 2021-SBB-0360).

This study used a pre-, and post-test control group quasi-experimental research design. The study was conducted in the Department of Nursing at a public university located in north-western Turkey during the fall semester of 2021/2022 academic year. Inclusion criteria were: (i) students from both first and second years who had not yet enrolled in the "Healthy Life and Environment" course, a second-year course which might affect the results, were excluded, (ii) students who provided informed consent. Any sampling method did not used due to all students from first and second years were recruited to the study. Students enrolled in the "Healthy Life and Environment" course assigned to intervention group, those who did not attend the course assigned to control group. We have reported findings according to the CONSORT 2010 Statement.[16]

Instruments

A survey form, Healthy Lifestyle Behaviour Scale II, Healthy Life Awareness Scale, and Development Awareness Scale were used for data collection. Data were collected two times, a 15-week interval when was at the beginning and end of the fall semester of 2021/2022 academic year.

Survey form

Survey form included questions related to nursing students' age, gender, income perception, tobacco and alcohol habits, and Body mass index (BMI). BMI was calculated by using the formula: BMI=(weight (kg)/height (m^2).[17]

Health-Promoting Lifestyle Profile II (HPLP II)

This scale was used to assess students' health-promoting lifestyle behaviours. The scale has 52 items and 6 subdimensions including health responsibility (3, 9, 15, 21, 27, 33, 39, 45, 51), physical activity (4, 10, 16, 22, 28, 34, 40, 46), nutrition (2, 8, 14, 20, 26, 32, 38, 44, 50), spiritual growth (6, 12, 18, 24, 30, 36, 42, 48, 52), interpersonal relations (1, 7, 13, 19, 25, 31, 37, 43, 49), and stress

management (5, 11, 17, 23, 29, 35, 41, 47). Each item is coded from 1 (Never) to 4 (Regularly). Total score of the scale ranges from 52 to 208. Cronbach Alpha coefficient of the scale was calculated as 0.92 for the Turkish population.[12] In this study, we calculated the Cronbach Alpha coefficient as 0.91.

Healthy Life Awareness Scale (HLA)

HLA was developed by Özer and Yılmaz. The scale consists of 15 items and 4 subdimensions including alteration (1, 9, 12, 19, 23), socialization (3, 10, 14, 22), responsibility (37, 38, 40), and nutrition (6, 18, 26). Each item is scored from 1 (strongly disagree) to 5 (strongly agree) and total score ranges from 15 to 75, which the highest score addresses high level awareness. Cronbach Alpha coefficient was 0.81 in the original study, we calculated it as 0.89 in this study.[13]

Sustainable Development Awareness Scale (SDA)

SDA was developed by Atmaca et al. This 5-point Likert-type scale consists of 37 items and 3 dimensions including economy (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13), society (14, 15, 16, 17, 18, 19, 20, 21, 22), and environment (23, 24, 25, 27, 28, 29, 30, 31, 32, 33, 34,35, 36, 37). Each item is scored from 1 (strongly disagree) to 5 (strongly agree) except 1, 8, 10, 24, 31 and 35 items that are reverse coded. One item (26) is not scored due to it is a control item. Total score ranges from 39 to 180, which the highest score addresses high level awareness. Cronbach Alpha coefficient was .91 in the original study, we calculated it as 0.95 in this study.[8]

Procedure

"Healthy Life and Environment" course, which is an optional course taken in the second year (third semester) in nursing program, was the intervention in this study. The course, which is two hours a week, was delivered using traditional teaching methods such as lecturing, discussion, and brainstorming. Outcomes of the course were: (i) defining environmental pollution nationally and internationally, (ii) defining impact of environmental pollution on health, (iii) defining associations between environment and health, and (iv) defining roles and responsibilities of nurses in environmental health.[18] In total, 189 first- and second-year nursing students were assessed for eligibility, 57 of all enrolled "Healthy Life and Environment" course. The first-year nursing students and 38 second-year nursing students who did not attend "Healthy Life and Environment" course assigned as control group. After excluding students who declined to participate the study (n=29), 55 students assigned in intervention group, 105 in control group (Fig. 1). Students only were blinded in this quasi-experimental study. Data was collected via a paper-based form, and face-to-face interviews with students lasted for roughly 15–20 minutes.

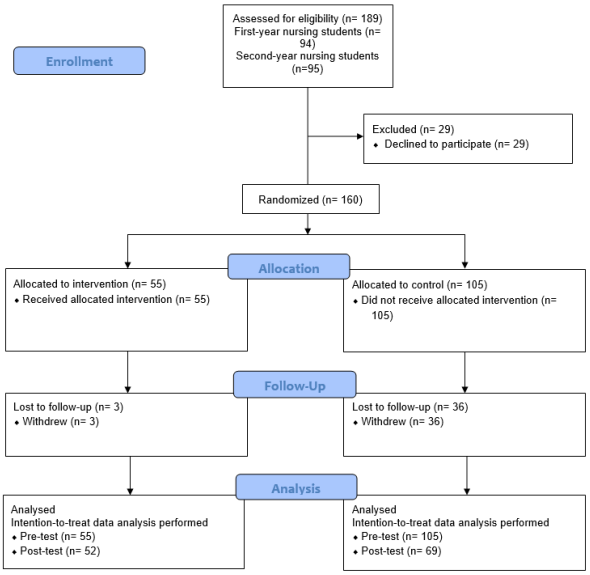

Fig. 1. CONSORT diagram of study procedure

Data analysis

A normal distribution of data was assessed by Shapiro-Wilk tests. Frequency distribution, mean, and standard deviation were used to report study participants' characteristics. Baseline characteristics of nursing students according to intervention and control groups were compared using a chi-squared test or Fisher Exact test that was interpreted in when one or more expected values are less than 5. Mann-Whitney U and Kruskal-Wallis test were used to determine differences between demographic characteristics for categorical variables, Health-Promoting Lifestyle Profile II, Healthy Life Awareness, and Sustainable Development Awareness. Wilcoxon signed-rank test and Mann-Whitney U were performed to determine the effectiveness of "Healthy Life and Environment" course on nursing students' health-promoting lifestyle, healthy life, and sustainable development awareness. Analyses of the effect of intervention were performed as intention-to- treat (ITT).[19] Data analysis was conducted using the IBM SPSS software version 25.0 (Armonk, NY, USA) considering $p < 0.05$ to be statistically significant.

Results

Sample characteristics

Baseline characteristics of nursing students was presented in Table 1. Majority nursing students (67.5%) were female, and the main age was 19.80±1.42. Half of the participants (50%) had income-expense-balanced and 64.2% of them were in the normal weight range. Almost all students did not use tobacco products (80.0%) or consume alcohol (79.7%).

Female students had higher level of health-promoting lifestyle profile (U=2115.500; p=0.012) and sustainable development awareness (U=2121.500; p=0.012) than

Table 1. Baseline characteristics of nursing students[a]

Characteristics	Overall (n=160)	Control (n=105)	Intervention (n=55)	X^2/ p value
Age, mean±SD*	19.80 (±1.42)	19.34 (±1.25)	20.09 (±1.30)	3.543/0.001
Gender				
Female	108 (67.5)	71 (67.6)	37 (67.3)	1.601/0.965
Male	52 (32.5)	34 (32.4)	18 (32.7)	
Income perception				
income>expenditure	21 (13.1)	12 (11.4)	9 (16.4)	0.807/0.668
income<expenditure	59 (36.9)	40 (38.1)	19 (34.5)	
income=expenditure	80 (50.0)	53 (50.5)	27 (49.1)	
Tobacco use				
Yes	32 (20.0)	21 (20.0)	11 (20.0)	0.000/1.000
No	128 (80.0)	84 (80.0)	44 (80.0)	
Alcohol consumption**				
Yes	15 (20.3)	14 (19.7)	1 (33.3)	0.330/0.499
No	59 (79.7)	57 (80.3)	2 (67.6)	
BMI**				
Underweight (less than 18.4)	22 (13.8)	14 (13.3)	8 (14.8)	1.434/0.715
Normal weight (18.5–24.9)	102 (64.2)	65 (61.9)	37 (68.5)	
Overweight (25–29.9)	21 (13.2)	15 (14.3)	6 (11.1)	
Obese (30–34.9)	14 (8.8)	11 (10.5)	3 (5.6)	

[a] SD – standard deviation; * – t-test; ** – Fisher's exact test

Table 2. Health-promoting lifestyle, healthy life and sustainable development awareness according to baseline characteristics of nursing students

Characteristics	Health-Promoting Lifestyle Profile II (Mean Rank)	Healthy Life Awareness (Mean Rank)	Sustainable Development Awareness (Mean Rank)
Gender			
Female	86.91	85.18	86.86
Male	67.18	70.79	67.30
U/p	2115.500/0.012	2303.000/0.066	2121.500/0.012
Income perception			
income>expenditure	68.12	64.14	78.48
income<expenditure	85.38	85.65	86.59
income=expenditure	80.15	80.99	76.54
KW/p	2.159/0.340	3.357/0.187	1.646/0.439
Tobacco use			
Yes	47.33	62.42	73.30
No	88.79	85.02	82.30
U/p	986.500/<0.001	1469.500/0.014	1817.500/0.325
Alcohol consumption			
Yes	27.73	25.63	39.60
No	39.98	40.52	36.97
U/p	296.000/0.049	264.500/0.017	411.000/0.672
BMI			
Underweight (less than 18.4)	82.43	65.11	82.07
Normal weight (18.5–24.9)	79.25	82.64	77.84
Overweight (25–29.9)	69.83	68.05	70.38
Obese (30–34.9)	96.93	102.11	106.93
KW/ p value	3.005/0.391	7.279/0.064	5.975/0.113

[a] U – Mann-Whitney U; Z – Wilcoxon Signed Rank test

male. Moreover, participants who did not use tobacco or consume alcohol had had higher level of health-promoting lifestyle profile (U=986.500; p<0.001 and U=296.000; p=0.049, respectively) and sustainable development awareness (U=1469.500; p=0.014 and U=264.500; p=0.017, respectively) compared to users or consumers (Table 2). It is not presented in any table, total scores of Health-Promoting Lifestyle Profile, Healthy Life Awareness, and Sustainable Development Awareness with subdimensions were found not to show significant differences according to intervention and control groups.

Table 3. Differences for subscale of health-promoting lifestyle between the control and intervention groups (n=160)[a]

Measurement	Control Mean±SD (n=105)	Intervention Mean±SD (n=55)	U/p
Health-Promoting Lifestyle Profile II Total Score			
Pre-test	2.43±0.38	2.45±0.32	1682.000/0.489
Post-test	2.52±0.37	2.48±0.36	
Z/ P value	-2.341/.019	-0.310/.757	
Health responsibility			
Pre-test	2.25±0.48	2.29±0.50	1661.500/0.423
Post-test	2.38±0.50	2.36±0.59	
Z/p	-1.316/0.188	-0.328/0.743	
Physical activity			
Pre-test	2.18±0.52	2.12±0.46	1742.500/0.705
Post-test	2.30±0.58	2.20±0.53	
Z/p	-2.452/.014	-1.221/.222	
Nutrition			
Pre-test	2.15±0.46	2.23±0.40	1770.000/0.814
Post-test	2.68±0.47	2.22±0.46	
Z/p	-2.190/0.029	-0.141/0.888	
Spiritual growth			
Pre-test	2.84±0.51	2.87±0.52	1764.500/0.792
Post-test	2.89±0.47	2.89±0.44	
Z/p	-1.528/0.126	-0.155/0.877	
Interpersonal relations			
Pre-test	2.77±0.52	2.80±0.43	1792.500/0.906
Post-test	2.79±0.42	2.79±0.442	
Z/p	-0.818/0.413	-0.099/0.921	
Stress management			
Pre-test	2.40±0.48	2.40±0.37	1633.000/0.342
Post-test	2.51±0.47	2.42±0.45	
Z/p	-2.393/0.017	-0.107.915	

[a] U – Mann-Whitney U; Z – Wilcoxon Signed Rank test

Health-Promoting Lifestyle Profile

The average pre- and post-test health-promoting lifestyle profile scores for the intervention group were 2.45 (SD=0.32) and 2.48 (SD=0.36), respectively, indicating an improvement of .03 points. On the other hand, for the control group, these scores were 2.43 (SD=0.38) and 2.52 (SD=0.37), respectively, indicating an improvement of 0.09 points. Mann-Whitney U test showed there was not a statistically significant difference between groups

at post-test measurement (U=1682.000; p=0.489). Moreover, all subdimensions of Health-Promoting Lifestyle Profile II scale did not indicate any statistically significant between groups at post-test measurements (Table 3).

Healthy Life Awareness

The average pre- and post-test healthy life awareness scores for the intervention group were 3.93 (SD=0.54) and 3.93 (SD=0.57), respectively, indicating no change. Alternatively, scores of control group were 3.84 (SD=0.63) and 3.33 (SD=0.55), respectively, indicating a drop off of 0.51 points. However, Mann-Whitney U test indicated there was not a statistically significant difference between groups (U=1575.500; p=0.489). Moreover, any four subdimensions of Healthy Life Awareness scale did not indicate any statistically significant at post-test measurements (Table 4).

Table 4. Differences for subscale of healthy life awareness between the control and intervention groups (n=160)[a]

Measurement	Control (n=105)	Intervention (n=55)	U/p
Healthy Life Awareness Total Score			
Pre-test	3.84±0.63	3.93±0.54	1575.500/0.212
Post-test	3.33±0.55	3.93±0.57	
Z/p	-0.466/0.641	-0.151/0.880	
Alteration			
Pre-test	4.04±0.68	4.07±0.63	1755.500/0.754
Post-test	4.03±0.53	4.07±0.62	
Z/p	-0.376/0.707	-0.034/0.973	
Socialization			
Pre-test	3.65±0.71	3.81±0.62	1622.500/0.309
Post-test	3.61±0.74	3.72±0.69	
Z/p	-0.234/0.815	-0.486/0.627	
Responsibility			
Pre-test	3.94±0.79	4.05±0.71	1635.500/0.335
Post-test	3.92±0.68	4.05±0.73	
Z/p	-0.474/0.636	-0.094/0.925	
Nutrition			
Pre-test	3.70±0.76	3.79±0.74	1602.000/0.256
Post-test	3.75±0.73	3.89±0.76	
Z/p	-0.748/0.455	-0.823/0.411	

[a] U – Mann-Whitney U; Z – Wilcoxon Signed Rank test

Sustainable Development Awareness

The average pre- and post-test sustainable development awareness scores for the intervention group were 4.11 (SD=0.59) and 4.12 (SD=0.60), respectively, indicating a slight improvement of .01 points. On the other hand, for the control group, these scores were 4.29 (SD=0.54) and 4.16 (SD=0.64), respectively, indicating a drop off 0.13 points. Mann-Whitney U test showed there was not a statistically significant difference at post-test measurement (U=1674.500; p=0.465). Moreover, all subdimensions of Sustainable Development Awareness scale did not show any statistically significant at post-test measurements (Table 5). Although it is not presented in a table, Spearman's rho correlation showed that SDA had a weak correlation with HLA (r= 0.298, p<0.001) and a very week correlation with HPLP II (r= 0.140, p<0.05) at baseline.

Table 5. Differences for subscale of sustainable development awareness between the control and intervention groups (n=160)[a]

Measurement	Control (n=105)	Intervention (n=55)	U/p
Sustainable Development Awareness Total Score			
Pre-test	4.29±0.54	4.11±0.59	1674.500/0.465
Post-test	4.16±0.64	4.12±0.60	
Z/p	-1.587/0.112	-0.034/0.973	
Economy			
Pre-test	4.15±0.57	3.97±0.58	1682.000/0.488
Post-test	4.04±0.63	4.00±0.56	
Z/p	-1.582/0.114	-0.323/0.746	
Society			
Pre-test	4.53±0.64	4.34±0.68	1788.500/0.887
Post-test	4.37±0.77	4.36±0.74	
Z/p	-1.615/0.106	-0.124/0.901	
Environment			
Pre-test	4.18±0.52	4.01±0.60	1676.500/0.470
Post-test	4.08±0.62	4.01±0.60	
Z/p	-1.082/0.279	-0.053/0.958	

[a] U – Mann-Whitney U; Z – Wilcoxon Signed Rank test

Discussion

The purpose of this study was to determine the effectiveness of "Healthy Life and Environment" course on nursing student's awareness of sustainable development, healthy life and health-promoting lifestyle behaviours by using a quasi-experimental research design. Our study findings demonstrated that certain characteristics of students including being female, not using tobacco or consuming alcohol positively effect on their health-promoting lifestyle behaviours. Contrary to our study's findings, other recent prior studies found no statistically significant differences between gender and a health-promoting lifestyle profile.[20-22] The present study showed a slight improvement of health-promoting lifestyle behaviours in intervention group despite not finding a statistically significant impact of the course on health-promoting lifestyle behaviours. Our study sample's overall health-promoting lifestyle score at baseline was greater than that of both the intervention and control groups, as reported in previous studies.[20,21,23,24] Having high score of nursing students at baseline in this study could be the reason for the slight increase of health-promoting lifestyle score in the intervention group.

In addition to the high-level of health-promoting lifestyle score, our sample had a high score of healthy life awareness, which is consistent with a recent study;

however, we did not find any impact of the intervention on students' healthy life awareness.[25] Supporting healthy lifestyle awareness and behaviours of students by the nursing curriculum is suggested in the literature to prevent unhealthy behaviours such as sedentary lifestyle patterns among students and to make lifestyle changes that might affect their health and wellbeing.[26] Our sample both intervention and control group had higher level healthy life awareness. Contrary to common belief that young people have unhealthy habits despite awareness of healthy lifestyle's effect on their health, only small number of our study sample used tobacco (20%) or consumed alcohol (20.3%).[27] Experiencing higher levels of stress by nursing students compared to students in other health science could consist of a part of the reason for unhealthy behaviours like smoke or consuming alcohol.[28]

The awareness of sustainable development was higher among female students in the present study, which is consisted with the results of a recent study.[29] Although our findings did not show an impact of the course tested on nursing student's awareness of sustainable development, there was a slight improvement of sustainable development awareness in intervention group and a drop-off in control group. The third of the SDGs directly target at individuals' good health and promoting their well-being for all at all ages.[3] Healthy lifestyle is one of the most important indicators of SDGs on a global aspect and an effort is needed for supporting awareness of healthy lifestyle.[30,31] This study findings illustrated that SDGs had association with healthy life awareness and health-promoting lifestyle profile. Moreover, tobacco users and alcohol consumers had low levels of awareness of sustainable development. Overall, our study findings confirmed that nurse educators need to be able to deliver information by using new teaching methods and upskilling their existing skill such as encourage students to be a part of a research.[14] Nurse educators are responsible for students' acquiring qualifications to be a role models in society with their healthy lifestyle and being a nurse having global perspective to achieve SDGs.[5] This result is necessary for integrating student-centered learning and sustainable development methodologies into nursing curricula.[14]

Study limitations

There are several limitations of the current study. First, health behaviours of nursing students were measured using a self-report instrument and we collected limited data about unhealthy behaviours of students including tobacco using, alcohol consuming, and BMI. Second, selection bias limits the generalizability of the results. This study did not apply any sapling method due to the study conducted in a single nursing college with a small sample. However, the study included a control group, and all students were blinded to manage this limitation and reduce bias. Lastly, it is possible that students in the control group improved their awareness during the assessment and they could communicate with students in the intervention group in dormitories or schools, and so this factor might have modified their awareness regarding SDGs, healthy life, and health-promoting lifestyle.

Conclusion

This study found that taking a course on "Healthy Life and Environment" did not increase nursing student's awareness of sustainable development and healthy life, and health-promoting lifestyle behaviours, but awareness of healthy life and health-promoting lifestyle behaviours had association with SDGs. Moreover, the present study defined certain sample characteristics including being female, not using tobacco or consume alcohol that impacted students' health-promoting lifestyle profile and only being female affected awareness of SDGs. The results of our study highlighted the need to adapt sustainable development methodology using student-centered learning into nursing curricula to increase the qualification of nursing students to be role models in society and nurses have global perspectives to achieve SDGs as well as the need for nurse educators' upskilling. Further, "Healthy Life and Environment" courses using innovative teaching strategies such as student-centered learning approaches are recommended to expand nursing students' awareness of sustainable development and healthy life, and health-promoting lifestyle behaviors and tested utilising randomized controlled trials method.

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Author contributions

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Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Bartin University (Reference number: 2021-SBB-0360).

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ORIGINAL PAPER

Utilizing machine learning to create a blood-based scoring system for sepsis detection

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ABSTRACT

Introduction and aim. Sepsis, a disease caused by inflammation as a response to infection, often goes undiagnosed due to its heterogeneity and lack of a single diagnostic test. Current sepsis detection scoring systems have low sensitivity and utilize biomarkers that are difficult to obtain from a single test. The goal of this research is to create a scoring system that outperforms current industry standards by utilizing blood-based biomarkers readily available in hospital settings.

Material and methods. Machine learning algorithms were run through Google Colab using Extreme Gradient Boost classifier. The dataset was obtained from NCBI website containing electronic hospital records of intensive care patients. A multivariate linear regression was applied to the dataset to determine statistically significant biomarkers in the detection of sepsis, and their β coefficients. Then, validation testing was performed, and the performance was compared to other scoring systems.

Results. This experiment reveals that a sepsis detection system that utilizes procalcitonin, white blood cells, C-reactive protein, neutrophil to lymphocyte ratio, and albumin can outperform other biomarkers and scoring systems with high sensitivity at a recall score of 0.7922.

Conclusion. These results demonstrate the potential of utilizing a blood-based scoring system for sepsis detection within hospital settings.

Keywords. blood-based, machine learning, sepsis

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.[1] It may lead to shock, multi-organ failure, and death – especially if not recognized early and treated promptly.[2,3] Sepsis is a Global Health crisis with 47–50 million cases each year of which 11 million people die, as of 2020. Most cases of sepsis are caused by bacterial infections, however, other infections like viral infections, such as COVID-19 or influenza, or fungal infections can also cause sepsis.[4] Sepsis is hard to diagnose because it is a heterogeneous syndrome whose course depends on different pathophysiological mechanisms, complexity in clinical context, and clinical phenotypes. Hospitals use scoring systems such as Acute Physiology and Chronic Health Evaluation (APACHE), Sequential Organ Failure Assessment (SOFA), and Quick SOFA (qSOFA) for sepsis detection; however they have low sensitivity and often require biomarkers from multiple different tests.[5] The SOFA score numerically quantifies the number and severity of failed organs. It provides potentially valuable prognostic information on in-hospital survival when applied to patients with severe sepsis with evidence of hypoperfusion at the time of emergency department presentation.[6] The Logistic Organ Dysfunction Score (LODS) is a tool to identify patients at high risk of developing postoperative severe sepsis and the need for early goal-directed therapy.[7] APACHE is a successful scoring system assessing severity

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of illness and prognosis of intensive care unit patients and has been evaluated and validated in patients for mortality outcome.[8] MODS refers to the clinical syndrome in which at least two systems or organs suffer from simultaneous or sequential dysfunction during major surgery, infection, shock, poisoning and severe trauma, thus undermining the stability of the internal environment.[9] Systemic inflammatory response syndrome (SIRS) is an exaggerated defense response of the body to a noxious stressor (infection, trauma, surgery, acute inflammation, ischemia or reperfusion, or malignancy) to localize and then eliminate the endogenous or exogenous source of the insult. SIRS with a suspected source of infection is termed sepsis.[10] There is still no current standard for sepsis detection scoring systems. Instead, blood culture is considered gold standard for the confirmation of bacteremia which can isolate and identify the causative agent and subsequently test the antimicrobial sensitivity, but the delayed process of bacterial culture emphasizes the importance of a reliable and fast sepsis detection system that utilizes only a few biomarkers that are readily available in hospital and clinical settings.[11]

Procalcitonin (PCT) is a protein that is synthesized in the thyroid gland as an immediate response to inflammation, making it a good biomarker for sepsis detection.[11,12] In everyday clinical practice, worldwide, white blood cells (WBC) and C-reactive protein (CRP) still represent the cornerstones on which is based the diagnosis and prognosis of infected patients together with other signs and symptoms like fever, tachycardia, and tachypnea, when a documented or suspected infection is detected.[13] PCT, CRP and WBC can be combined as effective indicators for the identification of acute bacterial or no-bacterial infections in children. Albumin can be another biomarker for sepsis as their levels are associated with short-term and long-term outcomes in sepsis.[15] C-reactive protein (CRP) and serum albumin (ALB) are useful markers that can predict morbidity and mortality among critically ill patients. This is because CRP effectively reflects acute-phase inflammation while ALB may reflect malnutrition among critically ill patients.[16] The neutrophil-lymphocyte ratio (NLR) is an inflammatory biomarker that uses two types of white blood cells (neutrophils and lymphocytes) that when out of equilibrium can indicate systemic inflammation. NLR serves as an effective biomarker, as neutrophils are typically an immediate response to infection, while lymphocytes are not.[17] Together, PCT, CRP, WBC, Alb, and NLR serve as the biomarkers used for sepsis detection in this machine learning model. As of recent, machine learning has been a point of focus in the biomedical research space, as it allows for automation in the healthcare industry, minimizing human error and maximizing efficiency. Still, current research on sepsis diagnostic scoring systems is limited. Previous studies have explored procalcitonin as a possible biomarker for sepsis detection, however sensitivity was below satisfactory, and the biomarkers chosen are not all blood-based and include comorbidities, which may create a less reliable model due to high correlation.[18]

Aim

The aim of this study was to create a sepsis detection artificial intelligence model utilizing only blood-based biomarkers readily available in hospital and clinical settings that outperforms current sepsis scoring systems.

Material and methods

Dataset

The dataset used in this study was obtained from the National Center for Biotechnology Information website.[18] The electronic health records were obtained from Saint Mary's Hospital Luodong, Taiwan and was approved by the Institutional Review Board of Saint Mary's Hospital Luodong (approval # SMHIRB_105012). The data were analyzed anonymously. The dataset consisted of 258 critically ill patients with sepsis group (n=115) and the non-sepsis group (n=143). It had 79 features including demographics, comorbidities, clinical variables, scoring systems, and the Sepsis label.

Preprocessing

The dataset was then preprocessed using Python on Google Colab. First, features irrelevant or highly correlated to sepsis were removed. These features include sex, comorbidities, and features specific to the hospital the data was collected in. Ultimately, 27 features were removed including 'sex', 'IPS' 'ICU_BilT', 'inhos_mortality', 'Reasons_ICUadm', 'Bacteremia', 'UTI', 'Pneumonia', 'Skin_infection', 'Other_infection', "Infection", 'CS_CADMI', 'CS_CHF', 'CS_CKD', 'CS_PADPAOD', 'CS_CVA', 'CS_Dementia', 'CS_CLD', 'CS_PUD', 'CS_HTN',CS_DM', 'CS_LD', 'CS_cancer', 'ICU_day', 'Division_ICUadm,"'VPAP_A"',"CPCR". After analyzing the dataset, it was evident that the feature of CRP had null values for several patients, so data imputation was utilized through filling in blank spaces with median values. The feature 'Sepsis_3', which is the label, was also removed from the dataset before performing the multivariate linear regression. The test procedure flow chart is shown in Figure 1.

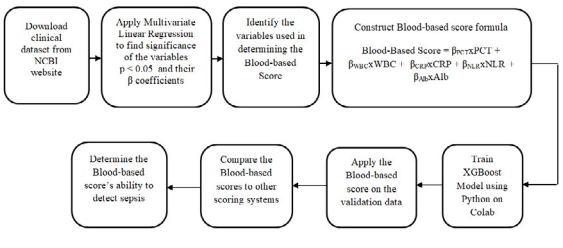


Fig. 1. Flowchart of procedure

Multivariate linear regression

Continuing on Google Colab, a multivariate linear regression analysis was applied to the dataset to determine the p values and the β coefficients for the different features. The five blood-based features that were determined to be statistically significant ($p<0.05$) in detecting sepsis include PCT, CRP, WBC, Alb, and NLR. The formula for the blood-based scoring system was set as Blood-based score=β_{PCT}xPCT + β_{CRP}xCRP + β_{WBC}xWBC + β_{Alb}xAlb + β_{NLR}xNLR. The blood-based scoring system was added back to the dataset as a feature and 'Sepsis_3' was added back as the label.

XGBoost model testing

Due to its speed, ease of use, and superior performance, XGBoost was the machine learning classifier used in this experiment.[19] The scoring system was then validated through the model and performance was compared to PCT alone and other sepsis scoring systems including SOFA, APACHE, LODS, MODS, and SIRS. The AUC curve was plotted, and the accuracy and recall scores were considered.

Results

Multivariate linear regression

The results of the multivariate linear regression along with the features and their p values and β coefficient values are shown in Table1. There were a total of nine features that were statistically significant ($p<0.05$) in detecting sepsis; however, excluding those that are scoring systems and biomarkers that can't be extracted from a blood test, only 5 biomarkers fit the criteria. These blood-based biomarkers include PCT, CRP, WBC, Alb, and NLR. They had p values of 0.042, 0.041, 0.039, 0.001, and 0.001 respectively. Additionally, the β coefficient values for these statistically significant biomarkers were 0.0202, -0.0002, -0.0155, -1.4525, and 0.0419 respectively.

XGBoost model performance

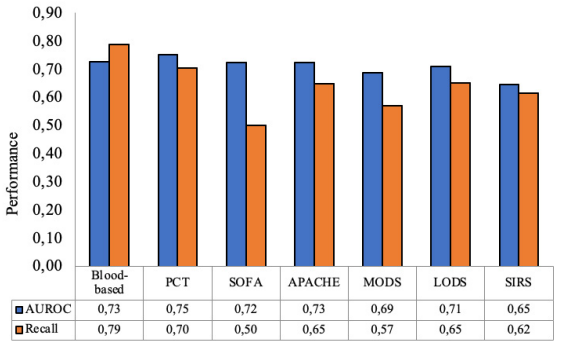
Once the formula β_{PCT}xPCT+β_{CRP}xCRP+β_{WBC}xWBC+β_{Alb}xAlb+β_{NLR}xNLR was applied to create the Blood-based score, training and validation testing was performed. The blood-based scoring system created, outperformed all other scoring systems, as it had a recall of 0.7922, while PCT alone had a recall of 0.7047, SOFA had a recall of 0.5044, APACHE had 0.6480, MODS had 0.5748, LODS had 0.6520, and SIRS had 0.6146. However, the blood-based score underperformed in other metrics with an AUROC of 0.7321, an accuracy of 0.6154, and a precision of 0.4615. The performance of the various scoring systems is shown in Table 2. A graph showing their AUROC and recall is shown in the Figure 2. The ROC (Receiver Operating Characteristic) curve is a graph of false positive rate, which is the x-axis, and true positive rate which is the y-axis. AUROC (Area Under Receiver Operating Characteristic Curve) is a metric for assessing an ROC curve's performance. A greater AUROC indicates a higher model performance, through minimal false positives and a higher rate of true positives. The greatest possible AUROC is 1. The AUROC curves of the scoring systems in shown in Figure 3.

Table 1. Multivariate linear regression results: p values and β coefficients for features

Feature	p	β coefficient
ICU_BT (body temperature)	0.473	0.1408
ICU_HR (heart rate)	0.463	0.0089
ICU_RR (respiratory rate)	0.516	0.0189
ICU_SPO_2 (oxygen Saturation)	0.002	0.1056
ICU_MAP (mean arterial pressure)	0.029	-0.0209
ICU_GCS (Glascow coma scale)	0.155	-0.1419
ICU_Cr (creatinine)	0.759	0.0336
ICU_PCT (procalcitonin)	0.042	0.0202
bCr (breakpoint cluster region)	0.97	0.4722
ICU_CRP (C-reactive protein)	0.041	-0.0002
ICU_BUN (blood urea nitrogen)	0.853	0.0015
ICU_eGFR (estimated glomerular filtration)	0.774	-0.0012
ICU_Ca (calcium)	0.539	0.1477
ICU_GLU (glucose)	0.968	-5.206e-05
ICU_Alb (albumin)	0.001	-1.4525
ICU_GOT (glutamic-ocaloacetic transaminase)	0.934	0.0002
ICU_GPT (glutamic-pyruvic trandaminase)	0.413	-0.0041
ICU_Na (sodium)	0.707	-0.0083
ICU_K (potassium)	0.661	-0.0938
ICU_PH (potential of hydrogen)	0.221	-2.8951
ICU_PCO_2 (partial pressure of oxygen)	0.403	-0.0151
ICU_ PO_2 (partial pressure of oxygen)	0.073	-0.0089
ICU_HCO_3 (bicarbonate)	0.401	0.0343
ICU_SO_2 (sulfur dioxide)	0.03	0.0380
ICU_WBC (white blood cells)	0.039	-0.0155
ICU_HB (hemoglobin)	0.665	0.2543
ICU_HCT (hematocrit)	0.784	-0.0530
ICU_NLR (neutrophil to lymphocyte ratio)	0.001	0.0419
ICU_PLT (platelet count)	0.514	0.0015
qSOFA (quick sequential organ failure assessment)	0.384	-0.3493
MODS (multiple organ dysfunction score)	0.656	-0.0873
APACH (APACHE II)	0.474	-0.0397
SIRS (systemic inflammatory response syndrome)	0.566	0.1769
LODS (logistic organ dysfunction system)	0.158	-0.2167
SOFA (sequential organ failure assessment)	0.02	0.3389

Table 2. Validation testing results for Blood-based Score, PCT and other scoring systems

Scoring System	Recall	AUROC	Accuracy	Precision
Blood-based Score	0.7922	0.7321	0.6154	0.4615
PCT	0.7047	0.7549	0.75	0.5909
SOFA	0.5044	0.7370	0.6731	0.5926
APACHE	0.6480	0.7370	0.5577	0.5833
MODS	0.5748	0.6851	0.7115	0.6522
LODS	0.6520	0.7144	0.7115	0.6429
SIRS	0.6146	0.6488	0.5769	0.5667



	Blood-based	PCT	SOFA	APACHE	MODS	LODS	SIRS
AUROC	0,73	0,75	0,72	0,73	0,69	0,71	0,65
Recall	0,79	0,70	0,50	0,65	0,57	0,65	0,62

Fig. 2. Graph of scoring systems' performance (AUROC in blue and recall in red)

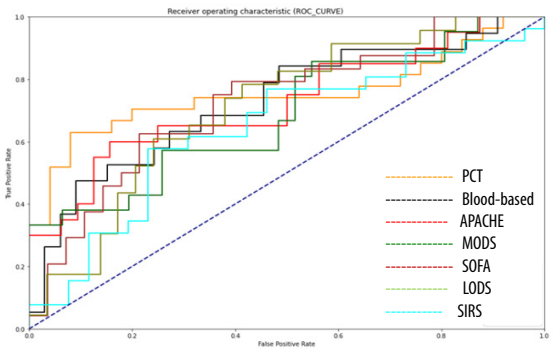


Fig. 3. AUROC Curve

Discussion

Hospital mortality from sepsis has ranged from 25% to 80% over the last few decades. Although mortality may be lower in recent years, sepsis is clearly still a very serious condition.[20] This study has developed a machine learning model that detects sepsis utilizing a combination of five blood-based biomarkers procalcitonin, white blood cells, C-reactive protein, neutrophil to lymphocyte ratio, and albumin which outperformed procalcitonin biomarker as well as other scoring systems such as APACHE, MODS, SOFA, LODS and SIRS with a higher sensitivity. A study conducted by Luo et al., 2009 found that LODS, SOFA and MODS show a good discrimination power, while maximum LODS is of the highest discrimination power to predict the outcome of patients with severe sepsis.[21] The blood-based biomarker developed in this study outperformed the LODS scoring system. A recent study conducted by Peipei Liang and Feng Yu supports the effective use of blood-based biomarkers in sepsis prognosis. They concluded that NLR, CRP, and PCT have important clinical applications in the assessment of the extent of disease and prognosis of patients with bloodstream infection and sepsis.[22] Interestingly, another study comparing the effectiveness of PCT and CRP concluded that PCT concentrations during multiple organ dysfunction syndrome provides more information about the severity and the course of the disease than that of CRP.[23] The current study is in line with the research conducted by Claudia Gregoriano. He concluded that PCT is very effective in detecting sepsis, but yet should be used as a complementary tool combined with available clinical and diagnostic parameters.[24] A similar study conducted by Tsui et al., 2021 used machine learning to develop a novel PCT-based score which outperformed existing sepsis biomarkers and scoring systems in sepsis detection. This PCT-based score was composed of five predictors, including higher serum PCT level, higher NLR, lower albumin level, diabetes mellitus, and necessitating vasopressor and performed at a sensitivity of 0.70.[18]

The blood-based scoring system developed in this study had a slightly lower AUROC than the PCT biomarker. But it had the highest recall score of 0.79, which is considered the most important metric of performance, as higher recall scores indicate minimal false negatives to minimize mortality. Even though the blood-based scoring system demonstrated satisfactory results relative to the other scoring systems, recall and AUC scores for the model were still considerably low. It also had lower precision than other scoring systems, which could lead to excessive medical intervention for healthy patients. To optimize performance, a greater balance between recall and precision scores must be met. The major limitation of this study is the limited data availability of sepsis patients. Further research would include testing of the model with larger datasets from Medical Information Mart for Intensive Care (MIMIC), which will lead to more accurate β coefficients, hence improving the performance. In addition to XGBoost, various other machine learning algorithms could be developed and tested utilizing the same blood-based biomarkers which may lead to higher performance.

Conclusion

This research has explored a novel blood-based scoring system based on PCT, CRP, NLR, WBC, and Alb. Because sepsis is a disease that is often acquired in hospital settings, this scoring system has the potential to be used within hospital and clinical settings, due to its sole use of blood-based biomarkers. This study also shows the potential for multivariate linear regression to be used as a tool for creating disease detection scoring systems. In conclusion, this study demonstrates the potential to utilize a blood-based scoring system to detect one of the deadliest and most widespread diseases: sepsis.

Declarations

Funding

This research received no external funding.

Author contributions

Conceptualization, S.A.; Methodology, S.A.; Software, S.A.; Validation, S.A.; Formal Analysis, S.A.; Investigation, S.A.; Resources, S.A.; Data Curation, S.A.; Writ-

ing Original Draft Preparation, S.A.; Writing - Review & Editing, S.A.; Visualization, S.A.; Supervision, S.A.; Project Administration, S.A.

Conflicts of interest
Author declares no conflict of interest.

Data availability
Data will be made available on reasonable request.

Ethics approval
Non applicable.

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ORIGINAL PAPER

Characterization of SUS-treated amputees at the Itanhaém Municipal Rehabilitation Center from 2012 to 2020

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ABSTRACT

Introduction and aim. Analyzing the factors related to amputations and understanding which factors are changeable can assist in the development of public policies for the prevention and education of the population. Objective: To describe the main etiological characteristics and symptoms after amputation in the Unified Health System (Sistema Único de Saúde – SUS) patients in order to contribute to the information for treatment planning by the multiprofessional team for these patients.

Material and methods. Retrospective study from 2012 to 2020, evaluating data from all amputation cases seen at the Itanhaém Municipal Rehabilitation Service, SP.

Results. 76 cases were studied, mostly middle-aged men, Caucasian, smokers, hypertensive, type II diabetics, some with dyslipdemia, cardiopathies, and the main cause of amputation was infection after wound.

Conclusion. The population needs to be warned about the risk of amputation due to infected wounds in patients with difficulty in controlling their comorbidities. Patients who have undergone amputation, should be oriented towards an early utilization of rehabilitation services, and municipalities should prepare their medical and rehabilitation professionals for the treatment of characteristic symptoms that amputees report and prosthetization.

Keywords. amputation, phantom limb, physical therapy, rehabilitation, single health system

Introduction

Before the descriptions by Hippocrates in Ancient Greece, the surgical procedure of amputation was already described in the oldest document in Hindu literature, Rigveda, dated 1,200 B.C. With technological evolution, it is seen as one of the first steps toward rehabilitation and early return to activities of daily living. Among the main causes of amputation, peripheral vascular disease is the most common in people aged 50 to 75 years, and more than half have associated diabetes mellitus.[1,2] Among young people and men, trauma is the most frequent cause, as it leads to lesions of soft tissues and vasculonervous structures that cannot be repaired, causing amputation. Besides the aforementioned causes, musculoskeletal tumors, congenital causes, and infections are also listed as conditions that may require amputation.[3–5]

The rehabilitation process for amputees takes into account the presence of symptoms such as pain, sensory alterations, phantom limb, functional evaluation, comorbidities, access to purchase and maintenance of the prosthesis by the municipality or privately by the patient, as well as considering the place where the patient lives and the possibility of periodic follow-up of the means of assistance, which generates the need for a multiprofessional team in their care.[6–8] Moreover, one must consider the patient's expectations in relation to prosthetization, if his idea corresponds to reality in activities of daily life, leisure and even sports.[3,9,10]

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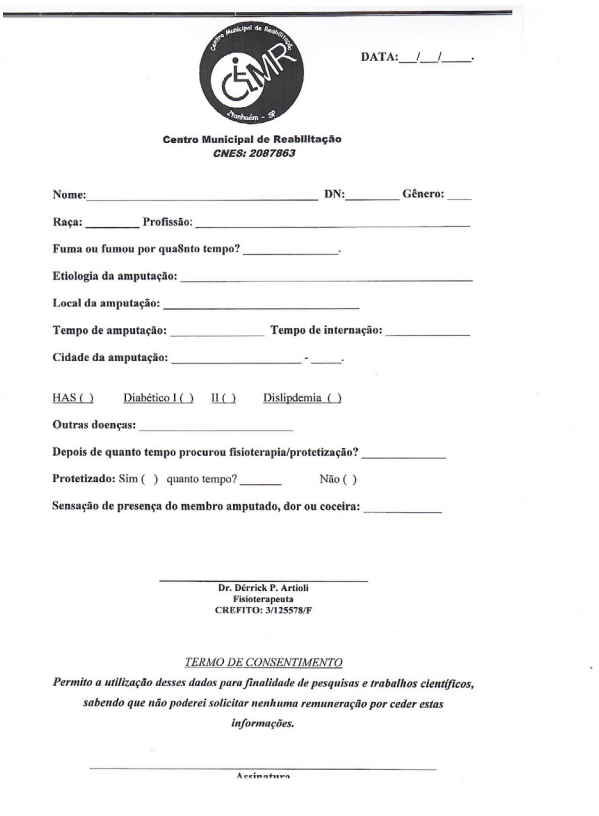
It is estimated that in the year 2050, approximately 3.6 million North Americans will suffer a lower limb amputation. It was estimated that in 2020 Brazil would be the sixth largest elderly population in the world, with 20% being diabetic.[11] The analysis of factors related to amputations and the understanding of which ones are changeable, can result in the development of public policies for the prevention and education of the population considering that many are due to complications of diseases that can be avoided, controlled with a low level of complexity and cost.[12]

Aim

Therefore, the objective of this study is to explore data from the health service responsible for these patients in a city in the state of São Paulo, showing the main etiological and postamputation characteristics. To contribute with information for planning the reception, orientations, and preparation of the multiprofessional team for this contingent.



DATA:__/__/____.

Centro Municipal de Reabilitação
CNES: 2087863

Nome:_____ **DN:**_____ **Gênero:** ___

Raça: _____ **Profissão:** _____

Fuma ou fumou por qua8nto tempo? _____.

Etiologia da amputação: _____

Local da amputação: _____

Tempo de amputação: _____ **Tempo de internação:** _____

Cidade da amputação: _____ - _____.

HAS ( ) Diabético I ( ) II ( ) Dislipdemia ( )

Outras doenças: _____

Depois de quanto tempo procurou fisioterapia/protetização? _____

Protetizado: Sim ( ) quanto tempo? _____ Não ( )

Sensação de presença do membro amputado, dor ou coceira: _____

_____
Dr. Dérrick P. Artioli
Fisioterapeuta
CREFITO: 3/125578/F

TERMO DE CONSENTIMENTO

Permito a utilização desses dados para finalidade de pesquisas e trabalhos científicos, sabendo que não poderei solicitar nenhuma remuneração por ceder estas informações.

_____
Assinatura

Fig. 1. Data collection form

Material and methods

This was an observational, retrospective study, evaluating the data of patients who were admitted to the Itanhaém Municipal Rehabilitation Center (CMR), which renders rehabilitation service via Unified Health System (Sistema Único de Saúde – SUS) for individuals who have suffered amputation, with approval by the Ethics Committee of the Lusíadas University Center (Opinion 5.815.874). The data collected was for the period 2012–2022, due to the COVID-19 Pandemic which changed the routine of care and reduced the number of elective surgeries. We collected information from 76 patients, using a form (Fig. 1), which collected information on the etiology, timing, anatomical site and place of amputation, comorbidities and related symptoms.

Principal Component Analysis (PCA) was performed, with a correlation matrix, to analyze the main explanatory variables. This test was used to analyze interrelationships between a large number of variables and explain them in terms of their components. The correlation of these factors was then analyzed using Pearson's correlation, with a significance level of 5%, adopting the r variation pattern between 0–0.3 insignificant, 0.31–0.5 weak, 0.51–0.7 moderate, 0.71–0.9 strong, and above 0.91 very strong.[13] The analyses were carried out using the Past 4.03 program (Oslo University, Norway).

Results

The mean age of patients was 57.6 years, most were men (58–76.4%), Caucasian (43–56.5%), smokers (60–78.9%) for 24.1 years on average, and the most common etiology was post- wound infection (37–48.6%) (Fig. 2). There were 41 (53.9%) with hypertension, 39 (51.3%) with type II diabetes, 29 (38.1%) with dyslipdemia and 12 (15.7%) with heart disease. The most common anatomical level of amputation was transtibial (30–39.4%), followed by transfemoral (28–36.8%) and to a lesser extent involving the ankle, foot, and phalanges (15–19.7%). Only 3 (3.9%) upper limb amputations were recorded. Most of the amputations were performed in the city of Praia Grande (28–36.8%), followed by São Paulo (19–25%) and only two (2.6%) in the city of origin, Itanhaém. The patients stayed in the hospital for an average of 40 days, but, on average, they sought help from the RCMP only 5 to 6 years after the amputation. However, they reported seeking physiotherapy for the first time, on average 20 months after the amputation. Just over half, 43 (56.5%) sought rehabilitation within the first 6 months. Of the total, only 12 (15.7%) had been prosthetized, and for an average time of 8.5 years. Of the amount analyzed, 50 (65.7%) had phantom limb sensation, 27 (35.5%) pain, 28 (36%) itching and other random symptoms such as burning, numbness, stabbing, shock, and tingling.

The PCA was performed, which is a multivariate analysis that transforms variables into the same dimension. When analyzing the figure, it was possible to observe inverse variables such as the time of prosthetization and hospitalization, with the etiology of the amputation, in addition to the site of amputation with pain (Fig. 3). For component 1, the variables that best explained the data were: time of amputation (0.33), hypertension (-0.37), diabetes (-0.37), use of prosthesis (0.40) and time of pros-

thetization (0.42). For component 2, the variables were: time of amputation (0.41), time to start physical therapy/protection (0.36), phantom limb (-0.35), and pain (-0.43). In other words, they are variables that are interrelated according to the component presented.

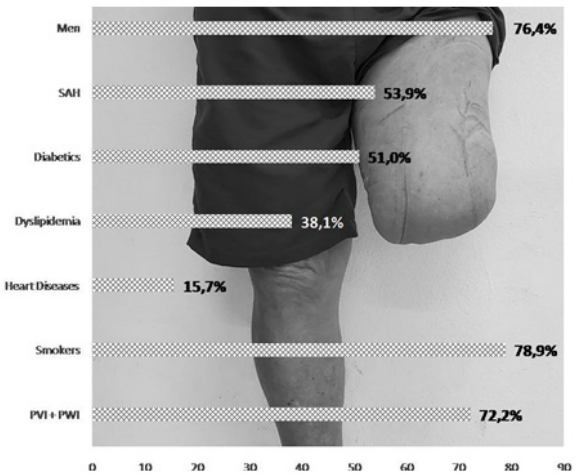


Fig. 2. Etiology of amputations

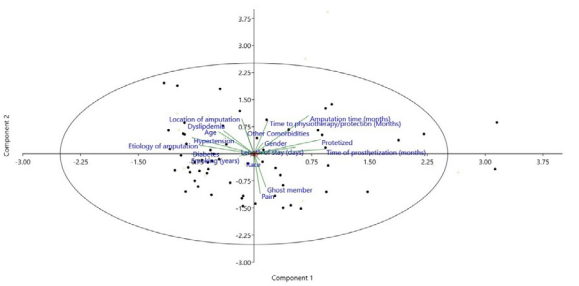


Fig. 3. Presentation of the PCA for the sample according to the analysis variables

The variables analyzed in the correlations, although significant, were weak for: age x hypertension (p=0.005, r=0.32), hypertension x etiology of amputation (p<0.001, r=0.40), diabetes x etiology of amputation (p=0.002, r=0.35),diabetes x time of amputation (p<0, 001, r= -0.38), diabetes x hypertension (p<0.001, r=0.47), dyslipidemia x hypertension (p<0.001, r=0.40), time to physical therapy/protection x time of amputation (p<0, 001, r=0.50),prosthetization x hospitalization time (p<0.001, r=0.40), prosthetization time x amputation time (p<0.001, r=0.40), prosthetization time x amputation etiology (p<0.001, r=-0.31), prosthetization time x diabetes (p<0.001, r=-0.31), pain x phantom limb (p<0.001, r=0.36).

In other comparisons, despite being significant, the correlation was considered negligible: age x smoking (p=0.004, r=0.32), smoking x time of amputation (p=0.030, r= -0.25), time x site of amputation (p=0.048, r=0.23), length of hospitalization x time of amputation (p=0.042, r=0.23), diabetes x age (p=0.003, r=0.25), dyslipidemia x gender (p=0.021, r= 0.26), other comorbidities x amputation site (p=0.043, r=0.23), other comorbidities x dyslipidemia (p=0.013, r=0.28), prosthetization x etiology of amputation (p=0.036, r=-0.24), prosthetization x time of amputation (p<0.001, r=-0.11), prosthetization x hypertension (p=0.028, r=-0.25), prosthetization x diabetes (p=0.047, r=-0.22), prosthetization time x gender (p=0.035, r=0.24), prosthetization time x hospitalization time (p=0.031, r=0.25), prosthetization time x hypertension (p=0.021, r=-0.26), pain x amputation site (p=0.012, r=-0.19).

Discussion

From 2012 to 2020 the patients admitted to the only rehabilitation SUS service in their municipality were middle-aged, mostly male, Caucasian and smokers for years, the main cause of amputation was infection after wound, more than half were hypertensive, type II diabetics and could still have related dyslipdemia and heart disease.

The data found are similar to those of Jesus-Silva et al. in which gender, age range and statements regarding the etiology of amputations are similar.[14] They highlighted the presence of chronic peripheral arterial disease and diabetes as the main causal factors. In Brazil, due to the high prevalence of atherosclerotic disease, diabetes, and the difficulty in controlling these factors there is the likelihood of higher levels of lower limb amputations, such as transtibial and transfemoral amputations, corroborating the findings of this study.[14] Local wound infections were the main factor found, being described as an important risk factor, thus, the immediate therapeutic approach in early skin lesions should be emphasized to minimize their progression and the resulting amputation. Infections after minimal wounds are more susceptible in diabetics and people with peripheral vascular insufficiencies due to the longer exposure time to external media due to the longer healing period required, increasing the risk of amputations.[2] Considering that most of the amputations were performed in other cities, similar findings pointed out in the study by Jesus-Silva et al., can be valid in which the reduced access to a physician and the poor socioeconomic level make it difficult to attempt revascularization and result in the impossibility of limb preservation and major amputations.[14] Barnes et al. pointed out low socioeconomic level as one of the criteria that point to greater chances of amputations, which emphasizes the importance of the services in the city that perform the related care to be more publicized and especially to be prepared to receive these patients.[2]

Approximately half of amputee patients sought physiotherapy in the first six months, the presence of a phantom limb was common and there were reports of pain, itching, numbness, burning, stabbing, shock, and tin-

gling. Mallik et al. described a 60–80% incidence of phantom pain and classified any other report in addition to pain as phantom sensation.[15] This sensation includes the other symptoms mentioned above by the patients in this study. The phantom sensation was more frequent (65.7%) than pain itself (35.5%); however, studies indicate that phantom pain can last from 2–30 years and that sometimes it is difficult for the patient to distinguish between local pain and phantom pain or sensation.[6,7,15,16] Aternali and Katz mention a high prevalence of phantom pain (50-80%), with high levels of pain and the difficulty with its chronicity, affecting quality of life.[16] These authors reported that the pain is typically of neuropathic origin, usually starts in the first week after amputation and decreases in severity and frequency over time. However, when investigating treatment methods such as mirror therapy, transcranial magnetic stimulation, virtual reality, desensitization therapy, and even surgery, there is neither a consensus nor superiority among these alternatives to justify their choices.[16] Neither the pathophysiology of phantom pain has been completely established, it is known that there is involvement of peripheral and central mechanisms, from the limb's encephalic representational zones to the pathways to the amputation site, but it still requires the establishment of relationships.[6,7] That is, in view of the complexity of phantom pain, initial orientations are important and the rehabilitation program must be emphasized during hospitalization and soon after amputation.

If we add the values of vascular insufficiency etiologies (23.6%) with those of infection after wounds (48.6%), which can be directly related, we get 72.2% of amputee patients analyzed. This value is 3.2 times higher than the traumatic cause (22.3%), without correlating it with the high incidence of comorbidities, which according to Varma et al. increases the risk of amputations.[5] Taking into account the estimate of approximately 69 million (32.3%) hypertensive patients and 20 million (9.4%) diabetics in Brazil, it sounds pertinent to prepare basic and specialized care in each city to educate and care for this population to prevent amputations from occurring.[17,18]

Given the data analyzed, the focus should remain on strategies that educate the population to stay away from risk factors, minimizing the number of complications and consequently amputation. The decrease in the number of amputations is related to better diabetes control, better governmental public health strategies, such as the implementation of a multidisciplinary team for the treatment of the diabetic foot with efficient coverage in the geographic area of care.[14] Investing in education and preventive means of care by SUS is necessary, as these initial expenses directly refer to the occurrence of an amputation, which, in addition to being costly for the patient, costs the public purses from the surgery to the end of the rehabilitation process. More than the value of a prosthesis, the municipalities must have a multidisciplinary team, composed of an orthopedist, a vascular surgeon, a psychologist, a physiotherapist, a prosthesis and orthesis technician, among other professionals, which is not the reality in most municipalities, making the prognosis of these patients more difficult. Thus, as Gailey et al. state, only a small percentage performs physical therapy after an amputation, either due to lack of guidance, interest, knowledge of treatment sites, or inadequate structures, which results in the impossibility to achieve maximum functionality.[19] Therefore, it is expected that the knowledge of the variables analyzed in this study will serve to plead for educational programs to inform patients and control their comorbidities, in addition to being the basis for the organization of public managers, together with health professionals, for the preparation of prevention programs and efficient treatment for those who have undergone an amputation.[6,16] A limitation is that the data was taken from a relatively small population sample, from just one city in the interior of the state of São Paulo – Brazil, and that studies with different populations are important for comparing other realities.

Conclusion

The population needs to be alerted to the risk of amputation due to inadequate control of their comorbidities. In case of amputation, patients should be oriented toward the early search for rehabilitation services, and municipalities must prepare their professionals for the treatment of common symptoms and prosthetization. Additionally, the process of purchasing the prosthesis and follow-up of the patient should be well structured, so that the prognosis of functionality and return to daily life, professional and leisure activities can be maximized.

Declarations

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Author contributions

Conceptualization, D.P.A. and G.R.F.B.; Methodology, D.P.A. and G.R.F.B.; Software, G.R.F.B.; Validation, D.P.A. and G.R.F.B; Formal Analysis, D.P.A; Investigation, D.P.A.; Resources, D.P.A. and G.R.F.B.; Data Curation, G.R.F.B.; Writing – Original Draft Preparation, D.P.A.; Writing – Review & Editing, G.R.F.B.; Visualization, D.P.A. and G.R.F.B; Project Administration, D.P.A.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

The data is in the possession of the authors and can be presented if necessary.

Ethics approval

Ethics Committee of the Lusíadas University Center (Opinion 5.815.874).

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ORIGINAL PAPER

Percutaneous sclerotherapy using polidocanol in the treatment of aneurysmal bone cysts of extremities – a report of 28 consecutive cases

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ABSTRACT

Introduction and aim. Aneurysmal bone cysts (ABC) are aggressive benign lesions with high rates of recurrence rendering its treatment uniquely challenging. We report the outcome in 28 consecutive patients of ABC of extremities treated with percutaneus sclerotherapy using 3% polidocanol over a period of 5 years.

Material and methods. In biopsy proven 28 ABC cases, Injection polidocanol 3% was used as sclerosing agent under fluoroscopy guidance. Injections were repeated at 6-week intervals till complete healing of cysts. Radiological and functional results were assessed by Rastogi et al. criteria, VAS score and Enneking scoring method.

Results. 25 out of 26 patients (89.28%) displayed good clinical response with an average 2.56 injections per patients. The mean follow-up period was 17.04±7.08 months. Reduction in cyst volume (mean 6.86±1.48 to 0.71±1.48.) and VAS score (mean 6.72±1.51 to 0.56±1.08) and improvement in Enneking score (17.35±3.14 to 28.92±1.35) at last follow up was significant. No recurrence was reported during the study period. Treatment failure, skin sclerosis and infection were reported in one case each.

Conclusion. sclerotherapy with polidocanol 3% for primary ABC is safe and effective alternate method with advantage of no surgical morbidity, having better functional score with lesser recurrence and minimal complications.

Keywords. aneurysmal bone cysts, Enneking score, polidocanol 3%, recurrence, sclerotherapy

Introduction

Aneurysmal bone cyst (ABC) was first described by Jaffe and Liechtenstein in 1942.[1] The lesion can be described as an eccentric, expansile, lytic lesion with cortical thinning, and a subperiosteal thin shell of bone located in the metaphysis of long bones and the spine. It occurs in the age group of 3 to 40 years, constituting about 1% of benign bone tumors.[2] The clinical presentation of pain and swelling, with or without pathologic fracture, is characteristic of ABCs. The predictable treatment goals are to halt the progression of the lesion, relieve pain, prevent pathological fractures and reduce recurrence rates.[3] To this end, various treatment modalities have been described, ranging from surgical procedures, selective artery embolization (SAE), heat-based ablations (microwave/radiofrequency/cryo-ablation), radiation to medical therapies.[4-9] Surgical treatment of ABC is associated with surgical morbidity, decreased range of motion and deformity due to potential physeal damage, along with a high recurrence rate of about 10% to 30%.[10] Recently, there has been a growing interest in less invasive approaches.

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Sclerotherapy is a newer modality that acts by causing damage to the endothelium of vessels and initiating a coagulation cascade that results in thrombosis and healing of the lesion.[11,12] Percutaneous sclerotherapy with polidocanol has been used in a few studies with promising results in terms of lesser recurrence and morbidity but safety and efficacy are still debated.[10-14]

Aim

We report the outcomes in 28 consecutive patients with ABC of the extremities treated with percutaneous sclerotherapy using 3% polidocanol over a period of 5 years.

Material and methods

Twenty eight patients of primary ABC of the extremities were treated with percutaneous sclerotherapy using 3% polidocanol between 2015 and 2020 at our tertiary level medical college center. Institutional ethics committee approval was obtained prior to the study's initiation (IEC NO. 1124/MC/EC/2015). All patients presenting with radiological evidence of primary ABC of the extremities at the orthopedics outpatient department (OPD) of the tertiary level medical college center underwent MRI and biopsy using the JAMSIDHI needle (J needle) no. 11, Aspiration and breaking of the septa were performed. A biopsy sample was taken from this aspirate and sent for histopathological confirmation.

A treatment holiday of 4 weeks was given to all patients after histopathological confirmation to allow for spontaneous healing of the lesion after bone puncture or trauma. During this 4-week period, patients were specifically instructed to note any increase in pain and swelling of the lesion. After four weeks, patients were followed up for clinical assessment of any change in pain intensity or size of swelling, and plain radiographs of the extremity were obtained to assess healing or progression of the lesion.

The approximate volume of the lesion was calculated using plain radiographs by multiplying the maximum length and breadth in the AP projection and the depth in the lateral projection. If, at the 4th week, the lesion did not heal or spontaneously reduce, then the patient was subjected to percutaneous injection of 3% polidocanol under fluoroscopy.

Technique of the polidocanol 3% injection application

All the procedures were conducted in the orthopedic operating theater under the guidance of the senior author. Patients under 12 years old, were given general anesthesia, while those over 12 years old were treated under local or regional anesthesia depending on the extremity. C-arm fluoroscopy was used to guide the injection site. The lesion was punctured with a J needle no. 11 and the contents of the cyst were aspirated to ensure proper positioning. The aspiration of a significant amount of blood from the ABC cavity was interpreted as a sign of remaining active disease. Before the injection of the sclerosant, Iohexol dye (7 mg/mL) in a 50 % dilution with normal saline (maximum 10 ml) was instilled into the lesion to identify the specific loculi to be injected especially in patients requiring multiple sitting. Prior to injecting the polidocanol, the drug was frothed using a 3-way cannula to reduce the chance of sclerosant spillage into soft tissue. Injection of Asklerol (polidocanol 3%, 60 mg/2 mL, Samarth Lifesciences Ltd.) was given at a ratio of 1 ml for each 1 cm^3 of the lesion, up to a maximum of 10 mL in a single session.[13] After the injection of the sclerosant, a1 ml saline flush was used, and the needle end was plugged for 1 minute. A purse-string suture was applied when the J needle was inside the lesion, and it was tightened immediately after the removal of the needle to prevent spillage of the sclerosant agent around subcutaneous tissue and skin. Patients were reviewed after 7 days to monitor for local complications of sclerosant injection. Thereafter, patients were followed up every 6 weeks with new radiograph and to assess the reduction of cyst volume, signs of cortical sclerosis, and opacification of the cavity. Patients who showed poor progression or had patchy opacification (loculation) were reinjected at 6-week intervals.

Radiographic healing and satisfactory progression were defined by opacification of the lesion with an increase in cortical thickness. Treatment completion was defined as opacification of the lesion (or all loculi) such that no additional injection was needed. Recurrence was defined as the development of a fresh area of radiolucency in a previously opacified cyst (treatment completed), with or without an increase in the size of the lesion. Treatment failure was defined as a lesion that failed to show opacification either altogether or in part after three sequential injections or in which any new communicating lesion appeared during treatment.

Radiological assessment of the reduction in cyst volume was done according to the criteria by Rastogi et al., with grade I representing residual lesion <25% of the initial lesion, grade II for residual lesion 25% to 49%, grade III for residual lesion 50% to 74%, and grade IV for residual lesion 75% or more.[13] Clinical assessment of reduction in pain was performed using the visual analogue scale (VAS) score. Functional score was assessed using the Enneking functional scoring method.[15]

For statistical analysis, qualitative data (age, bone site) were presented as percentages and proportions. Quantitative data (cyst size, VAS score, etc.) were calculated as means and standard deviations. Paired t tests were used to compare differences in cyst size, VAS score, and final functional score before and after treatment within a group. Microsoft Excel and Primer software (McGraw-Hill Global Education Holdings, LLC, NY, USA) for the analysis.

Results

A total 28 biopsy proven patients with ABC were included in the study. The male to female ratio was 4:3, and the mean age at presentation was 21.21±9 years, ranging from 3 to 40 years. The maximum number of ABC lesions was located in the radius and tibia (6 each), followed by the fibula (4), humerus (3), femur (3), pelvis (2), 5th metacarpal (1), and calcaneum (1) (Table.1). The lesion was situated at the metaphysis in 22 cases, at epiphysis in one case (Fig. 1), and five had meta-diaphyseal extension. Three cases had taken tablet ibandronic acid 150mg monthly for three months as conservative treatment at a previous center. Sixteen cases were operated under local anesthesia while general and regional anesthesia were applied in 6 cases each (Table 2). Out of 28 cases, 26 were injected with 3% polidocanol intra-lesionally, and they were followed up every 6 weeks. In one case where the lesion was situated at the tibial metaphysis in a 7-year-old child, spontaneous resolution of the cyst occurred after the biopsy procedure. In another case, where ABC was located at the 4th metacarpal of the right hand, an open biopsy was previously performed at another center. When we tried to inject the dye for confirmation, it spilled into the soft tissue and the procedure had to be abandoned. Therefore, no sclerosant injection was given further in both patients, and they were excluded from the final functional results. The mean number of injections per patient applied was 2.56±1.2, ranging from 1 to 6 (Table 3). The mean follow-up period was 17.04±7.08 months ranging 7 to 40 months. Radiological assessment of the reduction in the volume of the cyst was done according to Rastogi et al. criteria, we found that 88% of lesions (22 cases) fell into grade 1, while 12 % (3 cases) fell in grade 2. No cases were found in grades 3 and grades 4. Significant reduction in the size of the cyst was achieved, with the mean prior to treatment decreasing from 6.86±1.48 to 0.71±1.48 after treatment (Table 4). There was also a significant reduction in the VAS score which decreased from a mean of 6.72±1.51 to 0.56±1.08 after treatment, and 80% were pain free at the final follow up. The improvement in the mean Enneking functional score was also significant going from 17.35±3.14 prior to treatment to 28.92±1.35 after treatment. In one case where the lesion was located at the distal radius, there was no reduction in the volume of the lesion and opacification after three consecutive injections. Therefore, sclerosant injections were stopped, and curettage and cancellous bone grafting were performed. The Lesion healed in 8 weeks and was termed a failure. No recurrence was reported during the study period. Two patients reported complications, including skin sclerosis due to sclerosant spillage and pus discharge at the injection site each.

Table 1. Distribution of the cases according to bone involved

Bone involved	Number	Percentage (%)
Femur	3	10.71
Tibia	6	21.43
Fibula	4	14.29
Humerus	3	10.71
Radius	6	21.43
Ulna	2	7.14
Pelvis	2	7.14
Metacarpal	1	3.57
Calcaneum	1	3.57
Total 28		

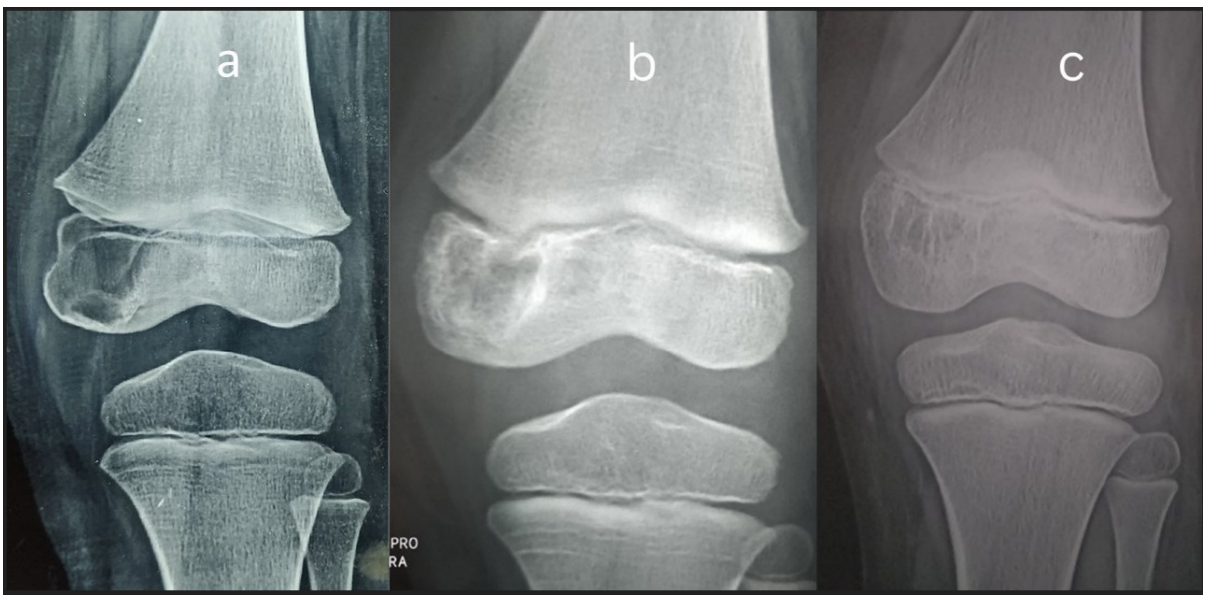

Fig. 1. Radiographs showing an ABC affecting the left distal femur in a 3-year-old female child contained entirely in epiphysis: (a) before treatment, (b) at completion of treatment, (c) at 39 months after sclerotherapy

Table 2. Distribution of cases according to type of anesthesia

Type of anesthesia	Number of patients	Percentage (%)
Local	16	57.14
Regional	6	21.42
General	6	21.42
Total	28	

Table 3. distribution of cases according to number of injections

No. of injections	No. of patients	Percentage %
0	1	4
1	4	16
2	8	32
3	7	28
4	4	16
6	1	4
Mean±SD	2.56±1.2	

Table 4. Paired sample t test showing results of sclerotherapy using 3% polidocanal

Variables	Pre op (mean)	Post op (at final follow-up) (mean)	Mean difference	p
VAS score	6.72±1.51	0.56±1.08	-6.16±1.99	<0.001
Volume of lesion	58.425±45 cc	1.974±3.54	56.45±44.99	<0.001
Enneking score	17.32±3.87	28.96±1.37	11.64±3.50	<0.001

Discussion

ABC is characterized an eccentric, expansile, osteolytic tumor like condition, located at the metaphysis of long bones and in the spine.[2] They are typically diagnosed during adolescence, and are equally rare in both sexes. Historically, ABCs were though to result from increased venous pressure causing extravasation of cellular and blood contents into cyst-like voids in the bone. More recently, a genetic driver has been identified – a translocation induced up-regulation of the ubiquitin-specific protease USP6 (Tre2) gene – defining at least a subset of ABCs as primary neoplasm.[16]

Treatment options for ABC include surgical removal of the lesion, curettage with or without bone grafting, optional adjuvant therapy, endovascular treatment using selective arterial embolization, external-beam radiotherapy, and percutaneous directed therapies such as sclerotherapy, ablation, cementoplasty, and percutaneous injections of bisphosphonates or doxycycline.[4-14,17,18]

En bloc excision, is associated with the lowest rates of recurrence with 95–100 % localized control but it comes at the cost of high patient morbidity.[4,17] Simple Curettage of the lesion with or without bone grafting has been linked with high and sometime unacceptable recurrence rate of 18 to 59%[18]. Various adjuvants including cement, high-speed burr, argon beam, phenol, and cryotherapy have been developed to reduce recurrence, but they still result in an approximate 15% recurrence rate.[8,10,19-22] Currently, there are no high-level controlled comparative studies regarding adjuvant efficacy, and the specific adjuvant strategy used varies based on the institution. Furthermore, surgical treatment of ABC is associated with surgical morbidity including postoperative pain, muscle weakness, decrease range of motion, limb length discrepancies and deformity due to potential physeal damage.[23]

Selective artery embolization (SAE) of feeding blood vessels has been used for surgically inaccessible such as the pubic bone, sacrum, spine, and to reduce intraoperative blood loss during curettage. It has been proven to be an effective treatment method.[24] Rossi et. al. treated 36 ABC patients with selective arterial embolization using N-2-butyl cyanoacrylate. In their study a total of 55 procedures (1.5 procedures per patient) were performed on 36 patients.[6] Among these, 22 cases (61%) required only one embolization, 9 cases (25%) required two and the remaining 5 patients (14%) required three. Seven patients (19.4%) eventually required surgery during the study period. However the efficacy of this procedure is limited as not all ABCs have major feeding blood vessels, and the risk of ischemia in vital neural and visceral structures remains a major concern.[13]

Radiofrequency ablation (RFA), cryoablation, and microwave ablation have emerged as effective treatments for ABCs in various studies. RFA utilizes high-frequency electrical currents to heat and destroy tumor tissue, cryoablation employs extreme cold to freeze and kill abnormal cells while microwave ablation generates heat through microwave energy to coagulate the cystic mass. Each modality has its advantages but complications such as thermal injury to adjacent structures, nerve damage, and infection can arise from these procedures. Collaborative decision-making among orthopedic surgeons, interventional radiologists, and oncologists is crucial to tailor the treatment strategy based on the patient's specific condition and anatomical considerations, ultimately leading to improved clinical outcomes.[25]

Radiotherapy has historically been used to treat ABCs primarily, as an adjuvant therapy in cases of recurrence, and in inoperable ABC lesions but Risk of malignant changes after radiotherapy and spinal deformity lead to abandoning this procedure.[3,5]

Denosumab has shown effectiveness as a rescue therapy for controlling ABCs but dangerous cases of hypercalcemia have been reported in children warranting its limited use to inoperable ABCs.[26] Systemic therapy with denusumab requires close observation to optimize treatment duration, a gradual discontinuation strategy and long-term monitoring for growth (deformities), serum calcium and mineral homeostasis.[27]

Sclerosants damage vascular endothelium, leading to in small vessels, and subsequent healing of the lesion.[11] Various sclerosing agent have been used to treat the ABCs including ethibloc, Methylprednisolone ace-

tate, calcitonin and radionuclides with healing rates of up to 92% and no recurrences.[3,28] However, a secondary procedure is required in 0% to 25% of patients. A meta-analysis by Cruz et al. reviewed 10 studies (294 patients) using Polidocanol, ethibloc, doxycycline, calcitonin, steroid or calcium sulfate as a sclerosant, reporting a recurrence-free survival of 94% at final follow-up (mean 41.1, range 23–58 months).[24] However, 68 out of 294 patients experienced complications including injection site induration, skin necrosis and fractures.

Polidocanal 3% injection has been used in the treatment of ABC in a few studieswith good results. In our study we treated 25 ABC patients with polidocanal 3% injection and followed up until complete healing.[10-14]

The mean age at presentation of ABC in our study was 21.21. years with a range from 3 to 40 years. Age distribution in our study remained almost equal in all age groups above 10 years. Children under 10 year had lower incidence of ABC, with the average age of patients being 21 years. These Findings are well consistent with studies conducted by Rastogi et al. and Varshney et al.[13,14]

Mascard et al. conducted a study and reported that ABCs are more commonly diagnosed in long bones in the (67%) than spine (15%) and pelvis (9 %).[29] In long bones, the tibia constitutes the most common site (40%), followed by the humerus (15 %) and femur (13%). Lower limbs are commonly affected (67%) than upper limb (20%). These findings are well correlated with our study, where we found that the incidence of ABC was more common in the lower limb (50%) followed by the upper limb (39%) and pelvis (7%). In our study the tibia and radius had an equal incidence of ABC (each 21%).

ABCs most commonly occur in the metaphyseal region of long bones. With time, large cysts may involve the diaphysis and become meta-diaphyseal (Fig. 2). Although epiphyseal extension has been reported in association with metaphyseal ABC (Fig. 3), Chan et al. found primary ABC entirely contained within the epiphysis which is a rare occurrence.[30] In our study, one patient aged 3years had an ABC completely within the epiphysis of the distal femur (Fig.1). For this patient, polidocanol 3% was injected through a trans-epiphyse-

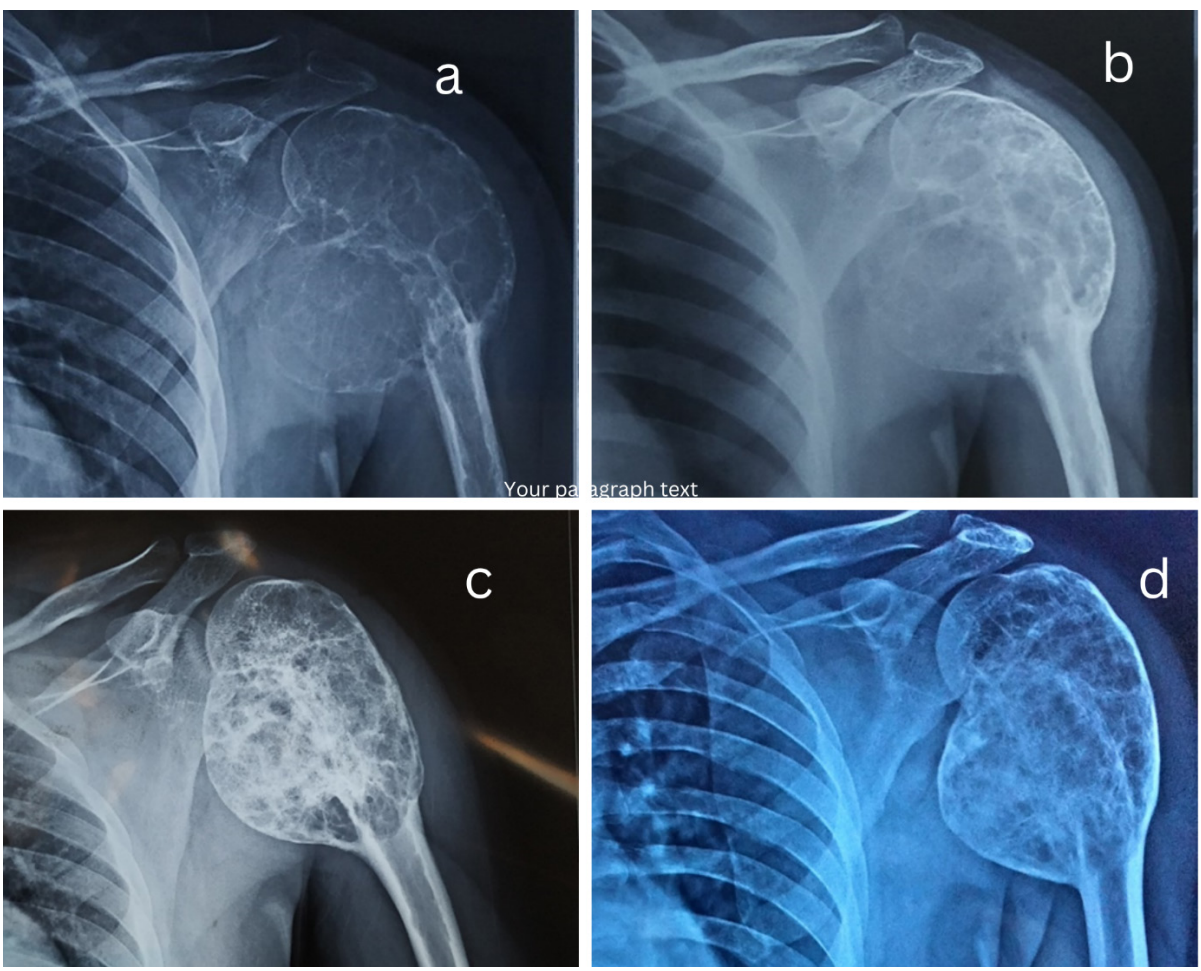

Fig. 2. Radiographs showing an ABC affecting the proximal humerus of the left side in a 25-year-old male with meta-diaphyseal extension: (a) before treatment, (b) An AP radiograph taken after two injections of polidocanol shows an unopacified loculation in the medial part of the lesion, (c) radiograph was obtained at completion of treatment after three injections, (d) AP and lateral view radiographs showing the lesion at 40 months with remodelling of the lesion

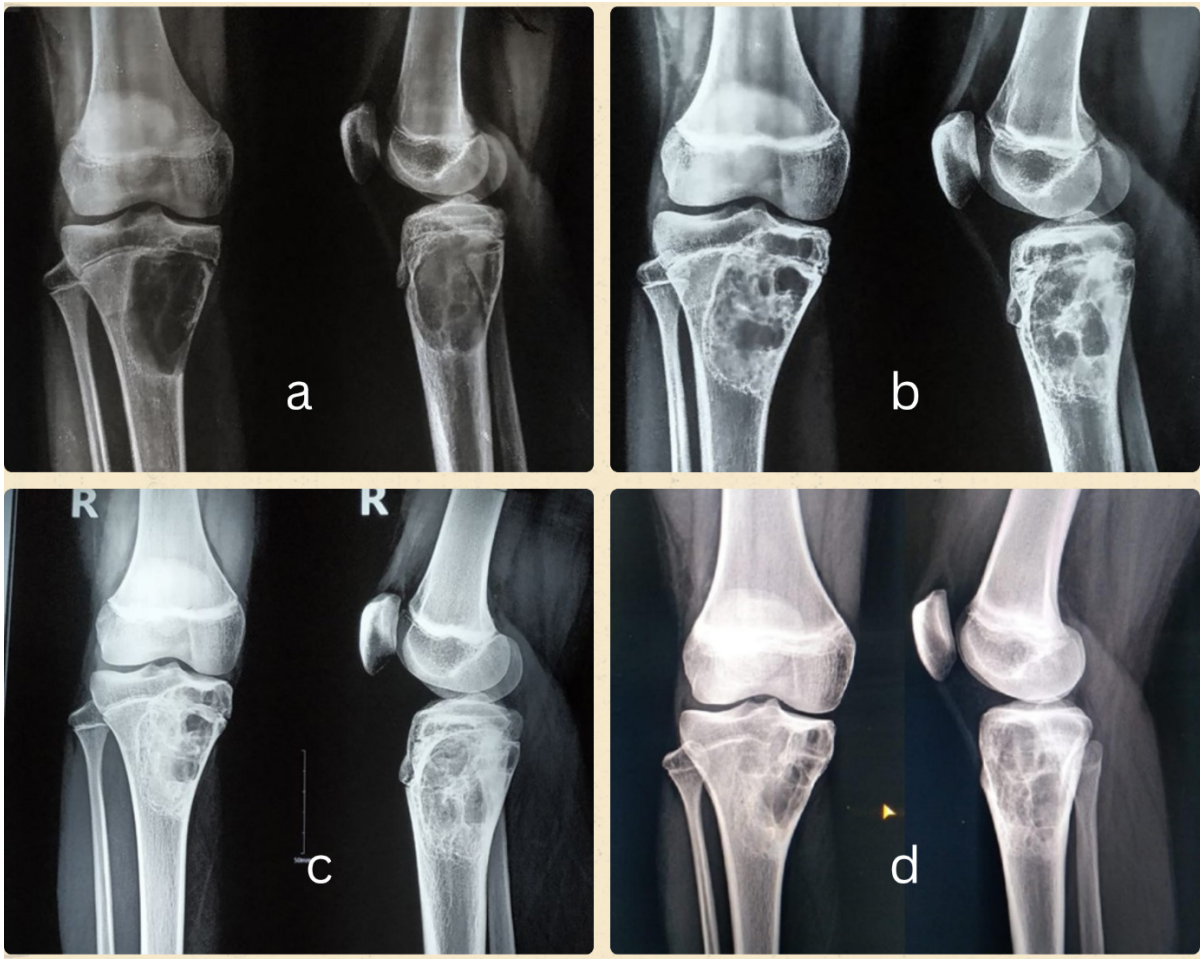

Fig. 3. Radiographs showing an ABC affecting the right proximal tibia metaphysis with epiphyseal extension in an 11-year-old female child: (a) before treatment, (b) after first polidocanol injection, (c) after second polidocanol injection at completion of treatment, (d) at 36 months after sclerotherapy

al route parallel to the physeal plate. We took great care to ensure that there was no damage to the physeal plate during the procedure. A single injection of sclerosant healed the lesion and there was no reported deformity or morbidity at the surgical site during the final follow up (39 months). This case highlights the importance of sclerotherapy in avoiding the morbidity associated with large surgical procedures and resulting epiphyseal damage and associated deformities. To the best of our knowledge, we did not find any previously reported cases of epiphyseal ABC treated with sclerotherapy in the literature

Reddy et al. introduced the concept of limited curettage using a J needle and a pituitary rongeur at the time of ABC biopsy and termed it curopsy.[31] They reported healing of lesion in 83 out of 102 cases (81%). In our study we also employed septal breaking with a J needle during simultaneous aspiration of blood. Only one case exhibited cyst consolidation and obliteration at 4 weeks, while the remaining 26 cases did not show any improvement with curopsy alone and leading them to be subjected to sclerosant treatment.

In a review of 72 ABC patients treated with polidocanol 3%, Rastogi et al. reported a clinical response rate of 84.5 % with an average of three injections per patient.[13] In a randomized trial, Varshney et al. compared polidocanol sclerotherapy with curettage, high-speed burr, and bone graft.[14] Polidocanol exhibited a healing rate of 93.3% compared to 84.8 % for curettage, with a mean of 2.3 injections per patient. In our study, 25 out of 26 patients (89.28%) completed treatment with polidocanol injection and displayed a good clinical response with an average of 2.56 injections per patient. 14% of patients achieved complete healing with a single injection only. These results underline the efficacy of polidocanol injection in cavity healing. The maximum number of injections required was six in a case where ABC was situated at the distal metaphysis of the femur. In this patient, even after consecutive two injections, the cyst volume did not reduce. Although the cyst was not large compared to others, it was situated at supracondylar level. Initially, this case was attempted without radio-opaque dye confirmation Subsequent four injections were administered by the senior author under C-ARM guid-

ance after dye confirmation, led to cavity healing. The use of Iohexol dye enabled precise polidocanol instillation in the remaining cavity that resulting in healing.

Polidocanol injection proved excellent in radiological regressing the cavity. In 22 cases (88%), cavity size reduced to <25 % (grade 1), and in 3 cases, the cavity size reduced to <50% (grade 2) on final follow up. a study by Rastogi et al., showed 66% cases achieving grade 1 healing, 30% grade 2 and 28% grade 3.[13] The mean Enneking score prior to treatment was 17.35±3.74, significantly increased to 28.92±1.35 after the final follow up. The improvement in functional score was associated with a reduction in lesion volume and decreased in pain. At the final follow up 80% of cases were pain-free. There was also a significant reduction in mean VAS score (from mean 6.72±1.51 to 0.56±1.08). In one case, after three continuous injections, the cyst did not show any reduction in volume or opacification. Therefore, it was deemed as failure. We utilized Iohexol 7mg/ml dye during sclerosant injection to confirm the exact site and ensured septa in every session, covering the entire cyst with sclerosing agent. This precise approach helped localize the cyst and sclerosant instillation, contributing to almost complete cavity resolution and excellent outcomes. This contributed to a higher number of pain-free patients and better functional scores at final follow-up.

Cornelis et al. reported varying degrees of lesion ossification and near-universal pain relief following bisphosphonate treatment for symptomatic, inoperable benign bone tumors including ABCs.[9] In our study, 3 out of 26 patients received bisphosphonate (Ibandronic acid 150 mg) 3 to 5 months prior to treatment. All three patients had received 2, 1, and 2 doses of polidocanol 3% respectively (average 1.7 doses, compared to 2.56 in the study), achieving complete cyst resolution and excellent functional scores at the final follow-up. Bisphosphonates are potent anti-resorptive agents that attach to hydroxyapatite sites on bone surfaces, particularly those undergoing active resorption. This inhibits osteoclast activity, preventing bone resorption and promoting osteoclast apoptosis. The anti-resorptive property may have contributed alongside polidocanol injection, leading to fewer injections and improved functional scores. This finding requires confirmation through further research.

Curettage of the lesion with or without bone grafting has been associated with a high and sometimes unacceptable recurrence rate ranging from 18% to 59%.[3,7,10] Other studies conducted by Varshney et al. (4.44%) and Rastogi et al. (2.8%) also demonstrated very low recurrence rates (4.44% and 2.8% respectively) when using polidocanol (Table 5).[13,14]

In our study, 57% of cases received polidocanol 3% injections under local anesthesia, well-tolerated, and were discharged on the same day after 3 hours of observation. General anesthesia was administered to only six children. This suggests that sclerotherapy is an excellent outpatient procedure, resulting in shorter hospital stays and minimal anesthesia-related complications.

Table 5. Results of treatment of ABC with sclerotherapy

Sr. no.	Study	Number of cysts	Average injection per patient	Recurrence rate	Failure rate	Complications
1	Rastogi et al. 2006	72	3	2	–	induration at the site of injection (18 cases), hypopigmentation (3), local inflammatory reaction (1), and an episode of dizziness (1)
2	Varshney et al. (2010)	47	2.3	2	3	local induration (37) hypopigmentation at the injection site (11), and dizziness episode (one patient)
3	Deventer et al. (2021)	32	5.7	0	10	Healing disorder (2 cases)
4	Puthoor et al. (2021)	34	1.09	0	–	Ulcerations (1) and hypopigmentation (1)
5	Rai et al. (2022)	43	3.11	5	–	Induration of skin (7), hypopigmentation (4)
6	Our study	28	2.5	0	1	Superficial infection (1) and induration (1)

Sclerotherapy should not be performed in cases where previous open biopsy or surgery has been conducted, as spillage of the sclerosant could harm adjacent neurovascular structures and soft tissue. In our study one case reported skin necrosis due to sclerosant spillage, but skin sclerosis healed within 2 weeks with the application of local antibiotics and silver sulfadiazine ointment. Another case exhibited swelling and discharge one week after the second injection. We had to debride the lesion and administer IV antibiotics for 2 weeks; the cyst healed after 2 polidocanol injections. By the final follow-up, the infection had subsided, and no patients reported recurrence. We did not observe any complications such as hypopigmentation, allergies, or episodes of dizziness during treatment in any of the patients.

The major limitations of our study include the small number of patients, a relatively short follow-up period, the absence of a comparative group for result comparison, and limited statistical data for analysis.

Conclusion

These preliminary results indicate that sclerotherapy with 3% polidocanol is safe, effective, and associated with fewer major surgical complications, yielding a better functional score and a lower recurrence rate. The procedure can be planned as a daycare procedure using local anesthesia in adults and mild sedation in pediatric patients.

Declaration

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Author contributions

Conceptualization, R.K.H. and D.S.M.; Methodology, R.K.H. and K.K.K.; Software, M.S.; Validation, R.K.H., K.K.K. and D.S.M.; Formal Analysis, N.S. and M.S.; Investigation, N.S. and M.S.; Resources, R.K.H.; Data Curation, R.K.H. and K.K.K.; Writing – Original Draft Preparation, R.K.H. and M.S.; Writing – Review & Editing, N.S. and D.S.M.; Visualization, M.S.; Supervision, R.K.H.; Project Administration, R.K.H.; Funding Acquisition, D.S.M.

Conflicts of interest

The authors declare that they have no conflicts of interest

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The protocol was approved by the institutional Ethics Committee (SMS Medical College Jaipur (IEC NO. 1124/MC/EC/2015).

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ORIGINAL PAPER

Evaluation of the antioxidant activity of *Berberis jaeschkeana* C. K. Schneid. fruits using the ABTS assay

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ABSTRACT

Introduction and aim. The assessment of the antioxidant activity of plant extracts is an important research direction in the search for new chemopreventive substances. The aim of this study was to demonstrate the antioxidant potential of the methanol extract of *Berberis jaeschkeana* C. K. Schneid fruit.

Material and methods. Spectrophotometric tests were carried out: testing of the total content of polyphenols (TPC) in extracts, testing of the total content of flavonoids (TFC) in extracts. Antiradical activity was determined using the improved ABTS+• decolorization test with modifications.

Results. TPC extracts was determined at the level of 43.52±2.37 mg/g, while TFC extracts was determined at the level 6.08±0.48 mg/g. The ABTS activity test showed 31.15 mg/g expressed as mg Trolox per g of dry extract.

Conclusion. It was assessed that the methanolic extract of the fruit of Berberis jaeschkeana C. K. Schneid. has an antioxidant properties, what makes it a potential chemopreventive agent in civilizational diseases.

Keywords. antioxidants, *Berberis jaeschkeana* C. K. Schneid., chemoprevention, civilization disease, fruit extract, traditional medicine

Introduction

Plants have been used for medical purposes for several thousand years. In ancient times, they were the only source of obtaining substances with medicinal properties. Botanical ingredients include chemical compounds that inhibit or delay the oxidation process and play a key role in chemoprevention. The search for new plant species as sources of antioxidants is one of the most important tasks in modern science.[1]

Free radicals are molecules or atoms that contain unpaired electrons. They arise through endogenous processes necessary for life, but also as a result of the influence of external factors, for example, radiation, air pollution, and pose a threat to the body and its proper functioning. Currently, there is a great demand for compounds with antioxidant properties that prevent the harmful effects of free radicals, both in the field of cosmetology and in the prevention of lifestyle diseases. Most antioxidants are produced synthetically, but natural antioxidants are more effective, including secondary plant metabolites.[2]

Berberis jaeschkeana C. K. Schneid. is a shrub that grows up to 1 m high. Leaves – oblong elliptical, serrated, 1-2 cm wide. Yellow flowers, gathered in clusters of 3-5. Fruit – red oblong-oval berries. Thick angular branches with spines 1-1.5 cm long. It is native to Assam, the East Himalaya, Nepal, Pakistan, Tibet, the West Himalaya[3,4] (Fig. 1, 2 and 3). All parts of the plant contain the alkaloid berberine most concentrated in the roots, stems and inner bark. In the fruits there is the

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lowest concentration of this alkaloid. The fruit of this genus *Berberis* L. are edible.[5,6]

Fig. 1. *B. jaeschkeana* C. K. Schneid. fruits

Fig. 2. *B. jaeschkeana* C. K. Schneid. shrub

Fig. 3. *B. jaeschkeana* C. K. Schneid. dried stems with fruits

Material and methods

Plant material and reagents

The plant material of *Berberis jaeschkeana* C. K. Schneid. fruits were obtained from the Maria Curie-Skłodowska University Botanical Garden in Lublin in October 2022. The raw material was separated and dried at room temperature in the shade with ventilation. The raw material was weighed and ground in an electric mill and portioned, vacuum packed, and stored in a closed package at –30°C until the start of the tests. Trolox, gallic acid, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (Stenheim, Germany); methanol and aluminium chloride hexahydrate of analytical grade were purchased from POCH (Gliwice, Poland).

Sample extraction and process

A 2 g amount of powdered *B. jaeschkeana* C. K. Schneid. fruits were extracted by accelerated solvent extraction (ASE). Accelerated solvent extractions with an 80% methanol concentration (3 cycles for 10 min each at 80°C) were performed on an ASE 150 system from Dionex Corporation (Sunnyvale, CA, USA). The extract was prepared in triplicate. The extract obtained was evaporated to dryness under reduced pressure and lyophilised in a Free Zone 1 apparatus (Labconco, Kansas City, KS, USA). Samples for testing were prepared immediately prior to analysis by dissolving them in an ultrasonic bath. A weighed amount of the extract after lyophilization was dissolved in a measuring volume of 80% methanol to obtain starting solutions with a concentration of 40 mg/mL. As required for the determinations, it was diluted with the same solvent to a specific concentration.

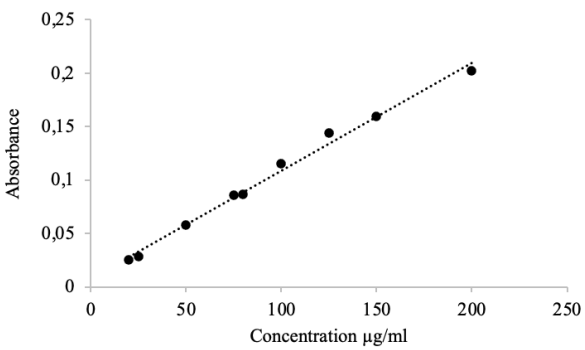

Fig. 4. Standard curve for gallic acid for TPC testing – a graph of absorbance versus gallic acid concentration

Determination of total phenolic (TPC) and total flavonoid contents (TFC)

The analysis of the total phenolic content was carried out using the modified Folin–Ciocalteu method.[8] The TPC was determined using a standard curve prepared for gallic acid (Fig. 4). The absorbance was read at 680 nm after a 20-min incubation using the Tecan microplate reader Infinite 200 Pro-Elisa with I-control Tecan system (Mannedorf, Switzerland). The results were expressed in mg of gallic acid per 1 g of dry weight of dry extract – gallic acid equivalent. The total flavonoid

content was determined according to the method proposed by Lamaison and Carret with modifications. The TFC was determined using a standard curve prepared for quercitin (Fig. 5). The absorbance was measured at 430 nm after a 30 min incubation against a blank containing methanol instead of the test sample. Results were expressed in mg of quercetin per 1 g of dry extract.

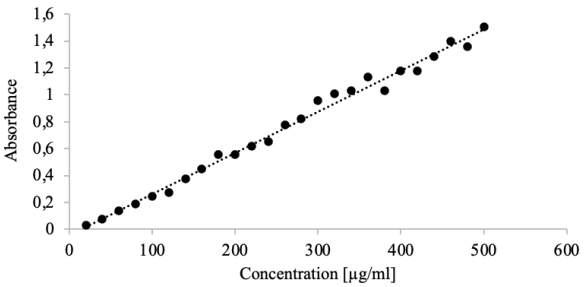

Fig. 5. Standard curve for quercetin for TFC testing – a graph of absorbance versus quercetin concentration

Antiradical activity analysis

Antiradical activity was determined with the ABTS+• discolouration test, with modifications.[9,10] The ability of the extract to quench ABTS+• free radicals was determined using equation:

$$\text{Capture \%} = [(AC - AA)/AC] \times 100,$$

where: AC is the absorbance of the control and AA is the absorbance of the sample. The absorbance was measured at 734 nm after a 6-min incubation. The results were obtained from measurements made for each sample and expressed as milligrammes of Trolox of dry extract (Trolox equivalents).

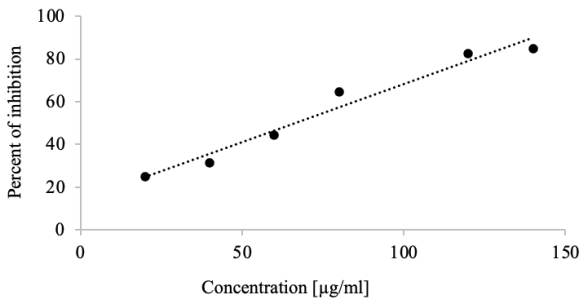

Fig. 6. Standard curve for Trolox for testing anti-radical activity – a graph of the dependence of the percentage of inhibition on the concentration of Trolox.

Results

The tests carried out allowed for the determination of the total polyphenol content in the TPC extracts (total phenolic content), expressed as the equivalent of gallic acid in plant extracts (mg/g) at the level of 43.52±2.37 mg/g. The analysis performed allowed the determination of the flavonoids in the TFC extracts, expressed as the equivalent quercetin in the tested extracts at the level of 6.08±0.48 mg/g. The ABTS activity test showed significant antioxidant potential of the *B. jaeschkeana* C.K. Schneid. fruit extract. Antiradical capacity (ABTS+•) expressed as mg Trolox per g of dry extract was 31.15 mg/g.

Discussion

Despite the long tradition of medicinal use of barberry species, relatively few of them have been tested in terms of chemical composition, chemopreventive potential and nutraceutical use. The content of polyphenols and flavonoids as well as antioxidant properties were determined only for selected species of *Berberis* L. For example, in fresh fruits of *Berberis heteropoda*, the TPC value was 68.55 mg GAE/g, expressed in milligrams of gallic acid equivalent per gram of fresh fruit weight, and the TFC value was 108.42 mg QE/g, expressed in milligrams of rutin equivalent per gram of fresh fruit pulp.[11] For *Berberis cretica*, the TPC value was determined to be 190 mg GAE/g, and for *B. sibirica*, the TPC value was determined to be 159 mg GAE/g.[12,13] Traditional eastern medicine knows genus from the antimicrobial activity of its roots rich in alkaloids. It is known that fruits of *Berberis* L. contain lower level of alkaloids so they are safe and eadible.[5,6] Until now antioxidant potential of this fruits has been very poorly examined. Bewal et al. has assessed the influence of extraction method on antioxidant metabolites in *B. jaeschkeana* C.K. Schneid. fruits and they obtained higher level of TPC and TFC using microwave extraction.[14,15] The fruits of *Berberis jaeschkeana* C.K. Schneid. examined in this study seem to be worth further research because it is as valuable in this respect as other species of the genus *Berberis* L. Thea are source of antioxidants which can be used as a chemoprevention in nutraceutics or can be potential as antioxidants in cosmetology.

Conclusion

B. jaeschkeana C.K. Schneid. are characterised by a high content of polyphenols and high antioxidant activity. Therefore, they can be considered as chemoprevetive agent. It can be also considered for potential use in cosmetology as natural antioxidants or for naturally extending the shelf life of food products.

Declaration

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Author contributions

Conceptualization, A.O.; Methodology, A.O.; Software, A.O; Validation, A.O. and A.C.; Formal Analysis, A.O.; Investigation, K.O. and A.Ć.; Resources, A.O.; Data Cu-

ration, A.C.; Writing – Original Draft Preparation, A.O.; Writing – Review & Editing, A.O. and K.O.; Visualization, A.O.; Supervision, A.O.; Project Administration, A.O.; Funding Acquisition, A.O.

Conflicts of interest

The author declare no competing interests.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Not applicable.

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REVIEW PAPER

Tuberculosis epidemic in India – a systematic review

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ABSTRACT

Introduction and aim. India, accounting for approximately 26% of the global tuberculosis (TB) burden with a significant proportion of 0.11 million (6%) pediatric TB cases. The goal of this systematic review paper is to provide a thorough analysis of the TB epidemic in India, including information on the severity of the illness and challenges associated with diagnosis and treatment, the risk factors for transmission and progression of the disease, and the advancements and difficulties encountered in efforts to control at community level.

Material and methods. The terms "Tuberculosis in India," "Drug-Resistant Tuberculosis," "Tuberculosis and Associated Co-morbidities," "Diagnosis of Tuberculosis," "Prevention of Tuberculosis," and "Treatment of Tuberculosis", keywords were used to search in number of international electronic databases, including "Google Scholar," "PubMed," and "DOAJ."

Analysis of the literature. Diagnosis and treatment of TB are significantly complicated by co-morbid condition such includes alcoholism, diabetes, HIV, undernutrition, diabetes and HIV. In addition, there may be treatment delays, and the extra-pulmonary or drug-resistant TB due to ignorance, misunderstandings, and lack of education among TB patients, and medical professionals, which ultimately increases morbidity and mortality.

Conclusion. Poverty, undernutrition, inadequate healthcare infrastructure, and co-morbidities, which frequently complicate TB diagnosis and treatment, must be addressed in addition to the critical need to prioritize TB research and development.

Keywords. drug-resistant tuberculosis, tuberculosis, undernutrition and tuberculosis

Introduction

For decades, tuberculosis (TB), caused by the bacteria *Mycobacterium tuberculosis*, has been a major global concern. *M. tuberculosis* bacteria divide TB into two types based on the organs infected: pulmonary tuberculosis (PTB), which primarily affects the lungs, and extra-pulmonary tuberculosis (EPTB), which primarily affects other organs such includes pleura, lymph nodes, intestines, genitourinary tract, skin, bones and joints, and brain in the body.[1-4] TB is a highly contagious and transmissible disease that spreads naturally from person to person through sneezing or coughing in the vicinity.[5–7] The World Health Organization (WHO) estimates that 10.6 million people worldwide will have TB (in 2021), including 6 million men, 3.4 million women, and 1.2 million children.[8] According to the Global Tuberculosis Report (2022) claimed that TB killed 1.6 million people and was difficult to diagnose and treat. Similar to this, in 2022 the high number of new cases (10.4 million) and fatalities (1.8 million) from TB, India's leading cause of illness and mortality, is made worse by these statistics (Fig. 1).[7,9,10] In the same vein TB is commonly prevalent in 0–9-year-old children in India, consisting 0.11 million pediatric cases especially among those who have immunological weaknesses.[11–15] The COVID-19 pandemic, on the other hand, has occasionally hampered the access to essential healthcare services, resulting in an increase

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in the incidence of various community-borne diseases, mortality, and morbidity.[1,2]

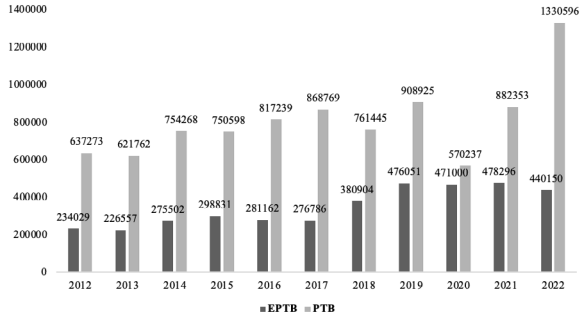

Fig. 1. Bacteriologically confirmed pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) cases in India (Source: Global Tuberculosis Report 2022, India TB Report 2023)

India has more than 26% of the world's TB cases (in 2022), and undernutrition and poverty are major contributors to the disease's rising prevalence.[1,2] As a result, treating TB is costly, time-consuming, and has a low success rate.[1,2,16–22] Co-morbidities that weaken the immune system and increase the risks of TB infection in people over a short period of time include undernutrition, diabetes, HIV, tobacco use, smoking, and alcohol consumption.[2,21–32] *M. tuberculosis* also has a diverse pattern of region-specific prevalence and susceptibility, genetic mutation over time, space, and environment.[23,33,34] Nearly one in every two TB patients' household contacts has latent TB, and the severity of the infection is related to the age of the household contacts.[28,35] Furthermore, old age group healthcare professionals are at a higher risk of developing a particularly latent TB infection due to their longer duration of employment and working in areas with high TB prevalence.[5,35,36]

Aim

The objective of this study is to provide a concise and comprehensive summary of the TB epidemic in India, with a focus on the disease burden, challenges in diagnosis and treatment, risk factors for TB transmission and progression, and progress and challenges in TB control efforts.

Material and methods

A systematic literature review was conducted using a variety of international electronic databases, including Google Scholar, PubMed, DOAJ, and Government Database including India TB Report, Govt. of India Central TB Division with the search terms 'Tuberculosis in India', 'Drug-Resistant Tuberculosis', 'Tuberculosis and Associated Co-morbidities', 'Diagnosis of Tuberculosis', 'Prevention of Tuberculosis', and 'Treatment of Tuberculosis'. Relevant technical reports and government databases were identified and reviewed according to predetermined inclusion criteria, which included descriptive studies, review articles, cross-sectional studies, and original survey studies. Government databases were utilized whenever required, along with the Global Tuberculosis Report (2022), to ensure comprehensive coverage of relevant literature and reports. This systematic review study covers literature and reports published between 20 May 2010 and 5 January 2023, and new studies were included during manuscript preparation and final revision. The search results were limited to full articles published in English, and a total of 216 publications, including titles, abstracts, and full texts, were initially identified using the present study's keywords and criteria. Duplicate scientific papers yielded from different search engines were excluded. The details of the literature search, inclusion, and exclusion are summarized in Fig. 2. A total of manuscripts (n=128) was identified and retrieved for detailed evaluation during the short-listing of published literature and manuscripts. Following a thorough review of the published research papers, and government database reports, n=80 were identified as appropriate and were considered for the present manuscript. The finalized manuscript, including both abstracts and full-length manuscripts, was downloaded to interpret the present study, revise it, and complete this review manuscript.

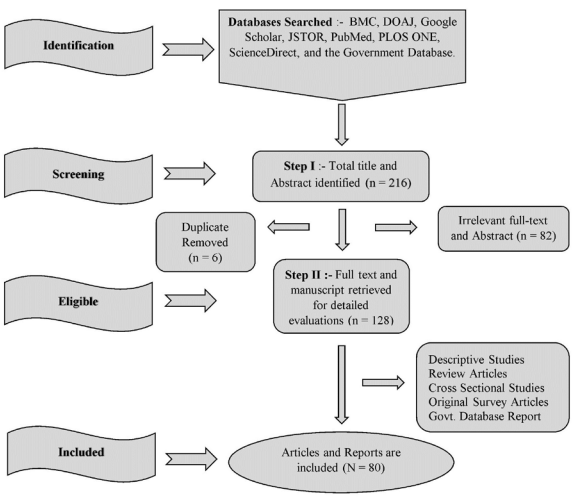

Fig. 2. Flow chart shows the research study selection process

Analysis of the literature

Occurrences of TB in India

The incidence of TB decreased by 18% from 2015 to 1.6 million cases in 2020, but following the COVID pandemic (in 2021), there was an 18% increase in cases to 2.14 million in India (Fig. 1), resulting in 40% of the population (1.9 million) having latent TB infection, with 10% of those cases expected to develop into active

TB.[21,31,37] Despite the massive TB burden, India is actively spending $23 billion a year to successfully reduce the global TB burden through the World Health Organization's End Tuberculosis Strategy and the Revised National Tuberculosis Control Program (RNTCP), as well as by establishing "Nikshay," an open web service that allows health organizations and medical councils to more easily track antibiotic use in real time as part of the Direct Observed Treatment Service.[38–40] However, there are still a lot of gaps in the fight against TB because of a lack of information, a lack of awareness, a lot of myths and misconceptions, and the failure to pay fees to accredited social health activists (ASHA) workers.[3,5,35] According to the National Family Health Survey-5 (NFHS-5) and several research investigations, certain social determinants are found to have the highest prevalence of TB (Fig 3).[5,29,31,37,41,42] Although it is evident that rural and urban slums areas have a comparatively greater TB incidence, the prevalence has decreased as people's socioeconomic position has improved.[31,40–44]

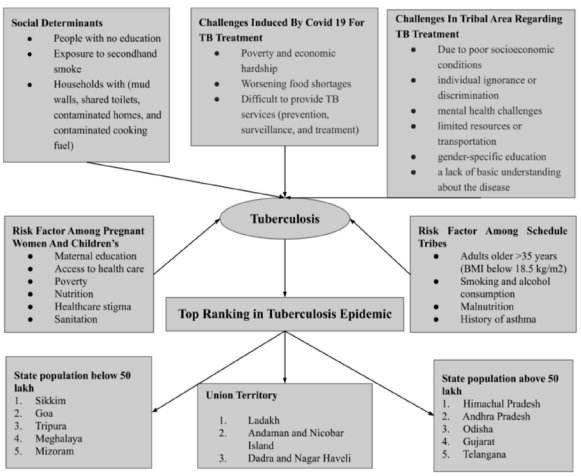

Fig. 3. Representing the risk factors, challenges and ranking (States and Union Territory) them with the tuberculosis epidemic in India

The burden of tuberculosis in different states of India

The vast geographic area and biological diversity of India have resulted in a number of epidemics that have harmed public health and caused catastrophic costs, such as TB, which accounts for a sizable portion of TB cases worldwide.[11,34,45] Similar to this, the Seasonal Waves of Respiratory Disorders study discovered strong correlations between seasonal variation and a high prevalence of TB, with symptoms worsening slightly in the summer and exacerbating in the autumn as a result of climatic variations in various parts of India.[46] Similarly, research has revealed that atmospheric temperature is an important factor and has a significant association with the progression of TB during the summer and monsoon seasons (Fig. 4).[47]

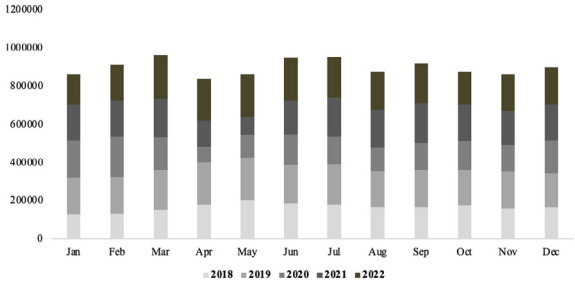

Fig. 4. Tuberculosis cases notified per month in the last five years (fluctuation of cases per month) (according to India TB Report 2019–2023)

According to the National Tuberculosis Reports (2022), (2023), the burden of TB in India is distributed across states, as shown in Fig. 5(a) and 5(b).[48,49] As shown in Fig. 3, the report also identify the top five states and territories with the highest rankings. Despite this difficult situation, India is committed to eliminating TB by 2025, with the assistance of the National Tuberculosis Elimination Program (NTEP), which collects the majority of TB statistics in India and was previously called the RNTCP.[22,48–53] According to the National Tuberculosis Report (2023), Kerala, Tamil Nadu, Assam, and Uttar Pradesh are the states that performed relatively better in terms of TB elimination than the majority of states in India in 2022.[49] Regarding performance Kerala created the System for Workplace Engagement to Eliminate and stop the spread of TB among employers in a variety of workplaces and industries.[48,49,51–53]

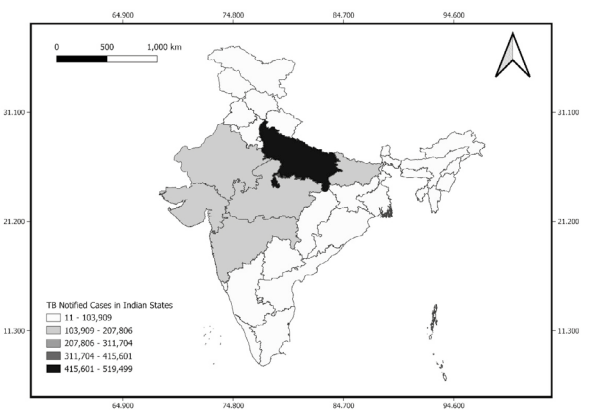

Fig. 5a. Distribution of the Tuberculosis Epidemic in Different States of India in 2021 (India TB Report 2022)

To understand the risk of TB among COVID patients and therefore help the program management design policies, the Tamil Nadu Post-COVID catch-up campaign was conducted.[48,49,51–53] Intersectoral engagement with the Assam Branch of the Indian Tea Association was conducted in Assam to identify TB cases in tea gardens and the transgender community.[48,49,51–53] In Uttar Pradesh, active case finding under the Dastak Abhi-

yan was carried out by going door to door to screen the community, eradicate communicable diseases, and inspire Gram Pradhan to actively participate in active case finding of TB.[48,49,51–53]

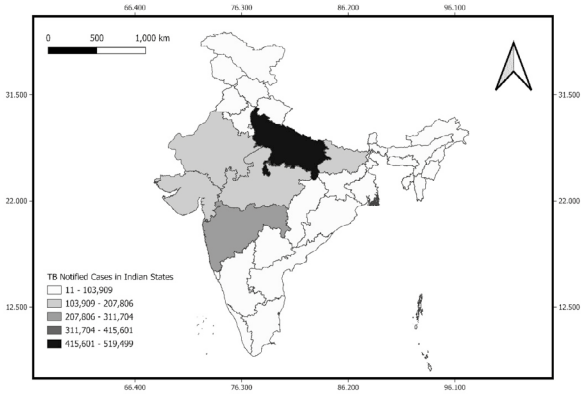

Fig. 5b. Distribution of the Tuberculosis Epidemic in Different States of India in 2022 (India TB Report 2023)

The scope of the TB epidemic in India was determined through prevalence studies. According to the National Tuberculosis Report (2023), men have a slightly higher prevalence of TB than women (1.37 million versus 0.78 million) (Fig. 6), which may be attributed to men's more intense outdoor activities and women's greater likelihood of accessing health services.[1,3,4,20,38,41,46,54,55] In fact, many PTB patients used dangerous sputum disposal techniques, which are dominant in male and illiterate patients from lower socioeconomic groups.[1,22,31,41–44,56]

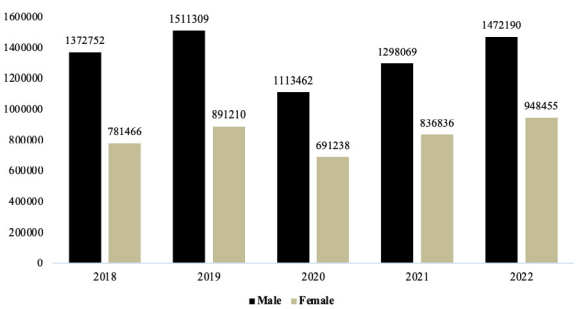

Fig. 6. Gender-specific data on TB-notified cases (India TB Annual Report 2019–2023)

TB is the most lethal infectious disease in the world, and genital TB is a common complication in infertile people who receive inadequate clinical care.[30,57] When a pregnant TB patient cannot adequately protect, medications like isoniazid prophylaxis (3-6 months) or the Bacillus Calmette-Guerin (BCG) vaccine are administered to protect the neonates or newborns.[11,57,58] Factors mentioned in Fig. 3 are the primary causes of an increase in risk factors among children and pregnant women, all of which contribute to the spread of TB.[11,57,58]

In addition to gender differences in TB prevalence, mortality rates increased dramatically with age, being 17 times higher in children under 15 years and more than twice as common in adults 60 and older than in those between the ages of 15 and 59.[1,3,4,10–12,20,28,38,41,43,46,59] Furthermore, the COVID-19 reaction-induced lockdown has resulted in poverty and economic hardship, worsening food shortages and making it more difficult to provide various TB services such as prevention, surveillance, and treatment to the vulnerable segment.[13,22,31,44,60] Children with TB had significant implications or problems during the COVID epidemic, such as requiring more mechanical breathing, being hospitalized for longer periods of time, and having poorer outcomes.[11,13,14]

Scheduled Tribes represent 8.6% of India's population with having high TB prevalence (703 per 1,00,000) as compared to national TB average cases (256 per 1,00,000).[6,15,16,22,61] Individual patients in tribal areas in India face major challenges due to poor socioeconomic conditions, socio-cultural variables, individual ignorance or discrimination, mental health challenges, limited resources or transportation, gender-specific education, a lack of basic understanding about the disease, and difficulty accessing the healthcare facility.[1,3,22,43,60–63] According to studies by Rao et al.[29], Bhat et al.[55], and Thomas et al.[16], PTB exposures were more prevalent among tribal groups, and 2.8 times higher prevalent among males than females.[16,22,29,55,63]

Drug-resistant tuberculosis

According to current trends, drug-resistant tuberculosis (DR-TB), which is resistant to a variety of anti-tuberculosis drugs, is a serious problem in the Indian population, and as a result, it has become a new impediment to India's efforts to combat the illness.[33,36,45,56,59,64,65] The four first-line antibiotics for tuberculosis are rifampicin, isoniazid, pyrazinamide, and ethambutol. They are typically used in conjunction with other TB medications for the first 6 to 9 months of a patient's treatment.[5,19,33,65] Unfortunately, all TB vaccinations that were previously effective in preventing the disease are no longer effective, and multidrug-resistant tuberculosis (MDR-TB) has begun to emerge.[56,59] There are number of factors, such as treatment failure, delayed diagnosis, financial difficulties, missed doses, drug side effects, dissatisfaction with services, lack of TB awareness, limited education and transportation, inconvenient clinic hours, prolonged treatment, non-adherence, alcoholism, illiteracy, other commitments during treatment, and insufficient support systems, have an impact on the spread of DR-TB.[5,38,56,64] Additionally, traditional healers, a non-allopathic provider, a private physician, and independent practitioners have a significant role in delaying the prevention, detection, and treatment of TB in both rural and urban regions, which ultimately leads

to DR-TB.[22,33–36,38] Furthermore, healthcare professionals can contribute to TB treatment failure due to various factors, including inadequate monitoring systems, a lack of courier services, insufficient knowledge and resources, increased workload, and a shortage of diagnostic kits.[3,5,6,64,66,67]

Resistance to frequently given anti-tuberculosis medications results in extensively drug-resistant tuberculosis (XDR-TB) and MDR-TB.[7,40,64] Furthermore, XDR-TB, a subtype of MDR-TB, is resistant to isoniazid, rifampicin, and any of three second-line injectable drugs (e.g., levofloxacin, moxifloxacin, and bedaquiline), whereas MDR is only resistant to drugs of the isoniazid and rifampicin types.[33,36,45,59,64,65] Risk factors for MDR-TB include male gender, age over 60, living in crowded or congested environment using indigenous stove (Chulha), prior TB treatment, contact with an MDR patient, and being from a hilly region.[40,45,59,68–70]

India is the country with the greatest rates of TB related morbidity and mortality, along with risk factors such as undernourishment, alcohol smoking, diabetes, and HIV infection (Fig. 7).[2,9,10,23,24,26,27,29–32,54,56,71] Similarly, these co-morbidities act as a risk factors for the activation of latent TB into active TB.[21,28,35,37,42] Moreover, misconceptions about TB transmission, such as the false beliefs regarding its spread through eating, sharing utensils, or touching patients, coupled with the social stigma faced more likely by women, contribute to social exclusion, avoidance, discrimination, rumors, verbal abuse, lost marriage prospects, and parental neglect.[12,25,62]

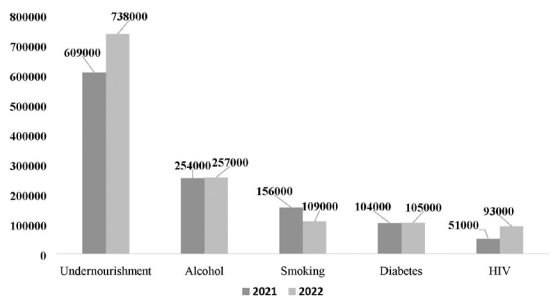

Fig. 7. Prevalence of tuberculosis-associated risk factors in India (Source: India TB Report 2022–2023)

Studies, highlighted that due to a lack of knowledge about TB, healthcare professionals either avoid dealing with TB patients or fear them, believing they will spread the disease, or lack trust and coordination with the government healthcare system.[5,62,67,72] In contrast, mental health problems are also a major concern usually present in TB patients due to rumors and misconceptions among them, healthcare professionals, and society.[5,12,62,67,72] Misconceptions about TB transmission, such as the false beliefs regarding its spread through eating, sharing utensils, or touching patients, coupled with the social stigma faced more likely by women, contribute to social exclusion, avoidance, discrimination, rumors, verbal abuse, lost marriage prospects, and parental neglect.[12,25,62]

Undernutrition and tuberculosis

Undernutrition, which affects 6.90 million people globally, is on the rise, negatively impacts people's immune systems, and is a risk factor in TB, although it is described as an individual's intake of insufficient calories and nutrients to meet their need to maintain good health.[8,32,73,74] Several studies have found that adults aged 15–49 years with a low body mass index (BMI) (<18.50 kg/m^2) are more likely to develop TB, experience poor treatment outcomes, and have a higher risk of death.[9,28,31,37,38,41,73,74] Rural areas, scheduled castes and tribes, other socially and economically backward classes, states with higher levels of poverty, and migrant workers all have high rates of undernourishment, which increases their risk of developing TB.[1,5,22,31,37,42] Nearly half of all active TB cases in women and all cases in males have undernutrition as a major risk factor.[9,31,73]

Diabetes and tuberculosis

India faces a dual challenge of TB and diabetes. Diabetes is a metabolic disorder characterized by inadequate insulin production by the pancreas, leading to treatment failure and mortality and potentially contributing to the spread of TB.[20,28,66,75] It is a fact that diabetes is a well-known major risk factor for more than 25% of TB patients, which also plays a major role in altering the disease pattern from PTB to EPTB or DR-TB (rifampin resistance) due to the weak immune system.[3,20,23,30,32,71,75] Patients with PTB over 50, generally men with poor psychological status, who had a higher BMI, sedentary occupation, and positive family history of diabetes and were receiving Category II treatment were more likely to acquire diabetes and hypertension.[62,66] Diabetes has been strongly associated with an increased risk of early mortality in TB patients and has created an enormous challenge for TB treatments.[20,60,66,75]

Human immunodeficiency viruses (HIV) and tuberculosis

India has the highest number of HIV infections in Asia and the third-highest total number of infected people worldwide.[16–20,54] Similarly, studies have revealed that participants' views toward people with HIV/AIDS were much more connected with their sentiments toward people with TB than with their attitudes toward people who act as sex workers.[14,17] However, it was shown that HIV-positive status, a low BMI or undernutrition (<18.5 kg/m^2), a male individual, and a history of prior TB treatment were substantially connected to PTB or EPTB and DR-TB.[4,6,41,54,65,73] TB has become more difficult to

treat as MDR-TB and XDR-TB forms have emerged, as has HIV coinfection.[2,58,76] The significant factors in the positive association of TB and HIV among sexually active age groups individuals is their poor knowledge of TB and its modes of transmission among.[2,24,54,58,76] In several studies, it is noted that due to HIV epidemics, deficiency of vitamin D and C, and greater use of immunomodulating medications, EPTB is becoming more common.[3,18–20,54,71]

Tobacco consumption and tuberculosis

In India, tobacco is a major health issue that contributes to a number of health problems, including mouth cancer, chronic bronchitis, and cardiovascular and lung disorders that ultimately cause millions of deaths annually.[21,33,46,56,65,69,77] In the TB patient, tobacco smoking affects the efficacy of TB treatment and raises the risk of infection, which increases the likelihood of DR-TB and ultimately leads to mortality.[28,33,65,77] Several studies revealed that smokers were four times more likely to get active or latent TB due to long-time of smoke exposure, not maintaining safe distance and following unhygienic lifestyle.[21,69,77] There is a strong association between TB and tobacco use among males, lesser educated individuals, older people, alcoholics, and residents of rural and slum areas, mainly in thatched houses.[29,69,71,77]

Alcohol consumption and tuberculosis

Alcohol consumption is observed as a serious public health concern on a global scale since it drastically raises morbidity and mortality as well as produces a variety of social issues and health concerns over the course of a lifetime.[26–28,41,71] In a similar vein, numerous researchers have discovered a positive correlation between alcohol use and TB patients. Risky alcohol consumptions have also been associated to a delay in seeking treatment for TB as well as a poor treatment outcome that can lead to DR-TB.[26,27,41,71] A father's history of alcohol consumption, residing in an area where illicit alcohol was being produced, and also belonging to a vulnerable segments of the population are all significant risk factors for consumption of alcohol and rising TB burden.[26,40] However, in several studies have revealed that TB patient consume alcohol mainly due to anxiety, depression, family burden, social stigma and misconceptions of TB epidemic.[12,25,62]

Adopted measures, challenges faced, and tuberculosis-related policies

According to the annual TB report, India is home to 26% of TB cases worldwide, and males are more likely to contract the disease than females (283 per 100,000 men versus 186 per 100,000 women) (162 per 100,000).[20,48,49] Therefore, the total mortality rate due to TB is 0.20 individuals per 1000 every day. However, not all strata bear the same amount of the TB burden; the tribal community is one of the most severely impacted groups in the country.[15,16,28,48,49,63] Additionally, several studies found that more than two-thirds of TB cases are observed in the economically productive age range (14–59 years).[1,3,4,9,10,20,31,37,38,40,41,43,46,59,73,74] The international health community, which has established multiple 5-year targets for TB elimination, has proposed a method combining improved diagnostics, medications, and vaccinations to identify and treat both latent and active TB infections in order to meet the 2050 target.[15,48,49,51] The Joint Effort for Tuberculosis Elimination Program, with TB professionals, NGOs, and private practitioners all playing crucial roles in assisting patients seeking TB treatment, has also proven successful in providing quality TB services and facilities to patients.[5,60,62,72] Additionally, the President of India established the Pradhan Mantri TB Mukt Bharat Abhiyan to hasten the elimination of TB by 2025. Through community support and the use of Corporate Social Responsibility initiatives, Ni-kshay Mitra and Ni-kshay Digital Porat were introduced as part of this project to provide patient support and enhance the treatment outcomes for TB patients.[48,49] In order to provide nutritional support for all TB patients, the Indian government introduced the direct benefit transfer (DBT) programme in March 2018.[22,39,48,49,51–53] This includes depositing INR 500 (roughly $7) into each TB patient's bank account, but delays were occurring in the DBT program due to the complexity of processes.[5,22,39,61] However, NTEP is also continuously taking steps to increase awareness and improve TB services, but its implementation is difficult, particularly in rural areas, because in rural areas various community have their own distinctive ways of addressing health issues, which is one of the main reasons for postponing the diagnosis and appropriate treatment of TB.[15,16,60,78] Additionally, studies have shown that social support is essential in order to improve the condition because patients with this chronic illness experience significant declines in their physical, social, economic, psychological, and emotional well-being even after therapy has ended.[70,79]

It has been discovered that people with no prior experience of TB are strongly associated with improved treatment outcomes.[4,40,79] But in the case of MDR-TB patients, they exhibited worse treatment results than the general TB patient and were unable to receive a proper diagnosis when tested at RNTCP diagnostic facilities.[33,45,65,80] Additionally, household members are at high risk of infection when having close contacts with individuals having MDR-TB or during treatment.[14,28,36]

The COVID-19 pandemic had a significant impact on TB notification methods, but several researchers have also reported that digital TB storytelling on Frequency Modulation radio has enhanced TB awareness

and knowledge. As a result, there has been an increase in active case-finding and better control of TB spread particularly among individuals belonging to vulnerable segments affected by poverty and undernutrition.[12,31,70,78] Similar to this, the most popular sources of up-to-date information about TB and the RNTCP were the health education programme, textbook content, and especially the internet and television campaigns.[12,31,70,78] However, despite people's awareness of TB diseases and knowledge of where to access free treatment and healthcare services provided by the government, there is still a lack of knowledge about the Directly Observed Treatment Service.[12,49,67,79] This knowledge gap can be attributed to poverty and limited awareness of the importance of the Directly Observed Treatment Service.[5,39,61]

Recommendations

Key measures should be implemented to reduce the prevalence of TB in India. First and foremost, there is a need for greater access to appropriate treatment and modern medical methods for diagnosis. Rapid and extremely sensitive testing processes, particularly in rural regions, should be expanded. Second, direct money transfers should be made available on a regular basis to TB patients registered on the Nikshay portal in order to provide financial assistance. Third, to address undernutrition among TB patients, enhanced distribution of resources and nutritional support should be ensured. Regular follow-ups with patients and social supports are also vital. Finally, thorough outreach campaigns utilizing traditional, and web-based forms should be established to improve knowledge of TB, its prevention, and treatment alternatives. These initiatives, particularly in impoverished areas, can assist to achieving TB eradication by 2025.

Conclusion

With a high case load and related problems, TB remains a major public health concern in India. Both genders are affected, with men having an admittedly higher occurrence. Undernutrition, diabetes, HIV, tobacco and alcohol usage, and mental health problems all contribute to the spread and progression of TB. Further, drug-resistant TB has emerged as a serious impediment to TB control efforts, worsening treatment outcomes even further. Despite the hurdles, India has introduced a variety of TB-fighting strategies and policies, such as enhanced diagnoses, treatments, Pradhan Mantri TB Mukt Bharat Abhiyan and nutritional support programmes.

Declarations

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Author contributions

Conceptualization, P.D. and N.M.; Methodology, P.D.; Software, P.D.; Validation, P.D. and N.M.; Formal Analysis, P.D. and N.M.; Resources, P.D.; Writing – Original Draft Preparation, P.D.; Writing – Review & Editing, N.M.; Visualization, N.M.; Supervision, N.M.; Funding Acquisition, P.D.

Conflicts of interest

Authors declare that there are no conflicts of interest.

Data availability

Data supporting the results of this study shall, upon appropriate request, be available from the corresponding author.

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REVIEW PAPER

Ascorbic acid in cancer management – time for a second look

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ABSTRACT

Introduction and aim. Over the past decades, the hypotheses that ascorbic acid (AA) can play a role as an anti-neoplastic therapy have generated many conflicting reports. Despite the controversies, mounting evidence has shown that AA has the potential to play a role as an anti-neoplastic agent. Recent studies have unraveled its pharmacokinetics and various mechanism of action on cancer cells. This has spawned different preclinical studies with reports of good activities against various cancers.

Material and methods. A review of the literature regarding ascorbic acid in the management of cancer was performed using the PubMed database. The research was limited to abstracts and available full-text articles.

Analysis of the literature. Clinical trials have also demonstrated its safety and tolerability across different dosages. AA has been noted as a multitargeting agent that acts as a pro-oxidative cytotoxic agent, anti-cancer epigenetic regulator and immune modulator. AA has also been shown act synergistically with standard chemotherapy regimens in different cancers. Despite its potentials, phase III clinical trials are seriously lacking. The recent phase III VITALITY study shows that AA may play a role as an adjunct targeted therapy for ras-mutated cancers. Therefore, there is need to for more standardized clinical trials to help identify cancer subtypes and AA combination regimens that can show the most benefits. In this review, the pleiotropic mechanism of action of AA was explored as well as various preclinical and clinical studies in cancer therapy. In addition, recommendations were also made for effective strategies towards an AA and standard cancer regimens in treatment as well as future directions. Ascorbic acid has been shown to induce cell death in various cancer types through different mechanisms of action. Several clinical trials and case reports have shown its efficacy in combination chemotherapy, and the pharmacological route of action can be either intravenous or oral. However, it can impair the actions of some drugs when given in combination. Also, dosage should be determined for maximal pharmacologic action.

Conclusion. Ascorbic acid has the potential to provide safe and cost-effective antineoplastic treatment option especially in combination therapy. Its potential needs to be further investigated through clinical trials.

Keywords. ascorbic acid, cancer, clinical trials

Introduction

Ascorbic acid (vitamin C, AA) is a six- carbon lactone that is synthesized from glucose and primarily acts as an electron donor at the physiological state. It is a known pleiotropic molecule that serves various functions in the human body ranging from collagen hydroxylation, metabolism of folic acid, tyrosine and tryptophan, synthesis of carnitine and catecholamines and neutralizing free radicals as well as protection of DNA damage (Fig. 1).[1]

Apart from its physiological roles, AA has been muted as a potential anticancer agent. Several experimental studies have shown that AA at pharmacological doses has some clinical effects on various types of cancer.[2-4] Since the 1950s, AA has been proposed as a

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cancer agent, however, in 1974 works carried out by the Scottish surgeon Ewan Cameron and his colleague Allan Campbell using high dose AA both intravenously and orally as a cancer treatment showed the drug as tolerable and safe, and with a complete remission in one case later reported.[5,6] Cameron and Pauling later on showed that AA significantly prolonged survival in terminal cancer patients.[7] Their work was met by criticisms which were largely procedural; which led to a second investigation by the men and this time the new study showed that patients on high dose AA had a mean survival time of about 300 days longer than the untreated controls.[8] However, two Mayo Clinic randomized, placebo-controlled prospective trials later disproved the efficacy of AA in cancer patients.[9-10] However, the Mayo clinic trial was faulted on two grounds; only patients with colorectal cancer were used in the trial which is not representative enough of the various cancer types. More importantly, unlike Cameron's subjects that received both intravenous and oral AA, subjects in the Mayo Clinic trials received only oral AA. The importance of the route of administration determines the degree of bioavailability of AA.[11]

Subsequently, a gradually increasing number of preclinical studies and some clinical studies have shown the efficacy of AA especially when given intravenously. Some experimental works have shown that high dose AA induce growth arrest in tumor cells both in vitro and ex vivo.[11-13] Padayatty et al. in their work showed that peak plasma AA concentrations were higher after administration of intravenous doses than after administration of oral doses, and this difference increased with the dose. When AA was given at a dose of 1.25 g orally, it produced a peak plasma concentration of 134.8±20.6 μmol/L, while the IV administration produced a peak plasma concentration of 885±201.2 μmol/L.[14] The IV administration is thus seen as the pharmacologic basis of action.

Aim

Material and methods A review of the literature regarding ascorbic acid in the management of cancer was performed using the PubMed database. The research was limited to abstracts and available full-text articles.

Analysis of the literature

Ascorbic acid homeostasis

Oral administration of AA is tightly controlled via intestinal absorption, accumulation and distribution in tissues, utilization and recycling, and renal excretion and reabsorption.[15-16] These processes are ensured through different ways including passive diffusion, facilitated diffusion, active transport and recycling.[17-19] After the ingestion of AA, it is absorbed into the bloodstream. The intestinal absorption of AA has been observed to be reduced with increased intake up to a certain dose; this is due to a decrease in the expression of the sodium AA transporter sodium-dependent vitamin C transporters (SVCT). AA is known to be taken up primarily into cells via SVCT 1 and 2, on the other hand, its oxidized form, dehydroascorbate (DHA), is taken up via the facilitated diffusion transporters glucose transporters (GLUTs).[15,19-20] The AA transporter SVCT1 which is expressed primarily in intestine, liver and kidney is known to mediate the renal reabsorption of AA. Mice lacking the SVCT1 gene have been reported to increase AA fractional excretion up to 18-fold with hepatic portal AA accumulation nearly terminated; however, the intestinal absorption was mildly affected.[21] SVCT2 on the other hand, which is expressed in almost all cells, contributes to the accumulation of AA in most tissues.[22] SVCT2 deficiency has been linked to perinatal mortality in mice, and elevated risk of spontaneous preterm births in humans.[22,23] This is probably as a result of poor AA accumulation.

Oral intake of AA is tightly controlled as a result of these regulatory processes. Whereas, the intravenous administration of AA have been shown to achieve a 70-fold higher plasma levels than even the highest oral tolerable dose.[14] Interestingly, at these higher plasma concentrations via intravenous administration AA is able to kill cancer cells making it an emerging potential anticancer therapy.

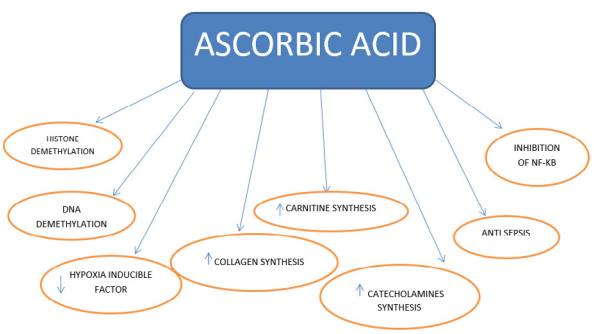

Fig. 1. Some of the different functions of AA

Antineoplastic activity of AA

AA has been shown to be an antioxidant through the suppression of free radicals generation, as well as attenuation of oxidative damages caused by the free radicals.[24-25] Examples of its antioxidant activity can be found in the prevention of low-density lipoprotein oxidation, and reduction in amyloid plaques in the nervous system.[26-28] AA supplementation is also reported to increase the levels of glutathione and thiols and negatively affects the levels of oxidative stress markers malondialdehyde, and nitrites.[29] AA also acts an anti-inflammatory in different pathological state, including cancer, sepsis, stroke etc.[30-33]

Antioxidant

Importantly, as part of its antioxidative properties AA is the reprogramming of the epigenome through the enhancement of the catalytic activity of the Jumonji-C domain-containing histone demethylases (JHDMs) and the ten-eleven translocation (TET) family of DNA hydroxylases which drive histone and DNA demethylation in somatic cells; thus AA can modulate embryonic stem cell function, enhance reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) and hinder the aberrant self-renewal of hematopoietic stem cells (HSCs).[34-37] In addition, AA also regulates the DNA methyl transferases (DNMT), the hypoxia inducible transcription factor alpha prolyl hydroxylases D (PHD) and the histone alpha/beta hydrolase (ABH).[38-40] This aforementioned activity of AA i.e. regulation of gene expression, is associated with its antioxidant function and has important role in cancer treatment. While antioxidant therapies have been promoted as potential antineoplastic agents, caution should be applied in the indiscriminate prescription of antioxidant supplements. Reactive oxygen species (ROS) are the primary targets of antioxidants, and are known to aid tumour growth.[41-42] This is not so in all cases; an excess of ROS can destroy cancer cells which is a mechanism used by some chemotherapy and radiotherapy.[42-44] However, based on the notion that ROS aid tumor growth, anti-oxidant therapies have been muted as likely antineoplastic therapies. This may not be so true, as some evidence shows that antioxidant activities are also deployed in tumourigenesis and metastasis.[45-48] Besides, Yasueda et al. in their systematic review were of the opinion that it is difficult to determine whether antioxidant supplements affect treatment outcomes.[59] This position was also muted by Watson.[50] Therefore, pro-oxidant therapies may be more amenable to some cancers.

Pro-oxidant

High-dose AA may just be a potential candidate for pro-oxidant therapy. Different mechanisms have been proposed for the pharmacologic action of AA on cancer cells. One of them is the pro-oxidant action of AA on cancer cells. According to the pro-oxidant theory, AA pro-oxidant activity occurs at higher concentrations in the presence of iron. Iron is reduced by AA to Fe^{2+} in the presence of oxygen which leads to the formation of hydrogen peroxide (H_2O_2) and reactive oxygen species. The H_2O_2 further reacts with Fe^{2+} to generate a highly reactive hydroxyl radical. However, in normal tissues H_2O_2 generated is quickly neutralized by the appropriate enzymes e.g. catalase. These enzymes in tumour cells can be defective which lead to the persistence of H_2O_2 and subsequent cell damage.[51-52] Another mechanism of cancer cell death involves DHA. DHA is an oxidized form of AA that is transported into the cell via the facilitated glucose transporters GLUTs.[53] Cancer cells take in DHA and it is reduced back to AA.[54] This intracellular reduction back to AA cause a depletion of glutathione in the cell, which consequently lead to an increase in ROS, oxidative stress, energy crisis and cell death.[55-56] In another study, DHA is also reported to reacts with homocysteine thiolactone (cancer cells have high levels of homocysteine thiolactone) converting it to the toxic compound, 3-mercaptopropionaldehyde and kills the cell.[57]

AA and intracellular labile iron

AA is also reported to target iron (Fe) for various physiological processes (Fig. 2). Iron is an important nutrient that plays various roles in the body such as oxygen homeostasis, cellular metabolism and DNA synthesis.[58-60] Iron is normally transported across the cells in the form of a transferrin (Tf)-Fe^{3+} complex through the cell surface receptor transferrin receptor 1 (TfR1) and then it is moved in via endocytosis. Fe^{3+} (ferric ion) is reduced to Fe^{2+} (ferrous ion) after acidification by the the endosomal six transmembrane epithelial antigen of the prostate 3 (STEAP3). Fe^{2+} is then transferred across the endosomal membrane by divalent metal transporter 1 (DMT1). The Fe^{2+} forms part of the labile iron pool (LIP) – a pool of chelatable and redox-active iron, which is serves as a crossroad of cell iron metabolism. LIP is known to promote formation of reactive oxygen species (ROS) via the Fenton chemistry.[60,61] Fe^{2+} can be oxidized back to Fe^{3+} via the Haber-Weiss reaction with the formation of hydroxyl radicals ($OH^{\bullet}$ and OH^{-}).[62] The presence of AA can prevent Fe^{2+} oxidation by reductive accelerating the Fe^{3+}/Fe^{2+} cycles, and lowering of the redox potential of Fe^{3+}/Fe^{2+} through chelating effect, which leads to enhanced ROS production with more lipid, protein, and DNA oxidation. With these, AA is seen to play a role in cell death through ferroptosis – a novel form of regulated cell death mediated by iron-dependent lipid peroxidation.[63] Ferroptosis have been muted as a promising approach in cancer therapy.[64,65] AA is thought to also play a role in the regulation of iron metabolism by the stimulation of ferritin synthesis, inhibition of lysosomal ferritin degradation and reduction of cellular iron efflux.[66]

Alteration in iron metabolism is generally associated with tumourigenesis which usually involves increased intercellular iron import and reduced iron export. For example, breast cancer patients have significantly higher levels of iron than normal controls.[67,68] Thus, cancers with high levels of iron might be more susceptible to AA through increased production of free radicals via LIP. In a research reported by Xia et al., high dose AA, in the presence of iron, leads to the formation of highly ROS resulting in cell death of multiple myeloma cells.[69]

AA and hypoxia

The iron containing alpha-ketoglutarate-dependent hydroxylases (α-KGDD) are another substrate for AA.

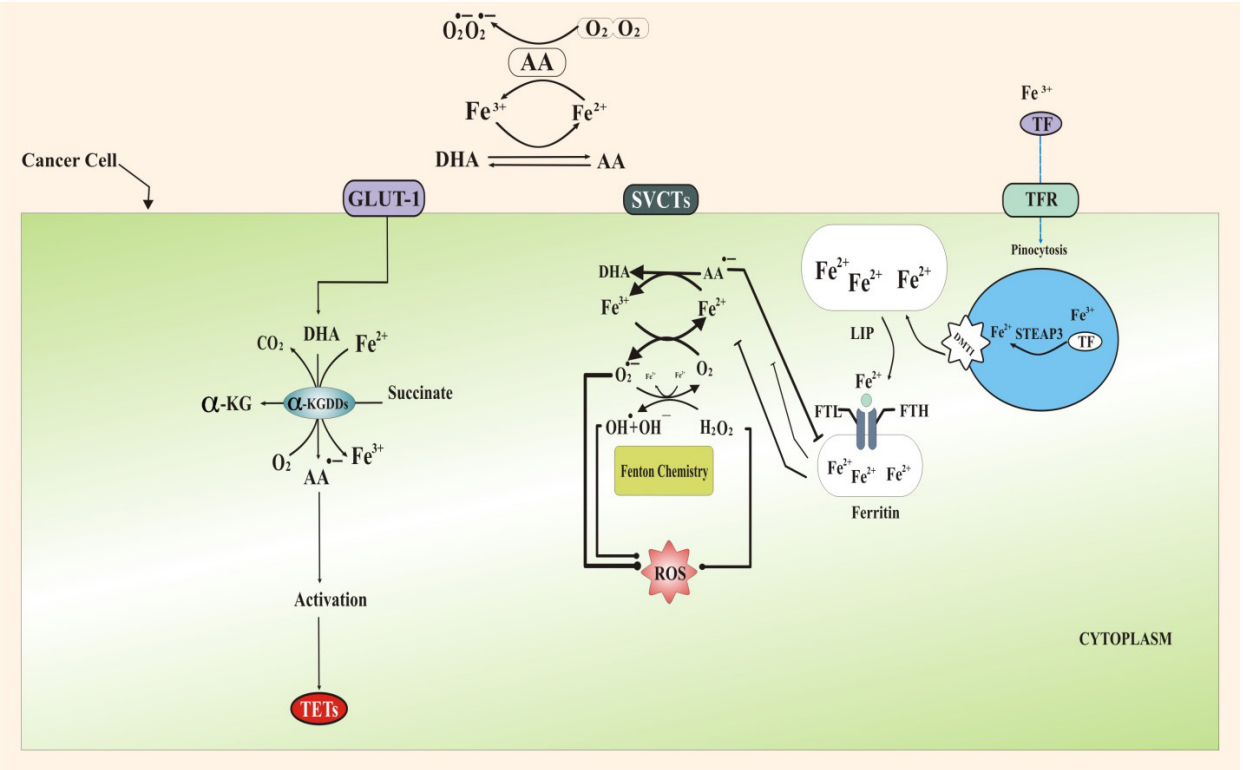

Fig. 2. AA is involved in the imbalance of LIP through the formation of the TF-Fe^{3+} complex which is later acidified by STEAP3, with Fe^{3+} reduced to Fe^{2+}. The accumulated Fe^{2+} generates LIP which interacts with AA and oxygen to produce DHA and Fe^{3+} which later produce ROS through the Fenton reaction. The ROS can lead to cancer cell death through the process of ferroptosis and apoptosis. AA can also act as a co-factor for TET enzymes leading to some epigenetic modifications

α-KGDD catalyze oxidation reactions by incorporating a single oxygen atom from molecular oxygen (O_2) into their substrates. Examples include asparagine hydroxylase and proline hydroxylase. They regulate the activity of hypoxia inducible factor 1α (HIF-1α). HIF-1α is a hetero-dimeric transcription factor that is regulated by hypoxia. They can also be activated by non-hypoxic pathways.[70,71] Prolyl hydroxylase domain (PHD) proteins are known to hydroxylate proline residues on HIF-1α in normoxic situations. After the hydroxylation, the von Hippel Lindau tumor suppressor protein binds to the prolyl-hydroxylated HIF1-α, activating an E3-ubiquitin ligase which targets it for proteasome degradation. On the other hand, asparagine hydroxylase hydroxylates HIF-1α at asparagine residues on the C-terminus, preventing the recruitment of p300/CBP co-activators, thereby making HIF-1α inactive. The reverse is the case in a hypoxic situation, with PHD and arginine hydroxylase suppressed leading to the translocation of HIF-1α into the nucleus where it dimerizes with HIF1-β (also known as aryl hydrocarbon nuclear receptor translocator (ARNT)). The HIF-1α- HIF1-β complex then binds to hypoxia response elements leading to the upregulation of a number of genes. HIF-1α is known to be up-regulated in various cancers.[72,73] HIF-1α also portends a poor prognosis in some cancers.[74,75] Due to its role in cancers, HIF-1α is seen as a viable target for cancer therapy. Most inhibitors work in an indirect mode such as bortezomib and camptothecin and its derivatives.[76] Recently, the FDA approved belzutifan, the first-in-class HIF inhibitor for adult patients with von Hippel-Lindau (VHL) disease – associated tumours.[77] AA has been shown to suppress HIF1-α-dependent cancer growth.[78,79] It does this primarily through the increase in activity of arginine and PHD hydroxylases, therefore decreasing HIF1-α action and cancer growth.[80] Research conducted on cancer patients show an association between AA, HIF-1 activation, and cancer growth. Tissues from these patients showed that cancers with the most potent HIF1 function were those lacking AA in its tumour microenvironment, and patients with higher levels of AA had better outcomes.[81,82] Thus, AA can prevent cancer development through HIF. More researches will be needed to validate this.

AA and NF-κB

Chronic inflammation is one of the hallmarks of carcinogenesis.[83] In the role of inflammation in cancer, one of the key players is the transcription factor nuclear factor-kappaB (NF-κB) which is responsible for signaling processes in immunity, inflammation, cell proliferation and survival. NF-κB consist of five structurally related members which are NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel, and together they are involved in the activa-

tion of certain target genes by binding to the κB enhancer – a specific DNA element, as hetero- or homo-dimers.[84] In the quiescent cell, NF-κB typically binds to its inhibitors IκB (IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100, and p105) in the cytoplasm making it transcriptionally inert. However, upon stimulation IκB is phosphorylated by IκB kinase (IKK), which cause the release of NF-κB and its translocation into the nucleus where it induce the upregulation and transcription of target genes involved in pro-inflammatory response including cyclooxygenase-2 (COX-2) and inducible nitric oxide (NO) synthase. Interestingly, NF-κB is involved in many aspects of cancer development and survival, and it is a target for many small molecules. NF-κB is also known to be regulated by redox control mechanisms; thus, its actions can be adjusted based on ROS concentration.[85-89] This activity is bidirectional.[90] It has been reported that low concentrations of ROS activate the IKK/IκB/NF-κB signal pathway, whereas high concentrations of those inhibit the activation.[91] Du et al. showed that AA via its oxidative product DHA, could inhibit NF-κB through massive ROS generation mediated by intracellular glutathione and copper ions.[91] Studies have shown that AA can inhibit NF-κB through other mechanisms. AA has been reported to block the activation of NF-κB by Tumor necrosis factor-alpha (TNF alpha) through the activation of p38 Mitogen-Activated Protein Kinase (MAPK), and also DHA directly inhibited IKKB and IKK alpha enzymatic activity in vitro independent of p38-MAPK, whereas AA did not.[92-94] These anti- NF-κB activities were ROS- independent. However, it is not all gloom. AA has been shown to be involved in the epigenomic and transcriptomic remodeling of monocyte-derived dendritic cells (DC). The P65 subunit of NF-κB is known to interact with TET2 protein of the epigenome in DCs, and during such interactions, AA triggers an extensive demethylation at NF-kB/p65 binding sites together with concordant upregulation of antigen-presentation immune response-related genes during DC maturation.[95] In addition, AA causes an increase in the production of tumor necrosis factor-beta (TNFβ) in DCs through NF-κB; the selective inhibition of NF-κB I DCs is reported to block maturation and proliferation of T cells.[96,97] These studies show that AA could play a role in some cancers with high NF-κB activity.

AA and epigenetic regulation

The TET proteins are a part of the α-KGDD – a family of non-heme proteins, that are involved in the hydroxylation of 5-methylcytosine (5mC) residues to 5-hydroxymethylcytosine (5hmC) leading to demethylation of DNA residues and activation of certain gene transcriptions (Fig. 3). This epigenetic regulation is an important hallmark in many malignancies. In solid tumours, TET2 mutation is uncommon.[98] However, it is frequently mutated in haematological cancers.[99] DNA hypermethylation as a result of TET2 mutation is associated increased risk of MDS progression, and poor prognosis in AML.[100,101] AA is an epigenetic regulator. It acts as a cofactor for optimal TET activities by reducing Fe^{3+} and Fe^{2+} which results in active DNA demethylation. In addition, mutations in the enzymes isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) lead to metabolic alterations and the formation of 2-hydroxyglutarate (2-HG), an oncometabolite that can inhibit the activity of α-KGDD such as ten-eleven translocation (TET) enzymes reducing 5hmC, boosting DNA methylation and leading to an inhibition of normal cell differentiation. In an AML model, AA was shown to induce an IDH-dependent reduction in cell proliferation and an increase in expression of genes involved in leukocyte differentiation.[102] Also, the hypomethylating agents, azacitidine and decitabine are cytidine analogs that cause DNA demethylation as a result of DNA methyltransferase-1 (DNMT-1) inhibition; including being active in TET2 mutated haematologic malignancies.[103-105] Gerecke et al. showed that addition of AA to hypomethylating agents caused the increased expression of the tumour suppressor p21 (CDKN1A), and induction of apoptosis.[106] A clinical trial (NCT02877277) involving MDS and AML patients showed that AA supplementation in patients on hypomethylating agents induced epigenetic changes.[107] While TET2 is known to have a pleiotropic role in hematopoiesis, it is equally known to promote leukemogenic predisposition especially in haematopoietic stem cells through its regulation of access of some key transcription factors to enhancers of target genes.[108] AA has been found to strengthen the DNA demethylation by TET2 in haematopoietic stem cells, thereby suppressing leukemogenesis and aiding lineage differentiation.[109-112] Thus, AA can play a role in the prevention and management of haematological malignancies. A very recent clinical trial published by Taira et al. showed that AA can also boost DNA demethylation in TET2 germline mutation carriers strengthening the case for AA supplementation in haematological malignancies.[113]

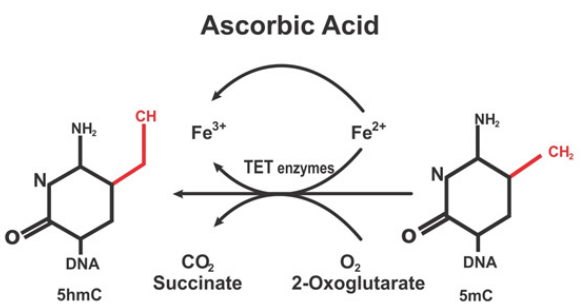

Fig. 3. The TET enzymes are involved in the catalysis of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) with AA as a co-factor through the transfer of an electron from Fe^{2+} to Fe^{3+}, and eventual activation of TET enzymes

AA and cancer immunotherapy

One of the modes of cancer resistance is immune evasion; this is a mechanism through which cancer cells camouflage themselves from immune cells of the body preventing their detection and destruction. These mechanisms are important for cancer progression and metastasis.[114] Furthermore, most subset of immune cells are fingered in cancer biology.[115] One way cancer cells impair immunity is through the high expression of immune checkpoint proteins such as programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). The immune checkpoint proteins naturally serve as costimulatory/coinhibitory molecules that provide the necessary checkpoint regulating T cells and other APCs' interactions as well as balancing immune homeostasis.[116] However, cancer cells express them as a way to suppress the immune system and escape from immune surveillance.[117] Anti-checkpoint inhibitors were therefore developed for the management of cancers that express them. Ipilimumab, an anti-CTLA-4 became the first checkpoint inhibitor to be approved in 2011 for the management of metastatic melanoma.[118] Others like nivolumab and pembrolizumab later followed. Currently, anti-PD-1 (nivolumab and pembrolizumab), anti-PD-L1 (Atezolizumab, avelumab, and duravulumab), and anti-CTLA-4 (ipilimumab, tremelimumab), are the FDA-approved checkpoint inhibitors for the management of a broad range of cancers. However, increased toxicities and treatment failures were some of the challenges encountered in the clinics.[119,120] Thus, reducing the toxicity of checkpoint inhibitors, while increasing their efficacy is an unmet clinical need. One way of achieving this, is the use of intravenous AA in pharmacological doses.[121]

AA has been shown to enhance cancer immunotherapies in both in vivo and in vitro studies. In a very recent study published, Burkard et al. were able to verify the efficacy of high dose AA to kill melanoma cells both in vitro and in vivo; and how it also exert their effect either alone or in combination with the anti-mouse checkpoint inhibitor antibody synergistically.[122] In another study, Luchtel et al. showed that the combination of AA with a checkpoint inhibitor could have significant activity in cancer treatment. Their evaluation was the combination of high dose AA with a checkpoint inhibitor in a lymphoma mouse model. Their findings were that AA i) increases immunogenicity of lymphoma cells; ii) enhances intratumoral infiltration of CD8+ T cells and macrophages; and iii) synergizes with anti-PD1 checkpoint inhibition in a syngeneic lymphoma mouse model via marked activation of cytotoxic cells (cytotoxic T cells and NK cells) and antigen presenting cells.[123] In their own study, Magri et al. showed that a combination of AA with anti-PD1 or anti-CTLA4 in mice resulted in a significant reduction of tumour volume in breast, pancreatic and colonic cancers.[124] Just like the Luchtel's group, they also reported an increase in T cell infiltration of the tumour microenvironment.[124] In the same vein, a recent study by Peng et al. showed that a combination of AA and PD-L1 inhibitor in murine renal cell carcinoma caused increased intramural infiltration of T cells as well as the expression of chemokines and cytokines.[125]

AA has equally been shown to contribute to immune defense especially the innate and adaptive immune system. The immune cells especially macrophages and neutrophils are known to accumulate AA which they use to protect themselves against reactive oxygen species and enhance chemotaxis and phagocytosis.[126-128] Neutrophil extracellular traps (NETs) – net-like structures composed of DNA-histone complexes and proteins released by activated neutrophils and are involved in many disease state, have been identified in neutrophil loss, tumour progression and metastasis as well as the promotion of T cell exhaustion.[129-131] A study by Mohammed et al. showed that in vitro administration of AA to human neutrophils caused a decrease in phorbol ester-induced NETosis.[132] In another study, AA incubated with human neutrophils from septic patients with reduced chemotactic and phagocytic activities showed a decrease in spontaneous NETosis formation and an improvement in neutrophil function.[133] Like the phagocytes, B, T lymphocytes and NK cells are also known to accumulate high levels of AA.[134,135] It is not certain why, though it is believed for antioxidant protection. AA has been linked to the development and maturation, proliferation and differentiation of the lymphocytes.[136-139] These activities of AA on immune cells have been muted to benefit chimeric antigen receptor (CAR) cells to enhance their efficacy against cancer cells.[140,141] Kouakanou et al. showed that addition of AA during CD19-CAR T cell production enforces a stem cell memory–like phenotype and enhance anti-tumour function.[140] Huijskens et al. showed this was also applicable to NK cell therapy.[142] Recently, γδ T cells have been shown to be potential effector cells in cell-based cancer immunotherapy.[143] This has attracted a fast track designation by the FDA for the allogeneic γδ T cells for the treatment of relapsed or refractory B-cell non-Hodgkin lymphoma.[144-146] AA has been shown to enhance the proliferation and effector functions of human γδ T cells.[147] This ability to expand γδ T cells as well as enhance their effector function have led to the first adoptive transfer of allogeneic γδ T cells expanded in vitro in the presence of vitamin C into patients with solid cancers, and which showed increased survival in those patients.[148] AA has effect on cancer immunotherapy, but more clinical studies would need to be carried out to determine just how useful they are.

AA and clinical studies

AA is used by many complementary and alternative cancer therapists, and for the past decade or more there

have been a steady rise in the evaluation of AA as an antineoplastic therapy. Some of these trials evaluated its use as a monotherapy or as combination therapy with standard chemotherapies. While the pre-clinical studies have shown potentials, the results of the clinical studies have been mixed. Zasowska-Nowak et al. in their review reported that from various studies done AA was ineffective in human studies conducted in advanced-stage cancer patients.[150] This was a bit different from a systematic review reported by Mohseni et al. who found that high dose AA with chemotherapy resulted in an increase in overall survival (OS); however, as a monotherapy, AA was tolerable and safe but without any objective tumour response.[151] Nielsen et al. in their study on prostate cancer patients reported no signs of disease remission.[152] This may have been so, because the dosage and frequency administered in this study was much lower compared to those reported by Stephenson et al. and Hoffer et al., who reported stable disease in 3 and 2 patients respectively, but also without objective tumour response.[153,154] While AA monotherapy may be less efficacious, however, a handful of random case reports have shown that AA monotherapy could possibly be efficacious in the treatment of some cancers.[155-157] This was best exemplified in a series of case reports by Raymond et al. among some patients treated in Singapore.[158] The case of an AML patient in palliative care who achieved complete remission for 2.5 years while on high dose AA also shows the efficacy of AA monotherapy.[159] Currently, some AA monotherapy studies are ongoing (NCT03613727) (NCT03682029).[160,161]

Clinical studies of AA in combination therapy

Some studies of AA as a combination therapy with chemotherapies have also been reported. However, they were carried out with only a limited number of patients, and no double blind randomzed trials. Some of the combinations have shown promise in some cancers. In a phase 1 clinical study of patients with metastatic gastric and metastatic colorectal cancers given IV AA at 1.5 g/kg once daily with mFOLFOX6 or FOLFIRI with or without bevacizumab in a 14 day cycle, Wang et al. reported a favourable safety profile and an objective response rate (ORR) of 58.3% with a disease control rate of 95.8%.[162] This result spurred a randomized, open label, multicenter phase 3 study of IV AA + FOLFOX ± bevacizumab (experimental group) vs. FOLFOX ± bevacizumab alone (control group). However, in the recently published result the experimental group failed to show superior progression free survival (PFS) compared to the control group [median PFS, 8.6 vs. 8.3 months; HR, 0.86; 95% confidence interval (CI), 0.70–1.05; P = 0.1]; but patients with RAS mutation had significantly longer PFS (median PFS, 9.2 vs. 7.8 months; HR, 0.67; 95% CI, 0.50–0.91; p=0.01) with AA added to chemotherapy.[163] Some other small successes have been recorded in AA plus chemotherapy combination therapy. A phase 1 clinical trial (PACMAN) of IV AA and gemcitabine in patients with metastatic or unresectable pancreatic adenocarcinoma showed good clinical safety and tolerability, and has now been escalated to a randomized phase 2 trial and it's currently ongoing (PACMAN 2.1) (NCT02905578).[164,165] Polireddy et al. also reported a phase I/IIa clinical trial (NCT01364805) in pancreatic cancer patients using IV AA and gemcitabine. The study showed an OS of 15.1 months with one of the participant showing significant tumour response.[166] Their study showed that AA has a multi-targeting mechanism of action on pancreatic cancer cells including ATP depletion and increased α-tubulin acetylation. AA has been shown to have good activity against RAS-mutated cancers, and since more than 90% of pancreatic cancers harbor the RAS mutation, AA could possibly have some activity against pancreatic cancers. In addition, AA has been shown to alter cancer metabolism.[163,167-170] A preclinical study of AA plus buformin on AML cell lines shows that AA inhibits glucose metabolism through interfering with hexokinase 1/2 and GLUT1 functions in hematopoietic cells as well as depletes ATP production.[171] Currently, a phase 2 clinical trial of AA in combination with metformin in some solid tumours is ongoing (NCT04033107).[172]

AA and haematological malignancies in clinical studies

A number of clinical studies have also been done with haematological malignancies (Table 1). Some of the studies involved the use of arsenic trioxide. In a clinical study (ChiCTR1800018811) by Qian et al. which aimed at comparing the efficacy and tolerability of an arsenic trioxide/bortezomib/ascorbic acid/dexamethasone (ABCD) regimen with efficacy and tolerability of a bortezomib/dexamethasone (BD) regimen in patients with newly diagnosed myeloma, the ABCD regimen showed a greater response rates (above VGPR) than the BD regimen. It also showed significantly improved PFS and better tolerability with lower bone marrow suppression especially in patients with low or standard risk disease.[173] In two similar phase I/II study by Berenson et al., arsenic trioxide/bortezomib/ascorbic acid (ABC) combination therapy in patients with relapsed/refractory multiple myeloma was well tolerated and showed an objective response rate (ORR) of 27% in the heavily pretreated study population; and also the melphalan, arsenic trioxide (ATO) and ascorbic acid (AA) (MAC) combination therapy for patients with multiple myeloma showed an ORR of 48% with good safety and tolerability.[174,175]

Similarly, in patients managed for acute promyelocytic leukemia (APL) given oral arsenic trioxide, all-trans retinoic acid and ascorbic acid (popularly known as the triple

Table 1. Clinical trials using ascorbic acid as anti-neoplastic therapy in hematological malignancies

Trial number	Cancer type	Phase	Study design	Combination	AA dose
ChiCTR1800018811[173]	Multiple myeloma	II	Randomized	Arm 1: AA + chemotherapy (ABD)	1000 mg IV days 1 to 3, 8–10, and 15–17 over 30 mins
				Arm 2: Chemotherapy (BD)	
NA[174]	Multiple myeloma	NA	Single arm	Bortezomib and Arsenic Trioxide	1 g IV on days 1, 4, 8, and 11 of a 21–day cycle for a maximum of 8 cycles
NCT04251754[176]	Acute promyelocytic leukemia	III	Observational	Arsenic Trioxide and All trans retinoic acid	1 g/day for 6 weeks (oral)
NCT03682029[160]	Myeloid malignancies	II	Interventional (randomized)	Placebo	1 g daily for 12 months (oral)
NCT03999723[185]	Myeloid malignancies	II	Interventional (randomized)	Arm 1: AA + Azacitidine	1 g daily (oral)
				Arm 2: placebo + Azacitidine	
ACTRN12621000223831[184]	Myeloid malignancies	II	Non-randomized, open trial	Arm 1: (TET2 mutation) azacitidine	IV 30g on days 1–5, 8–9 or days 1–7
				Arm 2: (RAS +/- TET2 mutation) azacitidine and lenzilumab	
NCT03418038	Lymphoma	II	Interventional (Randomized)	Arm 1: Ascorbic acid + combination chemotherapy	IV on days 1, 3, 5, 8, 10, 12, 15, 17 and 19
				Arm 2: Placebo + combination chemotherapy (rituximab + ifosfamide + carboplatin + etoposide D1–3; rituximab + cisplatin + cytarabine + dexamethasone if MR or SD after 2 courses)	
				Arm 3: Ascorbic acid + combination chemotherapy (ifosfamide + carboplatin + etoposide or cisplatin + cytarabine + dexamethasone or gemcitabine + dexamethasone + cisplatin or gemcitabine + oxaliplatin or oxaliplatin + cytarabine + dexamethasone)	

A regimen), the leukemia-free survival (LFS) and overall survival (OS) rates were 100% at 3 years and 94.1% at 5 years respectively.[176] In a similar study, APL patients who achieved first complete remission (CR) and were placed on triple A maintenance had a 5-year and 10-year rates of relapse-free survival (RFS) of 89% and 85%, and OS of 94% and 87%, respectively.[177] The triple A regimen was also shown to be safe and associated with long term survival in patients. The use of the Triple A regimen in APL as maintenance therapy is still ongoing in another study (NCT04251754).[178-180] AA is also known to induce DNA demethylation at the cellular level. In a clinical study by Zhao et al., 73 elderly AML patients treated with AA plus a combination of decitabine, cytarabine, aclarubicin and granulocyte colony-stimulating factor (DCAG) had a higher CR (79.92% vs. 44.11%; p=0.004) after one cycle of chemotherapy, and a median OS (15.3 months vs. 9.3 months, p=0.039) compared with the DCAG only group.[181] Welch et al., in a clinical phase 1 study of AA, decitabine and arsenic trioxide in patients with MDS and AML observed complete remission with incomplete blood count recovery (CRi) in one patient, and stable disease (SD) in five patients after four cycles of therapy.[182] A suggestion for a phase II trial was muted. TET2 is mutated in many haematological malignancies, and plays a major role in epigenetic modulation alongside prognosis of myeloid neoplasms.[99,101,183] Due to the role AA play as an epigenetic modifier, targeting demethylases with AA in combination therapy has been muted as therapeutic strategy.[34] Currently, the PREACH-M study a phase II trial of the use of AA with azacitidine and lenzilumab in CMML patients (ACTRN12621000223831) is ongoing.[184] Other similar clinical studies using CMML, MDS and AML patients (NCT03682029) (NCT03999723) are also ongoing.[160,185]

Time for a second look?

The jury is out, and after several pre-clinical and clinical studies on the potentials of AA as an antineoplastic agent, a verdict is yet to be reached. The pertinent question still remains, should randomized clinical trials be organized to test for the benefit of AA in cancer? For this author it is an affirmative yes. However, this should be within the confines of a well-designed randomized clinical trial (possibly, double-blinded) and preferably as a combination therapy; the phase (induction or maintenance) should also be determined.

Different issues needs to be resolved for a standardized randomized clinical trial, including dosage and frequency which can be dependent on the route of administration. Padayatty et al. determined that only an I.V administration can produce a pharmacologic dose for anti-tumour activity.[14] However, oral AA has been shown to have some activities.[107] So under which conditions oral AA can be used need also to be determined. In the VITALITY study by Wang et al., AA with chemotherapy was shown to induce a significantly longer PFS in CRC patients with RAS mutation than patients on chemotherapy alone.[163] In the EudraCT 2018-000155-41 clinical trial, Taira et al. showed that AA supplementation reinforces DNA demethylation in TET2 mutation carriers; Das et al. equally reported a complete remission in an AML patient with TET2 mutation on AA.[113,159]

This means clinical trials can be designed for AA as a targeted therapy, though AA is a known multitargeting agent in cancer.[186] This "promiscuity" as a multi-targeting agent can make the development of a biomarker as an indicator of response to AA therapy rather problematic. Clinical markers and response such as tumour size shrinkage, overall survival and improved quality of life may just be more durable measurements in such cases.

AA has also been shown to attack cancer by modulating the immune system.[187] Thus, pharmacological dose of AA can potentially serve as an adjunct anti-neoplastic therapy.[141,147] Clinical trials are required to test the efficacy of AA as a potential adjunct anti-neoplastic therapy. In pre-clinical studies, AA has been shown to have synergism with checkpoint inhibitors.[122,123] AA has also been shown to synergistically potentiate the cytotoxicity of targeted therapies ibrutinib, venetoclax and idelalisib in CLL.[188] Further clinical investigations would be needed to determine the clinical benefits. While AA is known to synergize with many anti-neoplastic drugs, it antagonizes in some cases.[189] Dhahri and Chhabra reported of a case of impaired effect of AA on imatinib in a CML patient.[190] Heaney et al. in their pre-clinical study also reported that AA antagonizes the cytotoxic effect of some commonly used chemotherapeutic drugs including imatinib.[191] This inhibitory effect is also reported with bortezomib. It has been reported that AA directly inactivates bortezomib activity by forming a tight but reversible complex through its vicinal diol group.[192] However, the dose used for this study was not pharmacological and it was in oral form. AA is also muted to be able to inactivate ixazomib because of the boronate moiety. However, in a clinical study reported by Bolaman et al., AA enhanced the cytotoxic effect of carfilzomib-lenalidomide-dexamethasone in relapsed/ refractory myeloma patients who initially did not respond to the treatment; thus AA may have no effect on carfilzomib at pharmacological dose.[193-199] In a phase I-II clinical trial (NCT01050621), Hoffer et al. recommended carrying out trials in higher numbers in order to identify specific clusters of cancer type, chemotherapy regimen and AA combination in which exceptional responses are observed to justify a more focused clinical trial.[196] This position is appropriate. The crosstalk between Cabanillas and his colleagues is a good example that a more focused randomized clinical trial is needed to put AA in a proper position for cancer therapy.[197-199] Further investigations of AA action on proteasome inhibitors will be needed. In general, the pharmacokinetics and pharmacodynamics of AA should be considered in the design of any clinical trial.

Conclusion

AA is reported to be generally low in cancer patients. The relationship between AA and cancer is a subject of intense study. While current reports on the anti-neoplastic activity of AA is mixed, it is known to be well-tolerated and possibly play a role in supportive care in cancer. Unlike recent innovative therapies, AA is more affordable for everyone. The use of AA in combination with standard cancer therapies should be further explored in randomized clinical trials. It is time for a second look.

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Author contributions

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Conflicts of interest

The author declare no conflicts of interest.

Data availability

Not applicable.

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REVIEW PAPER

Hematopoietic stem cell transplantation in the treatment of HIV infection – comparison of "Berlin patient", "London patient" and "Dusseldorf patient"

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ABSTRACT

Introduction and aim. Human Immunodeficiency Virus-1 (HIV-1) remains one of the major issues in global public health. The standard therapy for HIV-1 positive patients includes using antiretroviral therapy (ART). These medications ensure suppression of viral replication but do not lead to a cure for the patient. The aim of this study was to present hematopoietic stem cell transplantation (HSCT) as a malignant treatment method which led to cure for three HIV-1 positive patients.

Material and methods. Literature available in April 2023 was searched by using the PubMed and Google Scholar databases. Articles were selected using the following words: HIV, AIDS, HSCT therapy, ART therapy.

Analysis of the literature. In each case of these described HSCT, the donor of hematopoietic stem cells had a homozygous mutation in the HIV co-receptor CCR5 (CCR5Δ32/Δ32). This mutation leads to a permanent lack of the protein and prevents penetration of virus by using this receptor. After transplantation, all of these 3 patients remained virus-free despite discontinuation of ART therapy.

Conclusion. More research is needed to reduce the risk of using HSCT and perhaps in the future be able to use this therapy in all HIV-infected people.

Keywords. AIDS, ART therapy, HIV, HSCT therapy

Introduction

According to data from the World Health Organization (WHO), at the end of 2021 there were 38,4 million people infected with Human Immunodeficiency Virus-1 (HIV-1) infection living worldwide. Since the beginning of the pandemic about 40.1 million people have died because of complications of HIV and this is why HIV remains one of the major issues in global public health.[1] Currently the standard treatment includes using antiretroviral therapy (ART). Rapid initiation of this therapy is very important to improve a patient's own health and to prevent their risk of HIV transmission to others.[2] Unfortunately, there are still cases of late detection of HIV infection. These patients are more likely to have comorbidities such as opportunistic infections and have a poorer response to ART than those who were previously diagnosed with HIV.[3] In addition, it is estimated that in 2021 as many as 25% of infected people were not using antiretroviral treatment.[4] ART can successfully suppress viral replication but must be used for the life of the patient which is associated with costs.[2] Also, some of the antiretroviral medications can demonstrate

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a long-term toxicity such as hepatic failure, lipodystrophy, and lactic acidosis.[5] Therefore, the new methods of treatment are still in search of. In recent years there have been described three patients who received allogeneic CCR5Δ32/Δ32 hematopoietic stem cell transplantation (HSCT) to treat hematological malignancy. In each case after transplantation and interruption of ART the plasma HIV-1 RNA and the immunological correlates of HIV-1 antigen were undetectable, which provides evidence for HIV cure.[6–8]

Aim

The aim of this article was to compare these three patients and present HSCT as a probably new method of treatment for HIV infections in the future.

Material and methods

In April 2023 scientific articles were reviewed by using Medeline (PubMed) and Google Scholar. Articles were searched using keywords: HIV, AIDS, HSCT therapy, ART therapy. The available scientific articles were analyzed and 24 were selected for final analysis. The collected knowledge was completed and systematized to achieve the final effect of the review.

Analysis of the literature

Antiretroviral therapy

In the mid-1990s, therapy based on a combination of several drugs acting at different stages of the viral replication cycle began to be used to treat HIV infection. It has been successful in reducing the deaths of HIV patients by half. Currently, combination therapies consisting of at least 3 antiretroviral drugs are a routine method of fighting HIV used by 27.5 million people infected with HIV. Approved pharmaceuticals include: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, entry and fusion inhibitors and integrase chain transfer inhibitors.[9] According to the latest recommendations, it is suggested that antiretroviral therapy be started immediately after the diagnosis of virus infection. Initially, oral drugs containing an integrase chain transfer inhibitor are used. They are characterized by high safety and tolerability and cause rapid suppression of the virus. What is more, they have a high barrier to the development of resistance and a low potential for interactions with other drugs. If virus suppression is successful, patients may be offered bi-monthly injection therapy consisting of cabotegravir and rilpivirine. Typically, injections are started after oral medication to ensure tolerance. In a situation where, despite treatment, the viral load is high (RNA>200 copies/mL), it is necessary to modify the therapy individually for the patient, based on genotypic resistance testing.[10] Despite the great success of antiretroviral therapy, specifically reducing mortality and disease progression, virus reservoirs remain present in the body. It is necessary to use treatment for the rest of the patient's life, otherwise the virus will start to replicate and spread, which will definitely worsen the patient's clinical condition.[8,11] Additional motives to look for a different method of treatment are: long-term toxicity of drugs, the emergence of both acquired and transmissible drug resistance and possible interaction of antiretroviral drugs with drugs taken permanently by infected patients, whose number is increasing due to greater survival and thus aging population of HIV-positive patients. Some drugs may also cause metabolic complications such as weight gain, most commonly seen in females, black people and Latinos one year after starting treatment. Drugs causing weight gain include integrase inhibitors and tenofovir alafenamide. Weight gain may occur: in patients starting antiretroviral therapy with integrase inhibitors or tenofovir alafenamide, in patients switching to the above-mentioned agents due to viral suppression, and in those taking integrase inhibitors or tenofovir alafenamide as a consequence of pre-exposure prophylaxis.[10] For this reason, it is necessary to control BMI and perform annual tests to detect diabetes and vascular diseases. For years, the goal of scientists has been to find a treatment that would eliminate the virus from the body permanently and thus make it possible to resign from the use of antiretroviral therapy.[9,10]

Hematopoietic stem cell transplantation (HSCT) for HIV treatment

CCR5 coreceptor

HIV-1 can be transmitted through the bloodstream or through unprotected sexual contact. Entering the body, the virus infects cells that have the CD4 receptor on their surface. It exists mainly on the surface of Th lymphocytes, but also on other cells of the immune system, for example on monocytes. The viral glycoprotein gp120 binds to the receptor via coreceptors: CCR5 and CXCR4. Once the virus has entered the host cell, it uses the enzyme reverse transcriptase to create viral DNA. After being transported to the cell nucleus, the genetic material of the virus is translated and an infectious virion is formed, which leaves the cell by lysing it.[12] The vast majority of viruses use the CCR5 co-receptor during acute and early infection. As the disease progresses untreated, variants using CXCR4 emerge. Studies have shown that in some untreated patients infected with HIV-1 type B, 81-88% are 'R5-tropic' viruses. In people receiving treatment, "R5-tropic" viruses account for 49-78%, mixed variants are 22-48%, while "X4-tropic" viruses are only 2-5%. The emergence of virus variants using the CXCR4 co-receptor results in drug resistance to CCR5 inhibitors. One such drug is maraviroc, approved in 2007.[13] A natural deletion of the 32nd base pair in frame (CCR5Δ32) introduces a premature stop codon

and generates a shortened protein that does not appear on the cell surface, rendering the virus unable to enter it. The CCR5Δ32/Δ32 homozygous genotype leads to a permanent lack of the protein and grants natural resistance to HIV viruses that use the CCR5 receptor to enter the cell. People who are heterozygous for this gene have a 2-3 year lower disease progression.[11,14,15] In the Caucasian race, the frequency of the CCR5Δ32 allele (heterozygous form) is 10-20%, while homozygotes are only 1–2%. In contrast, in people of Mongoloid and Negroid races, this mutation is almost absent.[16] During the emergence of "X4-tropic" variants, a natural deletion of the 32 base pair in-frame reads (CCR5Δ32) will not prevent the virus from entering the body.[13]

HIV and malignant tumors of the hematopoietic system

The HIV-1 virus leads to cell lysis, resulting in the progressive destruction of the immune system. The effect of this is the occurrence of immunosuppression, which is responsible for the appearance of opportunistic infections or malignant tumors.[17] A study has shown that HIV-positive people are twice as likely to develop cancer as the general population.[18] In both high-income and low- or middle-income countries, the most common malignancy associated with AIDS is non-Hodgkin's lymphoma (NHL). Prior to the introduction of ART, the most common NHLs were: diffuse large B-cell lymphoma, Burkitt's lymphoma, and primary central nervous system lymphoma. They occurred more frequently in patients with more severe immunodeficiency. After the introduction of multidrug antiretroviral therapy, the incidence of NHL especially in developed countries has decreased but is still higher than in the general population.[17,18] In addition, thanks to the high effectiveness of ART therapy, HIV-infected patients live longer, which makes them more likely to suffer from malignant tumors unrelated to the disease, such as: acute leukemia, Hodgkin's lymphoma, myelodysplastic syndromes or multiple myeloma.[19] Studies have shown a significant disproportion in oncological care between the HIV-negative population and the infected population. Moreover, research into new cancer drugs usually does not include HIV-positive patients. This is due to the specific clinical picture of these patients, possible interactions between drugs included in ART, and chemotherapeutic agents and the expected number of post-treatment complications, including increased mortality in this group of oncological patients.[18,19]

HSCT scheme

The basis for qualifying HIV-positive patients with hematological malignancies for transplantation is the long-term use of ART therapy to significantly reduce and weaken virus populations. The first step is to search for an human leukocyte antigen (HLA)-matching donor who will also have the CCR5Δ32/Δ32 mutation, which gives a chance to completely eliminate the virus from the body.[20] Allogeneic stem cell transplantation begins with conditioning (myeloablative therapy). It involves high-dose chemotherapy and/or radiotherapy to kill cancer cells. Then, hematopoietic cells are transfused into the peripheral blood. Stem cells adhere to the endothelial cells of the bone marrow, followed by cell migration past the endothelium and extracellular matrix into the marrow niche. The bone marrow initiates the production of new cells.[21] After 12-16 months after transplantation, the number of lymphocytes returns to normal, which means that their number is within the range of 1-5 thousand/mm^3. The functional recovery of cells takes longer. After transplantation, it is necessary to use immunosuppressive drugs to weaken the immune response of the recipient to reduce the risk of graft-versus-host disease (GvHD). Supportive therapy involves the administration of drugs to prevent infections and eliminate the side effects of chemotherapy and radiotherapy.[22]

History of HSCT

Allogeneic stem cell transplantation is not a new concept in the fight against HIV-1. It was known that as a result of a transplant, the recipient's hematopoietic cells, including those carrying the HIV virus, are replaced by the donor's cells. With complete chimerism, the HIV-1 virus would be eliminated from the recipient's body. Two patients (known as Boston patients) with malignant tumors who had been HIV-positive for more than 30 years underwent a hematopoietic stem cell transplant in 2013. The donors were not carriers of the CCR5Δ32 mutation. Full past chimerism was established, and post-transplant viral DNA was not detectable in peripheral blood mononuclear cells or plasma. It is worth noting that the patients were treated with ART before, during and after the transplantation. Treatment was discontinued to determine whether drug-free, lasting HIV remission was possible. Viral relapse in the form of acute retroviral syndrome occurred after 3 and 8 months. Without transplantation, discontinuation of antiretroviral drugs resulted in relapse within 2-3 weeks. In addition, both patients developed an anti-host reaction.[11,20] Possibly latent proviruses remained in the bodies of the Boston patients and could multiply in the donor's cells without treatment.[11,22] Another case of a failed HSCT attempt in an HIV-positive patient is a patient from Essen. The patient was diagnosed with anaplastic large cell lymphoma. Decisions were made for HSCT from a donor who was homozygous for the allele CCR5Δ32. The recipient's ART therapy was discontinued 7 days prior to the transplantation. Post-transplant viral load was undetectable. The virus remission lasted 20 days followed by a viral rebound.[23]

Another HIV-positive patient with T-cell lymphoma received a transplant in 2019 from a donor homozygous for the CCR5Δ32 allele. The doctors hoped for a great success and complete elimination of the HIV virus from the patient's body. Unfortunately, the emergence of CXCR4-tropic viruses that could enter cells and were responsible for repopulation of the virus after transplantation was observed. Sequencing of the genetic material showed that viruses with this tropism were present 3 months before the HSCT was performed.[20] Detection of "X4-tropic" virus variants before starting treatment is therefore an important criterion when considering HSCT in HIV-positive patients, because if they are detected, a transplant from a donor with the CCR5Δ32 mutation will not be effective.

HSCT success- description of three cases

The first HIV-infected patient to see viral remission after allogeneic hematopoietic stem cell transplantation (allo-HSCT) was a 40-year-old man known as the "Berlin patient". He was diagnosed with acute myeloid leukemia (AML) in 2007, 10 years after being diagnosed with HIV-1 and 4 years after starting ART treatment. During AML treatment with induction chemotherapy, the patient developed severe adverse outcomes such as hepatotoxicity and renal failure. For this reason, ART therapy was discontinued, leading to a viral rebound. Treatment was resumed immediately before the virus reached a steady state. Despite the treatment, AML recurred after 7 months. The next stage of treatment was the transplantation of hematopoietic stem cells from a donor identical in terms of HLA (10/10), with a homozygous mutation in the HIV co-receptor CCR5 (CCR5Δ32/Δ32). Grade I graft-versus-host cutaneous disease was only observed within the first year after transplantation. AML recurrence was observed 332 days after transplantation. Before a second transplant of stem cells obtained from the same donor was performed, the patient underwent a single dose of whole-body irradiation. The patient was also treated with chemotherapy with an anti-CD33 monoclonal antibody conjugate (gemtuzumab ozogamicin). The second procedure led to remission of AML. In this patient the transplantation resulted in complete chimerism, and the patient's peripheral blood monocytes changed from heterozygous to homozygous genotype for the CCR5 delta32 allele. After just 159 days, no HIV-1 DNA could be detected in the rectal mucosa. During a 20-month ART-free follow-up, HIV-1 was undetectable in peripheral blood, bone marrow, and rectal mucosa as assessed by PCR tests.[24] 3 years after HSCT, while the patient was still off antiviral drugs, no evidence of viral replication was shown. The patient's CD4 cell count increased to over 800/μL and all hematopoietic stem cell-derived cells tested, including intestinal macrophages, became CCR5-negative.[6] The patient did not use ART and remained HIV-free until his death of a recurrence of AML in 2020.[8,25]

The second patient in whom the virus rebound was not observed after treatment for a hematological malignancy was a person known as the "London Patient".[26] Infection with the virus was detected in 2003, and ART treatment was started in 2012. Also in 2012, the patient was diagnosed with Hodgkin's lymphoma Stage IVB (nodular sclerosing). The patient received first-line chemotherapy and was given alemtuzumab (anti-CD52 antibody) before the transplantation in order to destroy T lymphocytes. The donor of hematopoietic stem cells was HLA 9/10 and CCR5Δ32 homozygous person. Full donor chimerism was obtained in fractions of all leukocytes and CD3+ T cells as early as day 30 post-transplantation and persisted in both cell fractions throughout. The host genotype was homozygous wild-type CCR5 before allogeneic HSCT and became CCR5Δ32/Δ32 after transplantation, with loss of CCR5 surface expression from circulating CD4 and CD8 T cells. On day 77 post-transplant, the patient developed fever and gastrointestinal symptoms. These were symptoms of GvHD grade I. However, they resolved without any intervention. Weekly analyses of plasma viral load were performed for the first three months and monthly thereafter. The plasma HIV-1 RNA remained undetectable with a detection limit of less than one RNA copy per ml. HIV-1 DNA associated with complete peripheral blood mononuclear cells also fell below the limit of detection. According to the treatment protocol, ART was interrupted 16 months after transplantation. Total DNA in CD4 T cells at posttransplant day 876 was undetectable in all replicates by ultrasensitive qPCR.[27]

The last HIV-free patient to date to stop taking ART after allo-HSCT therapy is the 'Düsseldorf patient'. The man, who was diagnosed with HIV-1 in 2008 and started ART in 2010, is now 53 and has not used treatment since 2018. In 2011, he was diagnosed with AML, which went into complete remission as a result of chemotherapy. However, after AML relapsed, the search for a donor began. A systematic search identified a 10/10 HLA-matched stem cell donor with a homozygous CCR5Δ32 mutation. After chemotherapy with antithymocyte globulin conjugate, the first transplant was performed in February 2013. After another relapse of AML and 34 days after the second HSCT, full donor chimerism was established and maintained. Following donor lymphocyte infusions, the patient developed mild chronic GvHD of the eyes with bilateral keratoconjunctivitis sicca, which persists to this day. ART was continued throughout, and HIV-1 proviral DNA and HIV-1 RNA remained undetectable. Negative in vivo growth tests using two different humanized mouse models confirmed the absence of replication-competent virus in the samples tested. ART was discontinued in November 2018, 69 months after

HSCT. After discontinuation of ART, the patient had no clinical or laboratory evidence of acute retroviral syndrome. Plasma HIV-1 RNA was still undetectable 48 months after treatment discontinuation.[28]

Comparing these 3 cases, it can be seen that cell therapy with allogeneic hematopoietic stem cell transplantation from donors with the homozygous CCR5Δ32 mutation may prove to be a breakthrough discovery in the treatment of HIV infection. So far, this therapy has been used in each of the patients to treat hematological cancer. The "Berlin patient" and the "Düsseldorf patient" required 2 transplants to achieve full recovery, while the "London patient" achieved remission after the first HSCT. There are more differences between these patients, the most important of which are listed in Table 1.[6,24,26–29]

Table 1. Differences between Berlin, London and Düsseldorf patients*

	Berlin patient	London patient	Dusseldorf patient
HIV diagnosed	1997	2003	2008
Years of ART treatment (pre-transplantation)	4	4	3
Hematological malignancy	Acute myeloid leukemia	Hodgkin's lymphoma	Acute myeloid leukemia
CCR5 genotype pre-transplantation	Heterozygous for 32 bp deletion	wild-type	wild-type
Donor HLA match	10/10	9/10	10/10
Conditioning regimen	1 HSCT: FLAMSA, CTX, ATG, TBI 2 HSCT: Ara-C, GO, TBI	LACE, anti-CD52	Flu, Treo
First transplantation	2007	2016	2013
Number of transplantations	2	1	2
GVHD	Yes	Yes	Yes
ART interruption post transplantation	Day of transplantation	16 months	69 months
Viral remission	Over 12 years	6 years	Over 4 years

* FLAMSA – fludarabine, arabinofuranosil citidina and amsacrine; CTX – cyclophosphamide; ATG – antithymocyte globulin; TBI – total body irradiation; Ara-C – arabinofuranosil citidina; GO – gemtuzumab; LACE – lomustine, arabinofuranosil citidina, cyclophosphamide and etoposide; Flu – fludarabine; Treo – treosulfan

Disadvantages of the method

Unfortunately, despite the great potential of HSCT, the complete recovery of patients from HIV infection has many limitations. First of all, hematopoietic stem cell transplants are very risky because mortality is between 40 and 55%.[16] In addition, baseline immunosuppression in HIV patients significantly increases the risk of opportunistic infections after transplantation.[19] Another difficulty of the method is the low frequency of CCR5Δ32/Δ32 homozygotes in the general population. Furthermore, a donor who is homozygous must be histocompatibility match to the recipient.[16] A way to avoid problems with HLA matching may be a haplo-cord blood transplant. Recently, the first case of HIV-1 remission and possible cure has been reported in a woman who received a CCR5Δ32/Δ32 haplo-core transplant (cord blood cells combined with haploidentical stem cells from an adult) for the treatment of AML. After 18 months without antiretroviral therapy there was still no viral rebound, however, this is a new method that needs more research.[30] There is also the possibility that the virus uses the CXCR4 co-receptor, chances of which increase with the duration of the infection. This will result in the repopulation of the virus after transplantation.[11] Finally, the side effects of conditioning regimens and the high risk associated with the development of GvHD mean that currently this therapy is used only in patients with hematological malignancy.[26]

Conclusion

Widespread use of antiretroviral therapy has resulted in increased survival, quality and life expectancy for HIV-infected people. The result is a growing number of HIV-positive patients with malignant neoplasms, especially hematological ones. Hematopoietic stem cell transplantation from a donor who is homozygous for the CCR5Δ32 allele not only causes tumor remission but can also cause permanent viral remission and the associated resignation from ART therapy. Currently, three such transplants have been successfully performed in the world. In the first patient, known as the "Berlin patient", virus load was undetectable for 12 years until the patient's death. However, this method is associated with numerous complications such as high mortality, toxicity, the low incidence of the CCR5Δ32 allele and the small chance of finding an appropriate donor. Currently, the use of this method is associated with more side effects than the use of ART therapy. To reduce the risk of HSCT treatment failure research is underway on autologous transplants using genetically engineered cells. Gene therapy is currently based mainly on the elimination of CCR5 in CD4+ T Cells or HSCs, which may contribute to the natural resistance to HIV and the lack of rebound. More research is needed to reduce the risk of using HSCT and perhaps in the future be able to use this therapy in all people living with HIV.

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Author contributions

Conceptualization, A.B. and J.B.; Methodology, A.B.; Software, J.B. and M.B.; Validation, J.B., M.B., and N.D.;

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Conflicts of interest

The authors declare no conflict of interest.

Data availability

Not applicable.

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REVIEW PAPER

The gut microbiota in development and treatment of depression

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ABSTRACT

Introduction and aim. Nowadays, almost 4% of people in the world suffer from depressive disorders, and the forecasts of further increase in incidence are alarming. The disease is debilitating and can lead to suicide, and available treatments are still imperfect. The aim of the study was to review the literature and present the potential role of the gut microbiota in the development of depression and to consider the use of prebiotics and probiotics as one of the therapeutic options in this disease.

Material and methods. Review of articles published on PubMed since 2015.

Analysis of the literature. The available reports point to a relationship between disturbances in the composition of the intestinal flora and the development of depressive disorders. In addition, more and more studies indicate the benefits of the influence on mood and clinical improvement, observed when using psychobiotics as an adjuvant treatment of depression, as well as monotherapy.

Conclusion. Further research is needed in this area, especially in humans, to gain a deeper understanding of the role of the gut microbiota in depression and the promising use of psychobiotics for its treatment.

Keywords. depression, gut microbiota, microbiome-gut-brain axis, prebiotics, psychobiotics

Introduction

Around 3,8% of the human population worldwide currently suffer from depression, and this disease affects women by approximately 50% more than men.[1] Unfortunately, the problem is constantly increasing, and the most important and dangerous complication of this debilitating disease is the occurrence of suicides. Annually 700,000 people commit suicide in the world, and in the group of people 15–29 years old it is the 4th leading cause of death.[1] Currently, depression is the fourth most serious disease and according to forecasts by the World Health Organization, will be the most widespread disease entity by 2030.[2] The clinical picture of the disease mainly consists of low mood, anhedonia and cognitive disorders. Concentration problems may also occur, and sleep and eating are disturbed, which can disrupt the daily functioning of such a patient.[3] Despite the existence of many therapeutic options, the waiting time for the treatment effect is quite long, sometimes even up to 6 weeks. Moreover, recovery is not facilitated by the occurrence of side effects of these substances, which often forces clinicians to change treatment or add more medications to get the desired result.[4] It is estimated, that only half of the patients respond to treatment, and the diagnosis is made mainly on the basis of the symptoms reported by the patient, which means that the therapy is not specific to the pathophysiological mechanisms of depression.[5] Therefore, further progress is needed to better

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understand the pathophysiological mechanisms of this disease and to improve its treatment.[6] This is very important because there are a number of causative factors that can lead to depressive symptoms, including biological, genetic, environmental and psychological factors.[7] Increasingly, the influence of the state of the intestinal flora is considered as contributing to the development of this disease. Endogenous microbial intestinal flora, which consists of trillions of microorganisms, plays a key role in maintaining the correctness of physiological processes occurring in the human body.[8] Thanks to the presence of effective two-way communication between the intestines and the brain, based on numerous mechanisms, it is possible to mutually transfer the essential information between the aforementioned structures necessary for their proper functioning. This correlation is referred to as the microbiome-gut-brain axis (MGBA).[9] In recent years, researchers have shown increased interest in both the composition of this ecosystem and the potential associations of microbiome disorders with the occurrence of psychiatric or neurological diseases.[8] In addition, due to the search for new therapeutic options, attention is drawn to the effect of supplementation of probiotics, prebiotics and synbiotics on the clinical improvement of patients with depressive disorders.[10]

Aim

The aim of this review is to highlight the importance of the problem of depression and to present the role of the intestinal microbiota in its pathomechanism, and in addition to consider whether probiotics and prebiotics can be one of the therapeutic options for this disorder.

Material and methods

This review article focuses on on the role played by the intestinal microbiota in the pathomechanism of depression, and, on the other hand, consider the potential benefits using probiotics and prebiotics in terms of clinical improvement in people with depression, based on a selective review of PubMed publications. We have considered reviews and original papers since 2015 and WHO report.

Analysis of the literature

The microbiome-gut-brain axis

The mammalian gastrointestinal tract, including humans, is inhabited by nearly 1000-1500 species of bacteria. Together with various fungal and viral species, they constitute the gut microbiota, a diverse and variable community. This composition is influenced by factors such as diet, age, lifestyle, and inflammatory status.[11] A stable and diverse microbiota plays a crucial role in regulating metabolic and immune processes within the human body.[12] In recent years, scientists have discovered a bidirectional communication between the gut microbiota and the brain, termed the gut microbiota-brain axis. It means that the state of the gut microbiota influences the behavior and function of the brain, and the brain, through its many neural properties, influences gut function.[13] The specific mechanisms and connections within the gut microbiota-brain axis are very complex and not yet fully elucidated, and further research is needed to unravel the complexities of these interactions and correlations.[13,14] There are reports that these two areas of our body are connected by several ways, including nervous, hormonal, metabolic and immunological, including mainly the autonomic nervous system (ANS), the enteric nervous system (ENS), vagus nerve, the hypothalamic-pituitary-adrenal axis (HPA), the sympatho-adrenal system, and descending monoaminergic pathways.[15,16] The extensive connections between the nervous and gastrointestinal systems explain the potential presence of depressive and anxiety disorders in individuals with abnormal gut microbiota composition.[17] Changes in the gut microbiota composition can also impact the nervous system through metabolic pathways. Microorganisms in our intestines are capable of producing neurotransmitters such as dopamine, GABA, noradrenaline, short-chain fatty acids (SCFAs) and serotonin that affect the proper functioning of the brain and mood.[15,18-20] Neurotransmitters that are produced in abnormal amounts by the intestinal microflora can affect the development of neurological and depressive disorders.[19,20] For example, certain bacterial strains such as *Escherichia* spp. and *Lactobacillus* spp. can synthesize gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter in the central nervous system.[18] Neurotransmitters such as kynurenine and serotonin also play a key role in the pathogenesis of depressive disorders. They are synthesized in the process of tryptophan metabolism, so a decrease in their level is most often associated with a decrease in the supply of this amino acid because it is obtained only from diet. Studies have shown that a diet increasing tryptophan levels can result in fewer depressive symptoms, while a diet low in this amino acid caused irritability and anxiety.[21] By also assessing the 3HKYN:KYN ratio, the level of destabilization can be estimated. This coefficient reflects the degree of conversion of kynurenines via the kynurenine pathway. Disturbances in kynurenine metabolism are reflected in a wide range of somatic and psychiatric diseases.[22] In addition, the vagus nerve plays a crucial role in maintaining the continuity of proper MGBA communication. Microorganisms regulate the activation of the vagus nerve, and then the signal is transmitted to the CNS, enabling healthy mental functioning. It is therefore assumed that disturbances in the composition of the intestinal ecosystem can affect the information transmitted to the brain via the vagus nerve, and

in consequence causing the symptoms of depression.[23] Each change in the composition of the intestinal microbiome causes the production of lipopolysaccharides (LPS) by microorganisms, which results in the activation of inflammatory reactions. The cytokines produced send signals to the vagus nerve, thus connecting to the HPA axis. There are reports suggesting that cutting the nerve X caused the disappearance of the influence of the microbiome on the behavior of animals tested for anxiety and depression symptoms.[24] The hormonal regulation of MGBA is mainly based on the HPA axis pathway. The intestinal microflora regulates the proper functioning of the HPA, for which it is responsible already in the first years of life. Numerous reports have shown that excessive activity in the HPA area is important for the development of depression, e.g. through excessive activity of adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH) and cortisol, which disturbs appropriate the body's response to psychological stress.[25] There are also reports suggesting a potential influence of the gut microbiota through Toll-like receptors 2 and 4 (TLR2 and TLR4).[16] Disruptions in the gut microbiota composition can compromise the integrity of the gut barrier, leading to increased permeability. This can result in the translocation of bacterial metabolites to mesenteric lymphoid tissues, potentially contributing to neurological disorders.[26] A prominent component of the gut microbiota, LPS, is recognized by Toll-like receptors mainly located on microglial cells. This activation leads to the release of pro-inflammatory cytokines, contributing to the development of neurological disorders.[27] These and many other mechanisms, not fully understood, contribute to the bidirectional relationship between the gastrointestinal and nervous systems known as the gut microbiota-brain axis. Changes in the composition of gut microbiota can lead to alterations in immune regulation and metabolic changes that influence the nervous system. Imbalance in the MGBA contributes to the development of many diseases, particularly depression and anxiety disorders.[28]

The potential impact of gut microbiota in the development of depression

In recent years, numerous studies have been conducted comparing the gut microbiota composition of individuals with depression and anxiety disorders to control groups. A consistent trend has been observed, showing an increase in the abundance of bacteria from the *Bacteroidetes, Protobacteria*, and *Actinobacteria genera*, as well as a decrease in the abundance of bacteria from the *Firmicutes genus* in patients with depressive and anxiety disorders. Additionally, reduced levels of bacteria from the *Faecalibacterium*, *Eubacterium rectale, Lachnospira, Butyricicoccus,* and *Sutterella* genera in individuals with depressive and anxiety disorders result in decreased production of short-chain fatty acids (SCFA).[29] Consequently, this leads to disruption of the gut barrier and dysregulation of the immune system, which impacts brain dysfunction.[29,30] Fecal samples of Polish women were analyzed for the presence of SCFA in order to investigate the role of this factor as a potential cause of women's mental health disorders. In the group of 11 patients who did not show depressive symptoms, higher concentrations of almost all short-chain fatty acids were observed compared to the group of patients who were diagnosed with worsening depression using the Beck Depression Inventory (BDI). Moreover, in the group of women who showed depressive symptoms, increased levels of isocaproic acid were observed, which may contribute to the development of these symptoms.[31] In many other studies comparing the gut microbiota composition of individuals with depression and anxiety disorders, a decreased level of bacteria from the *Prevotella* genus has been demonstrated compared to the control group.[32-35] Individuals with depression and anxiety disorders have also been observed to have a lower abundance of bacteria from the *Faecalibacterium* and *Sutterella genera*.[34] Furthermore, the presence of bacteria from the *Eggerthella* genus has been positively correlated with the occurrence of depressive and anxiety disorders.[33] Patients suffering from depression are often accompanied by intestinal disorders such as vomiting, abdominal pain, constipation, nausea, bloating or even irritable bowel syndrome (IBS).[36] It has been proven that the presence of disturbances in the composition of the intestinal microbiota may affect mood disorders. The meta-analysis showed that patients suffering from major depressive disorder (MDD) in the composition of their intestinal microbiota have less bacteria from the families: *Veillonellaceae*, *Prevotellaceae* and *Sutterellaceae* and less bacteria from the genus: *Coprococcus*, *Faecalibacterium*, *Ruminococcus*, *Bifidobacterium* and *Escherichia* compared to healthy subjects. In patients with depressive disorders there was an increased level of bacteria of the genus *Paraprevotella.*[37] Other scientific work has shown that the following are involved in the pathogenesis of depression: increased number of *Enterobacteriaceae* and *Alistipes* bacteria and decreased number of *Faecalibacterium*. What's more, anxiety and depression were accompanied by the presence of bacteria from the families: *Ruminococcaceae*, *Shewanellaceae*, *Halomonadaceae* and *Verrucomicrobiae*. A clinical study found elevated numbers of *Bacteroidetes* and *Proteobacteria* in MDD patients and decreased numbers of the *Lachnospiraceae* and *Ruminococcaceae* families.[36] Studies conducted on rodents have proven that animals exhibiting depressive behavior had a higher ratio of *Bacteroides* to *Firmicutes* bacteria in their gut microbiota. An experiment with mice exposed to chronic stress showed an increased population of *Clostridium* and a decreased

population of *Bacteroides* in their gut microbiota. Observing these relationships prompted scientists to look for biological relationships between intestinal dysbiosis and the occurrence of depression. It has been proven that changes in the intestinal microflora are associated with a change in the permeability of the intestinal barrier and with chronic inflammation. Modifications in the permeability of the intestinal barrier occur by reducing the number of strict proteins: claudin-5 and occludin. This is associated with changes in the secretion of intestinal peptides that have the ability to contact the central nervous system, taking a part in the gut-brain axis.[37] Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is also associated with leaky gut. HPA stimulates the immune system and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS can damage structures in cell membranes, causing leaky gut and chronic inflammation. Factors that activate the HPA axis include LPS, secreted by Gram-negative bacteria, and peptidoglycan, a component of the cell wall of most bacteria. Hyperactivation of HPA increases the secretion of cortisol, the high level of which can cause depression. Homocysteine is an amino acid synthesized from the breakdown of folic acid, vitamin B6 and vitamin B12. Scientific studies have shown that increased levels of homocysteine are associated with depression. Hyperhomocysteinemia disrupts the epithelial barrier and causes increased intestinal permeability, which causes inflammation. It has been proven that the bacteria that are the main producers of this amino acid are: *Subdoligranulum* sp., *Eubacterium* sp. and *Clostridiales* family XIII.[36] Gram-negative bacteria present in the intestines secrete endotoxin-lipopolysaccharide (LPS), which triggers the production of post-inflammatory cytokines such as: IL-6, IL-1β and TNF-α, contributing to the abnormal secretion of intestinal peptides and thus the pathological functioning of the intestinal-gut axis cerebral. Increased amount of the pro-inflammatory enzyme indoleamine 2,3-dioxygenase, secreted e.g. as a result of the action of LPS, reduces the level of serotonin, the deficiency of which is crucial in the development of depression.[36,37] In addition, intestinal dysbiosis triggers the loss of goblet cells, which results in a reduction in mucus secretion and thinning of its' layer. This may be the reason why toxic metabolites move into the bloodstream and cause inflammation.[36] Yuan et al. conducted an analysis of gut microbiota in 240 study participants, among whom 129 were suffering from active colitis ulcerosa (CU). In patients with coexisting depression and CU, a lower diversity of gut microbiota was observed compared to patients with UC without depression. Furthermore, individuals with both CU and depression exhibited a microbiota composition characterized by a reduced abundance of bacteria from the *Prevotella and Lachnospira* genus, along with an increased overgrowth of bacteria from the *Lactobacillales, Sellimonas, Streptococcus*, and *Enterococcus genera*, as compared to those with CU but without depressive disorders.[38] Depression and dementia are the most common diseases diagnosed in the elderly. Malnutrition is a massive problem in the geriatric population, which may be a cause of depression and may also exacerbate its symptoms. Depressive disorders in the elderly are associated with weakness, worse outcomes of chronic diseases and even higher mortality. A review of scientific articles proved that an adequate supply of nutrients has a positive effect on the occurrence of depression in this age group. Omega-3 fatty acid supplementation reduces oxidative stress, which is associated with depressive symptoms. In addition, fasting has a positive effect on the brain availability of serotonin, the deficiency of which is closely related to the disease. It has been proven that a low-calorie diet with the preservation of protein, minerals, water and vitamins also has a positive effect on well-being.[39] Depressive disorders are diagnosed twice as often in women. This is due to fluctuations in estradiol levels. Physiological decline in the level of this hormone occurs after menopause and after childbirth. Pathological causes include ovarian failure, hyperprolactinemia, congenital adrenal hyperplasia, and polycystic ovary syndrome. A scientific study aimed to detect microbes that break down estradiol in the intestines of women with depression in premenopausal age. Initially, estradiol was incubated with intestinal microbes taken from women suffering from depression and from healthy patients. It was found that this hormone decomposes within 120 minutes in 77.8% of women suffering from depression and in 19.3% of women without depression. Then it was established that the bacterium that degrades estradiol is *Klebsiella aerogenes* by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). Other bacteria that were detected in patients with the 3β-HSD enzyme were: *Bacteroides thetaiotaomicron* and *Clostridia*.[40] Diet also plays an important role in the proper functioning of the intestinal microbiota. It has been observed that individuals following a healthy and balanced diet are less prone to depression. Research conducted on the impact of diet on gut microbiota composition indicates that a low-fiber diet rich in saturated fats reduces the levels of bacteria from the Lactobacilli genus. On the other hand, the mediterranean diet reduces the number of pathogenic bacteria such as Escherichia coli, and increases the abundance of key commensal bacteria like *Bifidobacteria*, *Clostridium cluster* XVIa and *Faecalibacterium prausnitzii*.[9]

The use of probiotics and prebiotics as a potential therapeutic option for depression

Increasingly, the modification of the intestinal microbiota with the use of supplementation with probiotic

or prebiotic substances is being considered. Over the last decade, the concept of psychobiotics has appeared, which is defined as probiotic bacteria that, when used in appropriate doses, have an effect on the function of the intestinal microbiota and MGBA, contributing to the improvement of mental health.[24] Potential properties are attributed mainly to substances containing strains of bacteria from the Lactobacillus, Bifidobacterium, Streptococcus and Enterococcus groups. When it comes to prebiotics, these are food elements that have not been digested and thus contribute to the stimulation of growth and activity of bacteria in our intestines.[41] Eight-week study conducted on a group of 110 patients, of whom 81 with abnormal depressive symptoms, who were randomly assigned to the one of groups using prebiotic, probiotic or placebo. The therapeutic effect was compared by patients‘ subjective assessment of the severity of the effect using the BDI. It was observed that, compared to the use of placebo,benefits were incurred in connection with the use of a probiotic (*Lactobacillus helveticus and Bifidobacterium longum*), and in the case of a prebiotic (galactooligosaccharide) no effect on improving the well-being of the patients.[42] A post hoc analysis showed an improvement in the antidepressant response in depressed subjects who took the probiotic substance *L. helveticus* R0052 and *B. longum* R0175, compared to the groups using placebo and prebiotic. In addition, in this study assessed the level of neurotrophin, the deficiency of which may disturb the physiological processes of the central nervous system, and, according to recent reports, also contribute to the occurrence of mood disorders and symptoms of depression. There was a significant increase in BDNF due to the probiotic, and interestingly, a slight decrease in BDNF was observed in the prebiotic group. Further research is recommended to examine the impact of the use of psychobiotics and prebiotics on the level of BDNF and the correlation of this factor with the severity of depressive symptoms.[43] In another study, the effectiveness of the use of a probiotic containing Clostridium butyricum MIYAIRI 588 was tested and clinical improvement was demonstrated in 70% of the subjects, where the probiotic was used as a supportive treatment in therapy, without observing any disturbing side effects.[44] The use of probiotics as adjuvant treatment was also evaluated by Rudzki et al., who in a randomized study assigned patients to two groups, the first received a probiotic containing *Lactobacillus plantarum* 299v (LP299v) and an SSRI for eight weeks, and the second group received an SSRI and placebo. On a weekly basis, the severity of the symptoms of the disease and cognitive processes were assessed using the available depression scales, and what is more, biochemical tests were carried out, taking into account, among others, the level of tryptophan and kynurenine. In the group of people using a probiotic and antidepressant treatment, a significant improvement in cognitive processes was noticed, and what is more, a decrease in the concentration of kynurenine, i.e. a substance that under physiological conditions has a neuroprotective effect, was observed, and its high concentrations determine neurodegenerative and neurotoxic processes.[45] Another study also showed a positive effect of the use of probiotics in the treatment of depressive disorders. Patients were treated once with *Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium bifidum*. Patients suffering from depression and receiving probiotics achieved an improvement in their health condition (assessment according to the BDI questionnaire) compared to patients who received placebo.[46] In recent years, attempts have also been made to treat major depressive disorders using *Lactobacillus casei* strain Shirota (LcS). In a study involving patients with major depressive disorders, a decrease in the severity of depression symptoms based on the Hamilton Depression Rating Scale was observed after using the above-mentioned probiotic. These changes correlated with a change in the composition of the intestinal microbiota, which is crucial in the treatment of depressive disorders.[47] There is a need to conduct further studies to confirm the effectiveness of using probiotics in combination with antidepressants in patients with depression.[44-47] The use of a probiotic as the only therapeutic option for depression was considered by Wallace and Milev and conducted an open-label pilot study on a group of patients with major depressive disorder (MDD) who had not previously received treatment for major depressive disorder. 70% of the respondents were women. During the 8-week study period, patients took a probiotic once a day consisting of *L. helveticus* R0052 (90%) and *B. longum* R0175 (10%). Available clinical scales and self-report questionnaires were used to assess the effectiveness of treatment. Significant improvement in the area of symptoms was observed in the patients, as well as a reduction in the sense of anxiety after both 4 and 8 weeks, and in addition, the subjectively assessed quality of sleep significantly improved in the 8th week of the study. The use of the above probiotic was not associated with side effects and was well tolerated by patients. However, due to the fact that the study was conducted on a relatively small group of people, there is a need to conduct randomized, comprehensive studies on a larger number of people in order to justify the effectiveness of using probiotic substances as monotherapy in the treatment of depressive disorders.[48] The effectiveness of probiotic use was assessed in patients with various the composition of the microbiotawith various severity of depressive disorders. A placebo control was applied over an 8-week period, and compared the symptoms and composition of the ecosystem present in the intestines with a group of people who did not suffer from depression. It was noted that

Table 1. Clinical evaluation of probiotics on depression*

Strains	Population characteristics	Intervention	Duration	Clinical findings	Use of antidepressants	References
L. casei strain Shirota (LcS)	15 patients with MDD and 3 patients with BD	160 mL fermented milk containing at least 8.0×10^{10} CFU of LcS per day	12 weeks	↓HAMD - 17 0 weeks 17.7 ± 4.1, 12 weeks 10.9 ± 7.3) ↑ Bifidobacterium, Actinobacteria phylum	yes	Otaka et al., 2021.[47]
L. acidophilus , *L. casei, B. bifidum*	40 patients with MDD Probiotics (n=20) Placebo (n=20)	Oral capsule contained 2×10^9 CFU/g *L. acidophilus*, 2×10^9 CFU/g *L. casei*, 2×10^9 CFU/g *B. bifidum* per day	8 weeks	↓ BDI Probiotics(-5.7±6.4) placebo (-1.5±4.8)	yes	Akkasheh et al., 2015.[46]
Probiotics: *L. helveticus, B. longum*, Prebiotics: galactooligosaccharide	81 patients with MDD Probiotics (n=28) Prebiotics (n=27) Placebo (n=26)	Probiotics: 10 x 10^9 CFU per 5g sachet/day Prebiotics: galactooligosaccharide sachet/day	8 weeks	↓ BDI (probiotics 17.39-9.1), prebiotics (19.72-14.14), (placebo 18.18-15.55) ↓ kynureine/tryptophan ↑ tryptophan/isoleucine	yes	Kazemi et al., 2018.[42]
L. helveticus R0052, *B. longum* R0175 Prebiotics: galactalogosaccharide	78 patients with MDD Probiotic (n=28) Prebiotic (n=25) Placebo, (n=25)	Probiotics: ≥ 10×10^9 CFU *L. helveticus* R0052 and *B. longum* R0175 per 5 g sachet/day Prebiotics: galactalogosaccharide sachet	8 weeks	↑ BDNF (the biggest increase in probiotic group) ↓ BDI (probiotics 17.6 -9.8), (prebiotics 19.7- 14.1), (placebo 18.2-15.9)	yes	Heidarzadeh-Rad et al., 2020.[43]
C. butyricum MIYAIRI 588 (CBM588)	40 patients with MDD Probiotics (n=20) Placebo (n=20)	60 mg/d of *C. butyricum* CBM588 oraly per day	8 weeks	↓ HAMD-17, BDI, BAI (Probiotics: decrease 50% or greater)	yes	Miyaoka et al., 2018.[44]
L. plantarum 299v (LP299v)	60 patients with MDD Probiotics (n=30) Placebo (n=30)	2 capsules per day (each capsule contained 10x10^9CFU of L. Plantarum 299v)	8 weeks	↓ HAMD-17, SCL-90 and PSS-10 ↓ KYN (Probiotics 2,05-1,82), (Placebo 2,17-2,32) ↑ 3HKYN:KYN (Probiotics 15.88-27.68), (placebo 17.82 -15.26)	yes	Rudzki et al., 2019.[45]
L. helveticus R0052, *B. longum* R0175	10 patients with MDD	3×10^9 CFU of *L. helveticus* R0052 and *B. longum* R0175 per day	8 weeks	↓ MADRS (24.9 - 12.7), QIDS-SR16 (20.5 - 11.6), SHAPS (36.8-30.7)	no	Wallace et al., 2021.[48]
B. bifidum W23, *B. lactis* W51, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *Lactococcus lactis* W19 and *L. lactis* W58	71 patients with depression syndromes Probiotics (n=34) Placebo (n=37)	Ecologic®Barrier 2.5×10^9 CFU/g *B. bifidum* W23, *B. lactis* W51, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *L. lactis* W19 and *L. lactis* W58 2g per day	8 weeks	↓ BDI (Probiotics 28.91- 19.88) (placebo 27.97- 19.25)	yes	Chahwan et al., 2019.[49]
B. bifidum W23, *B. lactis* W51, *B. lactis* W52, *L. acidophilus* W22, *L. casei* W56, *L. paracasei* W20, *L. plantarum* W62, *L. salivarius* W24, *L. lactis* W19	61 patients with depression syndoms Probiotics (n=28) Placebo (n=33)	3g Omnibiotic Stress Repair® 7,5 x 109 CFU of *B. bifidum* W23, *B. lactis* W51, *B. lactis* W52, L. acidophilus W22, *L. casei* W56, *L. paracasei* W20, *L. plantarum* W62, *L. salivarius* W24 and *L. lactis* W19	28 days	↑ *Ruminococcus gauvreauii*, *Coprococcus* 3 (Probiotics group)	yes	Reininghaus et al., 2020.[50]

* MDD – major depressive disorder, HAMD–17 – Hamilton Depression Rating Scale, BDI – Beck Depression Inventory, BAI – Beck Anxiety Inventory, BDNF – brain–derived neurotrophic factor, SCL–90 – Symptom Checklist; PSS–10 – Perceived Stress Scale, KYN – kynureine 3HKYN:KYN – 3–hydroxykynurenine: kynureine, MADRS – Montgomery-Åsberg Depression Rating Scale,QIDS–SR16 – Quick Inventory of Depressive Symptomatology, SHAPS – Snaith–Hamilton Pleasure Scale

the use of probiotic substances – Ecologic® Barrier significantly improved cognitive functions compared to patients using placebo, but changes in microbiota were not so marked.[49] In people hospitalized with severe depressive episodes, there was an increase in the number of *Ruminococcus gauvreauii* and *Coprococcus* 3, which are perceived as potentially beneficial bacteria for the functioning of the human body, after treatment with probiotic with biotin compared to the control group that received biotin with biotin. The study confirms that the use of probiotic substances contributes to beneficial changes in the composition of the intestinal microbiota, already in the first weeks of treatment, in patients with major depressive disorders. There are reports that suggest that probiotics are effective in terms of balancing the composition of this ecosystem which prompts further considerations.[50] Much less research assesses the effect of prebiotics on the severity of depressive symptoms, and current reports suggest a rather small effect in the treatment of these disorders. However, there is a need to increase the research base that focuses on prebiotics in this aspect.[51] The results of studies on the use of probiotics in the treatment of depression are summarized in detail in Table 1. below, taking into account the

duration of the study, the composition and dose of probiotics used, changes in parameters assessing the therapeutic effect, characteristics of the study population and whether the patients were undergoing antidepressant treatment at the same time.

Depression is a disease entity that often poses a great diagnostic challenge among specialists, especially in the first phase of the disease. Disturbing forecasts regarding the increase in incidence, which may also result in an increase in the number of suicides as a result of it, indicate the need for a deeper understanding of many poorly understood mechanisms that can cause depression.[1-3] Recently, the interest of researchers has focused on the importance of the brain-gut microbiota axis, e.g. in terms of interactions on proper functioning through numerous mechanisms, including neuronal, hormonal or immunological pathways. Dysfunction within the composition of the intestinal ecosystem may contribute to a negative impact on the brain, which in turn may contribute to mood disorders and the appearance of a depressive episode.[25] Existing reports suggesting the influence of the microbiota on the regulation of brain functions as well as its impact on behavior and mood encourage further research that will go even deeper into determining the qualitative and quantitative changes in the microbiome in depressed patients as opposed to healthy people.[15] In patients with depressive disorders, changes in the composition of the intestinal microbiota were demonstrated, manifested by an increase in the population of *Bacteroidetes, Protobacteria, Enterobacteriaceae, Alistipes and Actinobacteria*, and a decrease in the share of strains of the genus *Firmicutes, Faecalibacterium, Eubacterium rectale, Lachnospira*, *Butyricicoccus, Prevotella and Sutterella*.[29-37] The observation of changes in the intestinal microbiota in patients with depressive disorders prompts further observations and expanding the existing knowledge on this subject. Therefore, attempts are made to modify this composition so as to alleviate depressive symptoms, which is why further research conducted with the use of psychobiotic supplementation is crucial to be able to use these substances as one of the therapeutic options or for supportive treatment with antidepressants.[42-50] In addition, it is worth paying more attention to prebiotics and testing them in terms of treating depression, because the current literature presents very few reports on this subject.[51] Further research and reports in this area will help to better understand the role of intestinal microbiota in the pathomechanism of depression, and on the other hand also its beneficial effect in the treatment of these disorders.

Conclusion

Disturbances in the composition of the intestinal microbiota may predispose to depression, but further research is needed in this area, especially with the participation of humans, which will allow for a deeper understanding of the role of this causative factor in the pathophysiology of depression. In addition, the use of an appropriate, well-balanced diet as well as the supplementation of probiotic and prebiotic substances, which can have a positive effect on balancing the composition of the intestinal microbiota and contribute to the clinical improvement of patients with depression. It is therefore encouraged to continue further research, as modifying the composition of the ecosystem in our intestines may be a potentially promising therapeutic option for a disease that is a threat to civilization, i.e. depression.

Declarations

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Author contributions

Conceptualization, N.W., W.S. and A.B.; Validation, N.W., W.S., A.B. and J.B.; Resources, A.B., J.B. and B.S.; Data Curation, N.W., B.S. and J.B.; Writing – Original Draft Preparation, N.W., W.S., A.B., J.B. and B.S.; Writing – Review & Editing, N.W., W.S., J.B., A.B. and B.S.; Supervision, H.P.C.; Project Administration, N.W. and H.P.C.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Not applicable.

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REVIEW PAPER

Exploring the versatility of ciclopirox – from anti-fungal to anticancer agent and beyond

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ABSTRACT

Introduction and aim. Ciclopirox has been treating fungal infections for decades. Recent studies suggest ciclopirox may be repurposed to treat cancer, viral infections, and neurological disorders. Ciclopirox exerts anticancer by inhibiting multiple pathways of cancer cell growth and survival and anti-viral actions by reducing viral replication and altering the host immunological response to viral infection. Recent research suggests that ciclopirox may protect against neurodegenerative illnesses including Alzheimer's and Parkinson's. This narrative review shows ciclopirox's potential to treat cancer, viral infections, and neurological diseases.

Material and methods. Current relevant research publications focused on ciclopirox and its repurposing medicinal potential, therefore a well-designed technique was used to find them. „Ciclopirox", „Anti-fungal", „Anti-cancer", „Repurposing", and „Therapeutic potential" were used to search PubMed, Web of Science, EMBASE, and Google Scholar.

Analysis of literature. Ciclopirox may reduce oxidative stress and inflammation, which may cause several illnesses. Overall, the repurposing of ciclopirox for the treatment of cancer, viral infections, and neurodegenerative disorders represents a promising avenue of research that warrants further investigation.

Conclusion. It was concluded that CPX and olamine derivatives as outstanding antifungal medications, as well as provide information on ongoing research to use them for other illnesses.

Keywords. AIDS, anti-fungal, cancer, cardiovascular diseases, ciclopirox

The list of abbreviations:

CPX – ciclopirox, **CPO** – ciclopirox olamine, **CPX-POM** – fosciclopirox, **HPCH** – hydroxypropyl chitosan, **VVC** – vulvovaginal candidiasis, **BBB** – blood–brain barrier, **AKT** – protein kinase B, PKB/AKT, **GSK3** – glycogen synthase kinase 3, **RR** – ribonucleotide reductase, **AML** – acute myeloid leukaemia, **DOHH** – deoxyhypusine hydroxylase, **HIF–1α** – hypoxia inducible factor–1α, **eIF5A** – eukaryotic translation initiation factor 5A

Introduction

In the current period of serious dermatological disorders, there is a demand for effective medications that can treat a variety of conditions. Ciclopirox (CPX) is

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a promising therapy choice for a number of dermatological conditions due to its broad-spectrum antifungal activity, capacity to target various stages of the fungal life cycle, and minimal toxicity. CPX is a unique formulation with an array of uses (Table 1 and Fig. 1). While it is primarily used as an antifungal drug, it can also be utilized as an anti-inflammatory medication. This antifungal drug unlike others belongs neither to the azole nor the imidazole class of chemical agents but is uniquely from the hydropyridone class, one which has been proven incredible to be incredibly effective against a wide spectrum of dermatological disorders. These include dermatophytes and those caused by microorganisms including yeast, fungi, bacteria, etc.[1]

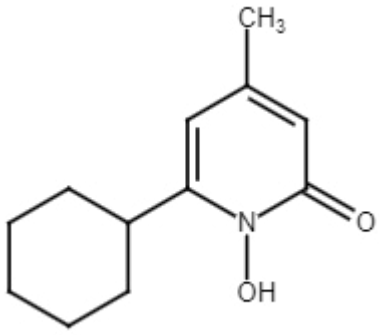

Fig. 1. Chemical structure of ciclopirox

Table 1. Chemical properties and names of ciclopirox

IUPAC	6-cyclohexyl-1-hydroxy-4-methyl pyridin-2-one
Generic	Ciclopirox
Synonyms	Batrafen, ciclopiroxolamine, cyclopirox, ciclopiroxium, HOE-296, Loprox, Penlac.
Molecular weight	207.27
Molecular formula	$C_{12}H_{17}NO_2$
Half-life	1.7 hours
Melting point	143°C-144°C

CPX antifungal topical drug therapy has a unique and different mechanism of action. This drug acts by penetrating the nails and other sites if administration and counters the ill effects of infection like brittle nails, scaly patches and many more by acting on the infection causing microbes. In minimal concentration, CPX acts by blocking the transportation of amino acids across the cell membrane.[2] Studies that have been undertaken on CPX prove that its sole use may not be restricted to its isolated antifungal action only but it can act for a variety of other purposes too. One of the many involve its action as an antibacterial. It's antibacterial action includes targeting both gram-positive (*Staphylococcus aureus, Enterococcus faecalis*) as well as gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae*).[3] Another of its many functions is as an HIV-1 inhibitor.[4] In this inhibitory action, the novel drug is believed to inhibit deoxyhypusine hydrolyse an inhibitor of the cell cycle through inhibition of DNA polymerase alpha. Research has also proved that CPX besides the above-mentioned uses, can also be efficacious in treating malignant leukaemia and a variety of cancers. These include myeloma, colorectal cancers and various types of tumours. CPX, a unique formulation of hydroxypirdone is an antifungal therapy that has been used for over three decades for various skin-related purposes as well as a nail lacquer. This Distinctive cream-based antifungal therapy gained USFDA approval in June 2004 and has been used Ever since for an array of functions.[5] CPX has been developed using unique drug innovation technology, which is rational drug designing. Researchers identified a specific enzyme (squalene epoxidase) that is essential for the growth of fungi and yeast. They then used this knowledge to design a molecule (CPX) that would bind to the enzyme and inhibit its function, thereby stopping the growth of the fungi. Onychomycosis is a typical infectious disease caused by fungi and may be incredibly painful and uncomfortable. Barring degradation of the patient's quality of life it may also cause immense physical impairment. CPX formulations are also used for the treatment and management of the disease.[6] Generally, an 8 % CPX hydroxypropyl chitosan (HPCH) formulation is considered most suitable and is used to treat mild to moderate fungal infections.[7]

The current scenario of dermatophytes is extensively alarming and calls for careful attention. Innumerable studies have been undertaken to look into the same and management still seems to be a challenging aspect to deal with. Whilst studies on different topical antifungals was being considered it was majorly concluded that CPX is not only a safe and efficacious option for antifungal therapy but also proves to be useful to patients who do not respond to other antifungal. Therefore, the considerations of this versatile antifungal in dermatological diseases is proving to be highly beneficial.[8] Furthermore, beyond its established use as an antifungal agent, CPX has shown potential in a number of different fields. Its possibility for use in the treatment of autoimmune diseases, cancer, and neurodegenerative disorders is currently being studied in researches that are ongoing.

Aim

It is a narrative review that reveals the repurposing potential of ciclopirox to cure cancer, viral infections, and neurodegenerative disorders.

Material and methods

Current relevant research publications focused on ciclopirox and its repurposing medicinal potential, therefore a well-designed technique was used to find them. „Ciclopirox", „Anti-fungal", „Anti-cancer", „Repurpos-

ing", and „Therapeutic potential" were used to search PubMed, Web of Science, EMBASE, and Google Scholar. The search method included applicable keywords, The search phrases, which were adapted to the study's specific goals, included „Ciclopirox," „Anti-fungal," „Anti-cancer," „Repurposing," and „Therapeutic potential," among other terms. After the initial search, retrieved articles were screened based on predetermined inclusion and exclusion criteria. The inclusion criteria encompassed includes up-to-date and pertinent information sources that are mostly vetted based on articles written during the last five years, Articles that helped provide information on drug repurposing based on clinical and preclinical trials conducted on ciclopirox were primarily screened using the titles of the subsections to be covered from research websites. Relevant and reliable information sources from reputable Journals were also used while the exclusion criteria were designed to exclude articles that did not meet the specific research focus.

Analysis of literature

History of ciclopirox

The German pharmaceutical company Merz Pharmaceuticals GmbH first developed the drug in the 1970s (Fig. 2). The development of CPX began with the screening of a large number of compounds for their antifungal properties. One of the compounds, named CPX-772, showed promising activity against a variety of fungi.[9] Further studies showed that CPX-772 had a unique mechanism of action, disrupting the function of the fungal cell membrane.[10] In 1985, Merz Pharmaceuticals obtained a patent for the use of CPX as an antifungal agent. The drug was subsequently approved for use in Europe and Japan in 1987, and in the United States in 1990. CPX is now available as a topical cream, lotion, or nail lacquer for the treatment of various fungal infections, including onychomycosis (fungal nail infection), pityriasis versicolor (a fungal infection of the skin), and seborrheic dermatitis (a skin condition characterized by red, scaly patches).[10,11] CPX has also been studied for its potential use in the treatment of other conditions, such as cancer and Alzheimer's disease. Some studies have shown that CPX may have anti-tumor and neuroprotective effects, although more research is needed in these areas.[11] CPX is generally well-tolerated, with mild and transient side effects such as burning or itching at the site of application. However, in rare cases, more severe allergic reactions or skin irritation may occur. Today, CPX is manufactured and marketed by several pharmaceutical companies around the world, and is widely used as a safe and effective treatment for fungal infections of the skin and nails.[12]

Structure activity relationship of ciclopirox

CPX is called 1-hydroxypyridin-2(1H), which has methyl and cyclohexyl groups at meta and para respectively as substitutes for hydrogen (Fig. 3). It is a cyclic hydroxamic acid.[13] The sixth position is set aside for a lipophilic substitution, which allows you to swap out the hexyl ring with another type of ring, such as phenol. The medicine becomes less oily as a result, but the strength remains unaffected. The medication loses between 10 and 30 times its potency when the hexyl ring is moved from position 6 to positions 5 or 4. This demonstrates that what you put on the carbon as well as being greasy is what makes it strong. The medicine might eventually be strengthened by adding a methyl group at position 4 along with another component, as removing the methyl group has no effect on the drug's potency. The hexyl ring can be lengthened by adding more components to the sixth position, or a benzyl group or ring can take its place. Even if the benzyl ring is modified with a 4-chloro or methoxy group, the drug's effectiveness is unaffected.[14-16]

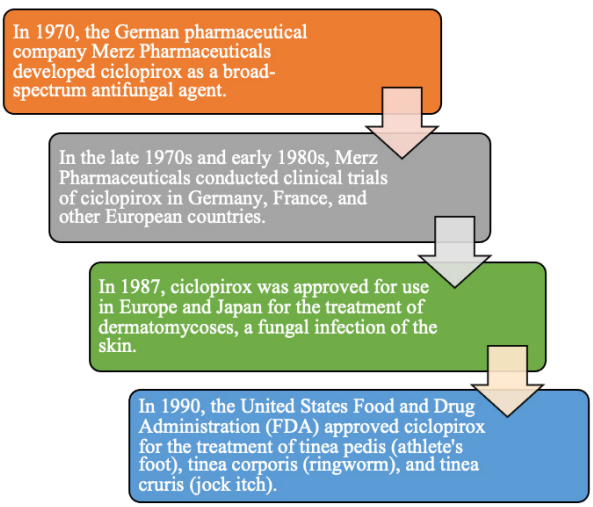

Fig. 2. Timeline of ciclopirox development starting from 1970 to recent uses

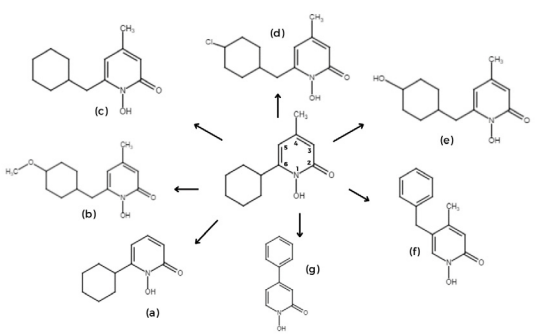

Fig. 3. Different substitution and modification to ciclopirox structure. (a) Desmethyl ciclopirox, cyclohexyl connected via methyl bridge replacement to (b) 4-methoxy phenyl, (c) phenyl, (d) 4- chlorine substituted phenyl, (e) phenol, (f) substitution on fifth position of benzyl connected by methyl bridge, (g) removal of side chain and substitution of methyl to benzene

General mechanism of ciclopirox

CPX acts by different mechanisms compared to normal antifungals like azoles, imidazoles, etc. CPX acts as a chelating agent for metal ions and inhibits metal-dependent enzymes confirmed by the genomic approach. Instead of intruding sterol biosynthesis of organisms like other agents it possesses a high affinity to trivalent metal cations like Fe^{3+} thus affecting cytochromes.[17] Not only cations but also radicals i.e., hydroxyl radicals are entrapped and provide relief from inflammation of the skin. These radicals are formed by superoxide anion which goes under dismutation by NADPH oxidase which also has an action against microbes.[18,19] Enzymes are degraded and electron transportation to mitochondria is hindered. creates an environment where it is hard for growing cells to take up amino acids that are essential for their growth.[17] Peroxides damage proteins, lipids, and nucleic acids, these peroxides degrading enzymes are inhibited by CPX as they chelate Al^{3+} and Fe^{3+} cations which affect the working of enzymes in low concentration.[3,18] It inhibits respiration in Candida albicans and at high concentrations, oxidation of NADH through mitochondria in yeast that are sensitive to inhibition that isolation of mitochondria.[3] CPX also acts on ferritin which is an iron complex involved in the activity of cysts in polycystic kidney disease. CPX inhibits the accumulation of ferritin in cysts via ferritinophagy. Ferritinophagy is ferritin degradation that results from its binding to nuclear receptor coactivator 4 present on autophagolysosome.[20] The anti-inflammatory action of CPX is likely due to the inhibition of cyclooxygenase and 5-lipooxygenase.

Pharmacokinetics and pharmacodynamics of ciclopirox

Pharmacokinetics

Absorption

Ciclopirox (CPX) absorption varies based on the formulation and route of administration (Table 2). Topical cream 1 % is equivalent to 0.77 % CPX as the olamine group has no activity.[5] In recent studies, CPX only partially penetrates deeper skin layers while maintaining larger concentrations on the skin's outermost layers. Peak blood levels of CPX are attained after a particular amount of time following topical application. For instance, peak serum levels of up to 0.01 mg/L are attained after 6 hours following the application of roughly 36 mg of the active component (CPX cream 1%). when studied using cadaverous skin, a horny layer showed the presence of more than the required inhibition concentration which is valued around 2300–2400 mg/cm^3.[21] To address specific medical needs, CPX is offered in a number of formulations. These compositions include shampoos, creams, gels, suspensions, and lacquers. Different amounts of CPX content and absorption properties are provided by each formulation. One recent development in pharmaceutical research is mucoadhesive films that contain CPX. These films stick to mucosal surfaces, enabling targeted drug delivery, increased local concentrations, and prolonged drug release. This advancement shows potential for boosting CPX's effectiveness in many therapeutic applications and promoting patient compliance.[22-24]

Radiolabelling CPX has shown that on the skin surface the most concentrated areas are the hair follicle and the upper dermis layer. It is safe to say that the absorption into the systemic region, the sebaceous gland, and the dermis which takes place through the hair follicles. Peak serum concentration is around 1.7 hours. Over 6 hours from administration, an average of 1.3 % of CPX (1%) was absorbed when applied on a 750 cm^3 area of skin.[25] Absorption is rapid when oral doses are administered. The penetration of CPX is dependent on how is the structure of the nail. The more damaged, the rougher, and the more fissured the nail is by myocyte the better and deep was the absorption of CPX.[17] Vaginal dosage gives 7 to 9 % absorption as 2 % douche for 6 days or 100 mg vaginal pessaries once daily for 3 days.[26]

Distribution

The concentration of CPX achieved in the nail bed exceeds the minimum inhibitory concentration required for onychomycosis compared to other fungal applications used (Table 2). The nail lacquer of CPX, after 24 hours of application, has shown a concentration ranging from eight μg/mg in the uppermost layer to 0.03 μg/mg in the deepest layer.[27] An open labelled study was carried out where five healthy subjects were asked to apply 8% nail lacquer for 24 to 26 weeks, once daily that is before bed on their toe nails and another group of 5 healthy individuals were asked to apply it on finger nails. CPX is highly keratinophilic and shows penetration into the nail bed through keratin like materials. CPX is distributed to all tissues but the highest concentrations are found in the kidney, liver, and smooth muscles of the stomach when given orally. The placental transfer is almost negligible.[28]

Metabolism

Protein binding is 94 to 97% by administration of CPX topically.[29,30] The main pathway of metabolism of the drug is through glucuronidation. Specifically, UGT1A1 and UGT1A9 are the main isoforms responsible for the glucuronidation of CPX. Other metabolic pathways of CPX include oxidative metabolism by cytochrome P450 enzymes, which leads to the formation of hydroxylated metabolites. CPX was extensively glucuronidated, consistent with substantial first-pass metabolism of the drug. The $t_{1/2}$ of CPX glucuronide was similar to that of the parent compound, ranging from 1.3 to 6.2 h.[31]

Excretion

The urine primarily containing 80% of the dose, which is converted into glucuronide metabolite, excretes CPX (Table 2). Bile juice excretion does not happen. These findings suggest that CPX has a low potential for drug interactions and is generally well-tolerated.

Table 2. Summary of ciclopirox pharmacokinetics

Aspect	Summary	Reference
Absorption	- topical: limited deep, high surface - rapid oral and hair follicle absorption - vaginal: 7–9% absorption	5,21-26
Distribution	- effective in nails, high in kidney, liver, smooth muscles of stomach when given orally.	27
Metabolism	- mostly glucuronidation, CYP450 metabolism	31
Excretion	- mostly in urine, minimal interaction risk	1

Pharmacodynamics

CPX works by inhibiting the fungal cell membrane synthesis, disrupting the function of membrane-bound enzymes and transporters, and reducing the synthesis of ergosterol. It also exhibits antibacterial and anti-inflammatory properties, which make it useful in the treatment of skin infections and inflammatory conditions.[32] CPX binds to the fungal cell membrane and disrupts its function, causing a loss of membrane integrity and leading to leakage of cellular contents.[33] This leads to the inhibition of fungal growth and ultimately results in the death of the fungal cell. The anti-inflammatory activity of CPX is thought to be due to its ability to inhibit the production of pro-inflammatory cytokines such as interleukin-1β, interleukin-6, and tumour necrosis factor-alpha.[34]

Pharmacological actions of ciclopirox

The diverse inhibitory activities of CPX and its derivatives allow for a wide range of pharmacological applications (Table 3). Due to the chelating effect of trivalent and other ions, these compounds have been used for their action as antifungal, antibacterial, anti-inflammatory, and many other purposes. It's fascinating because CPX has lately been discovered to have a significant amount of potential to act against many other diseases, such as cancer, diabetes, AIDS, cardiovascular issues, inflammation, and bacterial infection. These results suggest that CPX is a highly promising drug for the treatment and prevention of a variety of disorders.

Ciclopirox as an anti-fungal

The possibility of CPX olamine as an antifungal drug was originally raised in 1973. It has a very a broad spectrum of action that inhibits practically all clinically relevant dermatophytes, yeasts, and moulds, including the azole-resistant *Candida* species *Candida glabrata, Candida krusei,* and *Candida guilliermondii*.[11] Depending upon its contact time it could be fungistatic or fungicidal. After a week, CPX olamine starts to have fungicidal effects. Fungicidal tests with a chosen strain of *T. mentagrophytes* revealed that fungicidal concentrations of CPX olamine against non-proliferating and proliferating fungal cells were approximately 30 times the minimum inhibitory concentration after 1 day of exposure of the organism to graded concentrations of the drug.[24] On the other hand, fungicidal and inhibitory concentrations were comparable after a week of CPX olamine exposure.[35] The reduction in CPX's antifungal activity could be due to the presence of iron salts in some media, which can combine with CPX to create complexes that are microbiologically inactive. The growth medium has an impact on the MIC of CPX olamine for common strains of *C. albicans* and *Trichophyton mentagrophytes*. Selected fungi were inhibited by CPX olamine at a dosage of 1.95 mg/L in the medium employed by scientists to test the drug's *in vitro* activity, whereas in thioglycolate medium, the same strains required a concentration of 31 to 62 mg/L to be inhibited.[1,36] Using a widely available agar diffusion technique, the *in vitro* antifungal activity of CPO was compared to that of clotrimazole, econazole, ketoconazole, miconazole, tioconazole, fluconazole, itraconazole, and nystatin. (NeoSensitabsTM, RoscoTM, Taastrup, Denmark), which had previously been described and assessed in collaborative works by Casals (1979). In order to treat candidiasis, antifungal medications were employed as 9 mm diameter tablets (NeoSensitabs, Rosco, Taastrup, Denmark). These tablets were made and supplied by the manufacturer. Only one yeast strain (0.4 %) and four (1.8 %) were deemed moderately susceptible to CPX, while 220 strains were susceptible (97.3 %) out of 225 yeast strains.[37,38] Some of the strains and their susceptible "n" in percentage are *C. albicans* (n=75), *C. parapsilosis* (n=21), *C. glabrata* (n=25), *C. tropicalis* (n=25); "n" represents the number of strains of the yeast species used in the experiment. Total 225.[39]

Ciclopirox as anti-inflammatory

An antifungal drug that simultaneously has intrinsic anti-inflammatory effect would be highly desired since superficial fungal infections may be accompanied by significant inflammation. The anti-inflammatory properties of CPX and its derivatives have been examined in *in vitro* and *in vivo* studies. Since ciclopirox is non-toxic and unlikely to irritate skin, it has a number of advantages. In both biochemical and pharmacological settings, it also has weak anti-inflammatory capabilities, demonstrating excellent tolerance and a noticeable absence of serious side effects. *In vitro* studies have demonstrated that CPX inhibits the formation of 5-lipoxygenase metabolites (5-HETE and leukotriene LTB4) as well as prostaglandin E2 (PGE2) cellular release.[40] The test for arachidonic acid-induced ear edema can be used to

measure topical anti-inflammatory activity. According to the percentage difference from inflamed control ears, CPX effectively lowers arachidonic acid-induced ear edema. In comparison to the anti-inflammatory medicines indomethacin and desoximetasone, this occurred at a rate that was about two times greater than what was observed with naftifine, ketoconazole, fluconazole, or miconazole.[25] Significant inflammation might be brought on by cutaneous candidiasis. In a double-blind, randomised comparative investigation, 96 patients with cutaneous candidiasis were enrolled and randomly allocated to receive either CPX olamine cream 1% or clotrimazole cream l%. Both treatments were successful; at the final evaluation two weeks following the end of active therapy, the final clinical cure rates for those receiving CPX olamine and clotrimazole were 76 and 63%, respectively; mycologic cure rates were likewise equal. However, those receiving CPX olamine showed earlier clinical improvement; in comparison to those getting clotrimazole, a considerably higher proportion of these patients were evaluated as clinically cured at weeks 1, 2, and 3.[41] In a different comparison study, 1% CPX olamine cream was shown to be just as effective as 1% clotrimazole cream for treating *tinea corporis* and *tinea cruris*. In this experiment, both treatment groups saw a similar time course of recovery throughout the duration of 4 weeks of therapy. In a multicenter, double-blind trial of patients receiving therapy for 4 weeks, it was discovered that the inflammatory signs and symptoms of tinea pedis responded preferentially to CPX olamine cream 1% as opposed to clotrimazole cream 1%. Early on in the experiment, there were particularly noticeable disparities between the therapy groups. At week 1, the CPX olamine group's clinical response rate (improvement and cure) was noticeably greater than the clotrimazole group's (93% versus 71 %, $p<0.01$). Only 2 % of the 43 patients who received clotrimazole obtained a clinical cure, compared to 26 % of those who received CPX olamine. For the CPX group, clinical cure rates and combined clinical and mycologic cure rates were greater in weeks two and three after therapy and at weeks five and six after treatment ($p<0.05$). The two groups' final cure rates did not significantly differ from one another.[3]

Ciclopirox as anti-bacterial

Secondary bacterial infections can make superficial mycotic illnesses more difficult. Although other antifungal substances also possess antibacterial capabilities, CPX stands out from the competition due to its larger scope and more consistent activity, particularly its ability to combat Gram-negative bacteria. *Trichomonas vaginalis* and *Mycoplasma* species have both been shown to be sensitive to CPX.[40] Numerous Gram-positive and Gram-negative bacteria are susceptible to CPX *in vitro*. It is advantageous for CPX to be active against Gram-negative germs rather to certain azoles, which are more effective against Gram-positive bacteria. It is advantageous to treat macerated *tinea pedis* because CPX has a broad range of action that encompasses both gram-negative infections and fungal pathogens.[25] Recent research using a standardised microdilution method revealed that CPX was active against both Gram-positive and Gram-negative bacteria, with a MIC range of 0.06-2 mg/mL against B-haemolytic *Streptococcus* group A, *Proteus mirabilis, Escherichia coli, K. pneumoniae, S. aureus*, and Micrococci. MICs of 16-32 mg/mL for Gardnerella vaginalis and greater than 128 mg/mL for Lactobacillus species were found in more recent tests utilising bacteria isolated from clinical samples of vaginal swabs.[40] Minimal inhibitory concentrations (MIC) for bacterial isolates (n=45) were established using a standardised microdilution technique. The MIC range for CPX is 0.06-2 g/mL, and it shown action against every isolate tested, including Gram-positive and Gram-negative.[9]

Repurposing the forgotten anti-fungal CPX

An anticancer therapeutic

In terms of how it affects dermatophytes, yeast, filamentous fungus, and bacteria, CPX has a very broad range of activity. The methods by which CPX exerts these effects appear to be varied, involve altering membrane function in fungi or focusing on various metabolic (respiratory) and energy-producing activities in bacteria. CPX may also have an impact on the yeast *Saccharomyces cerevisiae* by interfering with intracellular transport, mitotic spindles, cell division signals, and DNA repair. In addition to its antibacterial and antimycotic properties, CPX also causes cell cycle arrest in the G1 phase in human cells and the G_2/M phase in yeast *S. cerevisiae*.[42-44] According to the most current research, CPX-induced cell death was linked to the chelation of intracellular iron and the suppression of the iron-dependent enzyme ribonucleotide reductase. Without causing severe organ damage or weight loss in NOD/SCID mice models, it appeared that CPX hindered the engraftment of primary acute myeloid leukaemia (AML) cells and appeared to cause cell death in primary human AML cells. Studying the *in vivo* impact of CPX on the growth of the human breast cancer MDAMB231 tumour in a mouse xenograft model was done in order to give more preclinical support for the development of CPX as an anticancer treatment. Results demonstrate that CPX potently reduced tumour development by preventing tumour cell proliferation and causing apoptosis *in vivo*. The preclinical anticancer potential of CPX against solid tumours such as rhabdomyosarcoma, breast cancer, prostate cancer, and colon cancer is still being evaluated.[45] According to research, there are two substances that reversibly halt the lymphocyte cell cycle. In late G_1, 1-2 h before the GUS boundary, which is determined by APH, the

compound mimosine (MIMO) reversibly stops the progression of B cells. Another substance, [2-(4-hydroxytoluene-3-yl)-4,5-dihydro-4carboxythiazole] (HTDCT; Hoechst 768159), inhibits T lymphocyte activation after the stimulation of cell surface transferrin receptors, suggesting that HTDCT may also operate in late G_1 phase.[46] The chemical CPX, which blocks the HL-60 promyeloid leukaemia cells' cell cycle reversibly at the same location close to the G_1/S phase boundary, produces the same results. After performing the flow cyclometer methodology, it was determined that, in contrast to the exponentially growing control, all arrest cell growth in the G_0/G_1 phase of the cell cycle. A batch of synchronously developing cells has advanced through the S phase around halfway after a 5 hours release.[47] CPX and HTDCT arrest HL-60 cells in late G_1 as determined by a series of drug release and readdition tests. Aphidicolin (APH), an inhibitor of DNA polymerase α activity, was also intended to show this. APH stops G_1 phase cells from entering the S phase just beyond the G_1/S barrier. It has been shown in the past using this strategy. The two chemicals CPX and HTDCT have an effect on HL-60 cells just before the early S phase arrest detected by APH, which takes place close to the G_1/S phase boundary. With no delay or a 1 h delay, cells discharged from CPX and HTDCT into APH do not reach the S phase.[46]

Additionally, by suppressing the production of vascular endothelial growth factor, CPX can prevent human umbilical vein endothelial cells from proliferating and forming new blood vessels.[48] Recent research indicates that CPX also prevents lymphatic endothelial cells from forming tubes, which may prevent lymphangiogenesis. These discoveries further underline the potential of CPX for the treatment and prevention of cancer since angiogenesis and lymphangiogenesis are essential for carcinogenesis and metastasis.[49] According to research, CPX prevents deoxyhypusine hydroxylase from working (DOHH). DOHH is an iron-dependent enzyme, just the same as RR.[50] Deoxyhypusine is transformed to hypusine via DOHH, which is necessary for the development of eukaryotic translation initiation factor 5A (eIF5A), an integral to maintaining translation elongation.[51,52] Deoxyhypusine hydroxylation was inhibited in a concentration-dependent manner when CPX and [3H]-spermidine were treated with exponentially developing HUVECs for 20 hours. Only [3H]-hypusine, not [3H]-deoxyhypusine, was found in the cellular protein hydrolysates of control HUVECs and HUVECs that had received 2.5 μM or less of CPX. At larger dosages (5-100 M), CPX inhibited deoxyhypusine hydroxylase, resulting in the formation of the intermediate [3H]-deoxyhypusine and a corresponding drop in [3H]-hypusine. Based on the fact that the IC_{50} for this inhibition was around 5 M and no [3H]-hypusine was identified at >10 M, the deoxyhypusine hydroxylase was totally inhibited. We compared the effects of CPX and the other test chemicals on DNA synthesis in HUVECs since deoxyhypusine hydroxylation and eIF5A have been connected to cell proliferation. All substances induced concentration-dependent suppression of DNA synthesis 18 hours into the therapy. A 10 μM IC_{50} for CPX demonstrated the highest inhibition.[53]

Cells were transfected with cDNA matching to the ribonucleotide reductase M2 subunit of ribonucleotide reductase (RRM2) or vector control in order to ascertain if inhibiting ribonucleotide reductase was functionally significant for CPX-induced mortality. Ribonucleotide reductase is the enzyme in charge of converting nucleoside diphosphates into deoxynucleoside diphosphates, so providing a consistent supply of deoxyribonucleotides for DNA synthesis. Because the ribonucleotide reductase M2 subunit of this enzyme contains an iron core that is necessary for the complex's enzymatic activity, it is iron-dependent.[54] After that, cells were exposed to CPX at progressively higher doses, and the MTS test was used to gauge cell viability. RRM2 overexpression prevented CPX-induced cell death, proving that ribonucleotide reductase inhibition is crucial for CPX's cytotoxic effects. Notably, CPX was more than 200 times more effective than hydroxyurea, a ribonucleotide reductase inhibitor that functions in a different way than CPX. As a result, CPX has an anticancer impact through at least one mechanism, which is the suppression of ribonucleotide reductase activity.[55]

Diabetes management

Diabetes causes endoplasmic reticulum (ER) stress to be induced on pancreatic β-cells, which is associated with pancreatic dysfunction. Because cells are unable to meet the increasing demands for insulin generation and secretion, endoplasmic reticulum (ER) stress is fundamental to the pathophysiology of diabetes. This loss of cell mass occurs over time. When treating diabetic mice with nutlin-3a, the proapoptotic effects of p53 are still present without p21, which decreases islet survival and function. Studies through experimentation reveal that the drug CPX, which promotes p21 expression, has an impact on insulin release in cultured pancreatic islets and glucose homeostasis in diabetic animals. Wild type mice's pancreas expressed p21 when CPX was administered at doses as low as 5 mg/kg. Inducing a decrease in blood glucose levels, CPX at 25 mg/kg once day significantly improved glucose homeostasis. A diabetic animal with blood sugar levels of 180 mg/dl is noteworthy since CPX treatment had a similar impact on p21, albeit at greater doses. Following CPX therapy, ferritin levels considerably reduced ($P<0.05$) from their high state in the diabetic mice's serum. Due to CPX injection having no effect on glucose levels in wild type non-diabetic mice, the effects of CPX on glucose homeostasis were

only seen in diabetic mice. CPX provides protection for pancreatic islets in the presence of p21/p53 expressions at glucotoxic levels.[56-59] The hypo-insulin condition results from islet cells, which are in charge of secreting insulin, malfunctioning or even dying. As a result of the pro-inflammatory cytokine's activation of inducible nitric oxide synthase expression and subsequent generation of nitric oxide, ATP synthesis is blocked, insulin secretion is inhibited, and cell death is induced.[60] According to one theory, eIF5A moves *Nos2* mRNA from the nucleus to the cytoplasm to encourage translation of the *Nos2* gene, which codes for inducible nitric oxide synthase. For eIF5A-mediated *Nos2* mRNA trafficking and the pathogenesis of islet cells, eIF5A must be hypusinated. Since CPX olamine has an IC_{50} of roughly 5 M and can inhibit DOHH, an essential enzyme for hypusination of eIF5A, it is promising to use CPX olamine for treating Type I diabetes.[61]

For acquired immune deficiency syndrome (AIDS)

CPX suppresses HIV replication in human peripheral blood mononuclear cells. In host cells, eIF5A is expressed and is implicated in the nucleocytoplasmic trafficking of viral mRNA and HIV replication. HIV replication is impeded by DOHH suppression, which interferes with hypusine formation on eIF5A. CPX olamine has been suggested to have a significant potential for the treatment and prevention of AIDS due to its powerful inhibitory action on the DOHH-eIF5A axis. Drugs hindered the maturation of eIF5A and inhibited substrate binding to DOHH.[61] At the RNA level, viral gene expression from HIV-1 molecular clones was inhibited independently of all viral genes. The inhibition took place at the stage of HIV-1 transcription beginning and was specific to the viral promoter. The suppression of HIV-1 gene expression caused by partial eIF5A-1 knockdown by siRNA was non-additive to medication activity.[62] Acute infection is suppressed and infected cells are preferentially eliminated when using the antifungal drug CPX, which inhibits retroviral gene expression in persistently infected T cells while concurrently activating the intrinsic route of death.[4] The 5'-untranslated region of HIV, which is not only necessary for HIV replication but also for the most conserved area of the HIV genome, is the focus of CPX's unique mechanism of blocking HIV gene expression. As a result, CPX could be able to counteract treatment resistance caused by HIV's variable nature.[63]

For anti-hepatitis B virus

HBV replication in cells and mice by blocking assembly of the HBV capsid. The crystal structure of the HBV core protein and the ciclopirox complex revealed a novel binding mode at dimer-dimer interfaces. It is also found that ciclopirox synergized with NAs to prevent HBV replication in cells and in a humanized liver mouse model. Orally administered ciclopirox may block HBV capsid assembly effectively and thus provide a novel opportunity to combat chronic HBV infection.[64] In an article written by Kang et al.[65] they performed experiments on mice using 978 FDA approved drugs using Cp149-Y132A as marker for core protein inhibition in HBV, it was discovered that ciclopirox showed inhibition of HBV capsid assembly and secretion of HBV DNA in infected cells in vitro and in mice. Due to particular residue and loop

Table 3. Summary of ciclopirox pharmacodynamics

Medical condition	Pharmacodynamic properties of ciclopirox and mechanism	References
Anti-fungal	Ciclopirox exhibits broad-spectrum antifungal activity by inhibiting fungal growth through interference with cell membrane integrity and transport processes. It targets various fungi, including dermatophytes, yeasts, and molds. Mechanism is various like reducing oxidating NADPH, inhibiting ferritin accumulation and chelating metal ions, etc	3,1
Anti-inflammatory	The anti-inflammatory action of CPX is likely due to the inhibition of cyclooxygenase and 5-lipooxygenase	40
Anti-bacterial	Ciclopirox demonstrates antibacterial properties by disrupting bacterial cell membranes and interfering with essential metabolic pathways. It has shown effectiveness against some gram-positive and gram-negative bacteria. Mechanism is quite similar to how it acts as an anti-fungal drug i.e., through ferritin and other metal ions chelation and inhibition. This affects cytochrome activities	3,20
Anti-cancer	Ciclopirox has been investigated for its potential anti-cancer effects due to its ability to inhibit cell proliferation and induce apoptosis in certain cancer cell lines. It may interfere with multiple signalling pathways involved in cancer growth and survival. It shows many mechanisms but suppression of ribonucleotide reductase activity is ultimate result	55,83,84
Anti-HIV	Studies suggest that ciclopirox may possess anti-HIV activity by blocking viral entry, replication, and maturation stages. It could inhibit enzymes and viral interactions required for HIV replication within host cells. Hinders the maturation of eIF5A and inhibited substrate binding to DOHH	62
Anti-HBV	Ciclopirox has been explored for its anti-hepatitis B virus (HBV) activity. It may inhibit HBV replication by interfering with viral polymerase activity and affecting viral protein synthesis, thereby suppressing viral load. It inhibits HBV capsid assembly and secretion of HBV DNA in infected cells. Also synergizes with NAs to prevent HBV replication in cells	65, 81
Anti-diabetic	Ciclopirox has shown potential in reducing insulin resistance by modulating cellular pathways involved in glucose metabolism. It may enhance insulin sensitivity and offer benefits in managing type 2 diabetes. CPX which promotes p21 expression which is vital for survival of pancreatic islet	56-59
Neurological disease	Emerging research suggests that ciclopirox might have neuroprotective effects by modulating signalling pathways involved in neurodegeneration. It could potentially offer therapeutic benefits in neurological disorders. Decreases the cell cycle and nitric oxide (NO) release in lipopolysaccharide (LPS)-induced BV-2 cells by phosphorylation of AKT and GSK3	67
Cardiovascular disease	Ciclopirox's impact on cardiovascular diseases is less studied. Some evidence indicates its potential to affect pathways relevant to cardiovascular health, but further research is needed to establish its direct effects in this area. CPX-induced HIF-1 promotes the increased production of urocortin 2, which has been shown to improve cardiac output and myocardial contractility	68,82

Table 4. List of documented preclinical studies of ciclopirox as a potential therapeutic molecule

Drug administered	Model	Dose	Duration of study	Study design	Results	Ref
Ciclopirox	Dermatophytes (110 strains; 98 from *Trichophyton* spp.), *Candida* spp. (14 strains)	0.003–2 µg /mL	7 days	Microbroth dilution *in vitro* susceptibility test as per National Committee for Clinical Laboratory Standards (NCCLS) M27-A proposed standard	Minimum inhibitory concentration 100% growth inhibition. MIC values) (µg/mL) for dermatophyte: (0.03–0.25), yeast: (0.001–0.25)	[9]
Ciclopirox	Non-dermatophyte moulds (nine strains)	0.003–4 µg /mL	72 h	Checkerboard microdilution method	The non-dermatophyte fungi MIC values (microg mL-1) (mean±SEM) were: ciclopirox (1.04±2.62)	[69]
Fulcare [ciclopirox hydroxypropyl chitosan nail lacquer]	Bovine hoof slices	75 µl	30 h	Transungal permeations of bovine hooves	Amount permeated (3.29±0.67%, wt/wt) in the slices. Works not only on nail plates but also on nail beds	[72]
Ciclopirox	*Escherichia coli*	Varied concentrations of ciclopirox	Not specified	*In vitro* study	- Ciclopirox inhibited the growth of *E. coli* in a dose-dependent manner. -The antibacterial effect was enhanced when ciclopirox was combined with low levels of iron	[73]
Ciclopirox Olamine	Murine myeloma model	Not specified	Not specified	*In vivo* experimental study	- It suppressed tumor growth and prolonged survival in the mice. - The drug reduced the expression of Wnt pathway-related proteins and inhibited myeloma cell proliferation	[74]

Table 5. Documented clinical trials of ciclopirox as an antifungal agent

Indication	Study type	Sample size	Treatment	Duration	Results	Refs
Onychomycosis	Randomized, double-blind, placebo-controlled	131	Ciclopirox cream	48 weeks	Significantly improved nail appearance and mycological cure compared to placebo	[75]
Onychomycosis	Randomized, double-blind, vehicle-controlled	131	Ciclopirox nail lacquer	48 weeks	Significantly greater complete cure of the target toenail compared to vehicle	[76]
Seborrheic dermatitis	Randomized, double-blind, placebo-controlled	43	Ciclopirox shampoo	4 weeks	Significantly reduced severity of seborrheic dermatitis compared to placebo	[77]
Tinea pedis	Randomized, double-blind, placebo-controlled	87	Ciclopirox cream	4 weeks	Significantly improved signs and symptoms of tinea pedis compared to placebo	[78]
Vulvovaginal candidiasis	Randomized, double-blind, placebo-controlled	297	Ciclopirox cream	7 days	Significantly improved clinical and mycological cure compared to placebo	[79]
Diaper dermatitis	Randomized, double-blind, placebo-controlled	90	Ciclopirox cream	14 days	Significantly improved diaper dermatitis compared to placebo	[80]

modifications, ciclopirox binds to three out of the hydrophobic pocket's six binding sites in the HBV core protein when it is complexed with it. Additionally, ciclopirox demonstrates synergistic actions that prevent HBV replication when coupled with TDF and ETV. This implies ciclopirox may be thought of as a supplement to TDF or ETV therapy for HBV-infected individuals. When cells were treated with 1 M ETV or TDF with varied doses of ciclopirox (0.1–10 µM), HBV DNA secretion was synergistically inhibited. Many other studies show a potential of ciclopirox to be used as an adjunct therapy with HBV medications.[66]

Neural diseases and cardiovascular

Acute cerebral thrombosis causes the serious disease known as an ischemic stroke. In a traditional rat model of ischemic stroke, CPX post-ischemic therapy reduced brain infarction, neurological impairments, and brain edema. A single dosage of CPX administered after an ischemic stroke had occurred had a long-lasting neuroprotective effect that might be strengthened by administering further doses. Additionally, CPX successfully repaired the blood-brain barrier (BBB) damage, glial activation, and neuronal loss brought on by ischemia. In oxygen glucose deprivation (OGD) exposed SH-SY5Y cells, CPX significantly increased the phosphorylation of AKT (protein kinase B, PKB/AKT) and GSK3 (glycogen synthase kinase kinase 3), and it significantly decreased the cell cycle and nitric oxide (NO) release in lipopolysaccharide (LPS)-induced BV-2 cells, which may help.[67] Through the activation of Hypoxia-inducible factor-1α, CPX is able to mitigate the lowered responsiveness to inotropic stimulation in aged myocytes (HIF-1). Additionally, CPX-induced HIF-1 promotes the increased production of urocortin 2, which has been shown to improve cardiac output and myocardial contractility, lessen peripheral resistance, and lessen the effects of ischemia.[68]

Pre-clinical and clinical trials of ciclopirox

Preclinical and clinical studies have been conducted to evaluate its efficacy and safety profile (Table 4 and 5). A study published by Gupta investigated the *in vitro* antifun-

gal activity of CPX against 51 strains of dermatophytes, which are fungi that cause skin infections. The study found that CPX was effective against all tested strains, suggesting its potential for the treatment of dermatophyte infections.[69] Another preclinical study published by Gupta A.K evaluated the efficacy of CPX in treating nail infections caused by dermatophytes. The study found that CPX penetrated the nail plate and reached therapeutic levels in the nail bed, indicating its potential as a topical treatment for onychomycosis.[70] In addition, CPX has been studied for its potential to treat other conditions, such as psoriasis and cancer. For instance, a study found that CPX could inhibit the growth of cancer cells and induce cell death *in vitro* and *in vivo*.[71]

Conclusion

An efficient anti-fungal medication called CPX has fallen out of favour over time. Because of its distinct mechanism of action, *in vitro* and *in vivo* effectiveness, broad-spectrum antimycotic coverage, additional antibacterial and anti-inflammatory activity, well-established safety, excellent tolerance, lack of drug resistance currently, with an extremely low likelihood of developing resistance in the future, and accessibility, CPO 1% cream may be the best topical antifungal for superficial cutaneous mycoses. RR, DOHH/eIF5A, Wnt/-catenin, HIF-1/VEGF, VEGFR-3/Erk1/2, mTOR, and CDKs are just a few of the enzymes or signalling pathways that CPX has the power to influence. The majority of these actions are associated with its chelation of iron. As a result, CPX has been discovered to have novel potentials, such as inhibiting the growth of tumours, reducing diabetes and its consequences, preventing HIV infection, and enhancing age-related cardiovascular abnormalities. Acute and latent nervous system infection, blepharitis development, and HSV-1 multiplication in the cornea are all decreased by topical therapy with CPX olamine. It is uncertain if CPX prevents infection of the neurological system only due to its effect on corneal replication, however this is doubtful given that low dosage CPX appeared to have a disproportionally high effect on accumulation of latent genomes. It is available worldwide in various formulations for the treatment of superficial fungal infections, including tinea pedis, tinea cruris, tinea corporis, cutaneous candidiasis, and tinea versicolor. Excellent bioavailability of CPX was seen after subcutaneous injection, showing the viability of this mode of administration should CPX-POM therapy prove effective in an outpatient or ambulatory cancer treatment context. For the treatment of both muscle-invasive and non-muscle-invasive bladder cancer, CPX-POM is currently being developed. Acute myelogenous leukaemia in humans, breast cancer, rhabdomyosarcoma, and colon carcinomas are among the illnesses for which CPX has recently been repositioned as a viable treatment drug. It is also used to combat viruses that cause HIV, HPV and other infections, as well as cyst formation in polycystic kidney disease. Its diversity is still underappreciated, particularly in light of the pharmaceutical industry's emphasis on creating and promoting „newer azoles". Most notably, CPX can help control treatment-resistant dermatophytic infections, tinea incognito, mixed infections, and recurrent VVC as well as the threat of steroid misuse. Ciclopirox's multi-faceted mechanisms of action make it an intriguing candidate for repurposing in the treatment of cancer, diabetes, HBV, neural degradation, and HIV. While preliminary evidence is encouraging. With continued research and exploration, CPX has the potential to bring significant benefits to patients suffering from various diseases and become a valuable addition to the pharmacological armamentarium.

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability

Not applicable.

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CASUISTIC PAPER

Demystifying the intricacies – a rare report of two cases of biradicular permanent mandibular lateral incisor and their endodontic management

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ABSTRACT

Introduction and aim. One of the imperative reasons for endodontic failure is an extra or missed root and root canals. Permanent mandibular lateral incisors generally present as monoradicular tooth with one or two canals, but aberrancies in the form of extra root are also rarely seen**.** There is insufficiency in the literary work regarding the presence of an extra root in permanent mandibular lateral incisor. We hereby report two illustrious cases of biradicular permanent mandibular lateral incisors and their endodontic management and brief review regarding the case reports published so far regarding the existence of an extra root in permanent mandibular lateral incisor in the literature. Also, we intend to apprise the scientific documentation with the aforementioned.

Description of the cases.

Case 1. A twenty-two-year-old male patient of Indian origin was referred to endodontic clinic for intentional root canal therapy. Pre-operative radiograph revealed 32 exhibited aberrancy in the form of an extra root, with normal periodontal structures and bone architecture.

Case 2. A sixty-two-year-old male patient came to the Department of Conservative Dentistry and Endodontics with the chief complaint of pain in the lower anterior tooth region**.** Radiographic examination revealed root caries on the mesial side and two roots with two canals with bifurcation at the level of middle third of root.

Conclusion. Clinicians should be well prepared in advance for management of any anatomical aberrancy in their clinical practice by having a scrupulous knowledge of root canal system.

Keywords. aberrancy, endo access bur, endodontic treatment, extra root, permanent mandibular lateral incisor, root canal anatomy

Introduction

Missed root(s) and/or root canal(s) is the major reason for endodontic failure as variations in the form of an extra root, root canal and morphology are not uncommon.[1] Such variations can be seen in any tooth of the dental arch with varying degree of incidence. When the clinician is unaware about these anatomic variations, these untreated canals may contain necrotic debris, tissue remnants or organic substrates that further facilitate growth of pathological microorganisms ultimately leading to endodontic failure. A comprehensive knowledge of tooth roots and root canal morphology is essential for a successful endodontic treatment, which includes pre and intraoperative knowledge about the landmarks as well as any aberrant anatomy facilitating chemo-mechanical cleaning and shaping followed by 3-D obturation of the root canal system.[2]

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Makkar S, Gupta S, Singh A, Rohilla S. Demystifying the intricacies – a rare report of two cases of biradicular permanent mandibular lateral incisor and their endodontic management. *Eur J Clin Exp Med.* 2023;21(4):909–913. doi: 10.15584/ejcem.2023.4.9.

Morphologically, permanent mandibular lateral incisor generally presents as a monoradicular tooth with one or two root canals. Various investigators have studied root canal system in mandibular anterior teeth. Earlier it was believed that permanent mandibular incisors have only one root and one root canal. Further studies revealed that 11–68% of mandibular incisors consists of two canals although it converges into one in the apical third.[3] Vertucci studied the root canal morphology of 300 mandibular anterior teeth and observed a second canal in 27.5% of mandibular incisors.[4] Miyashita et al. in another study observed that 12.4% of mandibular incisors contained two canals; however, only 3% had two foramina.[5] Saati S et al. studied CBCT scans of 207 patients to evaluate the morphology of their mandibular central and lateral incisors. They found that all mandibular central and lateral incisors were single-rooted and 78.2% of all mandibular lateral incisors had one canal.[6] Mashyakhy studied CBCT images of 822 well-developed mandibular incisors of 208 patients and found that two canals appeared in 26.3% of mandibular central incisors, 30.8% of lateral incisors, and 28.6% of all the 822 mandibular incisors.[7] However, there is a paucity in the literature regarding the presence of an extra root in permanent mandibular lateral incisor. Scientific literature shows only four such cases have been reported literature regarding the presence of an extra root in Permanent Mandibular Lateral Incisor.[8-11]

Hence, clinicians must be aware of the possible morphological variations in permanent mandibular lateral incisor with respect to number of roots and root canals.

Aim

This article, therefore, presents a fortuitous presence of an extra root in permanent mandibular lateral incisor and its endodontic management in two different cases.

Description of cases

Case 1.

A twenty-one-year-old male patient of Indian origin was referred to endodontic clinic from the Department of Prosthodontics for the intentional root canal treatment. The chief complaint of the patient was missing lower anterior teeth that he lost due to accident two years back and wanted to replace them. Patient's medical history was non-contributory. Clinical examination revealed missing mandibular left and right central incisor; normally placed 42 and rotated 32 with absence of any tenderness on percussion and mobility; and normal response to thermal and electric pulp sensitivity tests. Pre-operative radiograph revealed 42 with single root and root canal but 32 exhibited aberrancy in the form of extra root, with normal periodontal structures and bone architecture (Fig. 1a). The diagnosis of normal pulp with normal apical tissues in 42 and 32 was made. Treatment plan was formulated, and the patient was advised for intentional root canal treatment in 42 and 32 followed by prosthetic rehabilitation.

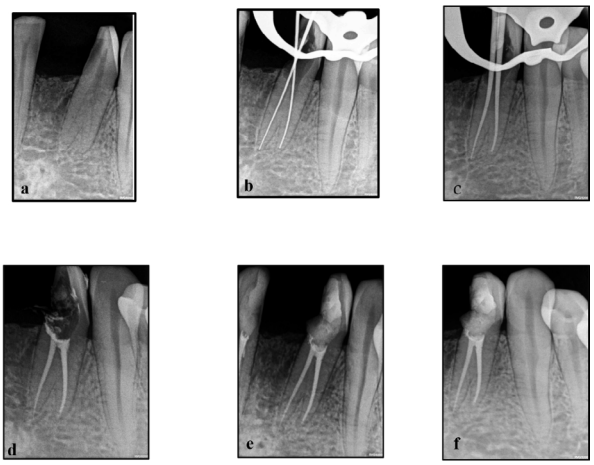

Fig. 1. Radiographic records: preoperative radiograph (a), working length determination (b), master cone (c), post obturation (d), post endodontic restoration (e), follow up (f)

On the day of appointment after taking consent, under proper sterilization protocol, the tooth was anaesthetized with 2% lignocaine (LIGNOCAD ADR, Cadila Pharmaceuticals) containing 1:200000 epinephrine solution followed by rubber dam isolation (Coltene Rubber Dam Kit Hygenic). Endodontic access cavity was prepared using an Endo Access bur (Maillefer-Dentsply, Switzerland) using a high-speed handpiece (NSK, Chicago, IL, USA). After deroofing, two canal orifices were located with DG 16 endodontic probe (**Hu-Friedy**) i.e., buccal and lingual canal. Canal patency was validated using No. 10 K file and No. 8 K file (Mani, Inc., Tochigi, Japan) and pulp was extirpated using barbed broaches. Working length determination (Fig. 1b) was done by means of radiovisiography and confirmed by apex locator (buccal canal: 21.5 mm and lingual canal: 20 mm) to bring off more predictable treatment outcomes. Thorough Cleaning and shaping of the root canals were done using hyflex (Coltene endo) files followed by sequential irrigation of the root canals using 5.25% sodium hypochlorite (Coltene CanalPro NaOCl), 17% EDTA (Coltene CanalPro) and saline. The canals were dried using sterilized absorbent paper points (Coltene Hygenic Spectra Point Absorbent Paper Points), obturation was done by cold lateral condensation technique using sealapex (Kerr Endodontics) as a sealer (Fig. 1c and Fig. 1d) and temporization was done with Cavit G (3M ESPE, Seefeld, Germany). RVG (Acteon Satelec Sopix) was taken at each step for confirming working length, master cone and subsequently obturation. Post endodontic restoration was done with nanocomposite resin (Ivoclar Tetric N-Ceram) (Fig. 1e) but further the contact was lost due to COVID-19. Patient was then tried to be con-

tacted later and a follow up IOPA was procured from the patient after almost 2 years. This case report has been prepared according to the PRICE2020 Guidelines.[13]

Case 2.

A sixty-two-year-old male patient came to the Department of Conservative Dentistry and Endodontics with the chief complaint of pain in the lower anterior tooth region. On clinical examination, 32 showed root caries on mesial side, elevated response to thermal and electric pulp sensitivity tests whereas it was not tender on percussion. Radiovisiography revealed two roots with two canals that is bifurcation at the level of middle third of root, with normal periodontium (Fig. 2a). Hence, a diagnosis of symptomatic irreversible pulpitis with normal apical tissues in 32 was made. Hence, root canal treatment was advised and explained to the patient.

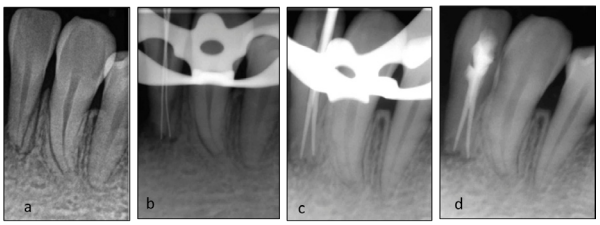

Fig. 2. Radiographic records: preoperative radiograph (a), working length determination (b), master cone (c), obturation with post endodontic restoration (d)

On the day of the procedure, informed consent was taken and the tooth was anaesthetized with 2% lignocaine containing 1:200000 epinephrine solution (LIGNOCAD ADR, Cadila Pharmaceuticals) followed by rubber dam isolation (Coltene Rubber Dam Kit Hygenic). Endodontic access cavity was prepared using an Endo Access bur (Maillefer-Dentsply, Switzerland) using a high-speed handpiece (NSK, Chicago, II, USA). The two canal orifices were located with DG 16 endodontic probe (Hu-Friedy) i.e., buccal and lingual canal. Subsequent to irrigation with sodium hypochlorite, 15 k-file (Mani, Inc., Tochigi, Japan) was used with watch winding motion to create a glide path for both the canals. Working length (Fig. 2b) was established with RVG (Acteon Satelec Sopix) in conjunction with electronic apex locator (buccal=17 mm; lingual=17.5mm). Cleaning and shaping of the root canals were done with (Coltene Gen Endo file system) along with constant irrigation using 5.25% sodium hypochlorite (Coltene CanalPro NaOCl), 17% EDTA (Coltene CanalPro) and saline. Calcium hydroxide as an intra canal medicament was placed for 7 days and patient was recalled.

On the next appointment, the intra canal medicament was removed by irrigating the canal with 5.25% sodium hypochlorite (Coltene CanalPro NaOCl), 17% EDTA (Coltene CanalPro) and saline along with instrumentation. The canals were dried using sterilized absorbent paper points (Coltene Hygenic Spectra Point Absorbent Paper Points) followed by obturation with gutta percha points and AH plus sealer (Figure 2c and Figure 2d). Post endodontic restoration was done with nanocomposite resin (Ivoclar Tetric N-Ceram) (Fig. 2d). Follow up taken telephonically for any signs and symptoms at 3 months and 6 months as patient had shifted to his native place due to COVID- 19. Follow up IOPA could not be obtained as patient had shifted to his native which is a very remote area. This case report has been prepared according to the PRICE2020 Guidelines.[13]

Discussion

Successful endodontic treatment depends upon requisite knowledge of the normal morphology and variations of the external as well as internal anatomy of the root canal system. Various studies have shown data regarding aberrancies in the form of extra roots and root canals, deltas, fins, calcifications, multiple foramina etc. in the dental arch are not infrequent.[12] For accurate diagnosis, proper clinical and radiographic examination with varying angles should be undertaken. It is essential that clinicians should have the knowledge about the clinical and radiographic signs that may suggest the presence of extra root.

According to literature search, various studies have been done to determine the internal as well as external anatomy of mandibular lateral incisors but very few cases have been reported so far regarding the presence of an extra root in permanent mandibular lateral incisor (Table 1).[8-11] We are fortunate enough to encounter two such noteworthy cases in our endodontic practice and also intend to add to the scientific literature.

Table 1. Case reports published so far regarding presence of extra root in permanent mandibular lateral incisor

S.No.	Author's Name and Year	Journal	No. of tooth reported
1.	Slowey, 1979	*Dental Clinics of North America*	1
2.	Loushine et al., 1993	*Journal of Endodontics*	1
3.	Mahajan et al., 2016	*International Journal of Medical and Dental Sciences*	1
4.	Aggarwal, 2016	*Journal of Natural Science, Biology, and Medicine*	1

Such anatomical variations in the form of extra roots and root canals can be attributed to genetic as well as racial variations and diagnostic techniques used. According to Weine et al, the present cases demonstrate Type III (two separate as well as completely distinct canals run from the pulp chamber to the root apex).[14] According to Ahmed et al. the tooth of concern presents $^{2}32\ B^{1}\ L^{1}$ configuration. As a corollary, a clinician must possess good

clinical skills and treat each tooth assuming to be having complex anatomy as a part of normality.[15]

Two roots may be seen separately in the radiograph when bifurcation is present either in the cervical or middle third of the root and is not present in the line of the x-ray beam or the tooth is rotated as seen in case 1. Similarly, bifurcation at the level of middle third of root with normal periodontium was noted in case 2 suggesting the presence of an extra root. The presence of continuous bleeding in teeth with pulpitis or normal pulp despite complete cleaning and shaping can suggest the presence of extra canals and the feeling of a "catch" on the canal wall during instrumentation of a wide and unobstructed main canal is indicative of same. Whereas in cases with necrotic pulps or pulpless teeth, inconsistent apex locator readings, the presence of an apical rarefaction and the offcentre location of an endodontic file in the radiograph indicates the presence of an extra root.[16]

At times it is imperative to detect such morphological and anotomic variations before starting the treatment to avoid any iatrogenic errors. It is also essential to modify access preparation as inadequate access opening into the tooth leaves a lingual shelf of dentine over the second (usually the lingual) canal.[17] Magnification using loupes and dental operating microscopes can be adjunct during endodontic therapy procedure. CBCT can be advised before or/and during the treatment procedure as it could be helpful in assessing the difficulty of the case and enhancing clinician's ability to correctly diagnose and execute a good endodontic therapy.

Conclusion

The present case reports highlight that the clinician should be aware of the fact that even the routine cases might have some deviations from the usual and should always be attentive to detect anatomic anomalies. Radiographs that are taken at different angles and Magnification using loupes and dental operating microscope can be a helping hand in locating extra root canal(s) to confirm any deviation for the normal root canal morphology. Patience, knowledge and judicious planning are the keys for successful endodontic treatment.

Declarations

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Author contributions

Conceptualization, S.G. and S.R.; Methodology, S.M.; Software, A.S.; Validation, S.M., S.G. and S.R..; Formal Analysis, S.M.; Investigation, S.R. and A.S.; Resources, S.R. and A.S.; Data Curation, A.S.; Writing – Original Draft Preparation, S.R.; Writing – Review & Editing, S.M. and S.G.; Visualization, S.G.; Supervision, S.M.; Project Administration, S.G. and S.R.

Conflicts of interest

The authors display no conflicts of interest.

Data availability

Data is available according to policy of the journal.

Ethics approval

Informed written consent was taken from the patients.

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CASUISTIC PAPER

Secondary Fahr's syndrome mimicking meningoencephalitis

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ABSTRACT

Introduction and aim. Fahr's disease is a rare neurological disorder characterized by bilateral basal ganglia calcifications. The aim of this report is to highlight a case of Fahr's disease with atypical features such as neck rigidity and peripheral nerve involvement.

Description of the case. Here, we present the case of a South Asian patient with secondary Fahr's disease whose clinical presentation mimicked that of meningoencephalitis. The patient had neurological and neuropsychiatric symptoms along with abnormal body movements. She also had restricted neck mobility. Computed tomography of the head revealed bilateral dense calcifications in the basal ganglia suggestive of Fahr's syndrome. Investigations revealed severe hypocalcemia, hyperphosphatemia, and low parathyroid hormone levels, which led to the identification of hypoparathyroidism as the underlying cause. The presence of pre-existing epilepsy and neck rigidity made the diagnosis difficult. The relatively rapid development of symptoms along with the presence of peripheral nerve involvement made this case even more unique. Calcium levels were corrected, and there was a marked symptomatic improvement.

Conclusion. Neck rigidity and restricted neck mobility may be present in cases of Fahr's syndrome due to calcifications of the nuchal ligament or other spinal ligaments and thus must be differentiated from meningoencephalitis. Although the symptoms of Fahr's syndrome are generally limited to the central nervous system, there may be involvement of the peripheral nerves as well.

Keywords. calcinosis, calcium metabolism disorders, Fahr's disease, hypoparathyroidism, intracranial calcification

Introduction

The deposition of calcium in the brain tissue or its blood vessels is referred to as intracranial calcification, whose occurrence varies from 1% in younger people to as high as 20% in the elderly population.[1] Depending on the site of involvement and the size of the lesion, intracranial calcifications can lead to a wide array of clinical presentations. Basal ganglia calcifications may be found incidentally during neuroimaging in asymptomatic individuals or may result from various neurological and metabolic diseases.[2] One such pathological condition is Fahr's disease, a condition characterized by bilateral intracranial calcifications, particularly in parts of the brain that control movement (including the basal ganglia).[3]

It is a rare condition that generally presents with extrapyramidal symptoms, cerebellar symptoms, problems with speech, dementia, etc.[4] Here, we describe a case of Fahr's syndrome secondary to hypoparathyroidism, mimicking meningoencephalitis with neuropsychiatric manifestations.

Description of the case

History and examination

A 32-year-old lady presented with complaints of generalized weakness for two months, inability to take oral

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feeds, inability to sit or stand without support, loss of voluntary control over defecation for one week, and altered sensorium for the last five days. She also had on-and-off finger paresthesia over the course of the last couple of months. She had a history of significant weight loss of around ten kilograms in the past six months. She had one episode of fever five days ago, which was resolved by taking medication. The patient had a known case of epilepsy and had been controlled on anti-epileptic medications levetiracetam and valproate for the past 20 years. She was diagnosed with dilated cardiomyopathy (with an ejection fraction of 35%) at another hospital and was taking digoxin for it. There was no history of loss of consciousness, focal neurological weaknesses, or any history of trauma, radiation, or neck surgery.

Physical examination revealed the presence of pallor, a blood pressure of 90/60 mmHg, and a normal body temperature. Bilateral coarse crepitations were noted on auscultation of the chest. The cardiovascular examination findings were unremarkable. A neurological examination was then conducted: higher mental functions could not be assessed owing to her poor sensorium; examination of the cranial nerves was unremarkable; motor examination showed a normal tone of all major muscle groups, but the power could not be assessed properly due to the poor sensorium and abnormal body movements; sensory examination could not be performed; all deep tendon reflexes were 1+, revealing global hyporeflexia; and signs of meningeal irritation were present in the form of neck rigidity and restricted side-to-side neck movements, while Kernig's sign and Brudzinski's sign were negative. She had continuous abnormal movements involving the entire body, mimicking generalized seizures, which were exaggerated on initiation of any voluntary motor activity but were present even at rest. The psychiatric evaluation revealed that the patient had emotional lability and echolalia.

Investigations

Her hemogram showed hemoglobin levels of 7 grams per deciliter and a leukocyte count of 18,000 cells/μL. Urine analysis showed no presence of pus cells, nitrite, or leukocyte esterase positivity. Both blood and urine cultures yielded sterile results, and the chest X-ray appeared normal. Kidney function tests revealed very low serum calcium levels of 3.5 milligrams per deciliter (mg/dL), hyperphosphatemia (9.1 mg/dL), and hypokalemia (3.2 mg/dL).

In view of the hypocalcemia, serum vitamin D and parathyroid hormone (PTH) levels were investigated, and it was seen that vitamin D levels were normal while serum PTH levels were low, indicating primary hypoparathyroidism. Computed tomography (CT) of the head and neck revealed the presence of bilateral symmetrical dense calcifications involving the basal ganglia and caudate lobes, suggestive of Fahr's syndrome and calcifications of the nuchal ligament (Fig. 1).

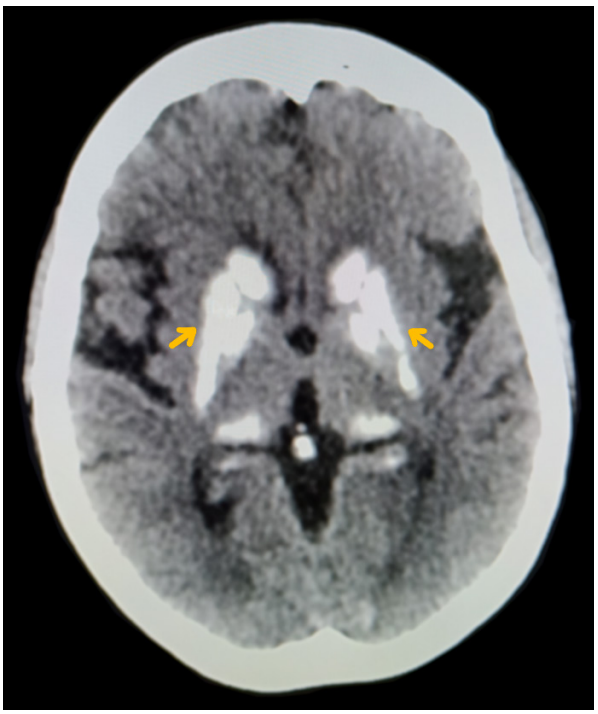

Fig. 1. Computed tomography (CT) of the head with arrows pointing to bilateral dense calcifications in the basal ganglia suggestive of Fahr's syndrome

Thus, a diagnosis of Fahr's syndrome secondary to primary hypoparathyroidism was established.

Differential diagnoses

Initially, meningoencephalitis with neuropsychiatric manifestations was suspected because of her altered mental status, abnormal body movements, neck rigidity, and elevated leukocyte count. A sample of the cerebrospinal fluid (CSF) was sent for biochemical, pathological, and microbiological analysis, but the results were unremarkable, which made meningoencephalitis unlikely. Digoxin toxicity was another differential to be considered as the patient had marked hypokalemia as a predisposing factor along with gastrointestinal disturbances and confusion, but a thorough medication history did not support this diagnosis.

Treatment

The patient was treated using intravenous calcium gluconate; five ampoules (50 milliliters) of 10% calcium gluconate were mixed in 100 milliliters of a 5% dextrose solution and infused slowly over an hour, three times a day. Intravenous magnesium sulfate, 2 grams, three times per day, was added as well. Her serum calcium levels improved from 3.5 mg/dL to 6 mg/dL, following which she was started on active vitamin D3 and hydrochlorothiazide. The calcium gradually reached

normal levels. Therapy with amantadine was started, and trihexyphenidyl was added later to avoid the extrapyramidal side effects. Transthoracic echocardiography revealed an ejection fraction of 57%, and digoxin was stopped. The patient was found to have subclinical hypothyroidism with a thyroid stimulating hormone (TSH) level of 20 micro-international units per milliliter (mIU/mL) and was started on low-dose levothyroxine.

Outcome and follow-up

The abnormal movements markedly decreased in intensity after her calcium levels were corrected but were still persisting, and thus, lamotrigine was prescribed in place of valproate as motor side effects are a known complication of it. There was a significant symptomatic improvement in the patient during her stay at the hospital. Her psychiatric symptoms also subsided gradually. She has been planned for teriparatide supplementation if her calcium levels continue to be on the lower side in the subsequent follow-up visits.

Discussion

Fahr's disease, named after Karl Theodore Fahr, is a disease characterized by bilateral calcifications in the brain, particularly involving the basal ganglia, and is thus also known as bilateral striopallidodentate calcinosis.[5] It is a rare neurological disorder that is classified as either primary or secondary based on the underlying etiology. It typically emerges in individuals aged between 40 and 50. The exact prevalence of the disease remains uncertain due to limited studies on the family members of affected patients.[6]

Secondary forms of the disorder are caused by an underlying systemic or metabolic disease. The etiologies that have been reported so far are calcium and phosphorus abnormalities like hypervitaminosis D, hypoparathyroidism, infections (including toxoplasmosis, tuberculosis, brucellosis, acquired immunodeficiency syndrome, and TORCH complex), toxic exposures (lead and carbon monoxide), astrocytoma, and immune disorders like systemic lupus erythematosus.[7] Hypoparathyroidism has been associated most frequently with Fahr's syndrome, even though it is not clinically common.[4] Thus, cases of basal ganglia calcifications with neurological signs and symptoms should be investigated for calcium and phosphate metabolic errors.

Hypoparathyroidism is a rare endocrine disorder caused by either a defect in the secretion or the effect of PTH.[8] It has a variety of clinical presentations, but neuromuscular irritability is one of the most common symptoms that occurs due to low blood calcium levels. The exact mechanism that leads to the calcification of the basal ganglia in hypoparathyroidism is not known, but a number of theories have been postulated to explain it. One potential explanation has been attributed to hyperphosphatemia, wherein the activation of the inorganic phosphate transporter (PiT1) due to elevated serum phosphate levels is thought to increase the expression of osteogenic molecules in the gray matter and the caudate nucleus.[9] Some studies also implicate the local destruction of the blood-brain barrier in abnormal calcification.[10] It is also postulated that metabolic dysfunctions cause an aberrant calcium/phosphorus ratio, which causes colloids to precipitate in cerebral arteries and lead to the formation of the calcified deposits.[11]

When it comes to clinical presentation, most individuals with intracranial calcifications are asymptomatic, and the discovery of these calcifications is often incidental, with no significant clinical consequences.[12] Even most cases of diagnosed Fahr's disease have minimal to no symptoms.[13] Among the relatively few cases of symptomatic Fahr's disease reported so far, the clinical manifestations are quite diverse and include neurological problems (like seizures, myoclonus, spasticity, gait disorder, speech impairment, dementia, coma, etc.), movement disorders (like unsteady gait, clumsiness, involuntary movements, muscle cramping, and fatigability), and neuropsychiatric symptoms (like psychosis, depression, deterioration of intelligence, inability to make decisions, etc.).[4] All three categories of symptoms were present in this case.

This patient was particularly unique because the history of epileptic disorder obscured some of the symptoms arising due to Fahr's syndrome, and it is difficult to point out from history alone which symptoms may have predated the onset of the calcifications. Another interesting finding was the presence of signs of meningeal irritation and the development of altered sensorium, coupled with the history of an episode of fever, which led to the initial impression of meningoencephalitis. Even though the CSF analysis ruled out that diagnosis, the findings of neck rigidity and restricted neck mobility are noteworthy. The neck rigidity could have been due to calcifications of the nuchal ligament or other ligaments around the vertebral column, which have been previously reported in a few cases of hypoparathyroidism.[14] This, along with the neuropsychiatric symptoms, were the reasons for this patient's clinical presentation mimicking meningoencephalitis.

Another important feature is that the clinical findings are generally limited to the central nervous system (CNS) in Fahr's disease,[15] but in this case, there was involvement of the peripheral nerves as well in the form of finger paresthesia, which was most probably caused by hypocalcemia. We found it intriguing that the patient's symptoms manifested in a relatively short amount of time and were quite severe, considering that Fahr's syndrome is typically a paucisymptomatic disorder that develops slowly and indolently, as previous reports have indicated.[16]

For the management of Fahr's disease, there have been various suggestions put forward that rely on limited clinical experience. The treatment of Fahr's syndrome is primarily symptomatic and focused on treating the underlying etiology of the secondary disease. It has been recommended that appropriate antiepileptic drugs be used for seizures, and patients who develop psychiatric symptoms should be treated with mood stabilizers or antipsychotic drugs.[7] Atypical antipsychotics may be preferred because of the presence of extrapyramidal symptoms in many cases of Fahr's disease.[17] Among the anti-epileptics, newer agents like levetiracetam and lamotrigine have been recommended, as the earlier anti-epileptic drugs such as phenytoin, carbamazepine, and valproate can impede the absorption of calcium and metabolism of vitamin D, resulting in a detrimental impact on calcium balance.[13]

For secondary forms of the disease, early diagnosis and treatment of the underlying cause may help stop the progression of the calcification and even reverse it in a few cases.[4] In this case, correction of the electrolyte imbalance arising due to hypoparathyroidism was our primary goal. Hypoparathyroidism is one of the very few hormone deficiency syndromes where the primary treatment is not simply the replacement of the hormone. Calcium and vitamin D supplementation, along with the use of thiazide diuretics, remain the mainstays for the long-term management of hypoparathyroidism, but maintaining the eucalcemic state while avoiding complications like hypercalciuria is a challenge.[8] The correction of the calcium and phosphate levels led to a significant improvement in the abnormal body movements in our patient, similar to the cases reported by Maghraoui et al. and Abe et al. who found that correcting the serum levels of these electrolytes led to an improvement in seizures and movement disorders.[18,19]

Conclusion

Cases of basal ganglia calcification with neurological signs and symptoms should be investigated for calcium and phosphate metabolic errors. Neck rigidity and restricted neck mobility may be present in cases of Fahr's syndrome due to calcifications of the nuchal ligament or other spinal ligaments and thus must be differentiated from meningoencephalitis. Although the symptoms of Fahr's syndrome are generally limited to the CNS, there may be involvement of the peripheral nerves as well. The management of Fahr's syndrome is generally aimed at providing symptomatic relief and treating the underlying cause.

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Author contributions

Conceptualization, K.V. and V.K.; Methodology & Patient Care, K.V., V.K., A.V.H., R.B., and V.S.O.; Investigation, K.V. and A.V.H.; Resources, V.K.; Data Curation, K.V., A.V.H., R.B., and V.S.O.; Writing – Original Draft Preparation, R.B.; Writing – Review & Editing, K.V. and V.S.O.; Visualization, R.B., V.S.O.; Supervision, V.K.

Conflicts of interest

The authors declare no competing interests.

Data availability

All data underlying the results are available as part of the article and no additional source data are required.

Ethics approval

Written informed consent was taken from the patient.

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CASUISTIC PAPER

Speech development delay in autism spectrum disorder – the perspective of using the "Talk To Me" speech therapy application

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ABSTRACT

Introduction and aim. The paper aims to present the prospects of using the „Talk To Me" application in the case of a child with this neurodevelopmental disorder.

Description of the case. The subject of the study was a 53-month-old boy who did not communicate verbally, but behaved forcefully. A child underwent a speech therapy using the „Talk To Me" application for a period of 12 weeks. The scale of acquiring language skills in the field of communication competence was used to monitor the progress of therapy. In the analyzed period, a decrease in the score on the scale from 23 to 15 points was observed, which means a result indicating an improvement in the communication sphere. The use of a newly developed therapeutic tool was an important motivational factor and, in the opinion of the boy's mother, significantly influenced the progress in therapy, which had not been observed before using a conventional approach.

Conclusion. The „Talk To Me" application is a therapeutic tool with potential effectiveness in the case of speech delays in children with autism spectrum disorder, and it is also compatible with behavioral therapy, a widely used therapeutic method with proven effectiveness in autism spectrum disorder.

Keywords. autism spectrum disorder, speech development delay, „Talk To Me" application, therapy

Introduction

Autism was decribed for the first time in 1943 in the article entitled „Autistic Disturbances of Affective Contact". Based on the analysis of 11 cases of children aged 2 to 8 years, it was concluded that the key symptoms occurring in this group of patients are stereotypical and repetitive patterns of behavior, preservation of sameness, restricted interest in activities and lack of communicative use of language.[1,2] Currently, according to the criteria of the Diagnostic and Statistical Manual of Mental Disorder (DSM-5) from 2013, autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by core symptoms such as deficits in social communication and social interaction, as well as restricted, repetitive behaviors.[3] Moreover, ASD often co-occurs with other deficits or disorders, including Attention Deficit Hyperactivity Disorder (ADHD), anxiety disorders, or tics and both core symptoms and other abnormalities that frequently co-occur in children with ASD may be characterized by very different intensities and co-occur with each other in various combinations.[4-7]

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The incidence of autism has increased rapidly since the 90's.[8] Currently, prevalence is estimated as approximately at 1 in 100 children worldwide. However, numerous authors provide much higher rates specific to defined areas or countries, and data from some low- or middle-income countries are frequently scarce.[9] Statistical data indicate higher incidence of the autism in boys than in girls – of course, the rates vary depending on the study group.[10] Early symptoms of ASD are observed as early as in the first two years of life. In terms of diagnosis, the role of specialists is crucial, as parents seek Professional assisstance for various reasons i.e. delayed speech development, abnormalities in motor development, „bizarre" behavior, lack of response to name, impaired sensory integration, problems with adaptation in kindergarten etc.[11-13] Parents and professionals who have daily contact with children in various care and educational facilities not necessarily recognize the need for an autism diagnosis. Therefore, it is important to detect symptoms that may indicate autism spectrum disorder early and refer the child to the diagnostic track. Hence, the education of doctors, educators, physiotherapists, occupational therapists, psychologists and speech therapists in recognizing the symptoms of autism is a very important issue.[14] However, autism is still diagnosed relatively late and the average age at diagnosis is 40 months.[15,16] In the United States, the average age of diagnosis is relatively late at 4-5 years of age.[13] Delayed diagnosis of ASD may be attributed to two main groups of factors: clinical and socioeconomic. From a clinical perspective, children who are high functioning and use speech to communicate are later diagnosed. In turn, socioeconomic factors that potentially negatively influence the time of ASD diagnosis include: gender, race, ethnicity, income and parents' educational level.[9,17-19] Often, an early symptom of autism that worries parents in young children is delayed speech development, which may occur in the case of ASD.[20] However, an important aspect that allows for differentiating speech delays from autism-related speech delays is trying to use other forms of communication to achieve the intended goal.[21] Motivating a child with ASD to communicate is an important factor determining the effectiveness of therapy, but it often requires the introduction of very creative solutions and appropriate waiting time for the child's reaction.[22] The literature often emphasizes that the lack of motivation to communicate is the main obstacle in teaching speech and language.[23]

Aim

Children with the ASD constitute a special group of patients requiring therapy due to speech development delay, therefore the purpose of this paper is to present the perspective of using the „Talk To Me" application in the case of a child with this neurodevelopmental disorder.

Description of the case

Presentation

The description of the use of the therapeutic tool (Talk To Me application) concerns a boy aged 4 years and 4 months diagnosed with ASD demonstrating delay in speech development. A child with a negative perinatal history: a cesarean section delivery due to lack of audible baby's heartbeat followed by resuscitation. At the age of 18 months, a clear developmental regression in speech was noted in the child. For the purposes of differential diagnosis, hearing was tested three times. In terms of gross motor skills - data from the child's observations: the boy walks on his toes, visible signs of excessive mobility and motor mannerisms occurring when excited: jumping, clenching his hand into a fist. The medical history indicated shows a great need to bite, knock on objects, a lot of vocalizations, attention deficit disorder, enjoying music and watching cartoons. Due to abnormalities in psycho-motor development and delayed speech development, from the age of 30 months he was subjected to comprehensive therapeutic treatment, including speech therapy. The boy did not communicate verbally, he satisfied his needs by presenting difficult behaviors.

The study was approved by the Bioethics Committee of the College of Medical Sciences of the University of Rzeszow No. 4/11/2020 and the child's parents gave written consent to his participation in the project. The duration of the intervention was 12 weeks, and the therapy using the „Talk To Me" application was conducted by a specialist (speech therapist) in the presence of parents. Before starting therapy, language functions were assessed in order to plan therapeutic procedures (including analysis of syllables articulated in spontaneous situations) – baseline assessment - test „0" before the start of the intervention, follow up in the middle of the therapy period after 6 weeks and after the end of therapy (after week 12). A new therapeutic tool was used in the child's therapy in the form of the „Talk To Me" application for the treatment of speech delays and acceleration of the acquisition of communication and language competences. The training time with the tool was 30 minutes/once a week. A simplified diagram of the application's operation is presented in Fig. 1.

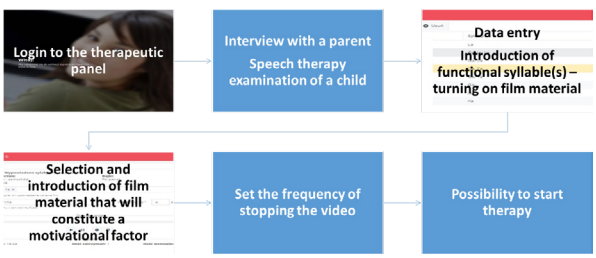

Fig. 1. „Talk To Me" application operation diagram

Therapy progress was monitored using the scale of language skills acquisition in the field of communication competence (LSAFCC). The tool allows for a quick and easy assessment of the examined aspects (communicating needs, speaking/responsible for the child, asking the child to say or repeat, the way of nodding/denying, saying/repeating syllables). Depending on the aspect, the assessment is made on a scale of 0-1 or 0-2 (where 0 means a correct result, and 1 or 2 – conditions deviating from the normal pattern), and the points obtained are summed up.

During the 12-week intervention, the boy obtained a score of 23 on the LSAFCC scale at the baseline measurement, which meant very low competence in the aspects examined by the tool. After the intervention, the result decreased by 8 points and amounted to 15 points, which is a 34% improvement compared to the result obtained during the diagnostic test. Table 1 presents descriptive statistics for the boy's outcomes during the intervention period.

Table 1. Descriptive statistics for LSAFCC scores

x	s^2	*S*	Min.	Max.	*R*
15.50	5.91	2.43	15.00	23.00	8

x – mean, s^2 – variance, S – standard deviation, Min. – minimum, Max. – maximum, R – range

The information received from the child's mother shows that the use of the tool in the form of the „Talk To Me" application was a factor that had a significantly positive impact on the development of the child's language skills. She assessed the therapy as more effective compared to conventional therapy in which the child had participated so far, and emphasized that the use of this type of solution resulted in greater interest of the child, improved concentration of attention, significantly extended the time of effective work and improved verbal communication in order to meet the need. In the studiem boy, the use of a motivational factor in the form of his favorite fairy tale was an important motivating factor.

Discussion

In the described case, the use of a modern technological solution in speech therapy in the form of the „Talk To Me" application for a child with autism spectrum disorder was associated with increasing its effectiveness and achieving a good therapeutic result. What is important is the fact that the application, thanks to its design enabling the playback of any selected film material (favorite fairy tale or program), was an important motivational factor, determining the child's involvement in therapy and, therefore, its better result – it motivates the child to talk (replaying the film is only possible in the event of uttering an appropriate voice command) and gives the speech effective power (meeting the need to watch the material). The literature on speech therapy in the autism spectrum emphasizes the need and importance of motivating the child to communicate verbally. One of the important aspects is the fact that children choose other, potentially easier ways of nonverbal communication, e.g. crying or mild tantrums in the early developmental period, which may potentially evolve later into difficult behaviors of varying intensity.[23,24]

It is a well-known fact that early detection of abnormalities, reporting to a specialist and making an early diagnosis of autism spectrum disorder is a very important factor. It determines the early implementation of therapeutic interventions and, consequently, the most optimal result that translates into the child's functioning in the later period.[25,26] Of course, this is influenced by the fact that the level of functioning of children and adolescents with ASD is very diverse: from mild to profound deficits visible in various contexts and areas of functioning.[27] The talk to me application, thanks to its simplicity (acting on the principle of positive reinforcement, building a sense of agency) and the possibility of introducing film material from any publicly available websites, can be successfully used both by younger and older children and children with ASD characterized by varying degrees of intellectual functioning. However, this will be the subject of further research and analysis.

Children and adolescents with autism spectrum disorder have specific needs in the field of upbringing, care, therapy and education. Ensuring their Leeds are met them should be the responsibility of the environment in which the child stays, i.e. the family environment and individual educational levels and care institutions (nursery, kindergarten and school). Unfortunately, education and therapy are still a common issues reported by parents of children with ASD.[28] The literature still raises problems in terms of cooperation, Communications of problems, insufficient knowledge and adequate support for this group.[29] Moreover, there is a visible need to implement interventions with scientifically proven effectiveness in various types of facilities in order to support the therapy and development of children and adolescents with ASD.[30] The „Talk To Me" application is a tool that potentially constitutes a solution that, in many cases, can improve the effectiveness of speech therapy in various institutional and non-institutional settings for this group of children.

Study limitations

As this is a case report, the conclusions cannot be generalized to all cases of children on the autism spectrum presenting a delay in speech development. It is therefore necessary to test the newly created tool on a sufficiently large group of children with this disorder.

Conclusion

The „Talk To Me" application is a therapeutic tool potentially effective in the case of speech delays in children with ASD also compatible with behavioral therapy, a widely used therapeutic method with proven effectiveness in ASD.

Declarations

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Author contributions

Conceptualization, W.A.W.; methodology, W.A.W., L.P. and J.P-B.; software, P.P. and J.P-B.; validation, L.P. and J.P-B.; formal analysis, P.P. and J.P-B.; investigation, W.A.W., J.P-B., L.P., P.P.; resources, L.P. and J.P-B.; data curation, P.P.; writing—original draft preparation, W.A.W., J.P-B. and L.P.; writing—review and editing, W.A.W., L.P. and J.P-B.; visualization, P.P.; supervision, L.P.; project administration, L.P.; funding acquisition, L.P. and J.P-B.. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data availability

The datasets analyzed during the current study are presented in this article.

Ethics approval

The study was approved by the Bioethics Committee of the College of Medical Sciences of the University of Rzeszów No. 4/11/2020. Written informed consent has been obtained from the patient to publish this paper.

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We reserve the right to reject a paper even after it has been accepted if it becomes apparent that there are serious problems with its scientific content, or our publishing policies have been violated.

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Authorship provides credit for a researcher's contributions to a study and carries accountability. Authors are expected to fulfil the criteria below (adapted from McNutt et al., Proceedings of the National Academy of Sciences, 2018, 201715374; DOI: 10.1073/pnas.1715374115):

— **Each author** is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it
— **AND** to have approved the submitted version (and any substantially modified version that involves the author's contribution to the study);
— **AND** to have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

The Eur J Clin Exp Med does not require all authors of a research paper to sign the cover letter upon submission, nor do they impose an order on the list of authors. Submission to the Eur J Clin Exp Med is taken by the publication to mean that all the listed authors have agreed to all of the contents. The corresponding (submitting) author is responsible for having ensured that this agreement has been reached, and for managing all communication between the publication and all co-authors, before and after publication.

Author contributions statements

Authors are required to include a statement of responsibility in the manuscript (at the end of the main text, before the 'References' section) that specifies the contribution of every author. For articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.".

Corresponding author – responsibilities

The corresponding (submitting) author is solely responsible for communicating with the Eur J Clin Exp Med and for managing communication between co-authors. Before submission, the corresponding author ensures that all authors are included in the author list, its order has been agreed by all authors, and that all authors are aware that the paper was submitted.

A confidential process

The Eur J Clin Exp Med treats the submitted manuscript and all communication with authors and referees as confidential. Authors must also treat communication with the Eur J Clin Exp Med as confidential: correspondence with the Eur J Clin Exp Med, referee reports and other confidential material must not be posted on any website or otherwise publicized without prior permission from the Eur J Clin Exp Med publishing team, regardless of whether or not the submission is eventually published. Our policies about posting preprints and post prints, and about previous communication of the work at conferences or as part of a personal blog or of an academic thesis, are described in the Confidentiality section.

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During the submission process, please suggest three potential reviewers (names and institutional e-mail addresses) with the appropriate expertise to review the manuscript, but please keep in mind that we are not obliged to follow these recommendations. The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may suggest reviewers from among the authors that you frequently cite in your paper. You may also name a limited number of scientists who should not review your paper (up to 3 named individuals or laboratories); these exclusions will be honored. The decision of the Editorial Board Member on the choice of referees is final.

Ethics, use of experimental animals, and human participants

For articles in the Eur J Clin Exp Med reporting experiments on live vertebrates and/or higher invertebrates, the methods section must include a statement: (i) identifying the institutional and/or licensing committee approving the experiments, including any relevant details; (ii) confirming that all experiments were performed in accordance with relevant guidelines and regulations.

For research involving human participants, authors must identify the committee that approved the research, confirm that all research was performed in accordance with relevant guidelines/regulations, and include in their manuscript a statement confirming that informed consent was obtained from all participants and/or their legal guardians.

Authors may be required to submit, on request, a statement from the research ethics committee or institutional review board indicating approval of the research.

Competing interests policy

In the interests of transparency and to help readers to form their own judgements of potential bias, authors must declare any competing financial and/or non-financial interests in relation to the work described. For the purposes of this policy, competing interests are defined as financial and non-financial interests that could directly undermine, or be perceived to undermine, the objectivity, integrity and value of a publication, through a potential influence on the judgements and actions of authors with regard to objective data presentation, analysis and interpretation. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Competing interests statement format guidelines

The statement included in the article file must be explicit and unambiguous, describing any potential competing interest (or lack thereof) for EACH contributing author.

Examples of declarations are:

— Competing interests: The author(s) declare no competing interests.
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The Eur J Clin Exp Med invites peer-reviewers to exclude themselves in cases where there is a significant conflict of interest, financial or otherwise. However, just as financial interests need not invalidate the conclusions of an article, nor do they automatically disqualify an individual from evaluating it. We ask peer-reviewers to inform the editors of any related interests, including financial interests as defined above that might be perceived as relevant. Editors will consider these statements when weighing peer-reviewers' recommendations.

Availability of materials and data

In order to maintain the integrity, transparency and reproducibility of research records, authors are encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Data availability statement format guidelines

The statement should be provided as a separate section (titled 'Data Availability') at the end of the main text, before the 'References' section. Data availability statements should include, where applicable, accession codes, other unique identifiers and associated web links for publicly available datasets, and any conditions for access of non-publicly available datasets. Where figure source data are provided, statements confirming this should be included in data availability statements. Depending on the data described in the manuscript, data availability statements commonly take one of the following forms, or can be a composite of the statements below:

— The datasets generated during and/or analyzed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].
— The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.
— All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).
— The datasets generated during and/or analyzed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.
— No datasets were generated or analyzed during the current study.
— The data that support the findings of this study are available from [THIRD PARTY NAME] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [THIRD PARTY NAME].

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Publishable amendments must be represented by a formal online notice because they affect the publication record and/or the scientific accuracy of published information. Where these amendments concern peer-reviewed material, they fall

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Publisher Correction (formerly Erratum). Notification of an important error made by the journal that affects the publication record or the scientific integrity of the paper or the reputation of the authors or the journal.

Author Correction (formerly Corrigendum). Notification of an important error made by the author(s) that affects the publication record or the scientific integrity of the paper, or the reputation of the authors or the journal.

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Once submitted, your manuscript will be assigned to a member of our Editorial Board, who will read the paper and decide whether it is appropriate for the journal. Manuscripts that are within scope and seem, on initial assessment, to be technically sound and scientifically valid, will be sent to external reviewers. Copies of any papers containing similar or related work under consideration or in press at other journals must be included with the submission.

Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission.

Peer review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. Reviewers will be able to access your manuscript securely using our online system, whilst maintaining referee anonymity. A double-blind review is applied, where authors' identities are unknown to reviewers and vice versa. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

Editorial decision

After considering the reviewer reports the Editorial Board Member will make one of the following decisions:

— Accept outright,
— Request a minor revision, where authors revise their manuscript to address specific concerns,
— Request a major revision, where authors revise their manuscript to address significant concerns and perhaps undertake additional work,
— Reject outright.

The final decision is made by the Editor-in-Chief.

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In cases where the referees or Editorial Board Member has requested changes to the manuscript, you will be invited to prepare a revision. The decision letter will specify a deadline for submission of a revised manuscript. Once resubmitted, the manuscript may then be sent back to the original referees or to new referees, at the Editorial Board Member's discretion.

A revised manuscript should be submitted via the revision link provided in the decision letter, and not as a new manuscript. Authors should attach a cover letter to explain, *point by point*, the details of the revisions to the manuscript and responses to the referees' comments. Cover letters should not contain information that could identify the authors. The destination of the cover letter file in the submission system is 'Supplementary File for Review'. Please ensure that all issues raised have been addressed in the first round of revision. Where the authors disagree with a reviewer, they must provide a clear response.

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When all editorial issues are resolved, your paper will be formally accepted for publication. Once accepted, the manuscript will undergo professional copy-editing, English editing, final corrections, pagination, and, publication on the http://www.ejcem.ur.edu.pl/. The Eur J Clin Exp Med reserves the right to make the final decision about matters of style and the size of figures.

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Even in cases where the Eur J Clin Exp Med does not invite resubmission of a manuscript, some authors may ask the Editorial Board to reconsider a rejection decision. These are considered appeals, which, by policy, must take second place to the normal workload. In practice, this means that decisions on appeals often take several weeks. Only one appeal is permitted for each manuscript, and appeals can only take place after peer review. Final decisions on appeals will be made by the Editorial Board Member handling the paper.

Decisions are reversed on appeal only if the relevant Editorial Board Member is convinced that the original decision was a serious mistake. Consideration of an appeal is merited if a referee made substantial errors of fact or showed evidence of bias, but only if a reversal of that referee's opinion would have changed the original decision.

Similarly, disputes on factual issues need not be resolved unless they were critical to the outcome.

If an appeal merits further consideration, the Editorial Board Member may send the authors' response and the revised paper out for further peer review.

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Cover letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Accepted file formats

Authors must use Microsoft Word to prepare their manuscript. Please insert your tables, graphics (schemes, figures, etc.) in the main text after the paragraph of its first citation.

In most cases, we do not impose strict limits on word count or page number. However, we strongly recommend that you write concisely and stick to the following guidelines:

— We encourage not exceeding 20 pages for original and review papers, and 8 pages for case reports of standard computer text (1800 signs on a page).
— The main text should be no more than 4,500 words (not including Abstract, Methods, References and figure legends).
— The title should be no more than 20 words.
— The abstract should be no more than 250 words.
— Recommended font: Times New Roman, 12 points.
— Manuscript text should be double-spaced. Do not format text in multiple columns.

Types of Publications

Manuscripts submitted to the Eur J Clin Exp Med should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information.

Reviews. These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.

The Eur J Clin Exp Med accepts also the following types of submissions: case reports, letters to the editor, commentaries, book reviews, and reports from scientific meetings and conferences.

Reporting guidelines

The guidelines listed below should be followed where appropriate. Please use these guidelines to structure your article. Completed applicable checklists, structured abstracts and flow diagrams should be uploaded with your submission; these will be published alongside the final version of your paper.

Please refer to existing guidelines for reporting methodology; e.g.:

— AGREE guidelines for clinical practice guidelines
— ARRIVE guidelines for *in vivo* animal studies
— CARE guidelines for clinical case reports
— CONSORT guidelines for clinical trials
— PRISMA guidelines for systematic reviews and meta-analyses
— SPIRIT for clinical trials
— STARD guidelines for studies of diagnostic accuracy
— STROBE guidelines for observational studies

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Your paper should consist of the following parts. Title page should be supplied as a **separate** file.

Research manuscripts should comprise:

— Title page: Title, Author list, Affiliations, Abstract, Keywords.
— Research manuscript sections: Introduction, Aim, Materials and Methods, Results, Discussion, Conclusions.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions,

Conflicts of Interest, Data Availability, Ethics Approval, References.

Research manuscript sections:

— *Introduction*

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

— *Material and methods*

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

— *Results*

Results should be clear and concise. The section may be divided into subsections, each with a concise subheading. Tables and figures central to the study should be included in the main paper. Do not use the term "significant" unless p-values are provided. Show p-values to 2 or 3 decimal places. The Results section should be written in past tense.

— *Discussion*

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

— *Conclusions*

Summarize the work's findings, state their importance, and possibly recommend further research.

Review manuscripts should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords, Literature review sections.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, References.

Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

Case reports should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords. Case reports should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, Ethics Approval, References.

Requirements for case reports submitted to Eur J Clin Exp Med:

— Patient ethnicity must be included in the Abstract under the Case Presentation section.
— Consent for publication is a mandatory journal requirement for all case reports. Written informed consent for publication must be obtained from the patient (or their parent or legal guardian in the case of children under 18, or from the next of kin if the patient has died).

Language Style

Manuscripts must be submitted in English (American or British usage is accepted, but not a mixture of these).

Title page

These sections should appear in all manuscript types:

Title: The title of your manuscript should be concise and informative. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.

Author list and affiliations: Authors' full first and last names must be provided. For each affiliation provide the details in the following order: department, institution, city, country. If available, the e-mail address of each author should also be provided. At least one author should be designated as *corresponding author*, and his or her email address and other details should be included at the end of the affiliation section.

Abstract: The abstract should be a total of about 250 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts: *Introduction and aim:* Place the question addressed in a broad context and highlight the purpose of the study; *Material and methods*: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. *Results:* Summarize the article's main findings; and *Conclusion*: Indicate the main conclusions or interpretations.

Keywords: Three to six pertinent keywords need to be added after the abstract in alphabetical order. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Back matter

Supplementary materials: Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.

Acknowledgments: Thank all of the people who helped with the research but did not qualify for authorship. Acknowledge anyone who provided intellectual assistance, technical help, or special equipment or materials.

Funding statement: All sources of funding of the study should be disclosed.

Author contributions: Authors must supply an Author Contribution Statement as described in the *Author contributions statements* section.

Conflicts of interest: Authors must supply a competing interests statement. For more details please see *Competing interests policy.*

Data availability: Authors must include a Data Availability Statement in all submitted manuscripts; see *Availability of materials and data* section for more information.

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References: References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, Reference Manager or Zotero to avoid typing mistakes and duplicated references.

References style

In-text citations and references should be prepared according to the American Medical Association (AMA) style. Each item should be listed in numerical order.

In-text citations

Each reference should be cited in the text using superscript arabic numerals. These superscript numbers should be outside periods. If you are citing sequential references, these should be indicated with a hyphen. Nonsequential references should be separated with commas. There should not be a space between numbers. For example: The degree of respiratory muscles fatigue depends on the applied exercise protocol and the research group's fitness level.[1,2] The greatest load with which a patient continues breathing for at least one minute is a measure of inspiratory muscles strength.[3] Diabetes mellitus is associated with a high risk of foot ulcers.[4-6]

Sample Reference

In listed references, the names of all authors should be given unless there are more than 6, in which case the names of the first 3 authors are used, followed by "et al.". If the source does not have any authors, the citation should begin with the title.

To find the proper abbreviation of journal go to the National Library of Medicine PubMed Journals Database at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Journals.

Page number(s) should be inserted in full (for example: use 111–112, not 111–2).

The following are examples of individual citations made according to the required rules of editing and punctuation:

— **Article from a journal, number of authors from 1 to 6**

Author AA, Author BB, Author CC. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Lee JC, Seo HG, Lee WH, Kim HC, Han TR, Oh BM. Computer-assisted detection of swallowing difficulty. *Comput Methods Programs Biomed*. 2016;134(2):72-78. doi: 10.1016/j.cmpb.2016.07.010

Morris A. New test for diabetes insipidus. *Nat Rev Endocrinol*. 2019;15(10):564-565. doi: 10.1038/s41574-019-0247-x

— ***Article from a journal, number of authors* more *than 6***

Author AA, Author BB, Author CC, et al. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Gonzalez ME, Martin EE, Anwar T, et al. Mesenchymal stem cell-induced DDR2 mediates stromal-breast cancer interactions and metastasis growth. *Cell Rep*. 2017;18:1215-1228. doi: 10.1016/j.celrep.2016.12.079

Jordan J, Toplak H, Grassi G, et al. Joint statement of the European Association for the Study of Obesity and the European Society of Hypertension: obesity and heart failure. *J Hypertens*. 2016;34:1678-1688. doi: 10.1097/HJH.0000000000001013

— **Websites**

Author AA (if indicated). Webpage title. Name of Website. URL. Published or Updated date. Accessed date.

Cholera in Haiti. Centers for Disease Control and Prevention Web site. http://www.cdc.gov/haiticholera/. Published October 22, 2010. Updated January 9, 2012. Accessed February 1, 2012.

Address double burden of malnutrition: WHO. World Health Organization site. http://www.searo.who.int/mediacentre/releases/2016/1636/en/. Accessed February 2, 2017.

— **Book**

Author AA, Author BB. *Title of Work*. Location: Publisher; Year:Page-Page

Doane GH, Varcoe C. *Family Nursing as Relational Inquiry: Developing Health– Promoting Practice*. Philadelphia, PA: Lippincott Williams & Wilkins; 2005:25-28.
London ML, Ladewig PW, Ball JW, et al. *Maternal & Child Nursing Care*. Upper Saddle River, NJ: Pearson Education; c2011:101-103.
— Chapter in a book
Chapter Author AA. Title of chapter. In: *Name of Book*. Edition Number. Editor AA, ed. Location: Name of Publisher; Year:Page-Page.
Grimsey E. An overview of the breast and breast cancer. In: *Breast Cancer Nursing Care and Management*. 2nd ed. Harmer V, ed. Chichester, UK: Wiley-Blackwell; 2011:35-42.
NOTE: The Editorial Board requires consistent and carefully made references prepared according to the above-mentioned AMA standards. Otherwise, the work will be sent back to the authors.

Preparing figures, schemes and tables

File for Figures and Schemes must be provided during submission and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure (not on the figure itself) or above the table. All figures, schemes, and tables should be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).

Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Tables must be provided in an editable format in appropriate place in the main text. Tables provided as jpeg/tiff files will not be accepted. Do not submit your tables in separate files.

Abbreviations

The journal requires using only standard abbreviations. Abbreviations should be defined in parentheses the first time they appear in the abstract, main text and in figure or table captions and used consistently thereafter. Ensure consistency of abbreviations throughout the article. Keep abbreviations to a minimum.

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SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.