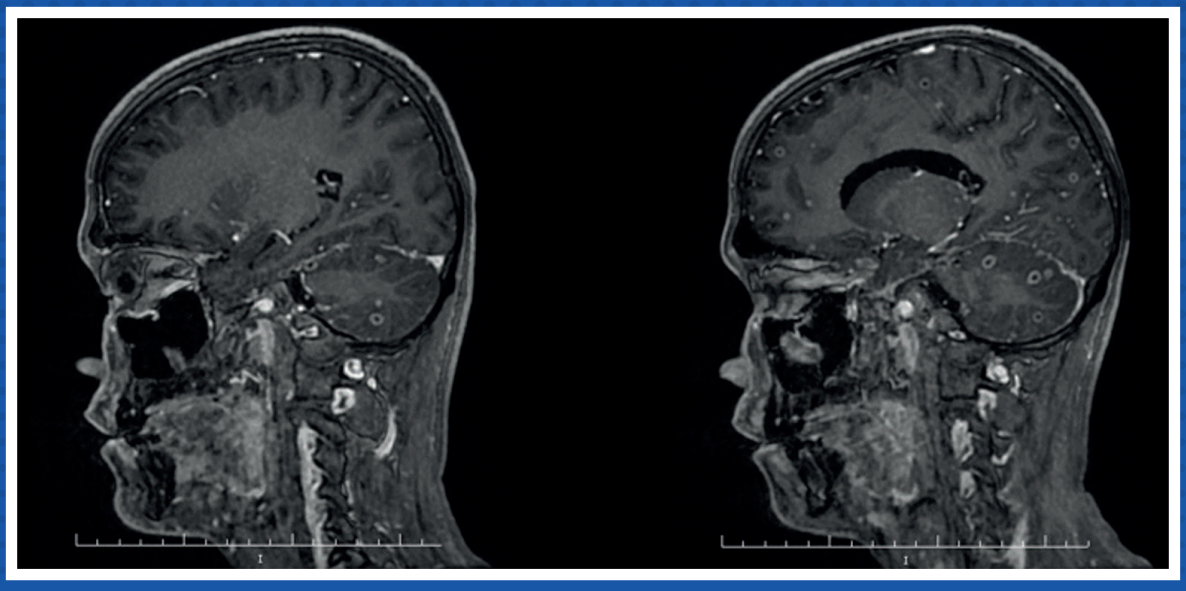
ISSN: 2979-9538

NORTHWESTERN MEDICAL JOURNAL

Year: 2025 ▪ Volume: 5 ▪ Issue: 2



Official Journal of Izzet Baysal Training and Research Hospital

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ISSN: 2979-9538

www.nwmedj.org

Official Journal of Izzet Baysal
Training and Research Hospital

Northwestern Medical Journal

ISSN: 2979-9538 (Online)

Owner
Owner on behalf of the Bolu İzzet Baysal Training and Research Hospital
Assoc. Prof. Süleyman Özsarı, MD
Director, Bolu İzzet Baysal Training and Research Hospital, Türkiye

Publication Type
International peer-reviewed journal

Publication Frequency and Language
Quarterly (January, April, July, October), English

Abstracting and Indexing
Northwestern Medical Journal indexed in TR Index, DOAJ, GALE, J-Gate, and TürkMedline.

Editor in Chief
Prof. Ahmet Ural, MD
Bolu Abant Izzet Baysal University, Faculty of Medicine, Department of Otorhinolaryngology, Head & Neck Surgery, Türkiye

Publisher
Izzet Baysal Training and Research Hospital

Publisher Address
Izzet Baysal Training and Research Hospital, Gölköy 14280 Bolu, Türkiye
Phone: +90 374 253 46 18 / +90 374 253 46 56
E-mail: nwmedj@gmail.com
Web: izzetbaysaleah.saglik.gov.tr

Publishing Services
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Editorial

Welcome to the April 2025 issue of our quarterly journal, a collection of ten compelling articles that reflect the dynamic and ever-evolving landscape of general medicine. As spring breathes new life into the world around us, this issue offers fresh insights and perspectives on a range of critical topics that directly impact clinical practice and patient care.

Within these pages, our dedicated authors delve into areas of significant contemporary relevance. You will find rigorous investigations into the early detection and management of prevalent chronic conditions, offering practical guidance for primary care physicians. Several articles explore innovative approaches to patient education and shared decision-making, underscoring the importance of empowering individuals in their healthcare journey. Furthermore, we present research examining the impact of emerging technologies on diagnostic accuracy and treatment strategies within the general practice setting.

We are particularly pleased to feature articles that address the complexities of multimorbidity and the challenges of providing holistic care to patients with multiple co-existing conditions.

The breadth and depth of the research presented here underscore the commitment of our contributors to advancing knowledge and improving patient outcomes in general medicine. Each article has undergone a rigorous peer-review process, ensuring the highest standards of scientific rigor and clinical relevance.

As editorial team, we extend our sincere gratitude to the authors for their dedication and scholarly contributions, and to our esteemed reviewers for their invaluable expertise and time. We trust that the findings and discussions within this issue will stimulate critical thinking, inform clinical practice, and ultimately contribute to the continuous improvement of healthcare delivery in the realm of general medicine.

We invite you to explore these ten insightful articles and engage with the knowledge they offer. Your ongoing support and readership are deeply appreciated as we continue our mission to disseminate high-quality research in general medicine every quarter.

We wish you a productive and insightful reading experience.

Sincerely,
Prof. Ahmet Ural, M.D.
Editor-in-chief

NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):61-76
DOI: 10.54307/NWMJ.2025.101

RESEARCH ARTICLE

In silico analysis of the whole genome of *Salmonella enterica*: genome assembly and annotation

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Cite as: Yavaş C, Şeflekçi Y, Eröz R. In silico analysis of the whole genome of Salmonella enterica: genome assembly and annotation. Northwestern Med J. 2025;5(2):61-76.

ABSTRACT

Aim: The goal is to classify *Salmonella enterica* using whole genome sequencing reads and explore their functional profiles. This approach simplifies resolving phylogenetic ambiguities in higher taxa compared to traditional methods.

Materials and Methods: Salmonella paired-end reads (SRA: SRR27334358) were obtained from the NCBI database and analyzed for quality using FastQC v0.12.1, with low-quality reads trimmed by Trimmomatic v0.36. De novo genome assembly was performed by using Unicycler v0.4.8, with subsequent gene annotation by using RAST. TYGS was utilized for taxonomic analysis. ResFinder v.2.1 identified antimicrobial resistance genes, and PathogenFinder v.1.1 was used for pathogenicity prediction. MLST analyzed the allele profile. CRISPR regions and proteins were identified by CRISPRCasFinder, while AntiSMASH 7.0.1 determined secondary metabolites. SPIFinder detected pathogenicity islands, and the genome map was created using the CGView server. RAST performed genomic functional classification.

Results: The genome, spanning 4,720,639 bp with 36 contigs, was analyzed by RAST, revealing 366 subsystems. TYGS showed a 100% dDDH with *S. enteritidis* ATCC 13076. The aac(6')-Iaa gene, conferring resistance to amikacin and tobramycin, was detected. PathogenFinder predicted *S. enterica* as a human pathogen with a 0.942 probability. MLST revealed 100% similarity with alleles of 7 housekeeping genes of Salmonella. CRISPRFinder identified eight Type I CRISPR-Cas proteins. AntiSMASH detected two secondary metabolites: enterobactin and O-antigen. SPIFinder identified 12 SPIs across the subspecies *S. Typhimurium*, *S. Typhi*, *S. Enteritidis*, *S. Choleraesuis*, and *S. Gallinarum*.

Conclusion: The genome showed 100% digital DNA-DNA hybridization (dDDH) with *Salmonella enteritidis* ATCC 13076 and was identified as a human pathogen. Recognizing pathogenic strains is crucial for timely intervention, control strategy design, and targeted vaccine development.

Keywords: *Salmonella enterica*, serovar, Salmonella Pathogenicity Islands, virulence

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Received: 13.03.2024 **Accepted:** 20.08.2024 **Published:** 28.04.2025

INTRODUCTION

Salmonella is a type of Gram-negative bacteria that can infect humans and various animals, leading to self-limiting enteritis or systemic disease. It is a member of the Enterobacter family, close to Escherichia, and consists of rod-shaped bacteria that do not form spores (1). According to the latest nomenclature accepted by the Centers for Disease Control (CDC), the genus Salmonella consists of *Salmonella enterica* and *Salmonella bongori* species (2). Among these species, *S. enterica* comprises six subspecies: *S. enterica subsp. enterica* (I), *S. enterica subsp. salamae* (II), *S. enterica subsp. arizona* (IIIa), *S. enterica subsp. diarizona* (IIIb), *S. enterica subsp. houtenae* (IV) and *S. enterica subsp. indica* (VI). *S. bongori* is rarely isolated from clinical specimens, it is found in cold-blooded organisms. Therefore, almost all Salmonella organisms that cause disease in humans and domestic animals belong to the *S. enterica subspecies enterica* (I) (3). Salmonella infections are usually caused by the consumption of contaminated food or water. More than 2600 pathogenic serovars/serotypes have been identified for the six subspecies of *Salmonella enterica* using the Kauffmann-White scheme (4). Among these serotypes, *Salmonella typhimurium*, *enteritidis*, *typhi*, *newport*, *heidelberg* and *paratyphi*, have been identified as important pathogens for humans and domestic animals (5). *Salmonella typhi* (*S. typhi*) and *S. paratyphi* cause typhoid fever, a systemic febrile disease that affects only humans. Other NTS serovars such as *S. typhimurium* and *S. enteritidis* infect many different hosts and cause diarrhea (6). Following ingestion, *S. enterica* (*S. enterica serovar typhimurium* and *S. enterica serovar enteritidis*) invades the intestinal epithelium in the colon and ileum and causes sepsis or spreads to systemic sites and causes neutrophilic gastroenteritis (7). Serotype cholerasuis is a host-compatible pathogen causing swine paratyphoid fever. It is also highly pathogenic for humans, usually causing a septicemic disease with little intestinal tract involvement (8). *Salmonella enterica serovar gallinarum* is a pathogen that is responsible for acute and chronic chicken typhoid fever (9).

S. enterica species are usually transmitted orally and cause four main syndromes: enteric fever (typhus), enterocolitis/diarrhea, bacteremia and chronic asymptomatic carriage. Disease manifestation relies on both susceptibility of the host and infectious *S. enterica serovar* (3). Pathogenic Salmonella species attack non-phagocytosing intestinal epithelial cells by transporting a specialized set of agents through an advanced mechanism called the type 3 secretion system (T3SS). This mechanism plays an essential role in the pathogenesis of Salmonella (10).

The Salmonella chromosome contains multiple virulence mechanisms to execute the pathogenic process. The most essential virulence genes are found in regions called Salmonella Pathogenicity Islands (SPIs) (11). Salmonella species use different virulence programs to interact with host defense mechanisms during infection, causing significant host immunopathology, morbidity and mortality (1). The ability of Salmonella to cause disease is related to its ability to survive in host cells. The genes used for this ability are located on pathogenicity islands such as SPI-1 and SPI-2 and encode two independent type III secretion systems (called T3SSSPI-1 and T3SSSPI-2). These mechanisms introduce effector proteins, which are essential throughout different phases of infection, into host cells. Currently, 17 SPIs thought to be acquired by horizontal gene transfers have been identified (12). Clusters of genes that contribute to specific phenotypes are typically found in pathogenicity islands, which usually manifest at particular stages during the infection process. Some SPIs are present in all *Salmonella serovars*, while others are present only in certain serotypes (13).

Today, with the advancement of sequencing technologies, the detection of genetic diseases has become both faster and more reliable (14-20). Therefore, sequencing methods and bioinformatics studies in humans and other organisms are becoming more and more important as time progresses. This research conducted a thorough examination of the complete genome of Salmonella enterica bacteria using sequence data acquired from the Illumina MiSeq platform. Within the framework of in silico analyses, the *Salmonella enterica* genome was assembled and annotated, and various in silico analyses such as taxonomic analysis, identification of antimicrobial resistance genes, identification of pathogenicity islands and CRISPR regions, and detection of MLST alleles and secondary metabolites were carried out.

MATERIALS AND METHODS

Acquisition of whole genome sequencing reads and analysis of genomic features

Raw Illumina paired-end reads of Salmonella with accession number SRR27334358 SRA were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/sra) in fastq format. Raw sequence reads were qualitatively evaluated using FastQC v0.12.1 (https://narrative.kbase.us). Low-quality reads were removed by Trimmomatic v0.36 (https://narrative.kbase.us). After this step, the quality of the reads was checked again with FastQC v0.12.1.

Genome assembly and annotation

The quality-controlled reads were de novo assembled by Unicycler v0.4.8 (https://narrative.kbase.us). Then, the quality of the sequence (genome size, contig number, N50 value, G+C content, etc.) was assessed using Quast v4.6.3 (https://narrative.kbase.us). Subsequently, the sequence was annotated using the Rapid Subsystem Technology (RAST) (https://rast.nmpdr.org/rast.cgi) tool.

Whole genome based taxonomic analysis

To conduct a comprehensive taxonomic analysis, the resulting assembled genome acquired by Unicycler v0.4.8 (https://narrative.kbase.us) was then submitted to the Type Strain Genome Server (TYGS) (https://tygs.dsmz.de). Then, the genome and phylogenetically related type strains were compared by Genome Blast Distance Phylogeny (GBDP). Inter-genome distances were estimated with 100 replicates utilizing the trim algorithm and the d5 distance formula. Using the recommended settings of the Genome-to-Genome Distance Calculator (GGDC) 2.1, digital DNA-DNA hybridization (dDDH) values and their associated confidence intervals were determined. The resultant inter-genome distances were utilized to build a balanced minimum evolutionary tree using FASTME 2.1.4 and SPR post-processing. The average nucleotide identity (ANI) of isolates was calculated using FastANI 0.1.2, utilizing the genomes of Enterobacter species that are closely related as reference genomes.

Identification of antimicrobial resistance determinants

Antimicrobial resistance genes in *Salmonella enterica* were identified from WGS data using ResFinder v.2.1 (https://www.genomicepidemiology.org).

Pathogenicity prediction

PathogenFinder v.1.1 (http://www.genomicepidemiology.org) was utilized to estimate the potential pathogenicity of *Salmonella enterica*.

Multiple locus sequencing typing (MLST) profile of *Salmonella enterica*

The MLST profile of *Salmonella enterica* was determined using MLST v.1.8 (http://www.genomicepidemiology.org) from WGS data using Illumina paired-end fastq files.

Predicting genomic islands and regularly interspaced short clustered palindromic repeats (CRISPR)

Genomic islands and regularly interspaced short clustered palindromic repeats (CRISPR) were predicted by CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr).

Secondary metabolite prediction

Gene clusters encoding secondary metabolites of known gene clusters were predicted by AntiSMASH 7.0.1 web server (https://antismash.secondarymetabolites.org/).

Prediction of pathogenicity islands

Salmonella Pathogenicity Islands (SPIs) were detected using SPIFinder (http://www.genomicepidemiology.org).

Circular genome mapping and gene prediction

The genome map of *Salmonella enterica* was predicted using CGView (https://cgview.ca). *Salmonella enteritidis* ATCC 13076 strain and its full sequence genome available in the NCBI database (S77744.1) were used as reference for comparison.

Genomic functional classification

The 4,720,639 bp draft genome was annotated with the help of the RAST (https://rast.nmpdr.org/rast.cgi) system. Genes involved in virulence, disease and defense mechanisms of Salmonella enterica genome and their functions were identified.

RESULTS

Genome assembly and annotation

The features of the draft complete genome sequence of *Salmonella enterica* were analyzed. The genome size of *S. enterica* is 4,720,639 bc with a G+C content of 52.1%. The draft genome consists of 4,744 coding sequences. The total number of RNAs is 79 and includes 366 subsystems (Table 1).

The RAST server provided information on 366 categorized subsystems, revealing the highest number of category features for carbohydrates (353), Amino acids and derivatives (346), protein metabolism (217) cofactors, vitamins, prosthetic groups, pigments (168), respiration (126), DNA metabolism and Stress response (88), Nucleosides and Nucleotides (82) (Figure 1).

Table 1. Genomic features of the genus *Salmonella enterica*

Description	Value
Chromosome size (bp)	4,720,639
GC content (%)	52,1
C366ontig (via PEG)	36
Contig N50	406,165
Contig L50	4
Number of Coding Sequences	4.744
Number of RNA	79
Number of Subsystems	366

Whole genome-based taxonomic analysis

According to the results of TYGS whole genome-based taxonomic analysis of *S. enterica*, the genome sequence analyzed is closer to *S. enteritidis* ATCC 13076 (Figure 2).

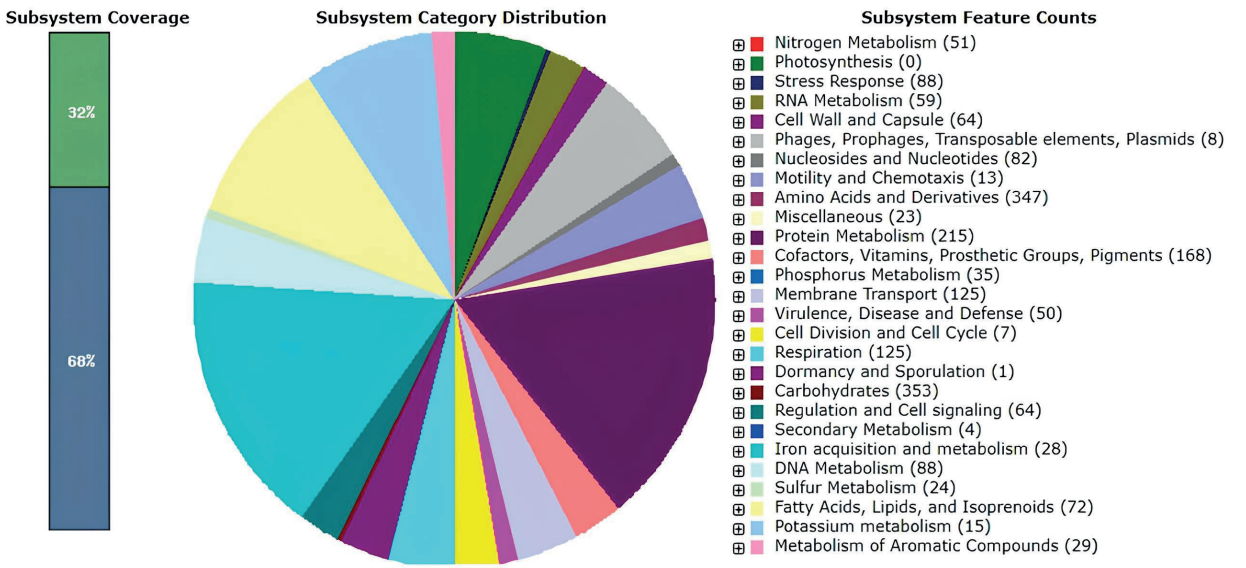

Figure 1. Subsystem distribution of the genus *Salmonella enterica* based on the RAST annotation server and functional classification of predicted genes in Salmonella.

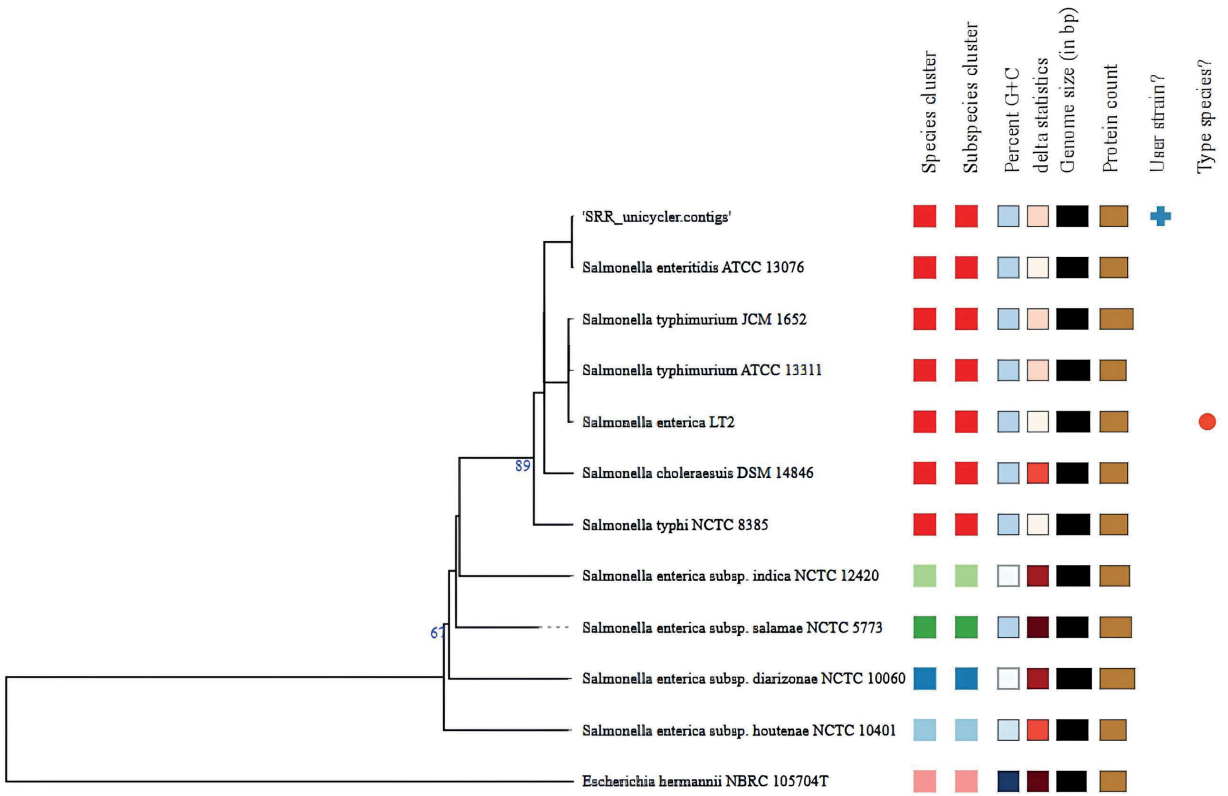

Figure 2. Tree inferred with FastME 2.1.6.1 from Genome Blast Distance Phylogeny (GBDP) distances calculated from whole genome sequences.

Table 2. Type Strain Genome Server (TYGS) calculation of digital DNA-DNA hybridization of *Salmonella enterica* and some closely related strains

Input Type	Output Type	dDDH (d4, %)	C.I. (d4, %)	G + C content difference (%)
SRR unicycler.contigs.fa	*Salmonella enteritidis* ATCC 13076	100.0	[99.9- 100.0]	0.02
SRR unicycler.contigs.fa	*Salmonella typhimurium* ATCC 13311	91.4	[89.3- 93.2]	0.01
SRR unicycler.contigs.fa	*Salmonella typhimurium* JCM 1652	91.2	[89.0- 92.9]	0.06
SRR unicycler.contigs.fa	*Salmonella enterica* LT2	90.9	[88.7- 92.7]	0.12
SRR unicycler.contigs.fa	*Salmonella choleraesuis* DSM 14846	90.3	[88.0- 92.2]	0.02
SRR unicycler.contigs.fa	*Salmonella typhi* NCTC 8385	86.1	[83.5- 88.4]	0.03
SRR unicycler.contigs.fa	*Salmonella enterica* subsp. salamae NCTC 5773	67.8	[64.8- 70.6]	0.04
SRR unicycler.contigs.fa	*Salmonella enterica* subsp. indica NCTC 12420	64.7	[61.7- 67.5]	0.55
SRR unicycler.contigs.fa	*Salmonella enterica* subsp. diarizonae NCTC 10060	62.0	[59.1- 64.8]	0.71
SRR unicycler.contigs.fa	*Salmonella enterica* subsp. houtenae NCTC 10401	60.5	[57.7- 63.3]	0.35
SRR unicycler.contigs.fa	*Escherichia hermannii* NBRC 105704T	21.1	[18.9- 23.6]	1.95

In addition to the resulting species and subspecies clusters, there is a taxonomic identification of *Salmonella enterica*. *Salmonella enterica* has 100% digital DNA-DNA hybridization (dDDH) and 0.02% G+C content difference with *Salmonella enteritidis* ATCC 13076, the closest strain genome in the TYGS database (Table 2).

Identification of antimicrobial resistance genes

Analysis with the ResFinder tool revealed aminoglycoside resistance genes. The aac(6')-Iaa gene, which has the potential to confer resistance to amikacin and tobramycin, was identified in *Salmonella enterica*.

Pathogenicity prediction

The pathogenicity of *Salmonella enterica* was estimated by comparing its proteins against a database of protein families associated with pathogenic and non-pathogenic bacteria. PathogenFinder predicted that *Salmonella enterica* is a human pathogen (probability of being pathogenic = 0.942). Proteins in *S. enterica* matched 1124 pathogenic families and 2 non-pathogenic families.

Multiple locus sequence typing (MLST)

It was revealed that Chorismate synthase (aroC_5), DNA polymerase III subunit beta (dnaN_2), uroporphyrinogen III synthase (hemD_3), histidinal dehydrogenase (hisD_7), phosphoribosylaminoimidazole carboxylase (purE_6), 2-oxoglutarate dehydrogenase decarboxylase (sucA_6), aspartokinase I (thrA_11) showed 100% similarity with alleles of the housekeeping gene in the *Salmonella enterica* genome.

Prediction of clustered regularly interspaced short palindromic repeats (CRISPRs)

The *Salmonella enterica* genome harbors two CRISPR sequences of 11 and 9 repeats, respectively, with a repeat length of 29 bc, matching two consensus sequences with evidence level 4 according to CRISPRFinder analysis. It also harbors 8 Type I CRISPR -Cas associated proteins (Table 3).

Secondary metabolites

In this study, two metabolite regions were detected using the AntiSMASH web server. Region 3.1 (location: 297,771- 352,985 nt) contained NRP-metallophore and NRP in contig 3; region 9.1 (location: 32,069-59,841 nt) contained thiopeptide in contig 6 (Table 4).

Table 3. Clustered regularly interspaced short palindromic repeats (CRISPR) sequences found in *Salmonella enterica* strain using CRISPRCasFinder

Element	CRISPR id/ Cas gene	Start	End	Spacer/ Gene	Repeat Consensus/Cas Gene	Direction	Level of Evidence
CRISPR	2_length_760858_1_14x_1	501789	502489	11	GTGTTCCCCGCGCCAGCGGGGATAAACCG	+	4
Cascluster	CAS-TypeIE	510092	518545	8	cas3_TypeI, cse1_TypeIE,cse2_TypeIE, cas7_TypeIE, cas5_TypeIE, cas6_TypeIE, cas1_TypeIE, cas2_TypeIE		
CRISPR	2_length_760858_1_14x_2	518642	519219	9	GTGTTCCCCGCGCCAGCGGGGATAAACCG	+	4

Table 4. Secondary metabolites identified by antiSMASH in *Samonella enterica*

Region	Type	Start	End	Most Similar Known Cluster	Similarity
Region 3.1	NRP- metalophor, NRPs	297,771	352,985	Enterobactin	100%
Region 9.1	Thiopeptide	32,069	59,841	O-antigen	14%

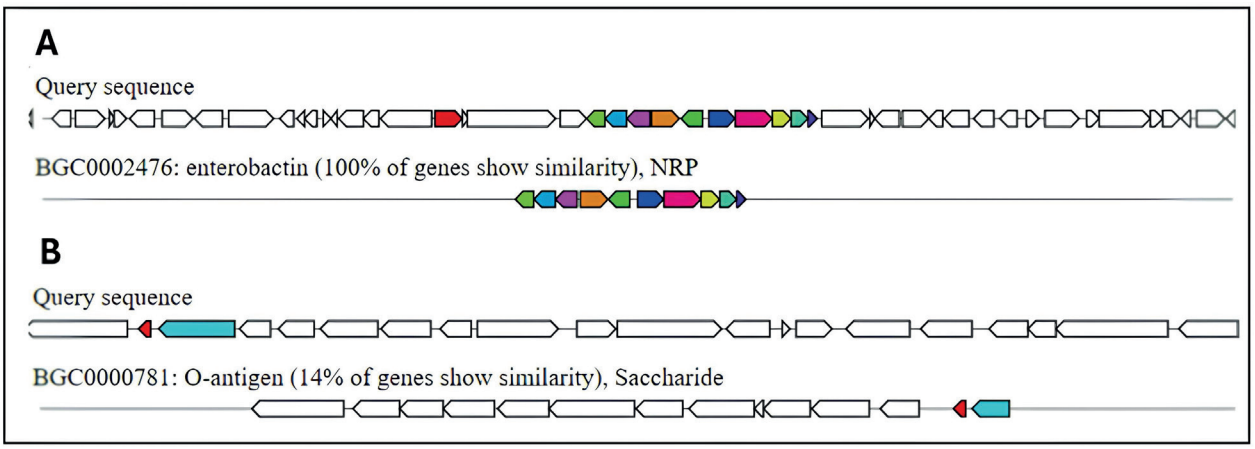

Figure 3. Biosynthetic gene clusters (BGCs) detected in the genome sequences of Salmonella enterica. A) Enterobactin biosynthetic gene cluster; B) O-antigen biosynthetic gene cluster.

Table 5. Pathogenicity islands of the *Salmonella enterica* genome analyzed with the SPIFinder tool

Subspecies	Serotype / Serovar	SPA	NCBI Number
Salmonella enterica - typhimurium	SL1344	C63PI	AF128999
	ATCC_14028	CS54_island	AF140550
	J4STEHO	SPI-1	JN982040
	SL1344	SPI-1	U16303
	SL1344	SPI-1	AF148689
	LT2	SPI-2	X99945
	Salmonella-enterica-Typhimurium-	SPI-2	Z95891
	14028s	SPI-3	AJ000509
	14028s	SPI-3	Y13864
	LT2	SPI-5	NC_003197
Salmonella-Typhi	CT18	SPI-9	NC_003198
Salmonella enterica- enteritidis	CMCC50041	Not_named	JQ071613
	C50041	SPI-2	JN673273
	Se11	SPI-2	JN673270
Salmonella enterica - choleraesuis	SC_B67	SPI-4	NC_006905
Salmonella enterica-gallinarum	SGB_1	SPI-1	AY956822
	SGE_2	SPI-1	AY956823
	SGB_4	SPI-1	AY956824
	SGB_8	SPI-1	AY956825
	SGE_3	SPI-10	AY956839
	SGD_3	SPI-13	AY956832
	SGA_10	SPI-13	AY956834
	SGA_8	SPI-14	AY956835
	SGC_8	SPI-14	AY956836
	SGB_10	SPI-2	AY956826
	SGC_2	SPI-2	AY956827
	SGC_9	SPI-2	AY956828
	SGH_1	SPI-2	AY956829
	SGD_8	SPI-2	AY956830

When the most similar gene clusters were analyzed by comparison with the MiBIG database, two cluster regions were identified. The first of these gene clusters was the NRP biosynthetic gene cluster from Escherichia coli (21) and the O-antigen biosynthetic gene cluster from Pseudomonas aeruginosa (Figure 3).

Salmonella pathogenicity islands (SPI)

As a result of the analysis performed with SPIFinder 2.0, 29 Pathogenicity Islands belonging to the total Salmonella genome were detected. As a result of the analysis, 6 pathogenicity islands belonging to 10 serovars of *S. typhimurium* subspecies, 1 belonging to one serovar of *S. typhi* subspecies, 2 belonging to 3 serovars of *S. enteritidis* subspecies, 1 belonging to one serovar of *S. choleraesius* subspecies and 5 belonging to 14 serovars of *S. Gallinarum* subspecies were identified (Table 5).

Circular genome mapping and gene prediction

The genome contains 4510 genes, 4427 protein-coding genes (CDS), 77 tRNA, 3 rRNA, 2 repetitive regions and 1 tmRNA copy. The physical genome map of *Salmonella enterica* was obtained by comparison with the reference strain *Salmonella enteritidis* ATCC 13076 (Figure 4). Prokka was used for position prediction, while BLAST was used to obtain information on function and identification in the nucleotide and protein sequence database against the assembled sequences.

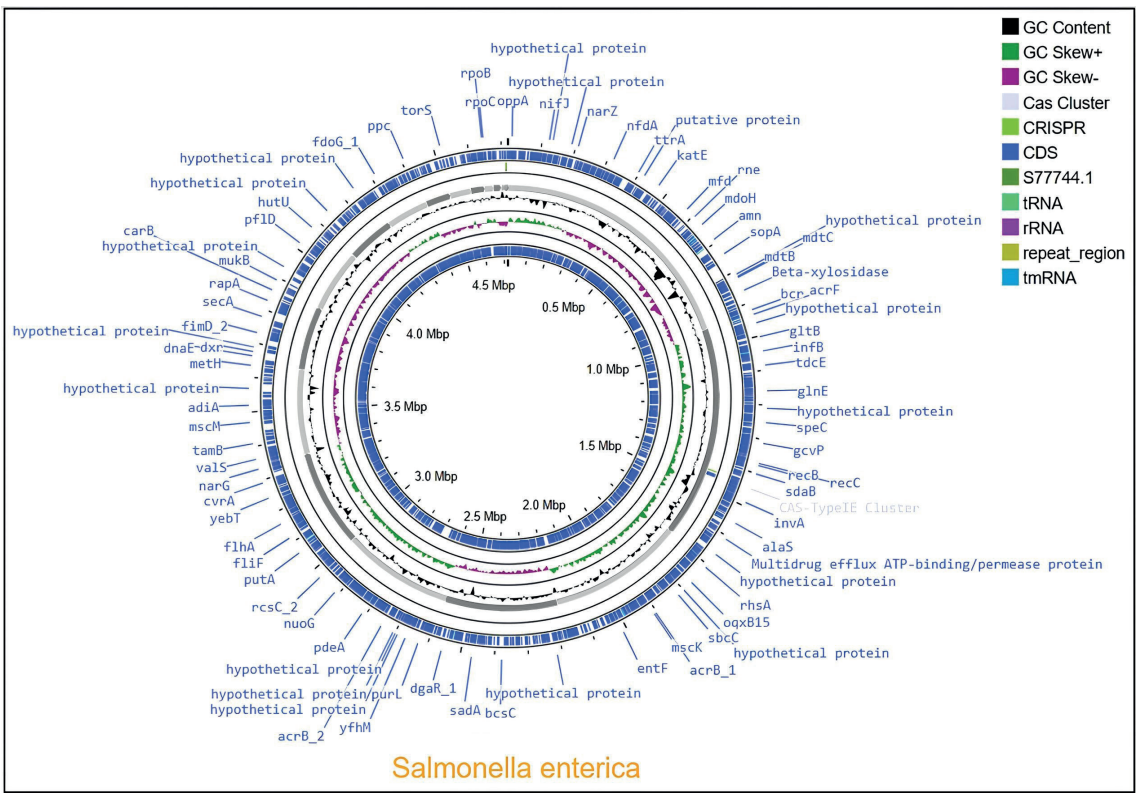

Figure 4. Circular map view of the *Salmonella enterica* genome generated using the CGView Server. The contents are organized starting from the outermost ring: the outermost first and last rings show the Prokka annotation (+/- helix) together with coding sequences (CDS), tRNA, rRNA, tmRNA and repetitive regions; the second ring represents the map of the reference taxon Salmonella enteritidis ATCC 13076; third ring shows CRISPRCasFinder anotation (+ helix); fourth ring shows contigs; fifth ring shows GC content; sixth ring shows GC skew information; seventh ring shows CRISPRCasFinder anotation (- helix).

Genomic functional classification

The genes of *Salmonella enterica* genome that play a role in virulence, disease and defense mechanisms and their functions were determined. It was determined that *S. enterica* has genes that function against bacteriocins, adhesion, invasion and intracellular resistance and antibiotics and toxic compounds (Table 6).

DISCUSSION

In this study, reads of Illumina sequencing of *Salmonella enterica* coded SRR27334358 in the Sequence Read Archive (SRA) database were merged and annotated. Salmonellosis is an essential public health problem caused by Salmonella bacteria and causes a significant increase in morbidity and mortality.

Table 6. Characteristics of Salmonella enterica that play a role in virulence, disease and defense mechanisms

Subcategory	Subsystem	Description				Function
		From	To (bp)	Size (bp:aa)	Contig	
Bacteriocins are antibacterial peptides that are synthesized by ribosome	Tolerance to Colicin E2	77300	76812	489;163	10	Conserved undefined protein CreA
		74628	73279	1350;450	10	Inner membrane protein CreD
Adhesion	YidE mediates hyperadherence in enterobacteria and in the conserved zone	403542	403114	429;143	4	16 kDa heat shock protein B
		404071	403652	420;140	4	16 kDa heat shock protein A
		405935	404709	1227;409	4	Uncharacterized YidR protein
		404417	404707	291;97	4	Outer membrane YidQ lipoprotein
Invasion and intracellular resistance	Mycobacterium virulence operon involved in DNA transcription.	4199	8227	4029;1343	15	DNA-directed RNA polymerase beta subunit
		8304	12527	4224;1408	15	
	Mycobacterium virulence operon likely involved in quinolinate biosynthesis	480054	481157	1104;368	3	Kinolinat sentetaz
		15968	14346	1623;241	5	L-aspartate oxidase
		123211	124104	894;298	8	Quinolinate phosphoribosyltransferase [decarboxylating]
	Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	3405	3031	375;125	4	SSU ribosomal protein S12p (S23e)
		2935	2465	471;157	4	SSU ribosomal protein S7p (S5e)
		2368	254	2115;705	4	Translation Elongation factor G
		2	82	81;27	15	Translation Elongation factor Tu
		26072	25992	81;27	16	
		1103	3	1101;367	18	
		182	3	180;60	4	
	Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	451004	450570	435;145	1	Translation initiation factor 3
		450474	450277	198;66	1	LSU ribosomal protein L35p
		450226	449870	357;119	1	LSU ribosomal protein L20p
Resistance to antibiotics and toxic compounds	Copper homeostasis	78317	80620	2304;768	3	ATPase transporting lead, cadmium, zinc and mercury; P-type ATPase transporting copper
		240386	237885	2502;834	3	
		142376	144574	2199;733	4	

Table 6. Continued

Subcategory	Subsystem	Description				Function
		From	To (bp)	Size (bp:aa)	Contig	
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper torelance	95582	93972	1611;537	8	CueO precursor to blue copper oxidase.
		935	3	933;311	17	Cytochrome c heme lyase subunit CcmL /
		3417	1486	1932;644	17	
		406164	406057	108;36	4	
		2	109	108;36	6	Cytochrome c heme lyase subunit CcmH
		210893	211267	375;125	6	Copper resistance protein CopC
		211268	212143	876;292	6	Copper resistance protein CopD
		64440	64802	363;121	6	Suppression of copper sensitization: putative copper-binding protein ScsA.
		64851	66737	1887;629	6	Membrane protein, copper sensitivity suppressor ScsB
		66734	67357	624;204	6	The secreted protein is the copper sensitization suppressor ScsC.
		67347	67853	507;169	6	Membrane protein, copper sensitivity suppressor ScsD
		151391	151738	348;116		Periplasmic divalent cation tolerance protein CutA
		179718	180464	747;249	6	Cytoplasmic copper homeostasis protein CutC
		7186	6485	702;234	8	Copper homeostasis protein CuffF precursor / Lipoprotein NlpE involved in surface adhesion
		404058	402469	1590;530	3	Apolipoprotein N-acyltransferase / Copper homeostasis protein CutE
		404905	404027	879;293	3	Magnesium and cobalt streaming protein CorC
	Mercury reductase	300227	301552	1326;442	3	Putative Dihydrolipoamide dehydrogenase; Mercury ion reductase; PF00070 family, FAD-dependent NAD(P)-disulfide oxidoreductase.
	Resistance to fluoroquinolones	377955	380591	2637;879	5	DNA gyrase subunit A
		22754	20340	2415;805	13	DNA gyrase subunit B
	Cobalt-zinc-cadmium resistance	482836	481898	939;313	3	Zinc carrier ZitB
		80617	81081	465;155	3	Transcriptional regulator, MerR family
	Zinc resistance	26727	28124	1398;466	15	Sensor protein of the zinc sigma-54-dependent two-component system
		28130	29455	1326;442	15	
	Adaptation to D-cysteine	138465	139451	987;329	6	D-cysteine desulfhydrase

There are more than 2600 serovars of Salmonella species and they are transmitted by fecal or oral route through contaminated water and food (4). Salmonella serotyping has an important function in diagnosis and detection. Serovar prediction by conventional serotyping may be limited due to lack of surface antigen expression or autoagglutination (22). Recently, serovar identification can be performed with the advancement of whole genome sequencing platforms. Many research have used whole genome sequencing-based genomic comparison to identify serovar-specific genes or DNA segments for serotyping (23). In this study, we tried to show that serovar identification by genome sequencing technology is more efficient than traditional methods.

To affiliate the species, the DNA-DNA hybridization threshold should be 70% as a main criteria (24). Digital DNA-DNA hybridization (dDDH) calculated by the Genome-Genome Distance Calculator (GGDC) between the analyzed genome and *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 13311, *Salmonella typhimurium* JCM 1652, *Salmonella enterica* LT2, *Salmonella cholerasuis* DSM 14846 and *Salmonella typhi* NCTC 8385 species was well above the threshold of 70%. In particular, the dDDH value obtained for *Salmonella enteritidis* ATCC 13076 was 100%. GGDC results have high average branch support and low delta values, indicating high phylogenetic accuracy. With whole genome sequencing, the functional profiles of taxonomic groups can be elucidated and species can be defined to a large extent and uncertainties in the phylogeny of high taxa, which may be difficult with traditional approaches, can be easily resolved (25).

The aac(6')-Iaa gene, which has the potential to confer resistance to the aminoglycoside antibiotics amikacin and tobramycin, was detected in the analyzed Salmonella genome. Enzymes that alter the drugs through acetylation, adenylation, or phosphorylation mediate resistance to these antibiotics. AAC(6′) enzymes make their subsrtaes inactive by acetylating them at the 6′ position of aminoglycosides. The aac(6′)-Iaa gene is also responsible for aminoglycoside resistance. Antimicrobial resistance genes to aminoglycosides are the most diverse and most frequently identified genes in Salmonella strains (26).

Identification of pathogenic bacterial strains and understanding their biological processes related with pathogenicity are essential for timely intervention, design of control strategies and development of targeted vaccines. The pathogenicity of the analyzed genome was predicted with the PathogenFinder algorithm (27). The analysis predicted that *Salmonella enterica* is a human pathogen (probability of pathogenicity = 0.942).

For Salmonella to exert pathogenicity, virulence genes assembled in its genome, called SPIs, must be expressed in a coordinated manner (28). As a result of the analysis performed with SPIFinder, a total of 29 pathogenicity islands belonging to the Salmonella genome were detected. The detected pathogenicity islands belong to *S. typhimurium*, *S. typhi*, *S. enteritidis*, *S. cholerasuis* and *S. gallinarum* subspecies. Among these subspecies, the highest number of pathogenicity islands (6) was detected in *S. typhimurium* serovar. *Salmonella enterica* serovar *typhimurium* is the primary enteric pathogen that infects both humans and animals (11). In addition, horizontal gene transfer is important for *S. typhimurium* serovars. Therefore, they can infect many cell types and cause severe infections with small changes in virulence genes by using pathogenicity islands (29). C63PI, CS54_island, SPI-1, SPI-2, SPI-3 and SPI-5 pathogenicity islands were detected in *S. typhimurium* serovars analyzed in silico. C63PI is the iron transport system in SPI-1 and mediates the entry of Salmonella into the host cell (30). The presence of C63PI in this serovar indicates that iron is indispensable for its survival. The CS54 island has an important role in intestinal colonization and persistence. The Type 3 Secretion System (T3SS) encoded by SPI-1 and SPI-2 (30) is required for Salmonella invasion of intestinal epithelial cells and is therefore essential for intestinal colonization causing to enteritis (13). Thus, the T3SS has an essentila function in Salmonella pathogenesis (10). So far, five SPIs (SPI-1-SPI-5) have been determined that are clearly included in *S. typhimurium* virulence. SPI-2 and SPI-3 are essential for the growth

and survival of bacteria within the host, which occurs during the systemic phase of the disease. The recently identified virulence factors encoded by SPI-5 appear to have a role in the inflammation and chloride secretion that characterize the enteric phase of the disease (31). SPI-9, detected in *S. typhi* serovars, is included in the transport and metabolism of specific nutrients essential for Salmonella survival in the host (32). SPI-10 is presumed to play a role in host specificity (33). SPI-13 and SPI-14 were first determined in avian-adapted *S. gallinarum*, the causative agent of typhoid fever in poultry (34).

The advantages of CRISPRs as a subtyping tool in Salmonella have been demonstrated in studies. Serotyping has been used as the reference method for Salmonella typing for almost 80 years (35). CRISPRs have been identified in the genomes of many archaea and bacterial species, including Salmonella (36). Salmonella has a type I E CRISPR-Cas system consisting of two CRISPR sequences (CRISPR-I and CRISPR-II) and a cas operon (37). Salmonella has two CRISPR loci, CRISPR1 and CRISPR2, separated by ~16 kb and sharing the same consensus direct repeat (DR) sequence (29 nt) and 32 nt long spacer (38). There are eight cas genes characteristic of the type I-E CRISPR-Cas system: cas3, cse1, cse2, cas7, cas5, cas6, cas1 and cas2. These genes are located at the CRISPR1 locus (39). Furthermore, all Salmonella analyzed to date harbor only the type I-E system (40). As a result of analysis with CRISPRFinder, the type I-E CRISPR-Cas system was identified in the Salmonella genome. In addition, cas1, cas2, cas3, cas3, cas7, cas5, cas6, cse1 and cse2 genes were identified.

The secondary metabolite analysis identified two gene clusters, non-ribosomal peptide synthase (NRPs) and thiopeptide, potentially related to the biosynthesis of secondary metabolites. Enterobactin from the NRPs gene cluster and O-antigen compounds from the thiopeptide gene cluster were identified. Since iron is an essential cofactor for processes such as energy production and DNA replication, iron retention offers an effective antimicrobial defense. Salmonella-infected macrophages increase iron export, thus Salmonella proliferation is limited by low iron levels in macrophages, highlighting the importance of identifying the iron uptake mechanisms of Salmonella in these iron-deficient environments. Enterobactin, a class of catecholate siderophores, are small iron chelators secreted by Salmonella that facilitate the transport of iron into bacterial cells (41). Lipopolysaccharides (LPS), the main part of the outer membrane of gram-negative bacteria, are important virulence factors of bacteria (such as Salmonella species) that are pathogenic in animals and humans. O-antigens are structures that are an important component of LPS that contribute to the diversity of the cell wall of gram-negative bacteria (42). O-antigen diversity is a fundamental criterion in Salmonella serotyping. O-antigen identification has resulted in the serologic identification of more than 2000 Salmonella strains. The presence of O-antigen is also important for the survival of bacteria in their natural environment and has a role in bacterial virulence. There is direct evidence that loss of the O-antigen sensitizes many pathogens to serum or otherwise severely impairs virulence (43).

Accurate typing and monitoring is important for microbial epidemiological research, food safety and public health. Bacterial typing methods are divided into phenotyping and genotyping. Among these, serotyping and multilocus sequence typing (MLST) are the most commonly utilized despite low resolution (35). MLST is an attractive method because it is an easily implemented protocol and is well associated with most lineages and serovars through eBGs. The advantages of MLST are more uniformity, better association with serotypes and accessibility to databases (44). MLST analysis resulted in 100% similarity with seven Salmonella housekeeping genes (aroC, dnaN, hemD, hisD, purE, sucA and thrA) (45). This indicates that the analyzed genome belongs to *Salmonella enterica*.

It was also identified 50 gene clusters of the Salmonella enterica genome are responsible for virulence, disease and defense mechanisms. Genes with functions such as bacteriocins, adhesion, invasion and intracellular resistance and resistance to antibiotics and toxic compounds were identified. In the subcategory of bacteriocins, ribosomally synthesized antibacterial peptides, the conserved, uncharacterized protein CreA, included in tolerance to colicin E2, needs to be characterized. Bacteriocins produced by enterobacteria (*E. coli*, Salmonella and relatives) are called colicins.

Colicins are bacterial protein toxins that has strong activity against sensitive strains in vitro (46). Such bacteriocins kill other bacteria with a high specificity (47). The tolerance of Salmonella to colicin E2 by RAST analysis indicates that the bacterium has the ability to withstand or resist the inhibitory effects of colicin E2. Thus, Salmonella bacteria can survive and continue to grow in the presence of bacteriocin. In addition, overexpression of creD protein, an inner membrane protein, is responsible for colicin E2 tolerance (48). Genes responsible for adhesion were also identified by RAST annotation. The mediator of hyperadhesion YidE in enterobacteria and its conserved region were predicted in the isolate. The pathogenesis of infections caused by *S. enterica* requires adhesion to various host cell surfaces (49).

Genomic functions associated with resistance to antibiotics and toxic compounds have also been determined. These functions include copper homeostasis and copper tolerance. Bacteria must maintain complete copper homeostasis to prevent copper-mediated toxicity while ensuring copper supply for copper-demanding proteins. In order to do this, copper-sensing transcriptional regulators must differentiate copper from other metal ions and, in reaction to levels above or below a threshold, initiate the proper physiological response, such as copper import, export, and detoxification. (50). Another defined function is mercury reductase. Mercury ion reductase (MerA), a mercury detoxification enzyme used by microorganisms, has high specificity for mercury ions (Hg2+) and functions to catalyze their reduction to a more volatile, less toxic elemental form (51). MerA (mercury reductase) is found in the mer operon responsible for the detoxification of inorganic (Mg(II)) mercury in many bacteria growing in Hg-contaminated environments (52). Fluoroquinolone resistance in bacteria arises as a result of changes in DNA gyrase enzymes found in gram-negative bacteria (53). Especially mutations in gyrA and gyrB genes contribute to fluoroquinolone resistance. Thus, some serovars continue to show their virulence effects by developing resistance or becoming less susceptible to fluoroquinolones, the antibiotics used against Salmonella (54). Resistance genes to cobalt, cadmium and zinc have also been found in the Salmonella genome. These heavy metals show toxic effects when used in high concentrations. Genes related to these heavy metal resistance have been detected in many isolates of Salmonella (55). Finally, there is adaptation to d-cysteine in the genome. Genes involved in adaptation to D-cysteine, a potent growth inhibitor, were obtained as a result of the analysis.

As a result, Illumina sequence reads of the *Salmonella enterica* genome were analyzed by in silico tools and annotation was performed. As a result of RAST analysis, 366 subsystems were identified. 50 of these subsystems are responsible for virulence, disease and defense. TYGS analysis showed that it has 100% digital DNA-DNA hybridization with *Salmonella enteritidis* ATCC 13076. The aac(6')-Iaa gene, which has the potential to confer resistance to amikacin and tobramycin, was found in the analyzed genome. The pathogenicity prediction predicted that Salmonella enterica is a human pathogen (probability of pathogenicity = 0.942). MLST analysis revealed 100% similarity with alleles of the Salmonella enterica housekeeping gene. CRISPR analysis identified 8 Type I CRISPR -Cas associated proteins. In addition, two secondary metabolites (enterobactin and O-antigen) were found as a result of secondary metabolite analysis. Finally, 6 pathogenicity islands belonging to 10 serovars of *S. typhimurium* subspecies, 1 serovar of *S. typhi* subspecies, 2 serovars of 3 serovars of *S. enteritidis* subspecies, 1 serovar of *S. cholerasuis* subspecies and 5 pathogenicity islands belonging to 14 serovars of S. *gallinarum* subspecies were identified.

Our primary goal in this work is to clarify the taxonomic classification and functional characteristics of *Salmonella enterica*, a pathogenic bacteria that causes serious illness in humans, using reads of whole genome sequencing. We also aim to easily resolve uncertainties in the phylogeny of higher taxa that are difficult to resolve by traditional methods. Identification of pathogenic bacterial strains and understanding the biological mechanisms associated with pathogenicity are also essential for timely intervention, design of control strategies and development of targeted vaccines.

Ethical approval

Our study is not in-vitro, in-vivo or survey, it was designed through a bioinformatics perspective. Therefore, there is no need for ethical approval.

Author contribution

Consept: YŞ; Data Collection or Processing: YŞ; Analysis or Interpretation: YŞ, CY; Design: YŞ, CY; Literature researh: YŞ, CY, RE. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):77-84
DOI: 10.54307/NWMJ.2025.120

RESEARCH ARTICLE

Pinna anthropometry in sex estimation: a machine learning-based approach

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Cite as: Söylemez E, Tokgöz Yılmaz S. Pinna anthropometry in sex estimation: a machine learning-based approach. Northwestern Med J. 2025;5(2):77-84.

ABSTRACT

Aim: The pinna, the hearing organ, also contributes to the aesthetic appearance of the face. We aimed to investigate the feasibility of sex prediction using anthropometric measurements of the pinna in machine learning.

Methods: The study included two hundred healthy individuals (104 women and 96 men). The pinna of these individuals were measured in eight different parts using a digital calliper. The data, which differed by sex, were processed in eight different machine-learning algorithms.

Results: Seven different measurements, such as pinna length, width and lobule length, were greater in men than in women ($p<0.05$). The K-Nearest Neighbor model showed the best success in sex prediction with an accuracy of 0.825 and a ROC value of 0.882.

Conclusions: Pinna's anthropometric measurement values can be used in machine learning to predict sex with a high success rate. Our study shows that ear prints may have potential use in forensic identification.

Keywords: anthropometry, machine learning, pinna, sex estimation

INTRODUCTION

The external ear consists of the pinna and the external ear canal. While the pinna captures the sound wave, the outer ear canal transmits the sound wave to the middle ear. In addition to hearing, the outer ear helps diagnose congenital anomalies and syndromes (1). Changes in the shape and size of the auricle and its position on the head help to identify anomalies and syndromes. While the pinna is large in individuals with Apert and Crouzon syndromes, the pinna is small in individuals with Down syndrome and cleft palate/lip (1). Additionally, pinna deformities can be seen in trisomy 13 and 18 (2).

The appearance of the pinna, its symmetry and harmony with age, sex and face are important for

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Received: 02.06.2024 **Accepted:** 27.08.2024 **Published:** 28.04.2025

facial aesthetics. Any deformity in the size, shape or position of the pinna, small size (microtia) or absence (anotia) of the pinna can be corrected by aesthetic and plastic surgery. For all of these surgical corrections and the creation of artificial ear prostheses, normative dimensions of the auricle are required (3). In addition, these dimensions are also crucial for the ergonomic production of hearing aids/cochlear implants used in hearing (re)habilitation.

Anthropometry refers to the study of the dimensions of different parts of the body. Due to its position relative to the head and its many indentations and protrusions, the auricle can be measured using direct or indirect anthropometric methods. Direct measurements use calipers or tape measures to measure the features of the pinna, such as width and length. In indirect measurements, measurements are obtained from various radiologic images and photographs (4). These anthropometric measurements of the pinna may vary between ethnic groups and sexes (1,5). Although the size of the pinna increases with age, the ratio between their sizes remains constant (4). Additionally, pinna measurements (indirect) require less spatial resolution and are less affected by light changes. For these reasons, pinna measurements perform better than facial measurements in terms of diagnosis and sex prediction (6). The Forensic Ear Identification Project is working to use earprints to identify criminals and suspects, similar to fingerprints, retinas and DNA (7). For this reason, it is believed that the pinna, which has distinct, permanent, and unpredictable biometric features, will be widely used for identification and verification purposes in forensic medicine.

Some studies have investigated the anthropometric characteristics of the pinna in the Turkish society (6,8,9). However, there are limited studies using anthropometric measurements of the pinna in machine learning for sex prediction (6). This study aims to investigate the feasibility of sex prediction with machine learning using anthropometric measurements of the pinna in Turkish people. The relationship between pinna and age, height, weight and body mass index (BMI) was also investigated.

MATERIAL AND METHOD

Permission was received from the ethics committee of Karabük University for this prospective study (2023/3 Decision no: 25). Written and verbal informed consent was obtained from all individuals included in the study.

Two hundred healthy individuals were included in the study. The study excluded participants with any pinna anomalies, deformities, microtia or anotia. The age, sex, height and weight information of these individuals were recorded. Participants were divided into two groups based on sex (Female: 104, Male 96). The participants' pinnae were measured in millimetres from 8 measurement points using a digital caliper (Figure 1). These measurement points are labeled a, b, c, d, e, f, g and h. The boundaries of the measurement points were created as stated by Petrescu et al. (10).

[a]: Superaurale- Subaurale
[b]: Protragion- Helix (Distance from tragus to helix) (11)
[c]: Incisura intertragica inferior- Postaurale
[d]: Incisura intertragica inferior- Otobasion inferior
[e]: Lobule anterior- Lobule posterior
[f]: Protragion- Strongest anthelical curvature
[g]: Incisura anterior auris posterior- Postaurale
[h]: Incisura anterior auris posterior- Stobasion superior

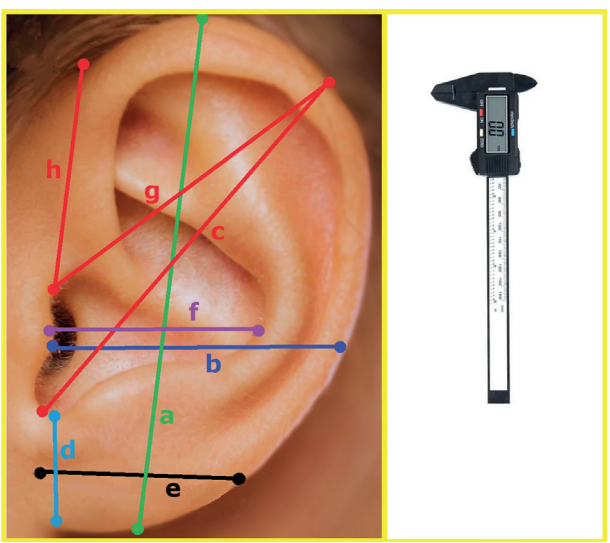

Figure 1. Measurement points taken on the pinna.

Statistical analysis and machine learning models

Statistical analysis was performed to determine the attributes to build machine learning models. Statistical analysis was carried out using the SPSS 21 IBM program. Shapiro-Wilk test was used to check the normality of the data. Student's t-test was used to analyze normally distributed data, and Mann-Whitney U tests were used to analyze non-normally distributed data. The statistical significance level was accepted as $p<0.05$. The significant data between the groups were used as input (feature) in machine learning models. Python (Version 3.7) programming language was used for machine learning models. Machine learning consists of supervised, unsupervised, and reinforcement learning types. Supervised learning algorithms are often used for classification and regression problems in medical and health sciences. In our study, K-Nearest Neighbor (KNN), Decision Trees (DT), Random Forest (RF), Support Vector Machine (SVM), Logistic Regression (LR), XGBoost, and Artificial Neural Network (ANN) were used as machine learning algorithms. We determined the best set of hyperparameters using the Grid Search method. While modeling the algorithms, 96 (80%) of the data were used in the training process, and the remaining 24 (20%) were used in the testing process. The success of the models was evaluated according to the accuracy in the testing phase and the area under the ROC curve (AUC).

RESULTS

The average age of the 104 women included in the study was 20.63±3.30 (range 18-35) years, and the mean age of the 96 men was 21.41±4.05 (range 17-40) years. There was no difference in age between the groups (p:0.137).

The values obtained from the measurement points of the right and left pinna based on sex are presented in Table 1. The number of pinnae (n: 400) was used to compare pinna measurements based on sex and to investigate the relationship between pinna measurements and age, height, weight, and body mass index (BMI). There was a positive correlation between age and lengths of [b], [c], [g] and [h]; a positive correlation between height and lengths of [a], [b], [c], [d], [f], [g] and [h]; a positive correlation between weight and lengths of [a], [b], [c], [d], [e], [f], [g] and [h]; and a positive correlation between BMI and lengths of [a], [b], [c], [d], [e], [g] and [h] ($p<0.05$). The relationship between pinna measurements and height, weight, and BMI is presented in Table 2. The lengths of [a], [b], [c], [d], [f], [g] and [h] were greater in men than in women ($p<0.05$). However, there was no difference in [e] length between the sexes ($p>0.05$). Pinna lengths by sex are presented in Figure 2.

Table 1. Values measured in the right and left pinna according to sex

	Female			Male		
	Right	**Left**	**Mean**	**Right**	**Left**	**Mean**
[a] mm	59.68±3.78	59.58±3.79	59.63±3.77	63.80±4.51	63.81±4.31	63.80±4.40
[b] mm	25.11±3.29	25.79±3.60	25.45±3.45	26.86±2.20	27.23±2.53	27.05±2.37
[c] mm	43.80±3.69	44.24±3.02	44.02±3.37	47.70±3.24	47.80±3.51	47.75±3.37
[d] mm	17.09±2.59	17.12±2.97	17.11±2.77	17.85±2.49	18.00±2.49	17.93±2.48
[e] mm	16.14±3.50	17.13±3.50	16.64±3.53	16.78±3.66	17.31±3.31	17.05±3.49
[f] mm	17.81±2.52	18.17±2.53	17.99±2.54	18.65±2.78	18.80±2.27	18.72±2.53
[g] mm	31.50±3.58	31.48±4.09	31.49±3.83	33.03±2.88	33.08±3.83	33.07±3.38
[h] mm	20.58±3.15	20.69±3.12	20.63±3.13	21.42±2.53	21.69±2.52	21.55±2.52

Table 2. Relationship between pinna measurement points and height, weight and BMI

		Age (year)	Height (cm)	Weight (kg)	BMI
	Mean±Sd	21.00±3.68	170.27±9.13	66.81±14.71	22.86±3.74
		Correlation coefficient (p value)			
[a] mm	61.63±4.58	0.6 (0.264)	4.6 (<0.001)	5.2 (<0.001)	3.8 (<0.001)
[b] mm	26.22±3.09	1.0 (0.047)	2.9 (<0.001)	3.1 (<0.001)	1.9 (<0.001)
[c] mm	45.81±3.78	1.9 (<0.001)	4.3 (<0.001)	5.0 (<0.001)	3.7 (<0.001)
[d] mm	17.50±2.66	0.1 (0.736)	2.0 (<0.001)	3.7 (<0.001)	3.6 (<0.001)
[e] mm	16.83±3.51	-0.7 (0.147)	0.8 (0.093)	2.0 (<0.001)	2.1 (<0.001)
[f] mm	18.34±2.56	0.5 (0.281)	1.0 (0.042)	1.2 (0.015)	0.7 (0.115)
[g] mm	32.25±3.70	1.3 (0.007)	2.4 (<0.001)	3.2 (<0.001)	2.5 (<0.001)
[h] mm	21.08±2.89	1.2 (0.011)	2.4 (<0.001)	2.6 (<0.001)	2.0 (<0.001)

BMI: Body Mass Index, Spearman Correlation Test

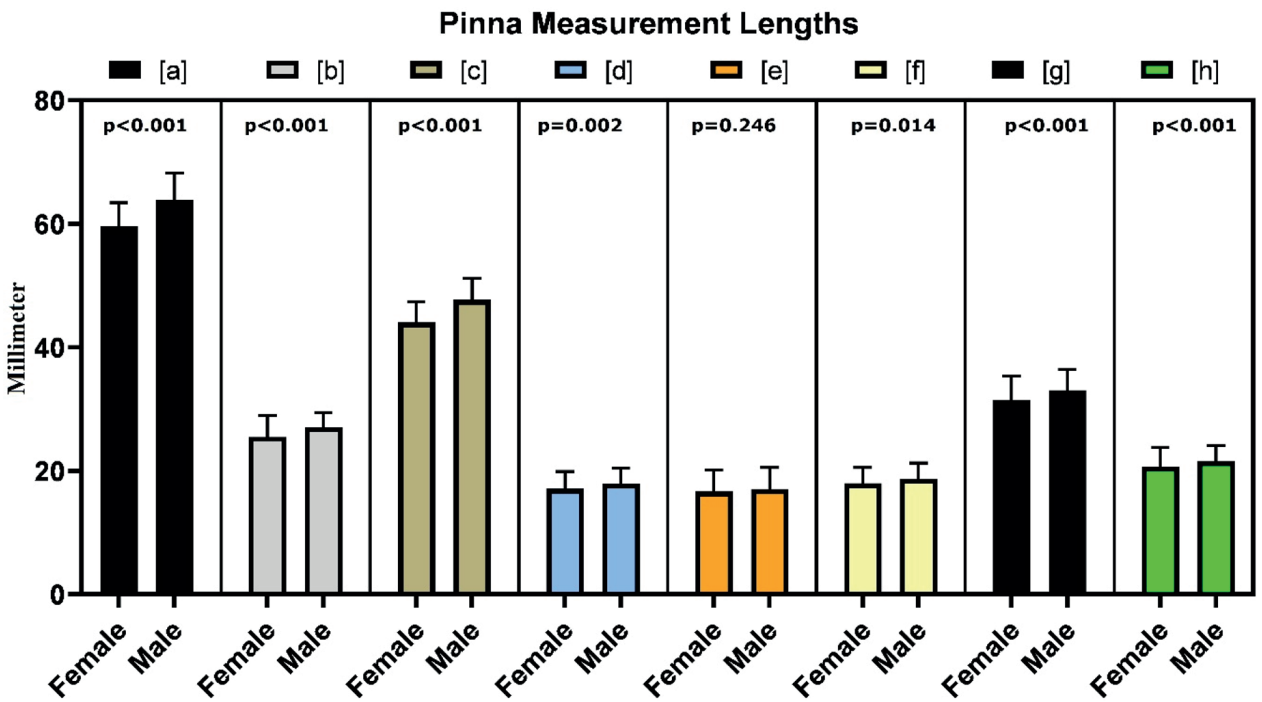

Figure 2. Measurement lengths by sex.

The lengths of [a], [b], [c], [d], [f], [g] and [h], which were found to be statistically significant, were used as features to build machine learning models. The heat map of the correlation matrix between the used features and the sex is presented in Figure 3A. KNN achieved the highest success rate among the created models with an accuracy value of 82.5% (Supplemental 1). The confusion matrix and ROC curve of the KNN algorithm are presented in Figures 3B and 3C. The performance of the eight algorithms used was shown in Table 3.

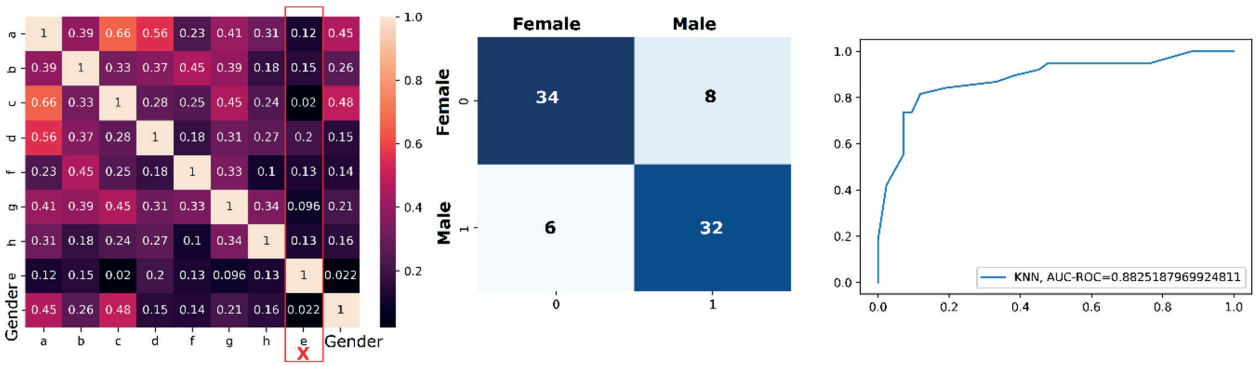

Figure 3. A: Heat map of the correlation matrix of features, [e] was not statistically significant between sexes. B: Confusion matrix of the K-Nearest Neighbor algorithm. C: ROC curve of the K-Nearest Neighbor algorithm.

Table 3. Success rates of the models

Algorithms	Precision	Recall	F1-score	Accuracy	ROC- AUC
K-Nearest Neighbor	0.80	0.84	0.82	0.8250	0.882
Naive Bayes	0.81	0.76	0.78	0.8000	0.827
Decision Tree	0.71	0.79	0.75	0.7375	0.741
Random Forest	0.84	0.84	0.84	0.7500	0.869
Support Vector Machine	0.72	0.76	0.74	0.7500	0.808
Logistic Regression	0.73	0.79	0.76	0.7625	0.808
XGBoost	0.72	0.74	0.73	0.7375	0.825
Artificial Neural Networks	0.73	0.87	0.80	0.7975	0.791

DISCUSSION

In addition to its functionality in providing hearing, the pinna also adds aesthetics to the face. Individuals want a normal and aesthetically pleasing auricle for social acceptance and self-confidence. We did not focus on the hearing benefit of the pinna and its aesthetics. We used seven pinna measurements, which were found to be longer in men than in women, in 8 different machine learning algorithms and aimed to predict sex with these features. The K-Nearest Neighbor model performed best with an accuracy of 0.825 and an AUC of 0.882.

Studies in the literature have shown differences in the pinna between ethnic groups and sexes. In a study conducted on Indians (11), the pinna length in males was 60.4 mm, with a width of 24.3 mm, while in females, the pinna length was 57.6 mm, and the pinna width was 23.3 mm. The researchers reported a difference between the sexes in all measured pinna qualities except lobule length. In a study conducted on Sudanese (2), the pinna length in males was 62.9 mm, with a width of 29.5 mm, while the pinna length in females was 60.9 mm, with a width of 28.8 mm. Researchers reported that the auricle length, width, base, and turbinate length of males are larger than those of females, while the width of the lobes and turbinates are similar. In a study conducted on Koreans (12), the pinna length in males was 62.2 mm and the pinna width was 46.3 mm, while the pinna length in females was 59.30 mm and the pinna width was 44.3 mm. In our study, the pinna length in males was 63.80 mm and the pinna width was 27.05 mm; the pinna length in females was 59.63 mm and the pinna width was 25.45 mm. In our study, all the measurements except the lobule width were larger in males than in females. In comparison with other ethnic groups, the pinna length of Turks in our study was larger than that

of Indians, smaller than that of Sudanese, and similar to that of Koreans. Pinna width was greater than that of Indians and smaller than that of Sudanese and Koreans. The differences in auricle length between societies or ethnic groups may be due to genetic, environmental, and nutritional factors.

The findings of studies investigating the relationship between pinna measurements and height, weight, and BMI are contradictory. Acar analyzed the pinna morphometry of 246 university students using the photo analysis method and examined its relationship with height, weight, and BMI (4). The authors reported that pinna length and width had a strong positive relationship with height, but not with BMI. Laxman reported that there is a positive correlation between pinna morphometric measurements and height and that the pinna can be used to estimate height ($r=0.728$, $p<0.001$ in girls; $r=0.815$, $p<0.001$ in boys) (13). In another study pinna and lobule length and width were associated with higher weight and BMI, but not with height (14). In our study, in contrast to the studies in the literature, a positive relationship was found between auricle anthropometric measurements and all three (BMI, height and weight) (4,13,14).

Maturation of the pinna cartilage is completed at the age of 13 in men and at the age of 12 in women (15). The legal system often requires two different types of corroborating evidence for identification. There are many structures on the pinna, and the distances of these structures from each other can be easily measured. Therefore, pinna anthropometry can be used for identification in forensic medicine and medicolegal cases. Sezgin et al. estimated sex using pinna measurements, height, and weight of 350 individuals in a binary logistic regression analysis (16). The researchers achieved a 68% success rate with the model created using only anthropometric measurements of the pinna, they achieved 88% success in the model developed using height, weight, and pinna measurements. Akyol et al. reported that the success rate with ANN in sex prediction using front face, side face, and age information in machine learning algorithms was 82.6% (6). It was reported that when pinna anthropometric measurements were added, this rate increased to 92.2%. Murgod et al. performed discriminant function analysis with pinna parameters and reported that these parameters accurately predicted sex at a rate of 68–71% (17). In our study, we used only anthropometric measurements of the pinna in machine learning and achieved an accuracy rate of 82.5% with the KNN model. To avoid going beyond our purpose, we did not include attributes such as height and weight in machine learning models. To the best of our knowledge, this study is the first to achieve the highest accuracy in sex prediction using machine learning based on pinna anthropometry. Machine learning-based mobile software can be developed in future studies, and sex prediction can be made with high accuracy.

We used eight different machine learning algorithms in our study. The performances of machine learning algorithms vary depending on data structures, problems, and application scenarios. The strengths and weaknesses of each algorithm depend on the specific problem context, dataset characteristics, and use cases. The discriminant function analysis used by Murgod et al. is a traditional statistical method (17). Although binary logistic regression is considered a machine learning algorithm, it is more suitable for situations where linear distinctions are evident, and its performance decreases in non-linear situations. In our findings, the accuracy of the logistic regression model was lower (76.25%). Unlike logistic regression, KNN can successfully solve non-linear classification problems and can be adapted to various data set structures. Moreover, KNN is also effective on multi-class and multi-dimensional datasets. This flexibility is especially advantageous for complex classification problems and large data sets (18). Therefore, the high success we achieved in our study by using only pinna morphometry is due to the use of many machine learning algorithms.

Our study presents anthropometric measurements of the pinna, the hearing organ, in Turkish people. Our data can serve as a database for otolaryngologists/plastic surgeons interested in pinna reconstruction. It can guide the ergonomic production of newly developed models of hearing aids/cochlear implants (19). As a contribution to the literature, we propose that the pinna can be utilized with high accuracy for

sex estimation, in addition to its primary functions of hearing and aesthetic appearance. By providing adequate infrastructure, creating data collection tools and standardizing measurement points, pinna anthropometry (ear print) can also be used in forensic identification.

This study has several limitations. The sample group in our study consists mainly of young adults. The pinna undergoes physical changes with ageng. Therefore, our machine learning model may produce different results in middle-aged and older individuals. In future studies, pinna anthropometric measurements from individuals in more homogeneous age groups or diverse age groups can be used in machine learning. In this way, models with higher accuracy rates can be developed.

CONCLUSION

Sexual dimorphism in pinna size is reflected in higher anthropometric measurements in males than in females. Pinna's anthropometric measurements can be used in machine learning to predict sex with a high success rate. Our study shows that ear prints may have potential use in forensic identification.

Ethical approval

This study has been approved by the Karabük University (approval date 29/03/2023, number 25). Written informed consent was obtained from the participants.

Author contribution

Surgical and Medical Practices: ES; Concept: ES; Design: ES, STY; Data Collection or Processing: ES; Analysis or Interpretation: STY, ES; Literature Search: ES, STY; Writing: ES, STY. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):85-92
DOI: 10.54307/NWMJ.2025.145

RESEARCH ARTICLE

Assessment of the effectiveness of micronutrient therapy in patients with neovascular age-related macular degeneration unable to receive intravitreal therapy due to COVID-19 pandemic constraints*

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*This manuscript has been previously published as a preprint on the ResearchSquare preprint platform.
https://doi.org/10.21203/rs.3.rs-4447522/v1

Cite as: Bozkurt E, Limon U, Tanındı Duman G, Seçgin Akçay Bİ, Toprak G. Assessment of the effectiveness of micronutrient therapy in patients with neovascular age-related macular degeneration unable to receive intravitreal therapy due to COVID-19 pandemic constraints. Northwestern Med J. 2025;5(2):85-92.

ABSTRACT

Aim: The aim of this study was to evaluate the efficacy of micronutrient therapy for individuals with neovascular age-related macular degeneration (nAMD) who were unable to undergo intravitreal therapy due to the COVID-19 pandemic.

Material and Method: Patients diagnosed with neovascular age-related macular degeneration (nAMD) between March 2020 and July 2021, who were ineligible for intravitreal therapy, had their medical records reviewed retrospectively. Those who met the inclusion criteria were divided into two groups. Group-1 received regular micronutrition therapy for at least six months, while Group-2 did not. Variables including age, gender, duration without intravitreal treatment, intraocular pressure (IOP), best-corrected visual acuity (BCVA), and central macular thickness (CMT) were recorded. Data obtained were compared between the two groups.

Result: Of the 183 nAMD patients screened, 125 were excluded due to missing data or irregular use of micronutrition tablets. Of the 58 patients who met the inclusion criteria, 27 were included in Group-1 and 31 in Group-2. The BCVA and CMT values at the beginning of the pandemic were 0.69±0.72 logMAR, 343.6±106.4 µm, respectively, for Group-1; and 0.85±0.82 logMAR, 381.3±93.7 µm, respectively, for Group-2 (p value 0.211, 0.153 respectively). The BCVA and CMT values obtained at the first examination were 0.74±0.76 logMAR and 330.3±148 µm, respectively, for Group-1; and 1.39±1.30 logMAR and 396.0±151.7 µm, respectively for Group-2 ($p<0.001$ and $p=0.102$, respectively).

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Received: 28.08.2024 **Accepted:** 06.11.2024 **Published:** 28.04.2025

Conclusion: The administration of micronutrient therapy did not lead to significant changes in the central macular thickness (CMT) levels between the groups. However, it did slow down the progression towards a poor prognosis in terms of best-corrected visual acuity (BCVA). We believe that for individuals with non-alcoholic fatty liver disease, micronutrition therapy could potentially serve as an adjunctive treatment to intravitreal therapy. These findings highlight the importance of adequate micronutrient intake in the medical treatment of patients with neovascular age-related macular degeneration (nAMD).

Keywords: central macular thickness, micronutrition, neovascular age-related macular degeneration, visual acuity

INTRODUCTION

Age-related macular degeneration (AMD) is formed by fibrovascular tissue that starts from the choriocapillaris and passes through the impaired Bruch's membrane and extends under the retinal pigment epithelium (RPE) or into the cavities in the subretinal space (1). The global prevalence of AMD is predicted to be 8.7%, and it is projected to double by 2040 (2). According to the AREDS study, the severity scale of AMD is divided into three stages as early, intermediate, and advanced. Advanced AMD is classified as either geographic atrophy affecting the fovea or exudative AMD (3). AMD is categorized into two types: the wet type, characterized by the development of choroidal neovascular membrane, and the dry type which features drusen and retinal pigment epithelial anomalies. The dry type constitutes 85-90% of AMD cases and the wet type 10-15%. Advanced AMD results in subretinal fibrosis or geographic atrophy, which can cause severe vision loss in 10-12% of patients with non-neovascular AMD and 88% of patients with neovascular AMD (nAMD) (4).

There are several studies in the literature that focus on nAMD, especially regarding the treatment of this disease. One of the most important steps in this regard is the use of anti-vascular endothelial growth factor (VEGF) drugs in recent years (5). However, the high cost of these drugs and the need for frequent re-administration are their most significant disadvantages. Combining intravitreal anti-VEGF with different treatment modalities aims to reduce the number of repeated intravitreal injections, costs, and associated complications.

In the pathogenesis of AMD, inflammation resulting from oxidative stress and reduced cellular antioxidant protective effects are important. Chronic oxidative stress and inflammation increase the accumulation of lipofuscin by reducing the RPE's capacity for phagocytosis, lysosomal activation, and autophagy. In contrast, omega-3 polyunsaturated fatty acids, especially in Mediterranean-type diets, prevent inflammation, angiogenesis, and apoptosis, thereby preventing the onset and progression of AMD (6,7). Molecules such as lutein and zeaxanthin, which are components of macular carotenoids obtained through a normal diet, absorb short-wavelength light and help protect against photochemical damage. In addition, they block reactive oxygen radicals that can affect the lipid and protein structures of cells, including the cell nucleus (8). In the AREDS study, high doses of oral antioxidant vitamins such as vitamins C and E, lutein, zeaxanthin, and zinc were shown to reduce the risk of late AMD progression in the fellow eye by 25% in patients with moderate or advanced AMD in one eye (9).

Some patients treated with anti-VEGF for nAMD were unable to visit the hospital for follow-up or treatment due to the COVID-19 pandemic conditions, resulting in severe vision loss in some cases. In this study, we aimed to analyze the effect of micronutrient therapy on the prognosis, anatomical and functional outcomes of nAMD in patients who could not receive intravitreal anti-VEGF treatment during the pandemic.

MATERIAL AND METHOD

This study was designed as a retrospective cohort study. The records of patients who were diagnosed with nAMD at Ümraniye Training and Research Hospital Eye Clinic between March 2020 and July 2021 but could not receive intravitreal treatment due to pandemic conditions, were retrospectively analyzed. Patients who met the inclusion criteria were divided into two groups: Group-1, consisting of patients receiving regular micronutrient therapy, and Group-2, consisting of patients not receiving regular micronutrient therapy. The data collected were compared between these two groups. Initially, changes in central macular thickness (CMT) and best-corrected visual acuity (BCVA) were compared and analyzed across both groups.

Approval was obtained from SBÜ Ümraniye Training and Research Hospital Clinical Research Ethics Committee with the number B.10.1.TKH.4.34.H.GP.0.01/247 on 05.08.2021. Patients were informed that the information contained in the medical records would be used for scientific research and that all personal information would be kept confidential. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Content and use of micronutrition therapy:

The patients in Group-1 were instructed to take a micronutrition tablet with the following contents at least once a day:

1- Omega-3, minimum 300 mg

2- Docosahexaenoic acid (DHA), minimum 360 mg

3- Vitamin C, 60 mg

4- Vitamin E, 10 mg

5- Zinc, 7.5, mg

6- Lutein, 5, mg

7- Zeaxanthin, 1 mg

Inclusion criteria: Advanced stage (stage 4) nAMD diagnosed according to AREDS criteria, at least three doses of anti-VEGF therapy for neovascular AMD, no history of intravitreal anti-VEGF treatment within the last six months during the pandemic period despite the presence of indications, regular micronutrition therapy applied according to AREDS-2 criteria for at least six months, for moderate or advanced AMD (category 3 or 4) in the fellow eye, age range 60-80 years.

Exclusion criteria: Non-neovascular AMD, irregular micronutrition therapy, presence of other concomitant ocular diseases (e.g., diabetic retinopathy, uveitis, glaucoma, and retinal vein occlusion), ocular trauma, previous pars plana vitrectomy surgery, and smoking.

Statistical analysis

Statistical Package for the Social Sciences (SPSS, Version 21, Chicago, IL) was used for statistical analysis at a 95% confidence level. Descriptive statistics, mean, and standard deviation were used to analyze the data. Quantitative data were analyzed using the chi-square test, independent-samples t-test, and paired-samples t-test. Statistical significance was considered at $p < 0.05$

RESULTS

Of the 183 screened patients with nAMD, 125 were excluded from the study due to missing data or irregular use of micronutrition tablets. Among the 58 patients who met the inclusion criteria, 27 were assigned to Group-1 (receiving micronutrition therapy) and 31 to Group-2 (not receiving micronutrition therapy). In Group-1, 12 patients were male and 15 were female, while in Group-2, 14 were male and 17 were female. The mean age was 74.5±7.6 years for Group-1 and 74.1±7.2 years for Group-2. No significant differences in age and gender were observed between the groups (p=0.803 and 0.178, respectively). The baseline and demographic characteristics of the patients are presented in Table 1.

No significant differences were observed between the groups in terms of BCVA, central macular thickness and intraocular pressure values at the patients' final examination while under intravitreal treatment (p=0.211, 0.153, and 0.402, respectively). The time

Table 1. Comparison of demographic characteristics, BCVA and CMT between the groups

Variables	Group-1	Group-2	p
Number of patients (male/female)	27 (12/15)	31 (14/17)	0.178
Age (years)	74.5±7.6	74.1±7.2	0.803
Time without IVI treatment (months)	9.19±3.10	8.42±2.77	0.325
IOP (mmHg)	13.9±3.5	14.7±3.7	0.402
BCVA [logMAR] (pre-pandemic)	0.69±0.72	0.85±0.82	0.211
CMT µm (pre-pandemic)	343.3±106.4	381.3±93.7	0.153
BCVA [logMAR] (post-pandemic)	0.74±0.76	1.39±1.22	<0.001
CMT µm (post-pandemic)	330.3±148	396.0±151.7	0.102

Group-1: patients receiving micronutrition therapy, Group-2: patients that did not receive micronutrition therapy, IVI: intravitreal injection, IOP: intraocular pressure, BCVA [logMAR]: best-corrected visual acuity, CMT µm: central macular thickness.

Independent-samples t-test, chi-square test.

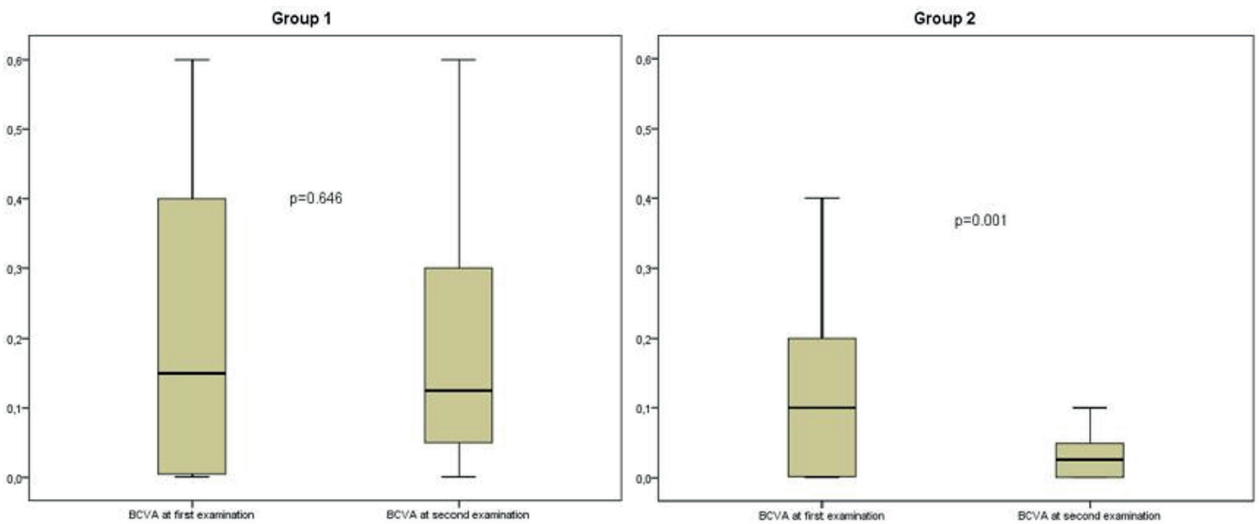

Figure 1. Best corrected visual acuity progression of Group-1 and Group-2 during the pandemic period.

from diagnosis of nAMD to the initiation of intravitreal treatment was 9.19±3.10 months in Group-1 and 8.42±2.77 months in Group-2. No significant difference was observed between the groups regarding the duration without treatment (p=0.325). At the first visit after the period without intravitreal treatment, the BCVA values of Group-1 and Group-2 were measured as 0.74±0.76 and 1.39±1.22 logMAR, respectively, indicating a significant difference between the groups (p < 0.001). No significant difference was observed in terms of CMT or intraocular pressure (p=0.102, 0.402, respectively) (Table 1).

Although there was a decrease in the BCVA values of both groups compared to the pre-pandemic period, the decrease in the BCVA value of Group-1 was not statistically significant (p=0.646). The BCVA value of Group-2 was significantly reduced compared to the pre-pandemic period (p=0.001) (Figure 1). The changes in the CMT values, as measured by optical coherence tomography, were not statistically significant for either group (p=0.542 for Group-1 and p=0.541 for Group-2) (Figure 2).

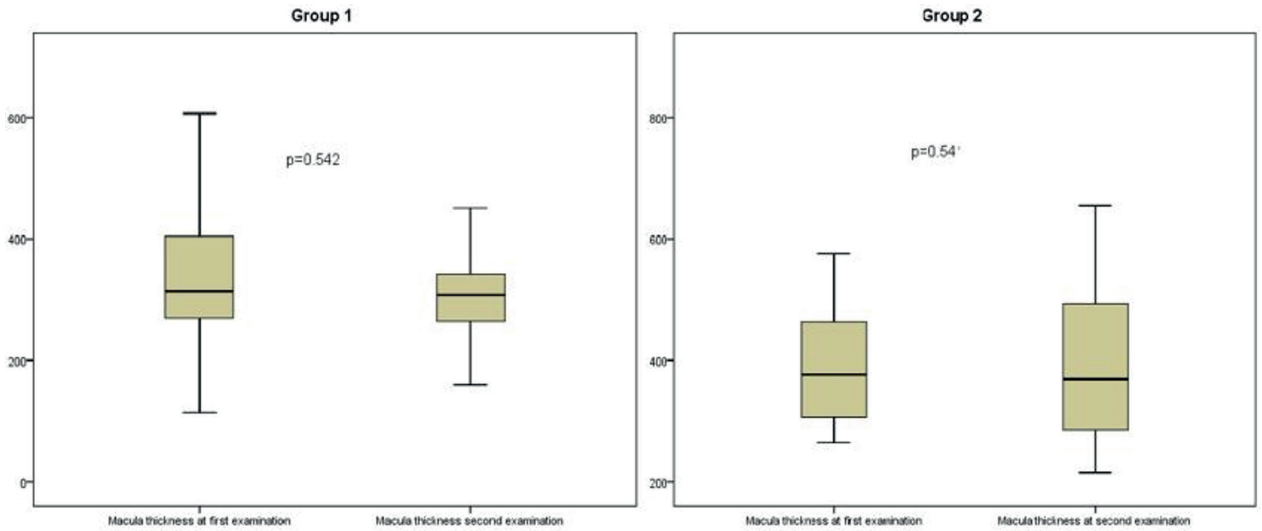

Figure 2. Macular thickness progression of Group-1 and Group-2 during the pandemic period.

DISCUSSION

Age-related macular degeneration is a degenerative condition that affects the macula, which is responsible for central vision, and is the leading cause of blindness in people over the age of 55 (10). The most important risk factors for AMD are advanced age and diet (11). It is known that diet also affects the aging process in the whole body. Similarly, age-related nutritional deficiencies may also predispose the retina to oxidative damage. This highlights the importance of adequate nutrition and nutritional supplements. In our study, we determined that micronutrition therapy had no effect on CMT but slowed down the decline in BCVA in these patients.

Inflammation and reduced antioxidant levels, induced by oxidative stress, are significant factors in the pathogenesis of AMD. Omega-3 polyunsaturated fatty acids have been shown to mitigate inflammation, angiogenesis, and apoptosis, thereby preventing the development and progression of AMD. Studies have shown that in societies with a high intake of long-chain omega-3 fatty acids, the risk of advanced AMD development decreases by approximately 30% (6,7). The AREDS 2 study showed that a combination of antioxidants (vitamins C, E, lutein and zeaxanthin) and zinc supplementation reduced the risk of AMD and vision loss in humans beyond what is achievable through diet alone (7,9). This combination was suggested to be the most valid supplemental therapy for atrophic AMD in patients with intermediate and late stage AMD.

Oxidative stress and inflammation play an important role in the pathophysiology of AMD; therefore, antioxidant vitamins are known to have protective effects. Lutein and zeaxanthin are potent antioxidants found in high concentrations in the retina and reduce oxidative damage by filtering phototoxic blue light, and protect the retinal pigment epithelium. Low levels of lutein and zeaxanthin increase the risk of AMD, while supplementation increases macular pigment density, improves visual function, and may slow disease progression. Other antioxidants such as zinc, vitamins C and E, B vitamins, and vitamin D also support retinal health and are effective in reducing the risk of AMD. In particular, the AREDS 2 study showed that a combination of these compounds reduced the risk of progression to late-stage AMD (12).

Anti-VEGF therapy protects retinal tissue in diseases such as AMD by preventing the development of abnormal blood vessels in the retina. This treatment prevents neovascularization and reduces inflammation by blocking VEGF. In particular, new generation drugs such as Aflibercept, Ranibizumab, Faricimab provide a more effective treatment by blocking Angiotensin

2, which has pro-angiogenic and pro-inflammatory effects together with VEGF. Micronutrition therapy protects retinal cells against oxidative stress with lutein, zeaxanthin, and antioxidant vitamins, reduces inflammation and supports vascular stability. In this way, micronutrition enhances the effectiveness of anti-VEGF therapy, provides better retinal protection, and may contribute to slowing disease progression (13).

In the AREDS 2 study, high doses of antioxidants (zinc, lutein, zeaxanthin, and vitamins C and E) in AMD patients led to a reduced risk of progression to an advanced stage of AMD in the fellow eye. For patients with advanced AMD in one eye, this treatment reduced the risk of developing advanced AMD in the fellow eye from 28% to 20%, and also reduced the rate of moderate vision loss from 29% to 23%. According to AREDS 2 report number 30, patients with high omega-3 intake had a 30% lower risk of developing geographic atrophy or nAMD (6). The AREDS 2 study, on the other hand, was designed to test whether the original AREDS formulation could be made safer and more effective by replacing B-carotene (15mg/day) with omega-3 fatty acids, lutein (10mg/day), and zeaxanthin (2mg/day). This study concluded that while the modified formulation was safe, it did not provide an overall additional benefit (14). In our study, the BCVA of the patients with nAMD received preparations containing at least 300 mg of omega-3, a minimum of 360 mg DHA, 80 mg EPA, 5 mg lutein, 1 mg zeaxanthin, 10 mg vitamin E, 60 mg vitamin C, and 7.5 mg zinc. Our findings showed that the decline in the BCVA of patients with neovascular type AMD who received micronutrition therapy was better preserved than the group that did not undergo this treatment.

According to the Eye Disease Case-Control Study Group, carotenoid intake reduced the risk of developing neovascular AMD by 43%, and the combination of lutein and zeaxanthin had a very strong association with reducing the risk of AMD (15). Similarly, the Waterford study found that macular pigment optical density was lower in all patients with AMD, but macular pigment density increased with oral lutein intake (16).

In the Nutritional AMD Treatment (NAT)-1 study, it was stated that the efficacy of EPA 720 mg/day and DHA 480 mg/day in patients with drusenoid pigment epithelial detachment was not significantly different from that of the placebo group (17). According to the NAT-2 study, the risk of developing nAMD was not different in the group receiving DHA (840 mg/day) and EPA (270 mg/day) compared to the group receiving a placebo. A 68% reduction in the risk of nAMD was reported in patients with high DHA+EPA levels (18). According to the Blue Mountains Eye Study, dietary intake of lutein and zeaxanthin reduced the risk of nAMD by 65%, high zinc intake reduced this risk by 44% for all stages of AMD and by 46% for early-stage AMD; and excess omega-3 intake reduced the risk of developing nAMD by 37% (19). In our study, the micronutrient formulation included a minimum of 5 mg lutein, 1 mg zeaxanthin, 7.5 mg zinc, and 300 mg omega-3. Although there were differences in intraretinal and subretinal fluid between patients receiving micronutrients and those not receiving this therapy, the lack of relative deterioration in BCVA raises concerns about the importance of decontamination therapy in maintaining retinal cell function.

A Mediterranean-style diet is linked to a reduced risk of developing advanced age-related macular degeneration (AMD) and large drusen, with fish consumption being a contributing factor to this protective effect (20). Other studies have indicated that low dietary zinc intake is associated with increased intraretinal fluid and macular thickness in patients with neovascular AMD (nAMD) (12,21). In our study, although no significant differences in central macular thickness (CMT) were observed between the two groups, we found that dietary intervention slowed down the progression towards a poor visual prognosis. These results suggest that adhering to evidence-based dietary recommendations may provide protective effects against nAMD.

Limitations of the study include the lack of a group of patients using micronutrients along with intravitreal therapy, the inability to compare patients with nAMD using micronutrient molecules with different contents (e.g., resveratrol, coenzyme Q, and astaxanthin), the small number of patients, and the short follow-up period.

In summary, we believe that micronutrition therapy has the potential to slow the progression towards poor prognosis in patients with neovascular age-related macular degeneration (nAMD) and may serve as a supportive treatment alongside intravitreal therapy. These results underscore the importance of micronutrient intake in patients with nAMD who are receiving clinical care.

Ethical approval

This study has been approved by the Ümraniye Training and Research Hospital Clinical Research Ethics Committee (approval date 05.08.2021, number B.10.1.TKH.4.34.H.GP.0.01/247). Written informed consent was obtained from the participants.

Author contribution

Concept: EB; Design: UL; Data Collection or Processing: BİSA; Analysis or Interpretation: GT, GTD; Literature Search: EB, GTD; Writing: EB, GT. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):93-97
DOI: 10.54307/NWMJ.2025.74

RESEARCH ARTICLE

Is there a difference in FSH levels between different age groups of infertile patients with poor ovarian reserve?

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Cite as: Peker N, Ege S. Is there a difference in FSH levels between different age groups of infertile patients with poor ovarian reserve? Northwestern Med J. 2025;5(2):93-97.

ABSTRACT

Aim: The aim of this study is to compare the diagnostic accuracy of follicular stimulating hormone (FSH) in different age groups of poor ovarian reserve (POR) infertile patients.

Material and Methods: The records of infertile patients who presented to a tertiary center hospital infertility outpatient clinic were retrospectively analysed. The patients were divided into two groups: those between the ages of 20-29 were considered as group 1, and those between the ages of 30-39 were considered as group 2. FSH specificity and sensitivity were compared in patients with (POR) with antimullerian hormone (AMH) values below <1 ng/mLThe specificity, sensitivity, and accuracy of the FSH assay as a diagnostic tool for determining reduced OR in females with POR were compared and predicted using ROC curve analysis.

Results: A total of 102 infertile patients were assessed. Those between the ages of 20-29 were considered as group 1, and those between the ages of 30-39 were considered as group 2. The avarages FSH levels for each group were 9.73 ± 3.17 and 10.06 ± 8.74, respectively (p value = 0.85). The sensitivity, accuracy, and specificity of the FSH assay were, 85%, 78%, and 68%, respectively (p=0,001). In group 2, FSH values may be more meaningful for evaluating the ovarian reserve.

Conclusion: FSH was not correlated with AMH and antral follicule count (AFC). In the age-specific FSH assessment showed that FSH is still not a specific marker in POR. This study concluded that FSH and age have a weak correlation with the number of follicles restored and the number of oocytes retrieved.

Keywords: age, antimullerian hormone, follicular stimulating hormone, infertility, ovarian reserve

INTRODUCTION

Primordial follicle granulosa cells generate the glycoprotein-structured antimüllerian hormone (AMH). Follicle growth is inhibited in response to follicular stimulating hormone (FSH). The number of antral follicles and the size of the primordial follicle pool are known to positively correlate with AMH (1-3). One of the endocrine tests used to assess ovarian reserve (OR) is the basal FSH measurement. The

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Received: 19.07.2023 **Accepted:** 07.02.2025 **Published:** 28.04.2025

pituitary secretes the glycoprotein FSH, which binds to receptors on the granulose cells of the ovaries. FSH levels and activity vary throughout a woman's menstrual cycle. A woman's menstrual cycle is often determined by measuring FSH serum levels on days 2-3 of menstruation. It is examined every day. At this stage of the menstrual cycle, low FSH levels are normal, but when follicles become smaller, FSH levels climb. Consequently, low OR is indicated by elevated FSH levels. While FSH has been employed as a marker to estimate OR, there are a number of intra- and inter-cycle variables that make it inconsistent to utilize FSH as a reliable indicator of OR. AMH levels that are more constant over the course of the menstrual cycle make it a more useful tool for OR prediction (4).

The aim of this study is to compare the diagnostic accuracy of FSH in different age groups of poor ovarian response (POR) infertile patients.

MATERIAL AND METHODS

The records of infertile patients who presented to a tertiary center hospital infertility outpatient clinic were retrospectively analysed. The study protocol was approved by the regional ethics committee (2020-459). FSH specificity and sensitivity were compared in patients with POR with AMH values below <1 ng/mL. One hundred two infertile patients were divided into two groups. Those between the ages of 20-29 were considered as group 1, those between the ages of 30-39 were considered as group 2. The evaluation of AMH tests and the cut-off values were difficult to determine because the AMH analysis varies. The cut-off values used for the evaluation of POR vary between 0.10-1.66 ng / mL (5-8). In this study, we evaluated patients with AMH less than 1 ng/mL. ROC curve analysis was used to compare and estimate the specificity, sensitivity, and accuracy of FSH testing as a diagnostic test to identify reduced OR in women suffering from POR Blood was collected (AMH, FSH, LH, E2) on days 2–4 of the menstrual cycle. Antral follicle count (AFC) was evaluated by transvaginal ultrasonography (TV-USG). The total of 2-10 mm follicles in the ovaries was defined as AFC (9). Those who had previous ovarian surgery, had endometrioma and ovarian cysts, have been exposed to pelvic radiation, have been on the contraceptive pill for the past 3 months, and patients older than 40 have been excluded from the study. Statistical analysis Descriptive statistics and (mean ± SD) SPSS were used to analyze statistical data. To evaluate quantitative data, the Student's t-test was used for normally distributed variables and Mann-Whitney U test was used for non- normally distributed variables. The chi-square test and Fisher's exact test were used to compare qualitative data. Statistical significance was determined as $p < 0.05$. ROC curve showing the balance between specificity (what is the false positive rate) and sensitivity (what is the true positivity rate) for FSH testing between the (20-29 years) and (30-39 years) range groups. We estimated the area under the ROC curve (AUC) using empirical methods. The ROC curve, which shows the balance between specificity (what is the false positivity rate) and sensitivity (what is the true positivity rate) for the FSH test, covers the age groups between (20-29 years) and (30-39 years).We estimated the area under the ROC curve (AUC) with the use of empirical methods.

RESULTS

Table 1 shows FSH levels evaluated in the 20-30 age group and 30-40 age group. The averages for each group were 9.73 ± 3.17 and 10.06 ± 8.74, respectively.

There was no significant difference in FSH levels between the groups (p value = 0 .85). The sensitivity, accuracy, and specificity of the FSH assay were, Sensitivity, accuracy and specificity of the FSH indicator 85%, 78% and, 68%, respectively ($p<0.01$) (Table 2).

The sensitivity, accuracy, and specificity of the FSH test were demonstrated by the ROC curve. In group 2, FSH values may be more meaningful to evaluate the ovarian reserve (Figure 1).

Table 1. Demographic and laboratory results and comparison of the two groups

	Group 1 (Age 20-29) n=22	Group 2 (Age 30-39) n=80	P value
Age (years)	26.45 ± 2.79	35.73 ± 2.33	0.43
AMH (ng/mL)	0.42 ± 0.33	0.47 ± 0.33	0.52
LH (mU/mL)	5.13 ± 1.14	7.58 ± 7.39	0.5
FSH (mU/mL)	9.73 ± 3.17	10 .06 ± 8.74	0.85
Antral follicle count (n)	4.32 ± 1.84	3.99 ± 1.86	0.45
Estradiol (pg/mL)	56.68 ± 47.53	66.79 ± 71.34	0.53

Study data (mean ± SD). Student's t-test was used for normally distributed variables, and the Mann-Whitney U test was used for non-normally distributed variables. A chi-square test and fisher exact test was used to compare qualitative data. Statistical significance was set at $p < 0.05$.

Table 2. ROC curve analysis for FSH assays comparing the two groups

Variable	AUC	P value	Sensitiviy	Accuracy	Specificiy
FSH	0.67	0.001	85%	78%	68%

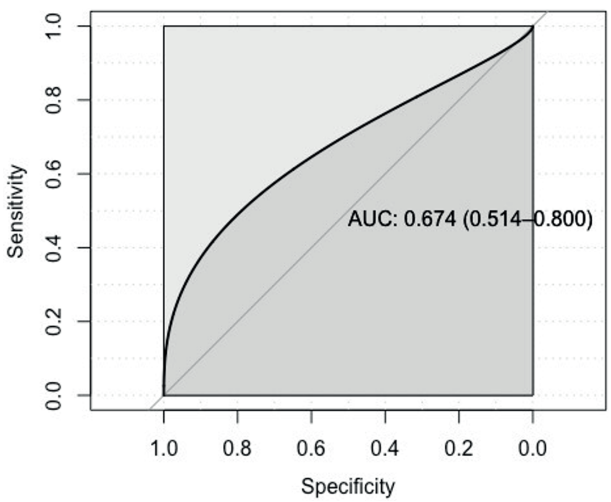

Figure 1. ROC curve analysis for FSH assays comparing the two groups.

DISCUSSION

Early diagnosis still remains an important issue in order to implement effective treatment protocols in infertility. Many tests for OR evaluation, including TV-USG, early antral follicle count, and second-day basal FSH levels, still maintain their current place. The main aim of this study was to determine the correlation between AMH values below 1 ng/mL and specific age ranges in patients. In this study, age-specific FSH evaluation suggests that FSH is still not a specific marker for POR. However, FSH values may be more meaningful for evaluating ovarian reserve in the older age group. AMH, FSH, and AFC are widely used as tests of the ovarian reserve. The AMH test shows better activity in POR estimation than AFC and FSH. These markers have their advantages and limitations. FSH has been reported to have high specificity and low sensitivity in POR estimation (5). FSH does not have a prediction for ovarian hyperstimulation syndrome and is known to have inter-cyclical variability. You cycle 2-4. Considered on the day, AFC has the advantage of generating urgent results and is useful for POR 's estimation and OHSS risk (10). However, AFC requires experienced sonographic experience by experienced specialists. Conversely, blood can be drawn for AMH even when TV-USG is not readily available (11). In this study, AFC was observed to be low in proportion to AMH values. However, it is seen that AFC is more valuable than FSH in POR patients. Abed et al. (12) predicting and comparing the specificity, sensitivity, and accuracy of AMH testing with FSH testing demonstrates the application of ROC curve analysis as a diagnostic test to determine OR in infertile women. When comparing the detection of premature ovarian failure (POF) using FSH or AMH tests between fertile female patients and POF patients, the AMH test remains a more sensitive and specific test than the commonly used FSH biomarkers in detecting POF. When comparing the POF group with the nonfertile control group, the sensitivity of FSH and AMH tests was the same.

However, the FSH test was more specific and accurate than the AMH test. On the other hand, in this study, two of our patients were evaluated as infertile POR, individuals with AMH levels below 1 ng/mL were included in the groups, and it was observed that FSH was not associated with AMH and AFC in both groups. Jamil et al. and Siddiqui et al. showed that FSH had a weak correlation between the number of follicles repaired and the number of oocytes retrieved (13,14). This creates negative differences with AMH, which has a strong relationship with the retrieval of oocytes.

AMH was reported to be more sensitive in detecting ovarian reserve when Parveen et al. (15) compared the diagnostic accuracy of AMH with FSH in the evaluation of ovarian reserve. When comparing individuals with poor ovarian reserve to those with normal to high response, the mean AMH was 0.74 ng/mL. According to Baker et al. (16), the receiver operating characteristic (ROC) curve's AMH cutoff point for determining POR was 0.93 ng/mL with 74.1% sensitivity and 90% specificity. AMH (area under the ROC curve [AUC] = 0.929) performed substantially better in POR prediction than FSH (AUC = 0.615; P<.0001), according to ROC analysis.

In this study, the sensitivity , accuracy, and specificity of the FSH test were demonstrated by the ROC curve.

The FSH test has a sensitivity, accuracy, and specificity of 85%, 78%, and 68%, in that order. It seems that FSH is still not a specific indication in POR based on age-specific FSH examination. For assessing ovarian reserve in the older age range, FSH readings might have greater significance. There has been no research done on the assessment of FSH based on age range. The primary limitation of our study is its retrospective nature. Another limitation is that the study was conducted at a single institute with a small sample size. AMH and AFC had no correlation with FSH. FSH is still not a specific sign in POR, according to the age-specific FSH assessment. This study demonstrates that oocyte number is a poor indicator of FSH and age.

Ethical approval

This study has been approved by the ethics committee of Health Science University Gazi Yasargil Education and Research Hospital (approval date 28/04/2020, number 459). Written informed consent was obtained from the participants.

Author contribution

Surgical and Medical Practices: NP, SE; Concept: NP, SE; Design: NP, SE; Data Collection or Processing: NP, SE; Analysis or Interpretation: NP, SE; Literature Search: NP, SE; Writing: NP, SE. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):98-103
DOI: 10.54307/NWMJ.2025.100

RESEARCH ARTICLE

Elderly suicides in Bolu province

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Cite as: Hösükler E, Erkol ZZ, Özdeş T, Turan T, Yazgı BK. Elderly suicides in Bolu province. Northwestern Med J. 2025;5(2):98-103.

ABSTRACT

Aim: The aim of this study was to analyze the characteristics of suicide cases in the elderly, identify risk factors and discuss preventive measures.

Methods: Forensic files of forensic death cases from the Bolu Chief Public Prosecutor's Office between January 1, 2003 and December 31, 2019 were retrospectively analyzed. Twenty cases aged 65 years and older who died by suicide were included in the study.

Results: Among the cases, 14 (70%) were male. The mean age was 77.1±9 (min=65, max=93) years, and 65% of the cases were 75 years or older. The most common method of suicide was hanging (n=12, 60%). Only two of the cases were employed. Eleven cases (55%) committed suicide at home. A suicide note was found at the scene in only one case. A history of psychiatric illness was found in eight cases (40%). In addition, relatives of five cases (25%) stated that the case had depressive symptoms before committing suicide.

Conclusion: Suicide attempts are more likely to result in death because elderly people use more lethal suicide methods than younger people. Practices aimed at reducing suicide attempts in the elderly population should be given more importance.

Keywords: autopsy, cause of suicide, elderly suicides, suicide note, suicide methods

INTRODUCTION

Elderly people may experience a psychological crisis triggered by the natural life process of loss, including loss of health, loss of social roles, loss of relatives or friends, and loss of meaning in life; this situation may lead to suicide, especially in those who lack family and social support and experience loneliness (1). In a 37-year retrospective study conducted in Northern Italy, pathological factors were identified in 427 cases (physical conditions: 194 cases, psychological states: 233 cases) and were found to be closely related to the risk of suicide due to mental illness (2). It is estimated that elderly suicide victims are nine times

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Received: XX.XX.2023 **Accepted:** XX.XX.2024 **Published:** 28.04.2025

more likely to live alone than adult suicide victims (3). Suicide attempts are less common in the elderly than in younger people, but are more lethal in the elderly population (4). Although people over the age of 65 generally have a higher suicide rate than any other age group, elderly males are more likely than elderly females to die following a suicide attempt (5). The total number of elderly people and their ratio of the overall population are both expected to increase significantly worldwide in the coming years. Therefore, the number of elderly suicides is also likely to rise in the future. In addition, the suicide rate has been reported to increase with age within the elderly population (6).

Our study aimed to investigate elderly suicides that resulted in death in Bolu province of Turkey between January 1, 2003, and December 31, 2019, and to discuss what should be done to reduce suicide attempts among the elderly.

MATERIALS AND METHODS

Study design

This retrospective study was conducted at the Department of Forensic Medicine, Bolu Abant İzzet Baysal Training and Research Hospital. The study was conducted in accordance with the principles of the Declaration of Helsinki. As this was a retrospective review, informed consent was not required. Written permission to conduct the study was obtained from the Bolu Chief Public Prosecutor's Office on April 8, 2020 (number 2020/3169 B.M.). Ethical approval was obtained from the Bolu Abant İzzet Baysal University Clinical Research Ethics Committee on May 29, 2020 (number 177).

Data collection and implementation

The forensic files of forensic death cases belonging to the Bolu Chief Public Prosecutor's Office between January 1, 2003 and December 31, 2019 were retrospectively analyzed. A total of 20 cases aged 65 years and older who died by suicide were included in the study.

These cases were evaluated for the following parameters: age, sex, marital status, lifestyle, employment status, suicide method, suicide site, presence of a suicide note, history of previous suicide attempts, presence of psychiatric symptoms or psychiatric diseases, and reasons for suicide.

Statistical analysis

The Statistical Package for the Social Sciences, version 21.0 (Statistical Software Package 21, IBM Corp., Armonk, NY, USA) was used for data analysis in this study. Descriptive statistics were presented as frequency, percentage, mean, standard deviation (SD), minimum (min), and maximum (max) values.

RESULTS

This study included 20 cases: 70% (n = 14) were male and 30% (n = 6) were female. The mean age of the cases was 77.1 ± 9 (min = 65, max = 93) years. Most cases were between 65-69 (n = 7, 35%) years of age, while 65% of cases were at least 75 years of age. Nine of the cases were married (45%). Five (25%) of the suicide victims had lived alone. Only two (10%) of the cases

Table 1. Distribution of cases by sociodemographic characteristics

Sociodemographic characteristics	n	%
Age Group		
65–69 years	7	35
75–79 years	6	30
80–84 years	3	15
85–89 years	2	10
≥ 90 years	2	10
Marital Status		
Married	9	45
Whose parter is ex	11	55
Individuals living together		
Alone	5	25
With partner	5	25
With partner and children	4	20
With children and/or descendant	6	30
Work status		
Working	2	10
Not working	18	90
Total	**20**	**100**

had been employed as farmers. The sociodemographic characteristics of the cases are presented in Table 1. Eleven of the cases (55%) committed suicide at home (Table 2).

The most common method of suicide was hanging (n=12, 60%) (Table 3). While seven male victims used the hanging method, four males used firearms, two males used stabbing, and one male jumped from a great height. Five female victims used hanging as the method, while the remaining case involved drug intoxication. There was no history of suicide attempts in any of the cases. A history of psychiatric illness was confirmed in eight cases (40%): two cases had depression, three cases had bipolar disorder, and three cases had schizophrenia. It was noted that five cases (25%) described symptoms of depression but did not seek psychiatric evaluation at any health facility. A single suicide-related cause was found in 12 (60%) cases, two suicide-related causes were found in six (30%) cases, and three suicide-related causes were identified in two (10%) cases (Table 4).

Table 2. Distribution of cases by scene

Scene	**n**	**%**
Home	11	55
Garden of the house	6	30
Forest	3	15
Total	**20**	**100**

Table 3. Distribution of cases by suicide method

Suicide methods	**n**	**%**
Hanging	12	60
Firearm injury	4	20
Stabbing	2	10
Medicine intoxication	1	5
Jumping from high	1	5
Total	**20**	**100**

DISCUSSION

The suicide rate has been increasing in both men and women with the general increase in the age of the elderly population worldwide (7). Karbeyaz et al. (8) reported that 63.5% of the 74 suicide cases that occurred in Eskisehir province, Turkey, were in people aged 75 years and older. In this study, the mean age of elderly suicide victims was 77.1 ± 9 (min=65, max=93) years, and 65% of the cases were ≥ 75 years. In a study conducted in China, 59.6% (n=62) of 104 elderly suicide cases were male (9). Seventy percent of elderly suicides in Malaysia were committed by men (10). Torresani et al. (11) stated that the suicide rate of men in Italy was 29.7/100,000 elderly persons, whereas

Table 4. Possible related factors of suicide

Related Factors	**n**	**%**
Psychiatric illness	5	25
Symptoms of undiagnosed psychiatric illness	2	10
Chronic disease	2	10
In need of care	2	10
Economic issue	1	5
Chronic disease + Psychiatric illness	2	10
Chronic disease + Symptoms of undiagnosed psychiatric illness	2	10
Living alone+ Economic issue	1	5
Living alone+ Loss of a loved one (spouse)	1	5
Loss of a loved one (spouse)+ In need of care+ Symptoms of undiagnosed psychiatric illness	1	5
Living alone+ In need of care+ Psychiatric illness	1	5
Total	**20**	**100**

the rate of women was only 9.1/100,000. In Türkiye, 74.3-74.5% of elderly suicide deaths were male (8,12). In this study, 70% of the cases were male.

Living alone has been identified as a risk factor for elderly suicide in many studies (13-15). Contrary to the literature, only five (25%) of the victims in this study lived alone. Of the 70 elderly who committed suicide in Hong Kong, only 20% were still actively working and only 54.3% were married (16). In the study conducted by Wiktorsson et al. (17), 36.9% of the elderly who attempted suicide were married. Karbeyaz et al. (8) found that only 6.8% of elderly suicide cases were still working and only 8.1% were still married. In this study, nine (45%) cases were married and only two (10%) cases were working as farmers.

Elderly people are more likely to spend time at home, and most elderly suicides occur at home (8,10). In this study, more than half of the elderly suicide cases (55%) preferred to end their lives at home.

A 37-year retrospective study conducted in northern Italy (2) reported that the most common method used by the elderly to commit suicide was hanging (32.5%). The most common method of suicide among the elderly in Malaysia was hanging (56.5%) (10). In Türkiye, both men and women over the age of 65 were most likely to attempt suicide by hanging (14). In this study, hanging was also the most preferred method regardless of gender (n=12, 60%).

In Italy, living alone, having a low level of education, visiting a doctor in the last month, and living in a nursing home were strongly associated with elderly suicide (11). In studies conducted in China, living alone, having a poor family status, having a physical illness, encountering negative events, and living in rural areas were closely related to elderly suicides (9,13). In the study conducted by Waern et al. of people over 75 years of age, family conflict, severe physical illness, loneliness, and both major and minor depression were found to be closely related to suicide in the elderly (18). The psychiatric disorders of anxiety, depression, and bipolar disorder have been associated with elderly suicide (19). In their controlled psychological autopsy study, Chiu et al. (16) reported that 86% of the elderly suicide cases and 9% of the elderly control group participants had experienced at least one psychiatric problem before suicide. The most common diagnosis was major depression; 77% of the elderly suicide cases had consulted a doctor less than a month before the suicide (16). In studies of elderly suicide in Türkiye, living alone, using alcohol, having been diagnosed with the disease for 11 years or more, having a history of hospitalization in a psychiatric clinic, losing a loved one, having a chronic physical illness, and being diagnosed with adjustment disorder, depression, or anxiety were identified as important risk factors for suicide (8,20,21). In this study, a history of psychiatric illness was found in eight (40%) cases. Although five victims (25%) had no psychiatric diagnosis, their relatives reported that depressive symptoms were present before the suicide. Psychiatric illness and symptoms appear to be the most important predictors of suicide in the elderly.

CONCLUSION

This study found that elderly individuals who committed suicide were generally male, over 75 years old, living alone at home, and most commonly used hanging as the method. The factor most associated with suicide was the psychological state. Considering that the number of elderly suicides will increase with the increase in the elderly population, necessary precautions should be taken before elderly people attempt suicide. To this end, a number of social and health measures should be taken.

Our recommendations for reducing elderly suicides are as follows:

- In the last month before a successful suicide attempt, elderly people often consult a physician at least one or more times (11,19). Therefore, clinicians must evaluate elderly patients for suicidal tendencies, even if they report only mild depressive symptoms (17). The rate at which elderly people visit psychiatrists is low in Türkiye (8). Considering the low rate of referral to psychiatrists among the elderly population, it is not possible for psychiatrists alone to perform follow-up and screening.

Therefore, educating family physicians about common suicide risk factors in elderly patients and providing them with training programs may contribute to the detection of even mild depressive symptoms in elderly patients and ensure that elderly people receive the psychological and social support needed to reduce suicide rates.

- Elderly people with chronic physical illnesses are already psychologically distressed due to their existing illnesses, and the possibility of co-existing mental illness is quite high. Therefore, all physicians need to be aware of the increased risk of suicide in older people who have both physical illnesses and psychiatric symptoms, and should develop specific strategies for them to address these issues. Healthcare professionals who treat the elderly should be particularly aware of the increased risk of suicide in this population. This includes not only primary care physicians, but also specialists who treat chronic conditions in the elderly. Elderly patients at risk for suicide should be referred to psychiatric clinics, and their follow-up and treatment should be planned in these clinics.
- Elderly suicides are often precipitated by living alone, experiencing social isolation, feeling worthless, and losing social status. Therefore, it is very important to increase social support in the elderly. This strategy may involve expanding home care services, establishing municipal recreational facilities where elderly individuals can spend time with their peers, organizing travel programs, and offering various educational courses to help prevent feelings of loneliness and worthlessness among the elderly.
- The elderly need more psychosocial support than adult psychiatric patients. In addition, the suicide attempts of older people represent a negative and impulsive approach to addressing problems. To correct this approach, better problem-solving strategies should be offered to the elderly. In addition, it would be beneficial to establish "geriatric psychiatry" as a separate specialty in medicine devoted exclusively to elderly patients, or to have "geriatric psychiatry" as a subspecialty within the field of "mental health and disease speciality".
- Until such a specialization is available, the creation of "geriatric psychiatry outpatient clinics" for the elderly population at every hospital and the efforts of psychiatrists specialized in geriatric psychiatry in these clinics will allow the elderly to be followed up and treated more professionally.
- Although living alone is defined as a risk factor in the literature, a significant portion of the elderly suicide cases in Türkiye lived with their spouses and/or relatives. For this reason, the family members of elderly people who are at risk of suicide about this subject may be educated.
- Using the power of the media, the public should be informed about elderly suicides via public service announcements, educational seminars, and television programs. Thus, elderly people with risk factors for suicide who are unwilling to seek medical help may be recognized by family members and provided with support before a suicide attempt occurs.

Ethical approval

This study has been approved by the Bolu Abant İzzet Baysal University Clinical Research Ethics Committee (approval date 29.05.2020, number 177). Written informed consent was obtained from the participants.

Author contribution

Idea: EH, ZZE, TÖ; Design: EH, ZZE, TÖ; Supervision: EH, ZZE; Resources: EH, ZZE, TÖ, TT, BKY; Materials: EH, ZZE, TÖ, TT, BKY; Data collection: EH, TT, BKY; Analysis: EH, ZZE, TT; Literature review: EH, TT, BKY; Writing: EH, ZZE, TÖ; Critical review: EH, ZZE, TÖ, TT, BKY. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):104-111
DOI: 10.54307/NWMJ.2025.122

RESEARCH ARTICLE

Relationship between depression levels and sexual activity in patients with temporomandibular joint disorder

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Cite as: Türken R, Büyük SK, Becet N, Abay F. Relationship between depression levels and sexual activity in patients with temporomandibular joint disorder. Northwestern Med J. 2025;5(2):104-111.

ABSTRACT

Aim: This study aimed to examine the relationship between temporomandibular joint (TMJ) problems, sexual disorders, and depression in male and female individuals.

Materials and Methods: A total of 228 participants (116 females and 112 males) participated in the study. The level of temporomandibular joint disorder (TMD), severity of depression, and sexual function were evaluated using the Fonseca Anamnestic Index (FAI), the Beck Depression Inventory (BDI), the Female Sexual Function Index (FSFI) and the International Index of Erectile Function (IIEF), respectively.

Results: The results revealed a statistically significant difference between the sexes, with severe TMD scores being significantly higher in females than males ($p<0.05$). The BDI scores for females (13.25±9.51) were observed to be higher than those for males (10.99±8.00). However, the difference did not reach statistical significance ($p=0.105$). We also observed a negative correlation between erectile function scores (IIEF) and both FAI ($r=-0.102$) and BDI ($r=-0.312$) in males. Similarly, a negative correlation was observed between sexual function values (SFV; IIEF, FSFI) and both FAI ($r=-0.122$) and BDI ($r=-0.019$) in females.

Conclusions: The results indicated a significant correlation between severe TMD and depression in females and males. Sexual dysfunction may also be associated with TMD and depression.

Keywords: depression, sexual dysfunction, survey, temporomandibular joint disorder

INTRODUCTION

Temporomandibular joint dysfunction (TMD), a disorder characterized by non-dental pain affecting masticatory muscles and the temporomandibular joint (TMJ), may manifest in a number of ways (1). The development of TMD is associated with a number of factors, including parafunctional habits such as teeth grinding or clenching, as well as stress, anxiety, traumatic injuries to the head and neck, occlusal

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Received: 07.06.2024 **Accepted:** 25.03.2025 **Published:** 28.04.2025

interferences, and central nervous system disorders. However, no single etiological factor has been identified as fully accounting for the underlying causes of TMD (2).

Psychological issues, particularly those pertaining to emotional distress such as depression, are pivotal in the etiology of TMD (3). Depression is a set of physiological and psychological disorders characterized by a loss of the will to live, accompanied by pessimism about the future, regret, guilt, and suicidal ideation (4). The presence of these symptoms is not necessarily indicative of depression; rather, they stem from the challenges posed by recurring depression, its duration, and its impact on daily life (5). Individuals suffering from myofascial pain and TMD tend to exhibit depressive symptoms, diminished performance in daily activities, and a reduction in the quality of life. Psychological disorders such as depression have an impact on the central nervous system that, in turn, reduce patients' pain threshold. Consequently, TMD is commonly associated with orofacial pain in patients with depression. This interdependence can contribute to a vicious cycle in TMD patients, which may result in increased functional limitations, such as those associated with smiling, speaking, or yawning (6). Therefore, TMD patients with depression or other psychological problems, as well as with central nervous system-related pain sensitivity, are recommended to undergo multidisciplinary treatment (7).

Sexual dysfunction (SD) is a complicated and multi-dimensional phenomenon that comprises both physiological and psychological factors (8). A number of risk factors, such as increasing age, bad-controlled diabetes mellitus, hyperlipidaemia, urinary tract symptoms, hypertension, psychological stress, and low physical activity may contribute to the etiology of this disorder (9). Despite the differences in female vs. male sexual physiology, SD in both sexes leads to alterations in the venous system of the genital area, primarily affecting endothelial tissues. Consequently, this may lead to issues such as inadequate wetting, delayed sexual arousal, diminished clitoral and vaginal sensation, as well as an inability to reach orgasm in females or to achieve or maintain erection in males (10).

Survey studies are a cost-effective method to evaluate the applicability of epidemiological research and patient follow-up. Moreover, as patients respond to survey questions without undue influence from the clinician, they are less susceptible to the impact of external factors, which helps reduce overall variability in the results (11). The Fonseca Anamnestic Index (FAI), which allows for the classification of TMD severity and the assessment of bruxism, is an effective method to gather epidemiological data. The FAI can also be used to determine the presence of TMJ pain, headaches, bruxism, mandibular movement limitations, malocclusions, and emotional stress (11,12).

In the literature, SD was reported to be related to depression and anxiety (8,13). However, physiopathology of this relationship remains poorly understood. Specifically, it remains unclear whether depression causes SD or, alternatively, whether SD is a common cause of emotional disorder. Similarly, there is no conclusive evidence demonstrating whether depression and anxiety, which play a part in the primary etiology of TMD, exert an influence on the development of TMD in a manner similar to that observed in SD. To the best of our knowledge, none of the previous studies analyzed the relationship between SD, TMD, and depression. To fill this gap in the literature, the present study evaluates the relationship between SD and depression levels of female and male patients with TMD.

METHODS

The sample size was calculated using G*Power Software version 3.1.9.2 with an alpha error probability of 0.05 and a power of 95% (14). The results of this analysis indicated that a sample of 86 would be necessary; however, to achieve more reliable results, we recruited a total of 228 participants (116 males and 112 females). The participants were recruited among the patients who presented at the Department of Orthodontics, Faculty of Dentistry, Ordu University, between 29 January 2022 and 22 March 2022, as well as their first-degree relatives who were willing to take part in the study. This study was approved by the Clinical Research Ethics Committee of Ordu University (Number: 2022/14). All participants signed informed consent forms.

The self-report survey was conducted after routine intra-oral and extra-oral examinations. Demographic data collected included age, gender, profession, monthly income, marital status, number of children, and harmful habits.

Inclusion criteria were as follows: (1) systematically healthy individuals; (2) age between 18 and 60 years old; (3) regular sexual intercourse in the last 6 months; and (4) no history of orthodontic or bruxism treatment. From the sample, we excluded (1) individuals with total edentulism; (2) those who had no sexual intercourse in the last 6 months; (3) pregnant or breastfeeding females; and (4) individuals receiving psychological treatment.

All eligible participants filled in the 21-item Beck Depression Inventory (BDI) and the 10-item Fonseca Anamnestic Index (FAI). Additionally, female participants completed the 19-item Female Sexual Function Inventory (FSFI), while male respondents filled in the 5-item International Index of Erectile Function (IIEF) form.

TMD assessment

The 10-item FAI questionnaire was administered to the participants to evaluate factors such as parafunctional habits, chewing, movement restrictions, sounds coming from the TMJ, and dizziness. Responses were scored as follows: "Yes" (10 points), "Sometimes" (5 points), or "No" (0 points) (11). The scores for all questions were then summed up to obtain the total score. Severity of the TMD was evaluated according to a scoring system where 0-15 points meant the absence of signs and symptoms of TMD, 20-45 points indicated mild TMD, 50-65 indicated moderate TMD, and 70-100 points were assumed to indicate severe TMD (12).

Assessment of sexual dysfunction

Sexual dysfunction among the female participants was evaluated using the Turkish version of the FSFI, originally developed by Rosen (15) and subsequently translated into Turkish by Öksüz and Malhan (16). This translation has been deemed reliable and appropriate for the purposes of this study.

The FSFI is a validated 19-item self-report questionnaire designed to evaluate sexual function based on six factors: desire, arousal, lubrication, orgasm, satisfaction, and pain. The FSFI was previously found to have high internal consistency and re-testing reliability and is extensively used to identify females with or without sexual dysfunction. Participants rated the FSFI items using a 6-point Likert scale, with each response assigned a value between 0 and 5. The total score ranged from a minimum of 0 to a maximum of 93.

Sexual dysfunction among the male participants was evaluated using the IIEF-5 form, a variant of the International Index of Erectile Function (IIEF) developed by Rosen (17). More specifically, we used the 5-item Turkish variant of the index developed by Deveci et al. (18) as this version has been proven to be valid and reliable.

The five items on the IIEF were rated using five options. The total index score ranged from a minimum of 5 to a maximum of 25 points.

Assessment of depression levels

The BDI is a 21-item self-report index that assesses the level of depression in individuals receiving and not receiving psychiatric treatment. Responses to items on the scale are scored between 0 and 3 points. Total BDI scores range from 0 to 63, with scores between 10 and 16 indicating mild depression, 17 to 21 indicating moderate depression, and 30 to 63 indicating severe depression (19,20). Hisli reported that the Turkish version of the scale is reliable and valid (21).

Statistical analyses

Statistical analyses were conducted using SPSS for Windows (version 20.0; SPSS Inc., Chicago, Illinois). The Kolmogorov-Smirnov test was employed to ascertain whether the data exhibited a normal distribution. The independent t-test was used for parameters showing a normal distribution; otherwise, the Mann-Whitney U test was used. Correlation tests were conducted to evaluate the impact of depression and sexual dysfunction on bruxism. The statistical significance was set at a p value of <0.05.

RESULTS

The mean age of male participants (35.76±8.45 years) was very close to that of the female participants (35.35±7.80 years). Table 1 illustrates gender-based distribution of the FAI scores. The results revealed that the probability of severe TMD was significantly higher in females than in males ($p<0.05$). Similarly, gender-based comparison of the individuals with respect to the absence of TMD problems revealed that the number of males (n=49) was higher than that of females (n=31).

Furthermore, a comparison of BDI scores revealed that the mean BDI score for females (13.25±9.51) was higher than that for males (10.99±8.00). However, this difference did not reach statistical significance ($p=0.105$; see Table 2).

In both groups, mild depression was identified with BDI scores between 10 and 16 points. The results of Kendall's tau-b correlation analysis revealed a positive correlation between FAI and BDI scores in male patients. The incidence of TMD increased in male patients with higher depression levels. However, no similar trend was observed in female patients (Table 3).

In addition, as shown in Table 4, the IIEF scores of males negatively correlated with both FAI (-0.102) and BDI (-0.312**) scores. Male participants with higher TMD severity scored lower on sexual function indexes (SFV; IIEF, FSFI). Similarly, a negative correlation was identified between SFV, FAI and BDI scores of female patients (Table 4).

Next, a comparison of FAI scores and SFV revealed a reduction in SFV scores among both male and female patients with severe TMD. However, this decrease was not statistically significant. The lowest IIEF score (19.00±8.49) was observed in males with severe TMD (maximum IIEF: 25.00). Similarly, females with severe TMD exhibited the lowest FSFI scores (44.38±17.30), which were less than half of the maximum score of 95.00 (Table 5).

Table 1. Distribution of TMD Scores by Gender

	No TMD	Mild TMD	Moderate TMD	Severe TMD	P-value*
Male	49	44	21	2	0.022
Female	31	42	31	8	

*Results of Fischer's Exact test. TMD: Temporomandibular joint dysfunction.

Table 2. Comparison of BDI Scores by Gender

	Male	Female	P-value*
	Mean (SD)	Mean (SD)	
BDI Scores	10.99 (8.00)	13.25 (9.51)	0.105

*Mann-Whitney U test. SD: Standard Deviation; BDI: Beck Depression Inventory.

Table 3. Comparison of Beck Depression Inventory (BDI) and TMD Scores by Genders Using Kendal Tau-b Correlation

	BDI Score	
	Male	Female
Fonseca Anamnestic Index Score	0.224*	0.096

*p<0.01. BDI: Beck Depression Inventory.

Table 4. Comparison of Erectile Function (IIEF) and Female Sexual Function (FSFI) Scores with TMD and BDI Scores

	Fonseca Anamnestic Index Scores [α]	BDI Score [β]
Female Sexual Function scores	-0.122	-0.019
Erectile Function scores	-0.102	-0.312**

[α] Kendall's tau-b correlation; [β] Spearman's rho correlation; *p<0.01. BDI: Beck Depression Inventory; IIEF: Comparison of Erectile Function; FSFI: Female Sexual Function Index; TMD: Temporomandibular joint disorder.

Table 6 shows the results of the comparison between BDI and sexual function scores by gender. A statistically significant difference was observed between BDI and IIEF scores ($p<0.05$). Likewise, in females, the FSFI scores declined as the severity of depression increased; however, this difference did not show statistical significance ($p>0.05$).

Table 5. Comparison of TMD Scores and Male Erectile Function / Female Sexual Function Scores by Gender

	Male Erectile Function scores	Female Sexual Function scores
No TMD	21.63 (3.70)	50.13 (12.39)
Mild TMD	20.95 (3.67)	47.86 (13.43)
Moderate TMD	21.00 (3.00)	45.10 (17.77)
Severe TMD	19.00 (8.49)	44.38 (17.30)
P-value*	0.606	0.443

*Kruskal-Wallis H test. TMD: Temporomandibular joint disorder.

Table 6. Comparison of BDI Scores and Male Erectile Function / Female Sexual Function Scores by Gender

	Male Erectile Function scores	Female Sexual Function scores
Minimal Depression	21.96 (3.31)	47.84 (15.32)
Mild Depression	21.30 (3.21)	46.89 (14.30)
Moderate Depression	20.14 (4.38)	46.54 (17.25)
Severe Depression	17.00 (3.74)	44.75 (16.80)
P-value*	**0.025**	0.920

*Kruskal-Wallis H test. BDI: Beck Depression Inventory.

DISCUSSION

The results of the present study showed a negative correlation between SFV and BDI scores. Similarly, both male and female participants exhibited a negative correlation between their SFV and FAI scores. A statistically significant relationship was identified between SFV and BDI scores exclusively among male participants. Likewise, a positive correlation was identified between FAI and BDI scores, which was statistically significant only in males. As males and females developed TMD problems, there was a concomitant increase in their respective depression levels. Yet, the association between depression and TMD problems reached statistical significance only in males.

Previous research have documented those symptoms of depression are more common in adult females. Compared to their male counterparts, females are more likely to experience psychiatric disorders, such as stress-related depression, throughout their lifetimes. In contrast, such as violence were reported to be more prevalent in males (22). Our analysis of the distribution of depression scores by gender revealed that females (13.25±9.51) had higher BDI values than males (10.99±8.00). Furthermore, previous research have documented that anxiety and depression reduce the pain threshold or alter pain perception in affected individuals (23), leading to symptoms such as pain in the TMJ and orofacial muscles. In addition, available evidence suggests that TMD patients exhibit similar psychological profiles and dysfunctions to those of individuals with chronic musculoskeletal pain problems (24). Our findings are consistent with those reported in a recent study (25). On the correlation between depression, stress and TMD. The authors found that 56% of TMD patients exhibited varying degrees of depressive symptoms, with 66.6% of this group being women. No statistically significant difference in the prevalence of elevated depressive symptoms was observed between genders. In our study, depression levels were higher in females than in males, and TMD severity increased with rising levels of depression.

Overall, TMD, SD, and depression can mutually reinforce one another in a complex manner. Specifically, depression may precipitate pain and muscle tension, thereby exacerbating TMD (26). Additionally, depression may impact libido and cause SD. Reduced libido may be a symptom of major depressive disorder. It is also a recognized consequence of depression, which can exacerbate both conditions (27). Individuals experiencing chronic pain related to TMD may subsequently develop depression. TMD can also impede sexual activity, thereby leading to the development of SD. Sexual dysfunction can adversely impact self-esteem and emotional well-being-which, in turn, may lead to or exacerbate depression. Moreover, stress, anxiety and depression associated with sexual dysfunction may also cause increased muscle tension, which could possibly exacerbate TMD symptoms (28). Laurent et al. (29) proposed that the relationship among sexual dysfunction, anxiety, and depressive disorders is multifaceted, complex, and often develops concurrently. Accordingly, and considering the pivotal role of psychological factors such as anxiety and depression in TMD, in this study, we aimed to elucidate the correlation between TMD and sexual dysfunctions. The findings revealed a negative correlation between TMD severity and SD values in both male and female

subjects, with correlation coefficients of -0.102 and -0.122, respectively. However, it is crucial to acknowledge that all three conditions can be affected by a broad range of factors, resulting in a complex network of interactions. This underscores the need for a holistic approach that addresses physical pain, psychological well-being, and sexual health in order to effectively manage these interconnected issues.

Previous epidemiological studies confirmed that psychiatric disorders are risk factors for sexual desire and arousal (30). Furthermore, recent studies reported strong associations between anxiety and depression, on one hand, and orgasm difficulties and sexual pain, on the other (13,31). Similarly, in a self-assessment study, Liu et al. (32) found that 61.9% of patients with major depressive disorder experienced sexual dysfunction. Of note, this prevalence was significantly higher in females (75.3%) compared to males (38.4%). In another relevant investigation, Galati et al. (33) found a significant association between SD and depressive symptoms in married couples, as well as a positive correlation between depressive symptoms and marital dissatisfaction. In our study, we observed a negative correlation between BDI and SD scores (male r=-0.312, female r=-0.019). The patients with advanced depression symptoms exhibited lower SD values, in both male (17.00±3.74) and female (44.75±16.80) groups. These findings are broadly consistent with previous research (34).

Furthermore, an increased level of muscle activity, the clenching and grinding of teeth, traumatic injuries to the masticatory system and postoperative complications following dental treatment are all factors that contribute to the etiology of TMJ problems (35). Over time, parafunctional habits have been reported to cause a number of adverse effects, such as pain in the masticatory muscles and neck, headaches, pain and loss of function in the TMJ, limitation of mandibular movement, decreased pain threshold in the orofacial and surrounding muscles, depression, anxiety and increased stress (36). Such physiological and psychological alterations prompt the adrenal cortex to secrete cortisol, a stress hormone that can lead to infertility, particularly in females. Moreover, elevated cortisol levels in the bloodstream can diminish libido and reproductive function by reducing dehydroepiandrosterone, a steroid hormone secreted by the adrenal glands (37,38). In the light of these considerations, a potential correlation between TMD, depression, and SD merits discussion. The results of the present study revealed that these three issues are interrelated, although the strength of the association between them was not statistically significant in all comparisons. This outcome may be attributed to physiological differences between males and females, as well as the relatively small sample size.

It is insufficient to rely on FAI alone to evaluate the severity of TMD. Considering that the clinical and radiological evaluation of TMD is of undeniable importance in its classification, its absence constitutes one of the limitations of the present study. Moreover, through diverse diagnostic techniques, it is possible to explore how parafunctional habits and other TMD etiologies affect the functionality of multiple physiological systems, including brain activity, muscle activity, heart function, and breathing. Therefore, it is thought that TMD is not caused exclusively by mechanical factors such as occlusal incompatibilities or psychological diseases such as stress, anxiety or depression or a combination of the two. Rather, it is now understood to be a condition with multiple etiologies (24,39).

Another limitation of the present study is a potential response bias in the survey data, which may have compromised the reliability of our data set. While our results enabled us to observe the interaction between males and females with varying degrees of TMD, depression, and SD, our findings do not provide sufficient evidence to establish a causality between these parameters. Consequently, it is not possible to determine whether these disorders have causality. However, based on the findings of the present study and previous research, it is reasonable to conclude that individuals with varying degrees of depression are more susceptible to TMD and sexual dysfunction. Nevertheless, further research is required to confirm this correlation.

CONCLUSIONS

The results of the present study indicate that elevated depressive symptoms are associated with an increased prevalence of TMD in both males and females, and that there is a correlation between sexual dysfunction and depression. In addition, we also observed that male gender is a strong predictor of the prevalence of TMD, depression, and sexual dysfunction.

Furthermore, our results suggest that as the severity of TMD increases, so does the prevalence of sexual dysfunction. However, when psychological factors are taken into account, it is difficult to determine whether TMD contributes directly or indirectly to this phenomenon.

Ethical approval

This study has been approved by the Clinical Research Ethics Committee of Ordu University (approval date 28.01.2022, number 2022/14). Written informed consent was obtained from the participants.

Author contribution

Surgical and Medical Practices: NB, FA; Concept: SKB; Design: RT; Data Collection or Processing: NB, FA; Analysis or Interpretation: SKB; Literature Search: RT; Writing: RT. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):112-121
DOI: 10.54307/NWMJ.2025.142

RESEARCH ARTICLE

Characteristics of the biological activities of the piperidine complex: an anticancer and antioxidant investigation

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Cite as: Sabancılar İ, Aydemir M, Kaya S. Characteristics of the biological activities of the piperidine complex: an anticancer and antioxidant investigation. Northwestern Med J. 2025;5(2):112-121.

ABSTRACT

Aim: To determine the anticancer and antioxidant activity levels of the synthesized heterocyclic molecule named 1-benzyl-1-(2- methyl-3-oxo-3-(p-tolyl)propyl) piperidin-1-ium chloride.

Methods: The molecule 1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl) propyl)piperidin-1-ium chloride was synthesized solvent-free via microwave synthesis. Piperidine purification involved dichloromethane extraction with 2 M HCl, followed by 5% NaHCO3 and precipitation with n-hexane. Anticancer activity on A549 lung cancer cells was assessed using the MTT assay. Antioxidant activity was evaluated by DPPH and CUPRAC methods at five concentrations (250-15.6 µM), with ascorbic acid as a control.

Results: The heterocyclic molecule dissolved in PBS was tested for anticancer activity on A549 cells at concentrations ranging from 6.25 to 100 µM. Cytotoxicity was highest at 66.90% for 100 µM and decreased to 5.57% at 6.25 µM, with an IC50 of 32.43 µM. In DPPH assays, the absorbance for AscA varied from 1.263±0.057 to 0.675±0.093, while the piperidine molecule ranged from 1.339±0.044 to 1.072±0.120. In CUPRAC assays, AscA absorbance was 0.227±0.052 and 1.768±0.176, and for the piperidine molecule, it was 0.132±0.042 and 0.142±0.031.

Conclusion: Piperidine is considered a saturated heterocyclic ring and possesses a wide range of biological activities. In this study, it was observed that the synthesized piperidine molecule showed limited DPPH radical scavenging activity. It also showed a high level of cytotoxic effect on A549 cancer cells and could be an important molecule for anticancer studies.

Keywords: antioxidant activity, cancer, cytotoxicity, drug synthesis

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Received: 11.08.2024 **Accepted:** 30.10.2024 **Published:** 28.04.2025

INTRODUCTION

Oxidants are reactive molecules that are formed during biochemical processes in the body and can damage cells. Free radicals are highly reactive oxidants that originate from endogenous or exogenous factors, are usually formed naturally during oxygen metabolism, and can damage cells when they exceed a certain level. Excessive production of these molecules or inadequate antioxidant defense mechanisms can lead to oxidative stress and cellular damage. Oxidative stress plays a role in the development of various chronic diseases such as aging, cancer, cardiovascular diseases, and neurodegenerative disorders by damaging cellular components such as DNA, proteins, and lipids (1,2).

Long-term oxidant exposure as a result of antioxidant deficiency has been associated with cell damage and many diseases (3). For this purpose, providing antioxidant support from exogenous sources is thought to prevent the occurrence of these undesirable conditions. Research continues to synthesize and discover new antioxidant molecules with different effects in order to prevent the occurrence of these diseases or to stop their progression (4).

Cancer, in which the antioxidant/oxidant imbalance plays important roles, is a group of diseases caused by the uncontrolled and abnormal growth and proliferation of cells. Normal cells are subject to a specific process of growth and division, which ends with programmed cell death (apoptosis) in the event of cell aging and damage. However, in cancer cells, these regulatory mechanisms are disrupted, causing the cells to proliferate uncontrollably (5).

Cancer can develop in almost any tissue in the body, can cause tumor formation and spread to other organs through metastasis (6). Both in the stages of cancer development and during treatment, depending on the tissues and systems it affects, it is accompanied by significant health problems. It is an important health problem worldwide and threatens the lives of many people. The most common treatment modalities are surgery, radiotherapy, chemotherapy, and immunotherapy. However, since the most effective treatment method may vary depending on the type of cancer and the individual, one or more of these treatment methods can be applied together or at different times (7). The nature of cancer and its ability to metastasize complicate the treatment process and necessitate the development of new treatment methods (6).

Lung cancer is globally prevalent and one of the deadliest cancers due to its significant impact on vital systems and the poor prognosis associated with the disease. This type of cancer is often diagnosed late because it does not show symptoms in the early stages, which reduces the chances for effective treatment (8).

Innovative approaches to cancer treatment aim to prevent the growth and metastasis of cancer cells (9,10). The complex nature of lung cancer and its potential for resistance to treatment necessitates the development of new and effective treatment strategies (11). In this context, the anticancer potential of piperidine and its derivatives emerges as a promising area of research. Piperidine and its derivatives have the potential to stop the cell cycle and induce apoptosis in cancer cells with their potent antioxidant and anticancer properties (12). Therefore, studies on the biological activities of piperidine complexes may contribute to the development of new and effective strategies in cancer treatment.

Piperidine is a saturated heterocyclic ring that is considered an important compound due to its various roles in biological activities. It is noted for its strong antioxidant properties and diverse biological activities. These activities include anti-microbial, anti-inflammatory, anti-viral, anti-malarial, general anesthetic, anti-depressant, anti-oxidant, anti-epileptic, anti-tumor, anti-convulsant and anti-hyperlipidemic activities (13). The planar structure of piperidine allows the addition of substituent groups at different positions of the ring, which can enhance its biological activities. For example, piperine (PubChem CID: 638024, Figure 1b), a piperidine derivative, is an alkaloid derived from the plant Piper nigrum L. (black pepper) that shows potent antioxidant activity due to its ability to inhibit or quench free radicals. Piperine shows antioxidant, antiplatelet, anti-inflammatory, antihypertensive, hepatoprotective, antithyroid effects (14).

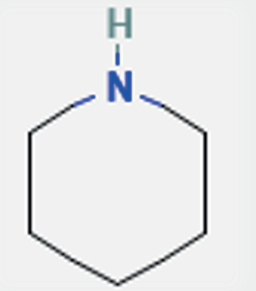
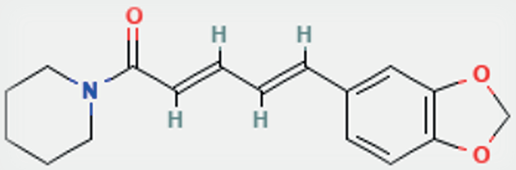
a) Piperidine	b) Piperine
	
PubChem CID: 8082 **Molecular Weight**: 85.15 g/mol Hexahydropyridine Cyclopentimine Azacyclohexane	**PubChem CID**: 638024 **Molecular Weight**: 285,34 g/mol

Figure 1. Some chemical properties of piperidine and piperine compounds

Piperidine (PubChem CID: 8082, Cyclopentimine, Hexahydropyridine) is a heterocyclic compound consisting of a six-membered ring containing five methylene groups (-CH_2-) and one amine group (-NH-) (Figure 1a). This compound can be found in barley (*Hordeum vulgare L., Poaceae*) and black pepper (*Piper nigrum L., Piperaceae*) and provides the characteristic flavor of black pepper and plays an important role in the pharmaceutical industry (12,15). Piperine, a piperidine derivative, has therapeutic potentials against cancers such as breast cancer, ovarian cancer, stomach cancer, glioma cancer, lung cancer, oral squamous, chronic pancreatitis, prostate cancer, rectal cancer, cervical cancer and leukemia (12). Research has shown that piperidine nitroxides, such as TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy radical), a piperidine derivative, exhibit potent antioxidant effects in various ways (16). The planar structure of the heterocyclic core of piperidine allows different groups to be attached at different positions of the ring (13). Due to the skeletal structure of piperidine, it can also be investigated as an important anticancer agent by acting on important receptors or with different derivatives to be created (17). These properties make it possible to study a wide range of biological activities of piperidine derivatives (13).

Piperidine and its derivatives contribute to various biological processes that induce cell apoptosis and inhibit the growth of cancer cells. Activation of cell apoptosis by contributing to anticancer biological processes such as activation of mitochondrial cytochrome C, release of Bax-protein from mitochondria and down-regulation of Bcl-2 protein (12). Its wide range of biological activities makes piperidine an important compound in pharmaceutical research and drug development. The aim of this study was to determine the anticancer and antioxidant activity levels of the synthesized heterocyclic molecule with the chemical structure of 1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl) propyl)piperidin-1-ium chloride (Figure 2).

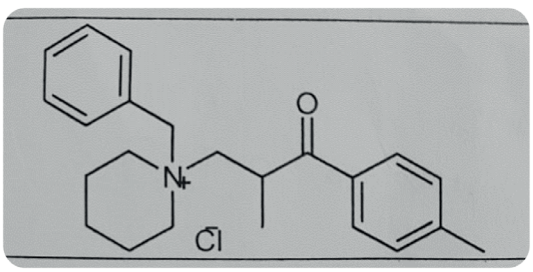


Figure 2. 1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl) propyl)piperidin-1-ium chloride

MATERIALS AND METHODS

Piperidine Derivative Synthesis

In the first step of the study, **1-benzyl-1-(2-methyl-3-oxo-3-(p-tolyl)propyl)piperidine-1-ium chloride** molecule was synthesized in a solvent-free medium using the microwave synthesis method (Figure 2). In the purification of piperidine, the amide dissolved in dichloromethane was first extracted with 2 M HCl followed by 5% sodium bicarbonate ($NaHCO_3$) and precipitated with n-hexane.

Cytotoxic Effect of Piperidine on A549 Cell Line

The lung cancer cell line (A549) was used for anticancer activities. The cytotoxic effect of the test compounds on the cells was determined by the MTT method, an enzymatic method widely used in the determination of cytotoxicity. This method is based on the ability of the MTT compound to cleave the tetrazolium ring. The compound (MTT) is absorbed into living cells and the reaction is catalyzed by mitochondrial succinate dehydrogenase and reduced to blue-violet, water-insoluble formazan (Figure 3) (18-20).

After thawing the stock A549 (Lung Cancer) cell line in a cryotube in a sterile water bath at 37 0C, studies were continued under sterile conditions in a laminar flow cabinet. Cells were homogenized by gentle pipetting. Dulbecco's Modified Eagle Media (DMEM) medium containing 20% (50 ml) Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA), 1% (5 ml) Penicillin/ Streptomycin (Sigma-Aldrich, USA) and 2.2 g/L

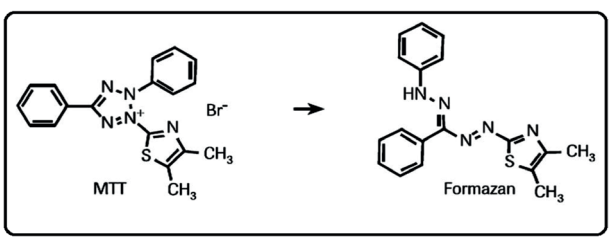


Figure 3. MTT-Formazan conversion via mitochondrial enzymes in living cells (20)

Sodium bicarbonate for A549 cell line was used. A549 cells were transferred to 25 cm2 sterile cell growth dishes (flasks) containing culture medium. The flask was placed in an incubator containing 5% CO2 at 37 oC. The flasks were checked daily under an inverted microscope, the medium was changed, and once the cells became confluent, they were passaged and the cells were multiplied and transferred to larger flasks. In 75 cm^2 culture flasks, the cells were cultured in DMEM medium prepared by adding 10% FBS, penicillin and streptomycin at 37°C in an incubator with 5% CO_2. Passaging was performed when the cells to be examined under the inverted microscope were confluent (when they reached 80-85% density). Tyrpan blue dye and cell suspension were mixed in a one-to-one ratio and cell counting was performed on a Thoma slide and the number of cells was determined as follows:

Equation 1:

$$Live\ cell\ count/mL = Mean\ live\ cell\ count\ x\ Dilution\ coefficient\ x\ 10^4$$

The respective serial dilutions of the piperidine complex were dissolved in 1% PBS (1ml) to a final concentration of 100, 50, 25, 12.5, and 6.25 uM and passed through a 0.22 µm filter (Millipore, USA). These samples were then added to 96-well plates in which cells were seeded and incubated for 24 hours.

For cytotoxicity assay, MTT solution was prepared in sterile phosphate buffer at a concentration of 0.5 mg/mL, and applied to each well and incubated for 3 hours. After incubation, the contents of each well were discarded and 100 µL dimethylsulfoxide (DMSO) was added to dissolve formazone crystals. The 96-well plate was covered with aluminum foil and mixed for 10 minutes. The optical densities (OD) of the cells in the wells were measured in an ELISA microplate reader (Thermo MultiskanGo, USA) at 570 nm wavelength (19). The absorbance values of the control group were averaged and this value was considered as 100% viable cells and cytotoxicity was determined by proportioning each well to which different concentrations of piperidine were added.

Determination of Antioxidant Activity of Piperidine

Assays used to evaluate antioxidant compounds can be classified into those associated with lipid peroxidation (thiobarbituric acid assay [TBA] and others) and those associated with electron or radical scavenging [2,2-diphenyl-1-picrylhydrazyl (DPPH) ABTS, FRAP, FTC and aldehyde/carboxylic acid assay] (13). DPPH and CUPRAC activities were evaluated to determine the antioxidant effects of piperidine.

Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

The DPPH antioxidant activity determination method developed by Blois (21) was modified and used. A 1 mM solution of DPPH- radical was used as a free radical. Different concentrations were prepared by dissolving the synthesized piperidine derivative molecule in water with final concentrations of 250, 125, 62.5, 31.25, and 15.63 µg/mL. Water was used as a blind and L-ascorbic acid (Merck, Germany) at the same concentrations was used as a positive control. 150 µL of DPPH- solution and 50 µL of sample were added to each well of the 96-well plate, incubated for 30 minutes at room temperature and in the dark, and the absorbance was measured at 517 nm. Three replicates were run for each different concentration and the data were calculated by the formula given below and presented as mean and standard deviation.

Equation 2:

$$DPPH\ Inhibition\% = \left(\frac{A_{Control} - A_{Sample}}{A_{Control}}\right) x100$$

$A_{Control}$: *Control Absorbance*

A_{Sample}: *Sample or Ascorbic acid Absorbance*

Determination of CUPRAC (Cupric Reducing Antioxidant Capacity)

CUPRAC is an analysis method used to determine the antioxidant capacity of a molecule. In this method, antioxidants react with copper(II)-neocuproin complex (Cu(II)-Nc) to form copper(I)-neocuproin complex (Cu(I)-Nc), which is determined by color change. This method is widely used because it is simple, rapid and sensitive and is useful in assessing the activity of various antioxidant compounds.

In order to determine the antioxidant activity, 0.01 M CuCl2 solution, 7.5x10-3 M ethanolic neocuprin solution and 1 M ammonium acetate buffer were prepared. 75 µL of each of these solutions were added to 96 wells. 75 µL of five different concentrations of piperidine or ascorbic acid solution were added, with a final concentration of 250 - 15.63 µg/ml. After a half-hour incubation in the dark, the absorbance at 450 nm was recorded. Increasing absorbance of the reaction mixture indicates increasing copper ion (Cu2+) reducing capacity. Three replicates were run for each concentration and absorbance values are presented as mean and standard deviation.

Statistical analysis

Statistical analysis results of the MTT study were obtained with GraphPad Prism 8 program. The data of the study were analyzed on a computer using SPSS 22 software. Two-way ANOVA test was performed to determine the differences between the groups studied in the MTT method. The significance level in different groups was determined according to $p<0.05$. The graphs and IC_{50} (sample concentration providing 50% inhibition) values of DPPH and MTT results were obtained using GraphPad Prism 8 program and A_{50} (sample concentration halving the maximum absorbance) values of CUPRAC results were obtained using the Microsoft Excel program.

RESULTS

Cytotoxic Effect of Piperidine on A549 Cell Line

The cytotoxic activity of the synthesized heterocyclic molecule in A549 cell line was determined by MTT method. At the highest concentration of 100 µM, 66.90% of A549 cells, 60.81% at 50 µM, 46.66% at 25 µM, 30.77% at 12.5 µM and 5.57% at 6.25 µM showed cytotoxic effect. According to these findings, IC_{50} value was obtained as 32.43 µM (Table 1 and Figure 4).

Table 1. Cytotoxicity ratio versus increasing piperidine concentration

	Concentration of Piperidine Complex (µM)				
NT	**6.25**	**12.5**	**25**	**50**	**100**
100	97.9	55.3	45.4	39	32.2
100	100	62.3	56.2	41.4	36.0
100	100	73	59.6	41.1	34.4
100	87.3	73.5	54.2	37.6	33.2
100	90.4	76.1	53.1	37.7	31.1
100	91	75.2	51.5	38.4	31.7
Mean±SD	94.4±5.05	69.2±7.72	53.3±4.37	39.2±1.52	33.1±1.68
	p<0.05	p<0.05	p<0.05	p<0.001	p<0.001

SD: Standard Deviation; NT: Non-Treatment.

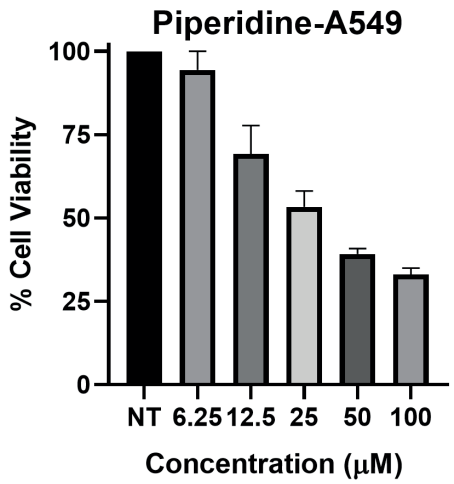


Figure 4. Plot of cytotoxicity rates against increasing piperidine concentration

(NT: Non-Treatment)

Antioxidant Activity of Piperidine

DPPH Radical Scavenging Activity

DPPH inhibition levels of ascorbic acid were determined as 58.2±0.8%, 52.8±0.6%, 48.0±0.4%, 35.1±0.8% and 17.6±1.1% at different concentrations between 250-15.63 µg/mL, respectively. For the piperidine complex synthesized under the same conditions, these values were 23.4±1.9%, 19.3±4.4%, 11.5±5.8%, 6.5±4.5% and 3.5±2.4%, respectively. Based on these findings, the IC_{50} value of ascorbic acid was calculated as 106.85 µg/mL, whereas the IC_{50} value was not calculated since the piperidine complex did not show 50% or more inhibition at the indicated concentrations and can be expressed as IC_{50} > 250 µg/mL (Table 2 and Figure 5).

CUPRAC Identification

The antioxidant activity of the synthesized piperidine complex was also evaluated by the CUPRAC method. During this evaluation, absorbance values for different concentrations of ascorbic acid between 250-15.63 µg/mL were read as 1.818±0.074, 1.244±0.012, 0.762±0.011, 0.578±0.014 and 0.227±0.037, respectively. Absorbance values for the piperidine complex synthesized under the same conditions were 0.142±0.025, 0.140±0.026, 0.142±0.023, 0.134±0.043 and 0.132±0.034, respectively. While the A_{50} value for ascorbic acid was calculated as 60.67 µg/mL, this value was not calculated since no significant activity was observed for the synthesized piperidine concentration (Table 3 and Figure 6).

DISCUSSION

Piperidine is a saturated heterocyclic secondary amine associated with various biological activities such as antimicrobial, anti-inflammatory, antiviral, antimalarial, general anesthetic, antidepressant, antioxidant, antiepileptic, antitumor, anticonvulsant and antihyperlipidemic activities (13).

Table 2. DPPH Inhibition Ratio Against Increasing Piperidine and Ascorbic Acid Concentration

Concentration (µg/mL)	Ascorbic A. (Mean±SD)	Piperidine (Mean±SD)
250	58.2±0.8	23.4±1.9
125	52.8±0.6	19.3±4.4
62.5	48.0±0.4	11.5±5.8
31.25	35.1±0.8	6.5±4.5
15.63	17.6±1.1	3.5±2.4
IC_{50}	106.85 µg/mL	> 250 µg/mL

Table 3. CUPRAC activity against increasing Piperidine and Ascorbic Acid concentration

Concentration (µg/mL)	Ascorbic A. (Mean±SD)	Piperidine (Mean±SD)
250	1.818±0.074	0.142±0.025
125	1.244±0.012	0.140±0.026
62.5	0.762±0.011	0.142±0.023
31.25	0.578±0.014	0.134±0.043
15.63	0.227±0.037	0.132±0.034
A_{50}	**60.67 µg/mL**	

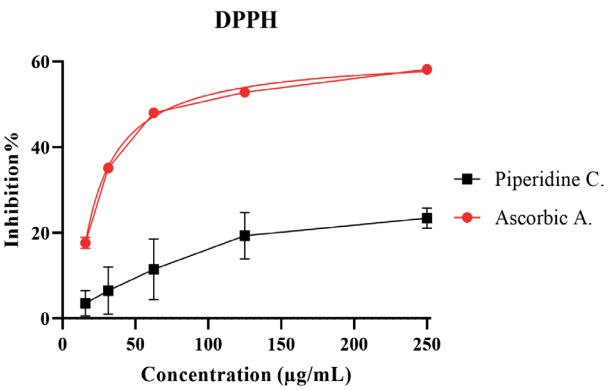


Figure 5. Plot of DPPH inhibition rates against increasing concentration of Piperidine complex and Ascorbic Acid

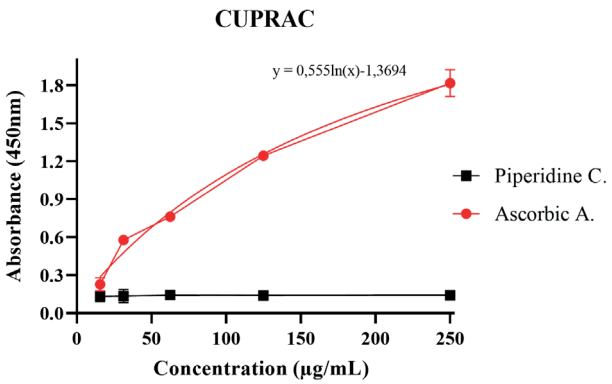


Figure 6. Plot of CUPRAC Activity against increasing Piperidine and Ascorbic Acid concentration

When the anticancer activities of the synthesized piperidine complexes were examined; Benaka et al. (22) synthesized new molecules containing more than ten piperidine groups. The antiproliferative activities of these compounds against HT-29 (colon carcinoma), HeLa (cervix-cervix carcinoma), MCF-7 (breast carcinoma) and HepG2 (hepatocellular carcinoma) cell lines at 100 µM concentration for 24 hours were evaluated by MTT assay. For these cell lines, 45.11-78.55%, 39.22-78.50%, 38.68-73.75% and 39.11-68.74% cell viability was maintained, respectively. In this study, the average survival for A549 cell line was 33.1±1.68% under the same conditions. It is understood that the molecules synthesized by Benaka et al. (22) showed a more cytotoxic effect on A549 cancer cell line. However, variables such as the structure of the molecules and the morphological structure of the treated cells should not be ignored.

Bezerra et al. (23) determined the cytotoxicity effects of piperine (Figure 1b), a piperidine derivative obtained from black pepper seeds and commercially available, on CEM and HL-60 (human leukemia), B16 (mouse melanoma) and HCT-8 (human colon) cell lines at increasing concentration by MTT method. They determined that these cell lines were cytotoxic with IC_{50} values of >87.6 µM, >87.6 µM, 69.9 µM and 66.0 µM, respectively.

Vinaya et al. (24) synthesized 9 different piperidine derivatives (4-[3-(piperidin-4-yl)propyl]piperidine derivative) at increasing concentrations and different incubation times and examined their MTT and some other cytotoxic effects on K562 and Reh (human leukemia cells) cell lines and concluded that they showed anti-leukemic effect with IC_{50} values of 2-125 µM. According to the investigations carried out in

this study and the studies presented in the literature, different piperidine derivatives can show anticancer effects at quite different levels according to the side groups that they confer.

Lahmidi et al. (25) with their study on determining the DPPH radical scavenging activity of three newly synthesized piperidine derivatives; they concluded that quercetin, which they used as a control, did not show significant activity, with IC_{50} values between 8.2±0.2 and 19.5±0.5 mM, compared to its IC_{50} values of 0.012±0.003 mM.

Prashanth et al. (26) determined the antioxidant activities of 10 newly synthesized piperidines and ascorbic acid by the same method. According to the results of this study, ascorbic acid showed an IC_{50} of 12.6±0.43 µg/mL, while piperidine derivatives showed IC_{50} values of 8.3±0.02 and 36.9±0.17 µg/mL. According to these data, some piperidine derivatives may show even better DPPH activity than ascorbic acid.

Karaman et al. (27) evaluated the DPPH activity of 19 different piperidine compounds and α-tocopherol as a control: IC_{50} value for α-tocopherol was calculated as 12.26±0.07 µM, while the IC_{50} value for piperidine compounds was calculated between 19.99±1.03 and 96.71±0.28. Thus, it was observed that none of these synthesized molecules showed DPPH activity as much as α-tocopherol.

In this study, a significant IC_{50} value (>250 µg/mL) could not be calculated since 50% inhibition of DPPH activity was not observed by the synthesized piperidine complex compound. Compared to ascorbic acid used as control, 58.2±0.8% inhibition was observed at the maximum ascorbic acid concentration (250 µg/mL), while 23.4±1.9% inhibition was observed at the same piperidine concentration.

Karaman et al. (27) evaluated the CUPRAC activity of 19 different piperidine compounds and α-tocopherol as a control: They calculated the A_{50} value for α-tocopherol as 40.48±1.87 µM, while the piperidine compounds were calculated between 4.16±0.04 and 92.13±0 µM. Thus, some of these synthesized molecules showed better CUPRAC activity than α-tocopherol. In this study, the compound showing the best CUPRAC activity compared to α-tocopherol (4.16±0.04 µM) did not show the same level of DPPH activity (74.83±0.46 µM, α-Tocopherol= 12.26±0.07 µM). Karaman et al. (27) synthesized 17 piperidine derivatives and α-tocopherol as control in DPPH and CUPRAC activity study; IC_{50} value between 31.35±1.07- 92.00±0.49 µM for DPPH activity of 17 piperidine derivatives and 17.72±0.03-87.82±0.01 µM A_{50} value for CUPRAC activity were calculated. For the DPPH and CUPRAC activities of α-Tocopherol, the data given in the other study are presented. In this study, ascorbic acid, which is used as a positive control for antioxidant studies, showed activity as expected with the CUPRAC method, and its A50 value was calculated as 60.67 µg/mL. While the synthesized piperidine compound exhibited some DPPH activity compared to ascorbic acid, no CUPRAC activity was observed.

CONCLUSION

Piperine and piperidine heterocyclic molecules have gained interest in the pharmaceutical industry as anticancer agents. Piperidine is considered as a saturated heterocyclic ring and is a structure with a wide range of biological activities. In this study, the synthesized piperidine molecule was also observed to exhibit limited DPPH radical scavenging activity. In addition, it showed a high level of cytotoxic effect in A549 cancer cells and it was concluded that it could be an important molecule for anticancer studies.

The cytotoxicity effects of this newly synthesized compound, which exhibits anticancer properties, can be investigated in healthy and other cancerous cell lines and its more comprehensive effects can be investigated with further in vivo studies. More comprehensive data can be obtained by investigating its antioxidant effects with other antioxidant methods other than CUPRAC and DPPH.

Ethical approval

Since the A549 cell line used in this study is a commercially available cell line, ethical approval was not required. Therefore, no ethical committee approval was obtained for this study.

Author contribution

Concept: İS, SK; Design: İS, SK; Data Collection or Processing: İS, MA, SK; Analysis or Interpretation: İS, SK; Literature Search: İS, SK; Writing: IS, MA, SK. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):122-126
DOI: 10.54307/NWMJ.2025.94

CASE REPORT

Direct contrast injection method: a novel approach to facilitate device crossing in peripheral artery lesions

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Cite as: Acar E, Dönmez İ, İzci S, et al. Direct contrast injection method: a novel approach to facilitate device crossing in peripheral artery lesions. Northwestern Med J. 2025;5(2):122-126.

ABSTRACT

It may be difficult to pass the device through the lesion due to moderate or severe calcification, tortuosity in or proximal to the lesion, excessive plaque load, the age of the lesion, and the characteristics of the device to be used. In such cases, the first thing that comes to mind is maneuvers to increase the support of the system. It may be preferable to choose low-profile and more flexible devices, or to angioplasty with small balloons and try again with a larger one. Intravascular ultrasound (IVUS) guidance and plaque debulking devices such as special balloons or atherectomy/laser/lithotripsy can be used to prepare the lesion. In addition, special lesion crossing devices can be used. However, the tools and equipment required for the special methods listed above are not available in every catheterization laboratory or they are not widely used because their use is not widely recommended and because of payment issues. We encountered a lesion that could not be passed through such a device, and we modified the plaque by injecting contrast directly into the lesion, as in the Carlino method.

Keywords: angioplasty, contrast agent, device, lesion, peripheral

INTRODUCTION

Percutaneous revascularization methods are most preferred in treating severe symptomatic lesions in peripheral arteries (1). The most common reasons for the failure of percutaneous procedures are the passage of the lesion with a guidewire, inability to pass the devices required for lesion preparation or guidewire exchange through the lesion, severe complications during lesion preparation, and inadequate preparation of the lesion (2). It may be difficult to pass a device through the lesion due to moderate or severe calcification, tortuosity in or proximal to the lesion, lesions with severe plaque load, age of the lesion, and features of the device to be used (3). There is no clear consensus on how to pass the device through the lesion. However, there are studies on some of the methods used in this regard (4). We presented a

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Received: 11.02.2024 **Accepted:** 03.09.2024 **Published:** 28.04.2025

method for such a condition, named Direct Contrast Injection into the Lesion for the Device Uncrossable Lesion, the Direct CIL method.

CASE PRESENTATION

A 67-year-old male patient was admitted with intermittent claudication in the left calf region. During the lower extremity arterial Doppler ultrasound (DUS), monophasic flow in the left popliteal artery (PA), arteria tibialis anterior (ATA), and arteria tibialis posterior (ATP) and total occlusion of the superficial femoral artery (SFA) were seen. Lower extremity peripheral angiography (PAG) was planned. Chronic total occlusion was observed at the left SFA, proximally just after the left common femoral artery (CFA) bifurcation (Figure 1, Video 1). A 7F 90 cm sheath (Flexor Shuttle-SL introducer guiding sheath, Cook, United States) was parked in the left CFA with a 6F right Judkins diagnostic catheter. A 0.035'' NAVICROSS® Support Catheter (Terumo, Tokyo, Japan) with a 0.035'' hydrophilic wire was attempted to cross the SFA occlusion. Then, several guidewires, including Gladius, Halberd, and Astato XS40, tried to cross but were unsuccessful despite the support catheter. So, as a last resort, we used the back of the 0.035'' hydrophilic wire, crossed the SFA occlusion, and finally successfully entered the true distal lumen (Figure 2, Video 2). However, we failed to cross the support catheter into the distal true lumen through the lesion. We exchanged the support catheter for a 4.0x120 mm peripheral balloon (Sterling, Boston Scientific, USA) to cross the lesion, with the aim of exchanging the wire back and performing balloon dilatation of the lesion in preparation for a larger balloon size. Unfortunately, the balloon could not cross the lesion (Video 3). We used simple solutions that can be done in such a situation. We positioned the access sheath at the closest location to the lesion. However, we could not use a special crossing device, atherectomy, laser, or lithoplasty because there was none in our laboratory. Again, we thought we could modify the lesion anterogradely with contrast injection before trying to do a second puncture in a retrograde way to pass the lesion and modify the plaque. We did not have a 0.035-inch compatible dual-lumen catheter. We also did not have an infusion catheter at the time. On the other hand, we could puncture a balloon and use it like an infusion catheter, but we didn't want to waste a new balloon as it was not yet a used balloon.

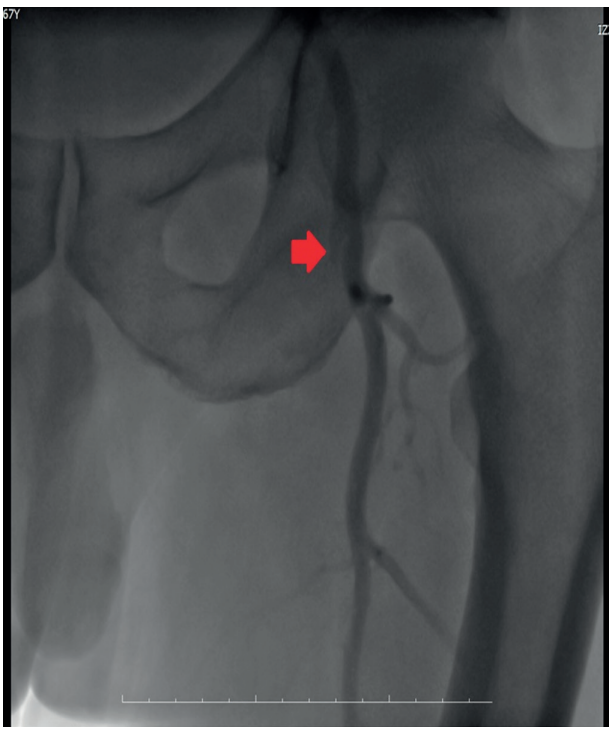

Figure 1. The occluded lesion proximal cap was showed with red arrow.

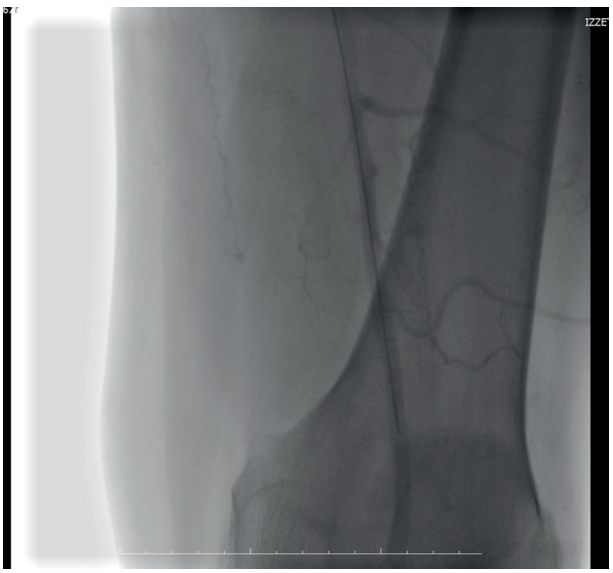

Figure 2. The back of the guidewire was in the distal true lumen.

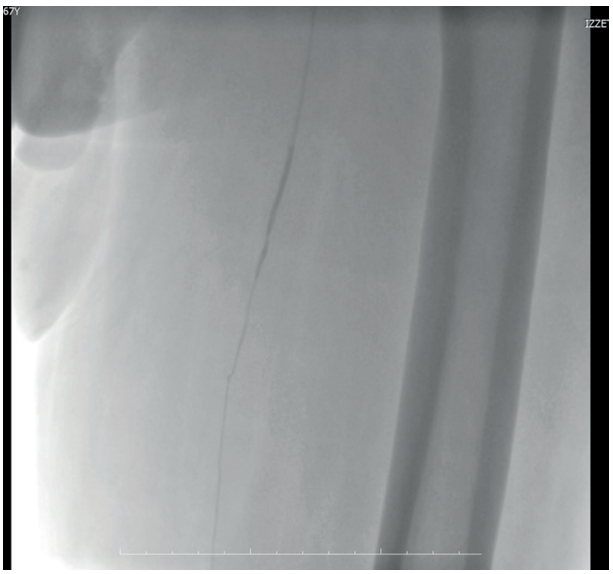

Figure 3. Direct CIL method.

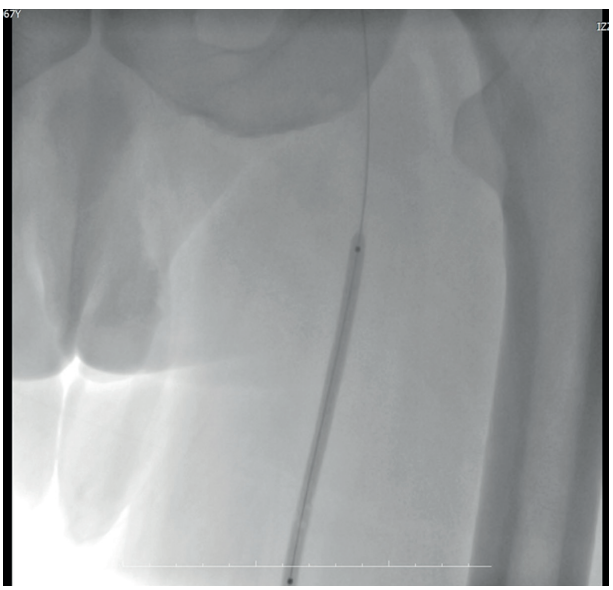

Figure 4. Balloon angioplasty with a 6.0 x 150 mm peripheral balloon.

We decided to soften the lesion with contrast agent-Direct CIL method. The support catheter was advanced as far as possible into the lesion with push and rotation. The guidewire inside was pulled, and 10 cc of contrast material (Iohexol 350 mg/ml) was injected into the lesion (Figure 3, Video 4). Then, the existing 0.035'' guidewire was passed through the support catheter crossed the SFA lesion again and into the true distal lumen. The lesion was dilated with a 4.0 x 150 mm peripheral balloon with balloon dilatation, followed by a balloon dilatation with a 6.0 x 150 mm peripheral balloon (Mustang, Boston Scientific, USA) (Figure 4). Afterward, we took an angiographic view (Video 5) to evaluate the lesion. We observed a heavy plaque burden at the distal lesion segment in addition to a long proximal dissection, which did not compromise the antegrade flow. So, we decided to watch the proximal dissection with medical treatment and employ a drug-eluted balloon only at the distal lesion segment for the bulky plaque burden. A 6.0 x 200 mm drug-eluted balloon (Extender, INVAMED, Ankara, Türkiye) was applied (Figure 5). It was concluded that the optimal result was obtained in the angiographic evaluation (Figure 6, Video 6), and the procedure was terminated without any complications. The patient was discharged one day after the procedure. The patient had no complaints at the 15th day control. It was observed that SFA was open on the DUS. The patient had no complaints at the 3rd and 6th month controls, and there was no serious obstruction in the SFA on Doppler USG.

Figure 5. Balloon angioplasty with a 6.0 x 200 mm drug eluted balloon at the distal lesion with bulky plaque burden.

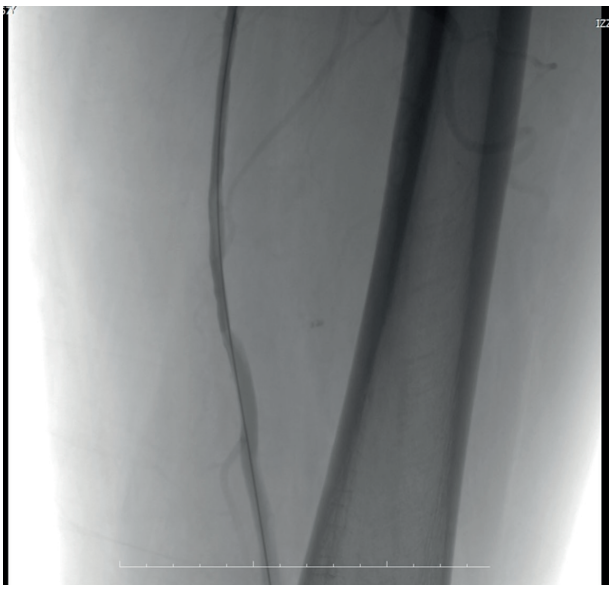

Figure 6. The final image after drug eluted balloon angioplasty.

DISCUSSION

Percutaneous revascularization is the preferred method for the treatment of severe symptomatic peripheral artery lesions (1). Procedural failure often results from guidewire passage issues, device advancement problems, lesion-related complications, or inadequate lesion preparation. Factors such as calcification, tortuosity, plaque load, lesion age, and device characteristics can hinder device passage (2,3). There must be a clear consensus on how to pass the device through the lesion. However, there are studies on some of the methods used in this regard (4). The first thing to do is to increase the support. Especially, the support features of guiding sheaths should be considered, and the most suitable sheath should be selected according to the vessel. Bringing the sheath to the position closest to the lesion is appropriate. The device features planned to be passed through the lesion are also important. A device profile as small as small can make the transition easier. Lesion preparation is also essential, especially for severe fibrocalcific lesions, to avoid futile effort. Crossing devices such as TruePath, Viance, Frontrunner, Crosser, Wildcat, and Ocelot can be used (5). However, the widespread use of these devices has yet to be established in every angiography laboratory and there may be particular financial barriers. In terms of lesion preparation, balloon dilatation with a special balloon catheter (cutting and scoring balloons), atherectomy (rotational and directional), endovascular laser ablation, and lithoplasty are other methods used (6). The last percutaneous method that can be used is the retrograde method (7). The lesion treatment can be started retrogradely; the lesion can be passed retrogradely, and the wire can be externalized and continued anterogradely. Again, a balloon can be inflated next to the wire crossing the lesion, and a retrograde distal anchoring balloon can be made, or this wire can be caught with a snare retrogradely, and the support of the wire can be increased retrogradely. Most of these methods can be considered as applying the methods used in the percutaneous treatment of coronary chronic total occlusion (CTO) to the peripheral arteries (8). In our case, we encountered an uncrossable lesion with a catheter and balloon. Although we successfully wired the lesion anterogradely, we could not pass the lowest profile balloon and support the catheter through the lesion. We used simple solutions that can be done in such a situation. However, we could not use a special crossing device, atherectomy, laser, or lithoplasty because none existed in our laboratory. We named this method "Direct contrast injection into the lesion-Direct CIL". We think the lesion can be used as a poor man's method in uncrossable situations. As a result of using the Direct CIL method in different centers and large patient groups, whether through device-based applications such as dual-lumen catheters or infusion catheters, or without a device as we did, this method will have an important place among the solutions for peripheral uncrossable lesions.

The Direct CIL method is not without some disadvantages. The first, and perhaps the most important one, is to pass the wire beyond the lesion and then administer the contrast agent. Microdissections caused by contrast administration may complicate rewiring. However, considering the lesion subtypes and conditions in which the Direct CIL method is used, this may be an acceptable risk. Secondly, severe dissection may develop during lesion modification caused by contrast injection, and there may even be a risk of perforation. Thirdly, microdissections caused by contrast injection may disrupt the collateral circulation that provides blood stream to the distal

vascular bed. Finally, the answer to the question of how much contrast material should be injected needs to be clarified. The amount used in coronary lesions is generally <5 cc (using the Carlino method) (8). We determined to inject 10 cc in the peripheral artery, which has a larger size than diameter of coronary arteries. However, the question of whether this is still an appropriate amount may be raised in future studies.

The Direct CIL method could be a simple solution for the peripheral arterial uncrossable lesions. However, the presented method is not without some disadvantages. In the future, we believe that the Direct CIL method will have an important place in the solution methods to be applied in peripheral uncrossable lesions by applying the large patient groups.

Ethical approval

Written informed consent was obtained from the participants.

Author contribution

Surgical and Medical Practices: EA, ID; Concept: EA; Design: EA, YG; Data Collection or Processing: EA, TD, EO; Analysis or Interpretation: EA, YG, IAI; Literature Search: EA; Writing: EA, SI. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):127-131
DOI: 10.54307/NWMJ.2025.98

CASE REPORT

Multiple cranial tuberculomas with meningitis and miliary tuberculosis in an immunocompetent adult

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Cite as: Doğan A, Gözdaş HT, Bal T, Şahin SB. Multiple cranial tuberculomas with meningitis and miliary tuberculosis in an immunocompetent adult. Northwestern Med J. 2025;5(2):127-131.

ABSTRACT

Miliary brain tuberculosis (TB) is a rare and highly lethal clinical form resulting from the extensive lymphohematogenous spread of Mycobacterium tuberculosis. Widespread brain involvement often suggests immunosuppression. However, cases have been reported in immunocompetent adults. Prognosis depends largely on prompt diagnosis and initiation of treatment. This case report presents an immunocompetent adult with miliary pulmonary TB diagnosed by bronchoalveolar lavage (BAL) fluid analysis and diffuse brain tuberculomas on brain magnetic resonance imaging (MRI).

Keywords: adult, immunocompetent, miliary tuberculosis, tuberculoma

INTRODUCTION

Miliary brain tuberculosis (TB) is still endemic in developing countries (1). It can affect any organ. Disseminated TB is a rare entity that often constitutes a diagnostic trap due to its misleading aspects and the frequent negativity of bacteriological samples. Subacute or chronic constitutional symptoms such as fever, weight loss, or night sweats are often observed and should prompt this diagnosis, particularly in endemic areas (2). It is mainly encountered in immunocompromised patients, but rare cases have been reported in immunocompetent patients (3).

CASE

A 19-year-old male from Afghanistan, with no history of chronic illness, presented to the emergency department with confusion and fever. Physical examination revealed a fever of >38°C. Neurological examination showed neck stiffness, with negative Kernig and Brudzinski signs. Extensive crackles were heard on lung auscultation. Other systemic examinations were unremarkable. Brain magnetic resonance imaging (MRI) showed lesions consistent with widespread supra and infratentorial tuberculomas (Figure 1). Chest computed tomography (CT) revealed

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Received: 01.03.2024 **Accepted:** 06.12.2024 **Published:** 28.04.2025

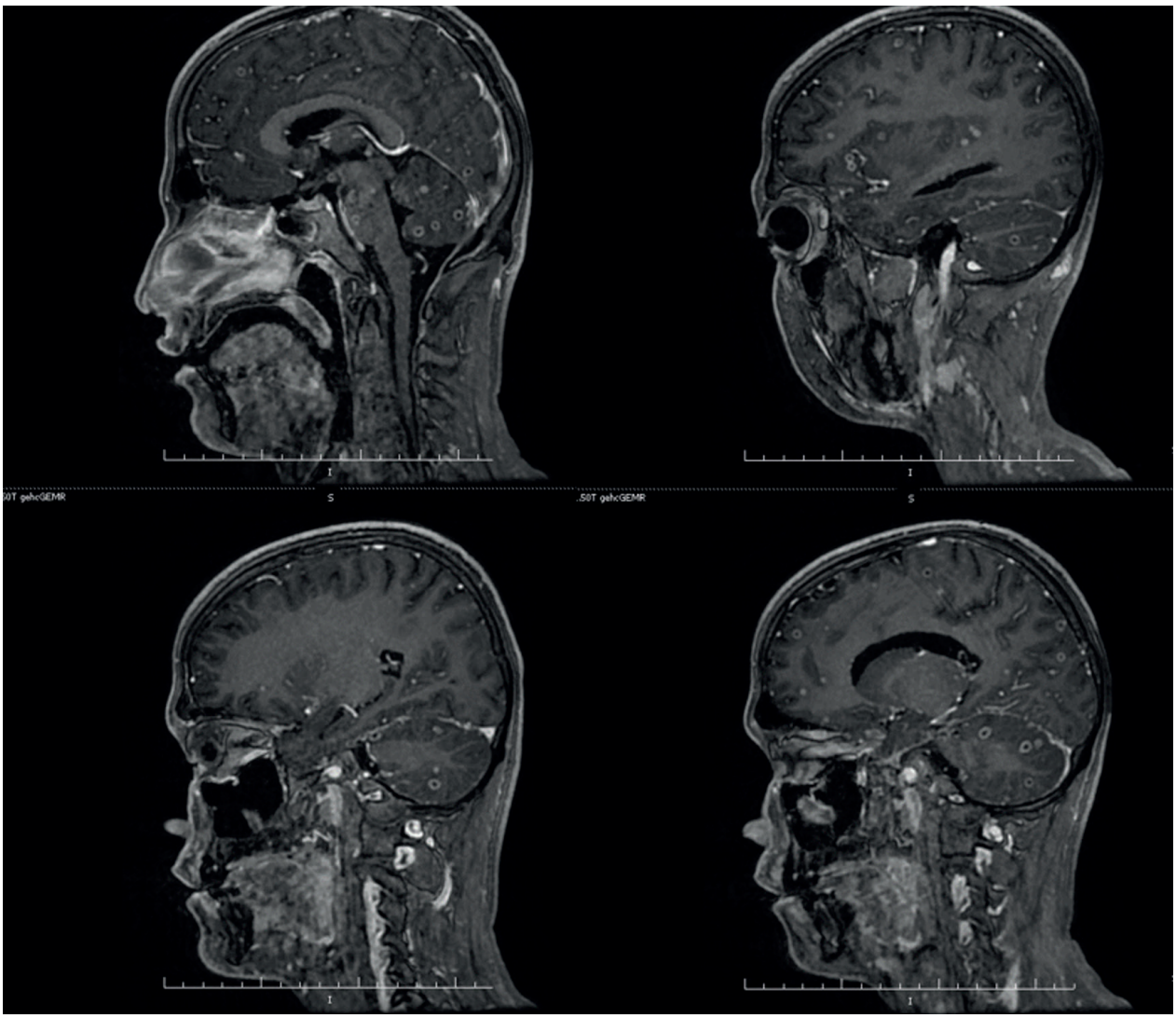

Figure 1. Appearance of tuberculomas from different slices on brain magnetic resonance imaging of the case.

bilateral miliary involvement and cavitation areas (Figure 2). Suspecting tuberculous meningitis, a lumbar puncture was performed. Cerebrospinal fluid (CSF) analysis showed white blood cells (WBC): 20/mm^3, red blood cells (RBC): 170/mm^3, protein: 1851 mg/L (150-400), glucose: 24 mg/dL (40-70) (simultaneous blood glucose: 91), chloride: 118 mmol/L (118-132). The purified protein derivative (PPD) test was anergic. Laboratory values are detailed in Table 1.

The patient was admitted to the intensive care unit (ICU) due to poor general condition. CSF, blood, and urine cultures showed no growth. Polymerase chain reaction (PCR) testing of the throat swab for respiratory viruses was negative. CSF acid-fast bacilli (AFB) staining was also negative. The CSF sample was sent to the laboratory for *M. tuberculosis* culture and PCR analysis. HBs Ag, Anti HCV, and Anti-HIV were all negative. Complement C3, C4, and immunoglobulin levels were normal. On the third day of ICU stay, the patient regained consciousness, and vital signs stabilized. He was transferred to the general ward. In coordination with the tuberculosis control program, a treatment regimen was initiated consisting of Isoniazid 1x300 mg/day, Rifampicin 1x600 mg/day, Ethambutol 1x15 mg/kg/day, and Pyrazinamide 1x25 mg/kg/day.

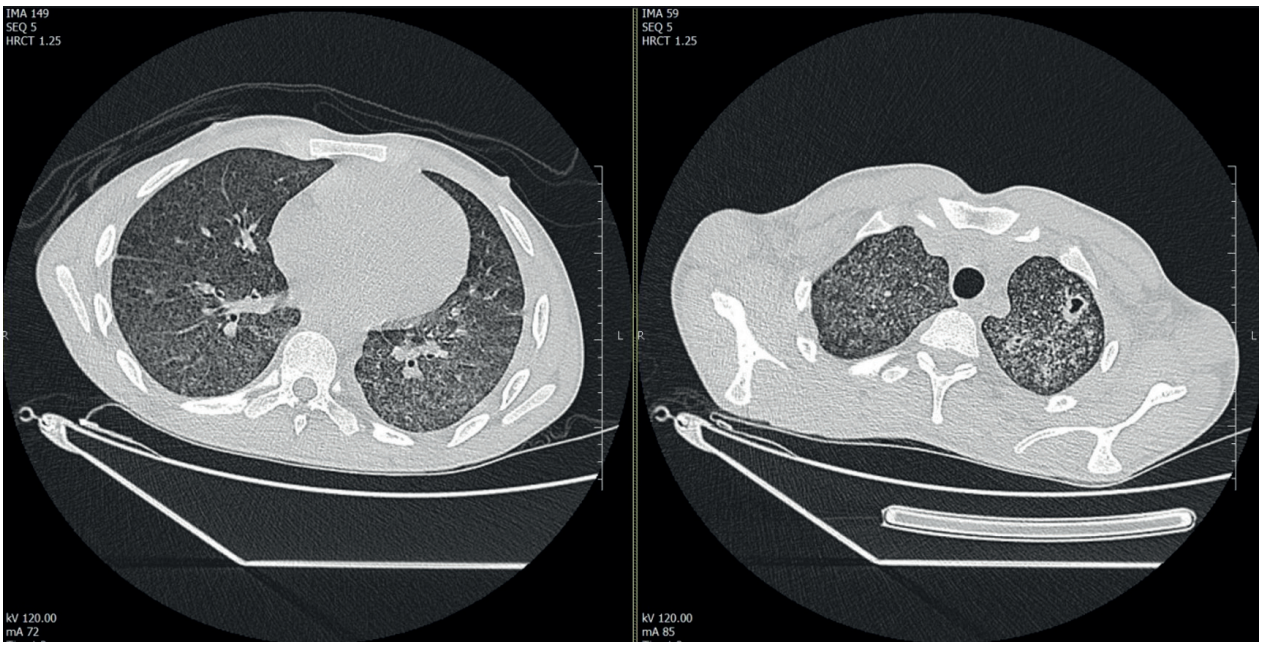

Figure 2. Diffuse miliary involvement and cavity areas on chest computer tomography of the case.

Table 1. Patient's Hemogram and Biochemistry Tests

Parameters	Results	Normal Range
Blood white blood cell (WBC)	6,61	4,5 - 11.0 K/uL
Hemoglobin (HGB)	11,8	11,5 - 17,5 g/dL
Platelet (PLT)	304	140 - 400 K/uL
Neutrophils (NEU%)	76,9	40 - 66 %
Lymphocytes (LYM%)	13,8	25 - 46 %
Fasting Blood Glucose	114	75 - 100 mg/dL
C-reactive protein (CRP)	14,9	0 - 5 mg/L
Urea	39	12 - 42 mg/dL
Creatinine	0,54	0,72 - 1,25 mg/dL
Alanine Aminotransferase (ALT)	27	0 - 55 U/L
Aspartat Aminotransferaz (AST)	39	5 - 34 U/L
Alkaline phosphatase (ALP)	90	40 - 150 U/L
Gamma Glutamyl Transferase (GGT)	47	12 - 64 U/L
Total bilirubin	0,36	0,2 - 1,2 mg/dL
Direct bilirubin	0,17	0 - 0,5 mg/dL
Lactate dehydrogenaz (LDH)	438	125 - 220 U/L

Dexamethasone was planned as 1x0.4 mg/kg/day for the first week, 1x0.3 mg/kg/day for the second week, 1x0.2 mg/kg/day for the third week, and 1x0.1 mg/kg/day for the fourth week, along with Pyridoxine 1x50 mg/day. Bronchoscopy was performed on the non-expectorating patient, and bronchoalveolar lavage (BAL) fluid was obtained. While AFB staining of BAL fluid was negative, *M. tuberculosis* PCR was positive. On the third day of antituberculosis treatment, AST and ALT values increased threefold, but the patient had no symptoms. Liver enzymes began to decrease by the end of the first week. On day 15, the patient was stable and symptom-free. The patient was discharged with detailed instructions for maintenance therapy and follow-up recommendations.

DISCUSSION

CSF findings are not always helpful in the diagnosis of tuberculous meningitis or cerebral tuberculoma without meningitis due to their variable nature. While lymphocytic pleocytosis is typically expected in CSF, neutrophilia may be present in early stages. Similarly, in our case, there was no significant leukocytosis in the CSF. Despite certain disadvantages, radiological imaging can contribute significantly to the diagnosis. Brain MRI is more advantageous than brain CT for the detection of tuberculomas, while chest CT is more advantageous than x-ray for the detection of miliary TB (4). However, CSF glucose and protein levels were indicative of bacterial meningitis. CSF AFB and CSF TB PCR negativity does not exclude TB meningitis. False negative results on the PPD test are another challenge in reaching a diagnosis. The contribution of CSF culture to diagnosis requires a long process, and its positivity rate remains around 30%. The positivity rate for AFB is approximately 10% (5). However, false negative results may lead to delays in treatment.

In smear-negative pulmonary tuberculosis cases, BAL fluid analysis is another useful diagnostic method. Studies have shown the diagnostic value of measuring interleukin 27 (IL-27), tumor necrosis factor alpha (TNF-alpha), IL-2, and interferon gamma (IFN-gamma) in BAL (6-8). In our case, the diagnosis was confirmed by the TB PCR result from BAL fluid. Despite extensive pulmonary TB with cavitations, meningitis, and widespread tuberculomas, other diagnostic tests were not helpful.

In tuberculosis-related brain involvement, clinical forms like meningitis followed by tuberculomas, brain abscesses, and miliary involvement, are common (9). However, cases of tuberculoma without meningitis have also been reported (10). Cases often present with mental status changes, meningeal signs, seizures, cranial nerve palsies, and focal neurological deficits (11). Even with effective antiTB treatment, about half of the cases may experience neurological sequelae (12). In a study, presenting TB culture-confirmed cases in Türkiye, the PCR positivity rate in TB cases was reported as 17.6%, while the PCR positivity rate in TB meningitis cases was reported as 12.5% (13). In a systematic review of 53 studies from 28 countries, the mortality rate of TB meningitis was found to be 42% (14). In our case, the patient regained consciousness with supportive care in the ICU before initiation of antiTB treatment, and vital signs returned to normal. On the third day of ICU admission, the patient was conscious and asymptomatic, and was transferred to the ward for the initiation of antiTB treatment plan. The patient's headache and fever lasted only two days and subsided without treatment. Despite the presence of tuberculoma foci spreading almost throughout the brain parenchyma and cerebellum, no seizures or neurological deficits were observed during follow-up. The young age of the patient and the absence of immunosuppression undoubtedly played a role in this outcome.

AntiTB-associated hepatotoxicity presents a significant challenge in patient management. Isoniazid, rifampicin, pyrazinamide, and their combinations are associated with hepatotoxicity (15). In our case, the ALT value tripled on the third day of treatment without symptoms. However, the treatment was continued without dose reduction or change. On the seventh day of treatment, the ALT value was observed to return to normal. It was concluded that in asymptomatic cases, continuation of the same treatment is necessary even with liver enzyme elevations up to three times the normal level.

CONCLUSION

Miliary TB is generally seen in infants or immunosuppressed patients, but it can rarely occur in young adults without immunodeficiency. Despite the availability of many tests for diagnosis, it is not always possible to diagnose with these tests. In such cases, BAL fluid may be helpful. In cases of brain involvement, tuberculomas may not always be accompanied by meningitis. Despite widespread tuberculomas in the brain, there may be no accompanying focal neurological deficit, cranial nerve involvement, or seizures. During antiTB treatment, especially in cases of ALT elevation up to three times without symptoms, it is not necessary to immediately stop the treatment, reduce the dose, or change the treatment regimen. Continuing the current treatment regimen with close monitoring may be sufficient.

Ethical approval

Written informed consent was obtained from the participants.

Author contribution

Surgical and Medical Practices: AD, HTG, TB, SBŞ; Concept: AD, HTG; Design: AD, HTG, TB; Data Collection or Processing: AD, HTG, SBŞ; Analysis or Interpretation: AD, HTG, TB; Literature Search: AD; Writing: AD, HTG. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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NORTHWESTERN MEDICAL JOURNAL

Northwestern Med J. 2025;5(2):132-134
DOI: 10.54307/NWMJ.2025.103

CASE REPORT

Tinea capitis profunda in an adult case

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Cite as: Sezer T, Şimşek FN. Tinea capitis profunda in an adult case. Northwestern Med J. 2025;5(2):132-134.

ABSTRACT

Tinea capitis is a dermatophyte infection of the scalp that occurs in childhood. Scalp erythema, scaling, pustules, and crusting are typical signs of tinea capitis. Tinea capitis is considered rare in adults. Adult tinea capitis may have polymorphic and atypical clinical presentations. Psoriasis, seborrheic dermatitis, and lichen simplex chronicus should be considered in the differential diagnosis of tinea capitis. Fungal examination should be performed for diagnosis. Early and accurate diagnosis of the disease prevents the formation of scars with the treatment administered. When diagnosing tinea capitis in adults, predisposing factors should be investigated. Here we present a case of tinea capitis profunda in a healthy adult.

Keywords: adult, dermatophyte, tinea capitis

INTRODUCTION

Tinea capitis (TC) is a dermatophyte infection characterized by scaling, alopecia, and pustules on the scalp and hair. It generally affects prepubertal children and is rarely observed in adults. The reason for this may be the fungistatic effect of saturated fatty acids in sebum with puberty. While TC is generally expected in adults with immunosuppression, recent reports have demonstrated that it also occurs in healthy adults. The present study presents a 42-year-old case of tinea capitis profunda without immunosuppression.

CASE REPORT

A 42-year-old female patient presented to our clinic with a three-week history of a painful sore on her scalp, which began as a pustule and spread. Before coming to our department, she was first treated with clobetasol lotion and ampicillin-sulbactam 1 g tablets 2x1 for one week. When there was no response, she received tetracycline capsule 100 mg/day 1x1 and mupirocin ointment treatment from another hospital. Her dermatologic examination revealed widespread pustules with yellowish crusts on the scalp and parietal region, approximately 8x8 cm in diameter, around the hair follicles on an erythematous alopecic area (Figure 1). There was no scutum and no mousy odor. On examination, she had painful lymphadenopathy in the right postauricular region. Wood's light examination did not show any reflection. The patient had a history of taking levothyroxine tablets for hypothyroidism. She was not menopausal. The infection was not present in family history. In the laboratory findings of the patient, erythrocyte sedimentation rate and C-reactive protein were normal. Hemoglobin was 9.5 g/dL and ferritin

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Received: 02.04.2024 **Accepted:** 31.05.2024 **Published:** 28.04.2025

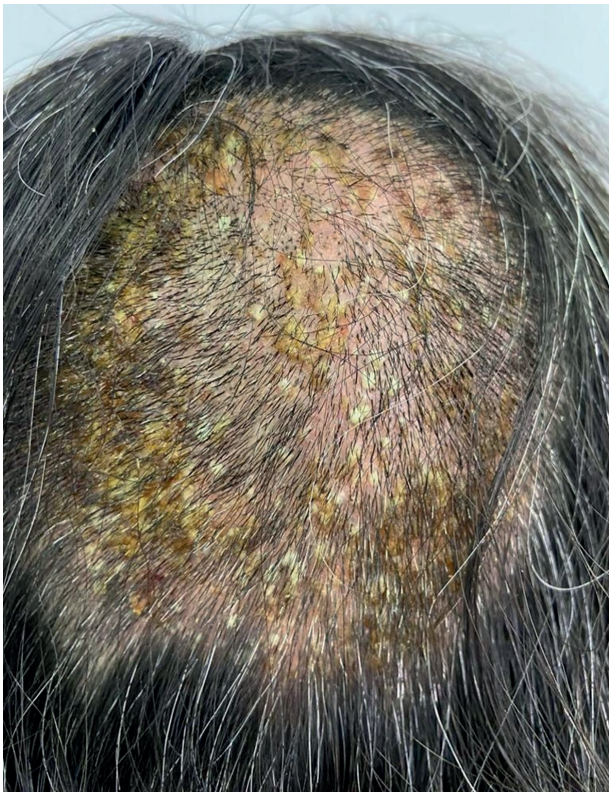

Figure 1. Widespread pustules with yellowish crusts on the scalp and parietal region.

was 3.37 microgram/L (10-204). Laboratory tests indicated no abnormalities except for low hemoglobin and ferritin levels in nonimmunsupressive patient. The sample taken from the patient's pustular lesions was assessed microscopically with potassium hydroxide and septate hyphae and spores was observed (Figure 2). There was no growth in the wound culture and fungal culture taken from the patient. Oral terbinafine 250 mg/day, isocanozole cream and ketocanozole shampoo were prescribed to the patient who was diagnosed with tinea capitis profunda. In the follow-up one month later, the lesions regressed and there was new hair growth (Figure 3).

DISCUSSION

TC is a superficial fungal infection caused by dermatophytes, and it is commonly observed in prepubertal children. The immune response of the host against dermatophyte infection determines the

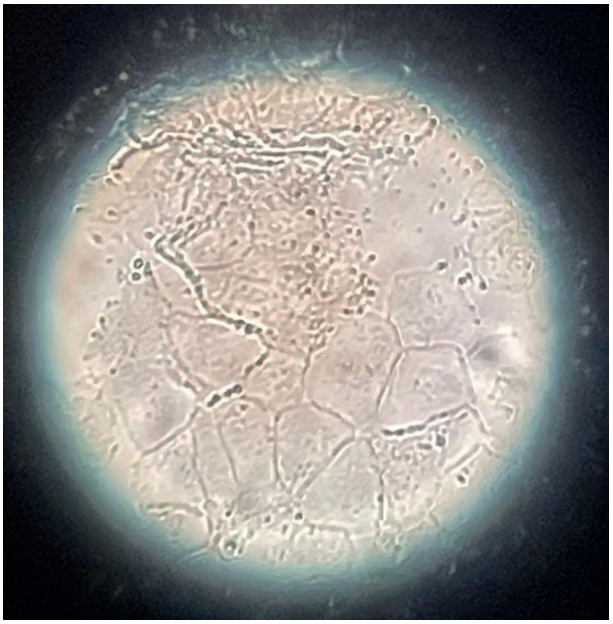

Figure 2. Septate hyphae and spores in the native preparation.

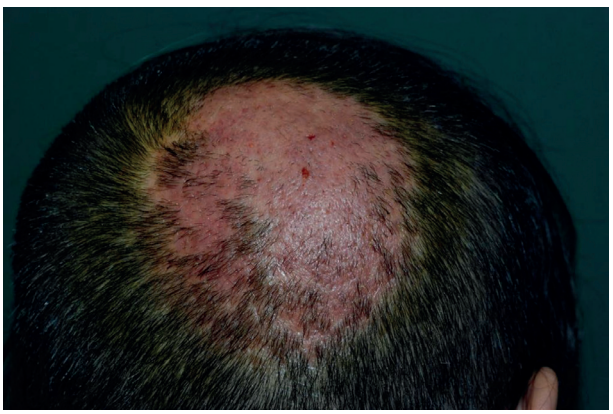

Figure 3. At the end of the first month, the lesions regressed.

clinical presentation and appears in three main clinical forms: tinea capitis superficialis, tinea capitis profunda (kerion celsi), and favus. Tinea capitis profunda is an inflammatory scalp lesion that occurs due to a delayed-type cellular immune response against fungal elements. The follicles that pustulate on the scalp merge over time and become painful inflammatory nodules. TC is rarely observed in adults, which may be attributed to the fungistatic effect of short- and medium-chain fatty acids present in sebum, the maturation of hair follicles,

and the effectiveness of the immune system during the post-pubertal period (1,2).

Risk factors for the development of TC in adults include menopausal status, age over 50 years, diabetes mellitus, and the use of topical steroids on the scalp (2). According to a study conducted in China between 2000 and 2019, only six of the 269 diagnosed TC cases were immunosuppressive. The majority of patients with TC were 45 years of age or older.

The study determined that women were more likely to be diagnosed with the disease than men, and that postmenopausal women were at higher risk of TC (3). In the study by Khosravi et al., 25 out of 121 patients with TC were adults. The disease-causing immunosuppression was observed in 80% of the adult patients and the remaining adult patients were found to be healthy (4). During the examinations of our patient, there were no abnormal clinical and laboratory findings except for iron deficiency.

The clinical presentation of TC may be atypical, which may lead to misdiagnosis and delay in treatment (3,4). It has been reported in the literature that the diagnosis of TC may take anywhere from 20 days to 30 years. A delay in diagnosis and treatment may lead to scarring alopecia on the scalp (3). In our case, it took 3 weeks to get the right diagnosis.

In the differential diagnosis, the possibility of diseases such as folliculitis, folliculitis decalvans, dissecting cellulitis, seborrheic dermatitis, and psoriasis should be taken into account (5). In suspected patients, potassium hydroxide (KOH) examination and fungal culture tests should be performed. In the study by Liang et al., KOH examination was positive in all patients, while the growth rate of fungal culture was detected as 90.7% (3). In our patient's KOH examination, septate hyphae and spores were observed, but no fungal growth was found in the culture.

Some systemic antifungals such as griseofulvin, terbinafine, itraconazole and fluconazole are recommended as first-line treatment alternatives for TC. Topical agents may be added to the treatment to prevent the spread of fungal spores (6).

Treatment must be continued for at least 6 to 8 weeks. Short-term glucocorticoids may be applied to suppress inflammation in the kerion. If secondary infection occurs, appropriate use of antibiotics and epilation of the hair around the lesion may increase the effectiveness of treatment (1,6). We used systemic terbinafine, isoconazole cream, and ketoconazole shampoo in treatment of our patient.

Ethical approval

Written informed consent was obtained from the participants.

Author contribution

Concept: TS; Design: TS; Data Collection or Processing: FNŞ; Analysis or Interpretation: TS, FNŞ; Literature Search: TS, FNŞ; Writing: TS. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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ISSN: 2979-9538