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The relationship between sexual health literacy and sexual health attitudes in young adults

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MAIN POINTS

- This study found a positive correlation between sexual health attitudes and sexual health literacy among young adults.
- Sexual health attitudes accounted for 19% of the variance in sexual health literacy scores among young adults.
- Gender, sources of sexual health information, and receiving sexual health education were significantly associated with higher sexual health literacy and more positive sexual health attitudes.
- In this context, nurses must consider the key determinants of sexual health literacy when designing educational and counseling strategies for young adults.

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ABSTRACT

Aim: This paper investigated the relationship between sexual health literacy (SHL) of young adults and attitudes toward sexual health.

Materials and Methods: This study was descriptive and correlational in design. The sample size included 281 young adults. Data were collected using a personal information form, the Sexual Health Literacy Scale (SHLS), and the Sexual Health Attitude Scale (SHAS). The data were analyzed using the independent-samples t-test, one-way analysis of variance (ANOVA), Welch ANOVA test, Pearson's correlation coefficients, and simple linear regression analysis.

Results: Participants' mean SHLS and SHAS scores were 59.60±10.86 and 149.68±12.43, respectively. Both SHLS and SHAS scores were significantly higher among women compared to men, and among those who had received sexual health education compared to those who had not ($p<0.01$). Participants with master's or higher degrees had higher mean SHLS score than other groups ($p<0.01$). When examining sources of sexual health information, participants who learned from healthcare professionals, books, and personal experiences scored higher on SHLS than those who did not ($p<0.01$). For SHAS, participants who gained knowledge from friends/acquaintances, siblings, and personal experiences had higher mean score than others ($p<0.01$). A positive correlation was found between total SHLS scores and both total SHAS score and all SHAS subscale scores ($p<0.05$). Additionally, SHLS "sexual attitude" subscale score was positively correlated with total SHAS score and all SHAS subscale scores ($p<0.05$). Similarly, SHLS "sexual knowledge" subscale score showed a positive correlation with total SHAS score and all SHAS subscale scores, except for the "gender roles" subscale ($p<0.05$). Finally, SHAS score explained 19% of the variance in SHLS score ($\beta=0.433$, $p<0.001$).

Conclusion: Sexual health attitude, higher education, being a woman, and having received sexual health education are important determinants of SHL. Young adults with reliable sources of information on sexual health have high levels of SHL.

Keywords: Sexual health, Health literacy, Attitude, Knowledge, Young adult

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INTRODUCTION

Health literacy plays a crucial role in maintaining health and well-being in contemporary societies. It is also an important area of public health that is overlooked [1]. The definition of health literacy remains unclear and lacks a universally accepted consensus. However, it is defined as the degree to which one acquires, processes, and understands the basic health information and services one needs to make the right health decisions [2]. The World Health Organization (WHO) defines health literacy as "both a means and an outcome of actions aimed at promoting the empowerment and participation of people in their communities and of people in their health care" [1]. The World Health Organization (WHO) recognized health literacy as a key action plan for reducing health inequalities in the Shanghai Declaration [3]. Sexual and reproductive health (SRH) literacy, which encom-

passes the ability to access, understand, evaluate, and apply SRH-related information to address related issues [4], is therefore crucial for overall sexual health [5,6].

Sexual health literacy (SHL) is the ability to access, understand, evaluate, and apply sexual health information, encompassing one's knowledge, beliefs, attitudes, motivations, and skills [7]. Individuals with more SHL are better at understanding and assessing sexual health risks. They postpone their first sexual experience until they find a reliable spouse/partner. Therefore, they have a safe sex life free from unplanned pregnancies. They also suffer fewer sexually transmitted diseases and share tasks and responsibilities in sexual life. Therefore, sexual health literacy helps improve family and community health [8].

Sexual health literacy is a critical part of improving SRH for young people in low-income countries [4]. During adolescence and young adulthood, people develop SRH literacy skills, adopt healthy living behaviors, and take responsibility for their own health [9,10]. However, individual, sociocultural, and economic factors affect how young adults develop SHL. Some young adults know little about SRH because they are bombarded with different sources of information and have limited access to reliable ones [4]. People who receive sexual health education tend to have more SHL. Research also suggests that individuals with more SHL have more positive attitudes toward sexual health [11]. Furthermore, the extent to which people have SHL is determined by gender, sexual health education [11,12], first sexual experience, place of residence, and beliefs [13,14].

People with more SHL are more empowered in terms of SRH. They are also more satisfied with their marriage/partners and enjoy a better quality of life, resulting in strengthened family and community health [5,6,8]. In this regard, healthcare professionals are responsible for assessing and strengthening individuals' sexual health and referring them to counseling, clinical services, and specialists when necessary [15,16]. There is a large body of research into SHL in young adults [11,12] and other age groups [5,17,18]. However, only a few researchers have investigated what kind of attitudes young adults with SHL have toward sexual health. Therefore, this study investigated the relationship between SHL and attitudes of young adults toward sexual health. Specifically, the level of SHL among young adults, their attitudes toward sexual health, and the factors influencing both were examined. In addition, the relationship between SHL and sexual health attitudes in this population was investigated.

MATERIALS AND METHODS

Research type

This study adopted a descriptive and correlational research design.

Population and sample

The study population comprised young adults with social media accounts in Turkey. According to the American Psychological Association, people between the ages of 20-35 are young adults [19]. The sample size was calculated based on young adults' sexual health knowledge (23%) reported by Özcan et al. [20]. Convenience sampling method, one of the non-probability sampling methods, was used. The sample size was calculated using "Sampsize Program" with an unknown population [21]. In the calculation, the target sample was 273 participant (precision: 5%; prevalence: 23%; level: 95%). The final sample consisted of 281 young adults. In post hoc analysis, the study's achieved power was computed as 99% [alpha= 0.05, constant proportion= 0.23, effect size (g)= 0.26] based on the status of "receiving sexual health education (49.5%)" (G*Power (3.1.9.7. v.). The inclusion criteria were (1) volunteering, (2) being 20-35 years of age, (3) speaking Turkish, (4) being literate, and (5) having at least one social media account. Foreign national participants were excluded from the study.

Ethical considerations

The study was approved by the Health Sciences Ethics Committee of Ankara Yıldırım Beyazıt University (Date: 01.07.2024, No: 06/801). An online survey served as the data collection instrument. Before beginning, all young adults were thoroughly briefed on the research purpose, procedures, and confidentiality. They were also explicitly informed of their right to withdraw at any point without penalty. Consent to participate was indicated by clicking an "Agree" button, after which participants proceeded to complete the data collection tools. This research strictly adhered to the principles outlined in the Declaration of Helsinki. Furthermore, necessary authorization was secured from the developers of the scales used. Participation was voluntary.

Data collection tools

The date were collected using a personal information form, the Sexual Health Literacy Scale (SHLS), and the Sexual Health Attitude Scale (SHAS).

Personal information form

To gather participant data, the researchers developed a personal information form [4,6,8,9,11,12,18,20,22,23]. This 15-item instrument covered sociodemographic details (such as age, gender, marital status, education, and level of development of the place of residence) and sexual health information (including receipt of sexual health education and sources of information). A key variable, the level of development of the place of residence where participants lived until age 12, was categorized [24]. This variable was included because attitudes and behaviors are largely shaped in childhood, with the period up to age 12 being crucial for identity development. Given that family, social structure, culture, environment, friends,

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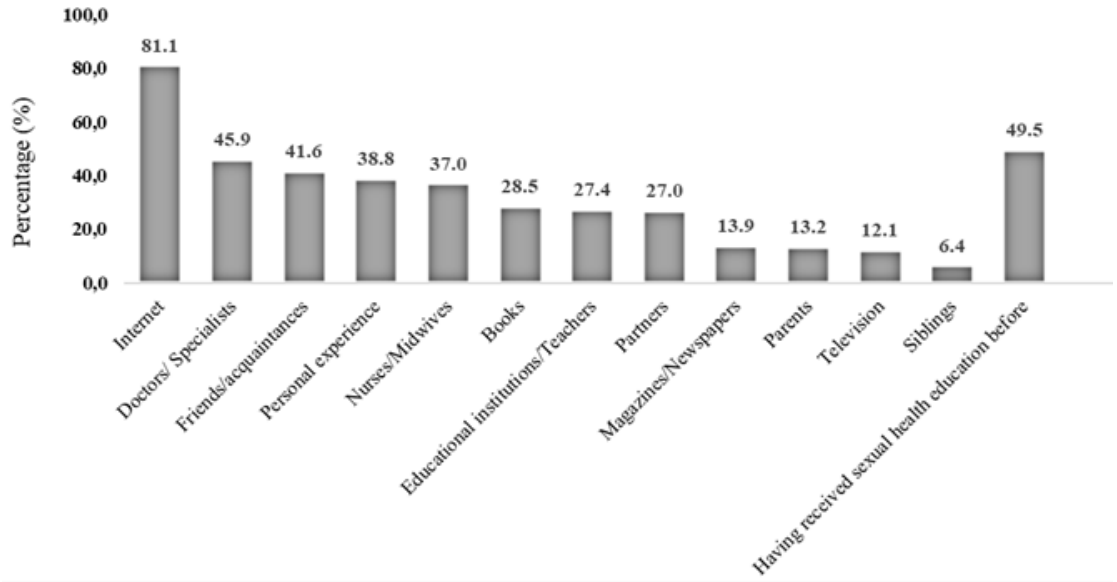

Figure 1. Distribution of sources for sexual health information and sexual health education status (n=281) (*Participants gave more than one answer).

and school can all influence personality, attitudes, and behaviors during childhood [25], it was important to investigate the sociocultural environment experienced by participants up to that age.

Sexual health literacy scale (SHLS)

The Sexual Health Literacy Scale (SHLS) was developed by Üstgörül (2022) [26]. The instrument consists of 17 items rated on a five-point Likert-type scale ("strongly disagree: 1," to "strongly agree: 5"). The total score ranges from 17 to 85. The scale has two subscales: (1) sexual knowledge and (2) sexual attitude. The first subscale comprises 12 items, with a potential total score between 12 and 60. The second subscale consists of five reverse-scored items, resulting in a total score ranging from 5 to 25. In both instances, elevated scores signify a higher level of sexual health literacy (SHL). The scale's reported Cronbach's alpha in the original study was 0.88 [26], which was consistent with the 0.89 observed in this study.

Sexual health attitude scale (SHAS)

The Sexual Health Attitude Scale (SHAS) was developed by Köprülü (2022) [23]. The instrument consists of 33 items rated on a five-point Likert-type scale ("strongly disagree: 1," to "strongly agree: 5"). The total score ranges from 33 to 165. The scale has seven subscales: (1) decision-making and responsibility (11 items), (2) communication and rights (five items), (3) safe sex (four items), (4) sexual rights (four items), (5) gender roles (four items), (6) awareness (two items), and (7) self-confidence (three items). Higher scores indicate more positive attitudes toward sexual health. The original scale has a Cronbach's alpha score of 0.94 [23], which was 0.93 in the present study.

Data collection

The researchers contacted young adults through social media platforms (WhatsApp, Instagram, Facebook etc.). They used Google Forms to create an online survey. They sent a link to the survey on the social media platforms. The survey informed the young adults about the research purpose, procedure, and confidentiality. Each participant took 10-15 minutes to complete the survey. The data were collected between October 2024 and December 2024.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (IBM Corp. SPSS Statistics Version 21.0, Released 2012. Armonk, NY) at a significance level of 0.05. To assess normality, the Shapiro-Wilk test was conducted, complemented by an examination of skewness and kurtosis values, with a range between -1.5 and +1.5 indicating a normal distribution. Homogeneity of variances was determined via Levene's test. Descriptive statistics are presented using frequencies, percentage distributions, mean ± standard deviations, and medians (min-max). Group comparisons were performed using an independent-samples t-test for two groups, and one-way analysis of variance (ANOVA) or Welch ANOVA for more than two groups. Post-hoc comparisons were further analyzed with the Dunn-Bonferroni test. Pearson's correlation coefficients were utilized to ascertain the relationships between scale scores. A simple linear regression analysis was subsequently performed to predict SHAS scores from SHLS scores.

RESULTS

Participants had a mean age of 25.69±4.49 years. 72.2% of the participants were women, and 31% were married. 54.4% of the participants had a neutral income (income = expense).

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Table 1. Sociodemographic characteristics (n=281).

Sociodemographic characteristics	MD(min-max)	M±SD
Age (year)	24.00(20-35)	25.69±4.49
Partner age* (year) (n=87)	31.00(21-42)	31.41±4.34
	n	%
Gender		
Woman	203	72.2
Man	78	27.8
Marital status		
Married	87	31.0
Single	194	69.0
Income		
Negative (income < expense)	74	26.3
Neutral (income = expense)	153	54.4
Positive (income > expense)	54	19.2
Place of residence until age 12		
City/big city	172	61.2
District	65	23.1
Village/Borough/Town	44	15.7
Developmental level of place of residence until age 12		
First tier	122	43.4
Second tier	48	17.1
Third tier	31	11.0
Fourth tier	26	9.3
Fifth tier	26	9.3
Sixth tier	28	10.0
Family type		
Nuclear	232	82.6
Extended	49	17.4
Education (degree)		
High school	71	25.3
Associate's	37	13.2
Bachelor's	125	44.5
Master's and ↑	48	17.1
Employment status		
Unemployed/ Housewife	30	10.7
Public employee	95	33.6
Private sector employee	64	22.8
Student	92	32.7
Partner's education (degree)*		
High school	19	21.8
Associate's	11	12.7
Bachelor's and ↑**	57	65.5
Partner's employment status*		
Unemployed/ Housewife	10	11.5
Public employee	41	47.1
Private sector employee	36	41.4

MD: Median; Min: Minimum; Max: Maximum; M: Mean; SD: Standard deviation. * Eighty-seven participants with partners were included. **The number of partners with postgraduate education is 9.

A significant proportion of participants (61.2%) reported living in cities or major urban centers until the age of 12. Furthermore, 43.4% resided in first-tier settlements based on development level. Educational attainment revealed that 44.5% of participants possessed a Bachelor's degree. In terms of employment, 33.6% were employed in the public sector, and 47.1% had partners working in the public sector. Among married individuals, 65.5% had attained a Bachelor's degree or higher (Table 1). In total, 49.5% of the participants had received sexual health education before. Participants learned about sexual health from the internet (81.1%), specialists/doctors (45.9%), friends/acquaintances (41.6%), personal experiences (38.8%), nurses/midwives (37.0%), books (28.5%), educational institutions/teachers (27.4%), partners (27.0%), magazines/newspapers (13.9%), parents (13.2%), television (12.1%), and siblings (6.4%) (Figure 1).

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Table 2. The distribution of scale scores (n=281).

Scales and Subscales	Item	Score Ranges	MD(Min-Max)	M±SD
SHLS Total Score	17 items	17-85	60.00(32-85)	59.60±10.86
Sexual knowledge	12 items	12-60	41.00(15-60)	39.77±8.71
Sexual attitude	5 items	5-25	20.00(7-25)	19.83±4.03
SHAS Total Score	33 items	33-165	153.00(98-165)	149.68±12.43
Decision-making and responsibility	11 items	11-55	52.00(29-55)	50.70±4.63
Communication and rights	5 items	5-25	23.00(14-25)	22.74±2.14
Safe sex	4 items	4-20	19.00(12-20)	18.34±1.95
Sexual rights	4 items	4-20	20.00(12-20)	18.63±1.73
Gender roles	4 items	4-20	18.00(4-20)	17.41±2.93
Awareness	2 items	2-10	8.00(3-10)	8.29±1.30
Self-confidence	3 items	3-15	14.00(9-15)	13.57±1.27

MD: Median; Min: Minimum; Max: Maximum; M: Mean; SD: Standard deviation. SHLS: Sexual Health Literacy Scale. SHAS: Sexual Health Attitude Scale.

Participants had a mean SHLS score of 59.60±10.86. They had mean SHLS "sexual knowledge" and "sexual attitude" subscale scores of 39.77±8.71 and 19.83±4.03, respectively. They had a mean SHAS score of 149.68±12.43. They had mean SHAS "decision-making and responsibility," "communication and rights," "safe sex," "sexual rights," "gender roles," "awareness," and "self-confidence" subscale scores of 50.70±4.63, 22.74±2.14, 18.34±1.95, 18.63±1.73, 17.41±2.93, 8.29±1.30, and 13.57±1.27, respectively (Table 2).

Female participants (60.73±11.07) had a higher mean SHLS score than males (56.65±9.75) ($p<0.01$). Participants with a neutral income (61.51±10.35) had a higher mean SHLS score than those with a negative income (55.88±11.21) ($p<0.01$). Participants with master's degrees (65.17±10.89) had a higher mean SHLS score than others ($p<0.01$). Participants who had received sexual health education before (63.16±10.12) had a higher mean SHLS score than those who had not (56.11±10.45) ($p<0.01$). Female participants (151.80±11.02) had a higher mean SHAS score than their male counterparts (144.17±14.16) ($p<0.01$). Single participants (151.13±12.04) had a higher mean SHAS score than their married counterparts (146.44±12.74) ($p<0.01$). Participants who had received sexual health education before (151.98±11.57) had a higher mean SHAS score than those who had not (147.43±12.86) ($p<0.01$) (Table 3).

Participants who had learned about sexual health from doctors/specialists (61.78±10.06), personal experiences (62.08±10.35), nurses/midwives (62.87±9.52), and books (63.80±10.08) had high SHLS scores ($p<0.01$). Moreover, participants who had learned about sexual health from friends/acquaintances (151.50±10.47), personal experiences (152.32±11.16), and siblings (156.33±8.41) had high SHAS scores ($p<0.05$) (Table 4).

A weak positive correlation was observed between SHLS total score and SHAS "gender roles" subscale score ($r= 0.150$, $p<0.05$). A moderate positive correlation was present between SHLS total score and SHAS total score and SHAS "decision-making and responsibility" ($r= 0.376$), "communication and rights" ($r= 0.446$), "safe sex" ($r= 0.386$), "sexual rights" ($r= 0.354$), "awareness" ($r= 0.305$), and "self-confidence" ($r= 0.378$) subscale scores ($p<0.001$). There was a moderate positive correlation between SHLS "sexual knowledge" subscale score and SHAS total score ($r= 0.350$) and SHAS "decision-making and responsibility" ($r= 0.304$), "communication and rights" ($r= 0.366$), "safe sex" ($r= 0.344$), "awareness" ($r= 0.307$), and "self-confidence" ($r= 0.319$) subscale scores ($p<0.001$). A weak positive correlation existed between SHLS "sexual knowledge" subscale score and SHAS "sexual rights" subscale score ($r= 0.270$, $p<0.001$). A moderate positive correlation was detected between SHLS "sexual attitude" subscale score and SHAS total score ($r= 0.412$) and SHAS "decision-making and responsibility" ($r= 0.356$), "communication and rights" ($r= 0.411$), "sexual rights" ($r=0.370$), and "self-confidence" ($r= 0.330$) subscale scores ($p<0.001$). A weak positive correlation was determined between SHLS "sexual attitude" subscale score and SHAS "safe sex" ($r= 0.297$), "gender roles" ($r= 0.252$), and "awareness" ($r= 0.159$) subscale scores ($p<0.01$) (Table 5). Regression analysis indicated that SHLS total score had a significant effect on SHAS total scores ($R= 0.433$, $R^2 = 0.188$, $F= 64.521$, $p<0.001$) (Table 6).

DISCUSSION

Sexual health and reproductive health (SRH) and sexual health literacy (SHL) are fundamental components of overall health and health literacy. Fostering SHL is essential for strengthening SRH and contributing to a healthier society [5,6,8,26]. Young adults are particularly susceptible to risky behaviors, making SHL competence especially critical for this demographic [4]. While there's no universal consensus on the factors influencing SHL [11], our study found that gender, education, prior sexual health education, and sexual health attitudes significantly affected participants' SHL.

Young adults with high SHL are better equipped to access reliable sexual health information. Interestingly, individuals holding more positive sexual health attitudes primarily gather

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Table 3. The distribution of scale scores in accordance with various variables (n=281).

Characteristics	SHLS			SHAS		
	M±SD	Analysis†	p value	M±SD	Analysis†	p value
Gender						
Woman	60.73±11.07	2.852	**0.005****	151.80±11.02	4.285	**<0.001*****
Man	56.65±9.75			144.17±14.16		
Marital status						
Married	60.26±10.67	0.721	0.492	146.44±12.74	2.969	**0.003****
Single	59.30±10.95			151.13±12.04		
Income						
Negative	55.88±11.21[a]			149.08±11.02		
Neutral	61.51±10.35[b]	7.023	**0.001****	151.07±11.921	2.270§	0.108
Positive	59.28±10.60[a,b]			146.56±15.01		
Place of residence until age 12						
City/big city	60.24±10.20			149.33±12.72		
District	59.54±11.83	1.419	0.244	149.91±12.94	0.236	0.790
Village/Borough/Town	57.16±11.73			150.73±10.57		
Developmental level of place of residence until age 12						
First/second tier	59.77±10.55			150.12±12.38		
Third/fourth tier	60.35±11.73	0.567	0.568	151.33±11.49	2.337	0.099
Fifth/sixth tier	58.26±10.96			146.56±13.18		
Family type						
Nuclear	59.82±10.72	0.756	0.450	149.69±12.67	0.016	0.987
Extended	58.53±11.54			149.65±11.33		
Education (degree)						
High school	57.17±10.86[a]			149.99±11.65		
Associate's	57.73±11.10[a]	6.084	**0.001****	150.51±12.67	1.206	0.308
Bachelor's	59.39±10.11[a]			148.31±12.86		
Master's and ↑	65.17±10.89[b]			152.15±12.11		
Employment status						
Unemployed/ Housewife	58.87±10.75			147.40±13.86		
Public employee	61.38±11.14	1.579	0.195	149.67±13.30	0.437	0.726
Private sector employee	59.61±10.31			149.72±12.53		
Student	57.99±10.86			150.40±10.97		
Partner's education (degree)						
High school	59.53±7.53			145.42±12.41		
Associate's	60.73±9.66	0.097§	0.908	145.00±12.41	0.193	0.825
Bachelor's and ↑	60.42±11.82			147.05±13.08		
Partner's employment status						
Unemployed/ Housewife	56.30±6.80			143.60±8.50		
Public employee	59.80±11.10	1.148	0.322	144.37±13.49	1.927	0.152
Private sector employee	61.89±10.94			149.58±12.44		
Having received sexual health education before						
Yes	63.16±10.12	5.738	**<0.001*****	151.98±11.57	3.117	0.002**
No	56.11±10.45			147.43±12.86		

M: Mean; SD: Standard deviation. SHLS: Sexual Health Literacy Scale. SHAS: Sexual Health Attitude Scale. †Independent samples t-test was used for paired groups, while One Way Anova or Welch Anova§ test was used for more than two groups. a-b: No difference between groups with the same letter for each measurement (Dunn Bonferroni Test). *p<0.05, **p<0.01, ***p<0.001.

information from informal sources such as friends, acquaintances, personal experiences, and siblings.

Our participants achieved a mean total SHLS score of 59.60±10.86. This contrasts with previous research, which typically reports young adult SHLS scores ranging from 45 to 55 [11,12,27-29]. Similarly, our participants' mean total SHAS score was 149.68±12.43. For comparison, Köprülü (2022) found a mean SHAS score of 143.63±19.37 among college students [23]. Our participants' relatively higher SHLS and SHAS scores likely stem from our sample's inclusion of individuals with bachelor's or higher degrees, not just college students.

Our findings indicate that education, gender, sexual health education, and income influenced participants' SHLS scores, while gender, sexual health education, and marital status affected their SHAS scores.

Previous research aligns with some of these findings, suggesting women often exhibit significantly higher SHLS and

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Table 4. The distribution of scale scores by sexual health information sources (n=281).

Sexuality-Related Information Sources	SHLS			SHAS		
	M±SD	Analysis†	p value	M±SD	Analysis†	p value
Internet						
Yes	59.58±10.35	0.041	0.968	149.99±12.00	0.785	0.435
No	59.66±12.91			148.34±14.17		
Doctors/Specialists						
Yes	61.78±10.06	3.144	**0.002****	150.29±12.16	0.763	0.446
No	57.75±11.20			149.16±12.67		
Friends/Acquaintances						
Yes	58.43±10.36	1.530	0.127	151.50±10.47	2.170	**0.031***
No	60.43±11.15			148.38±13.54		
Personal experience						
Yes	62.08±10.35	3.146	**0.002****	152.32±11.16	2.904	**0.004****
No	57.98±10.91			147.96±12.97		
Nurses/Midwives						
Yes	62.87±9.52	4.132	**<0.001*****	151.16±12.67	1.537	0.125
No	57.68±11.16			148.81±12.24		
Books						
Yes	63.80±10.08	4.212	**<0.001*****	150.55±11.67	0.740	0.460
No	57.93±10.72			149.33±12.73		
Schools/Teachers						
Yes	60.22±11.37	0.590	0.556	151.71±11.53	1.691	0.092
No	59.36±10.68			148.91±12.69		
Partners						
Yes	60.62±9.17	1.060	0.291	149.30±12.44	0.309	0.757
No	59.22±11.42			149.82±12.45		
Magazines/Newspapers						
Yes	61.05±11.06	0.900	0.369	148.82±11.75	0.871	0.643
No	59.36±10.83			149.82±12.55		
Parents						
Yes	59.08±9.94	0.310	0.757	151.19±12.15	0.792	0.429
No	59.68±11.01			149.45±12.48		
Television						
Yes	58.64±9.99	0.375	0.708	150.00±11.89	0.160	0.873
No	59.69±10.99			149.64±12.52		
Siblings						
Yes	60.11±9.17	0.207	0.836	156.33±8.41	3.339	**0.003****
No	59.56±10.98			149.22±12.54		

M: Mean; SD: Standard deviation SHLS: Sexual Health Literacy Scale. SHAS: Sexual Health Attitude Scale. †Independent samples t-test was used. *p<0.05, **p<0.01, ***p<0.001.

Table 5. Correlation of the scores used in the present study (n=281).

Scales and Subscales	SHLS Total Score		SHLS Subscales			
			Sexual knowledge		Sexual attitude	
	r†	p	r†	p	r†	p
SHAS Total Score	0.433	**<0.001*****	0.350	**<0.001*****	0.412	**<0.001*****
Decision-making and responsibility	0.376	**<0.001*****	0.304	**<0.001*****	0.356	**<0.001*****
Communication and rights	0.446	**<0.001*****	0.366	**<0.001*****	0.411	**<0.001*****
Safe sex	0.386	**<0.001*****	0.344	**<0.001*****	0.297	**<0.001*****
Sexual rights	0.354	**<0.001*****	0.270	**<0.001*****	0.370	**<0.001*****
Gender roles	0.150	**0.012***	0.070	0.242	0.252	**<0.001*****
Awareness	0.305	**<0.001*****	0.307	**<0.001*****	0.159	**0.008****
Self-confidence	0.378	**<0.001*****	0.319	**<0.001*****	0.330	**<0.001*****

SHLS: Sexual Health Literacy Scale. SHAS: Sexual Health Attitude Scale. r†: Pearson's correlation analysis was used. *p<0.05, **p<0.01, ***p<0.001.

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Table 6. Efficiency of SHAS Scores in terms of Predicting SHLS Scores (n=281).

Variable	B (95% CI)	Std. Error	β	t	p
Constant	2.925 (-11.011 -- 16.862)	7.080		0.413	0.680
SHAS	0.379 (0.286 -- 0.471)	0.047	0.433	8.032	**<0.001**

SHLS: Sexual Health Literacy Scale. SHAS: Sexual Health Attitude Scale. B: Unstandardized coefficient, CI: Confidence interval, Std. Error: Coefficients Standardized Error, β: Standardized coefficient. R= 0.433, R^2= 0.188, F= 64.521, p<0.001, Durbin-Watson= 1.930.

SHAS scores than men [27,30]. Furthermore, prior sexual health education is consistently identified as a key determinant for both SHL and positive sexual attitudes [27,30]. However, there are conflicting results in the literature; for instance, Özcan et al. reported that two-thirds of female college students had inadequate SRH knowledge [20], and Yeşil and Apak found no gender difference in SHLS and SHAS scores [11].

The impact of perceived income on SHL and sexual attitude remains underexplored, with some studies suggesting minimal to no effect [22], while others indicate higher SHL among individuals with higher incomes. Similarly, the literature on marital status presents mixed findings: Dişsiz et al. observed no significant difference in SHLS and SHAS scores between married and single nursing students [22], yet Yeşil and Apak reported significantly higher SHLS and SHAS scores among married midwifery and nursing students compared to their single counterparts [11].

Collectively, these findings highlight that receiving sexual health education is a crucial determinant of SHL and sexual attitudes. However, further research is needed to comprehensively investigate how gender, marital status, and income consistently affect SHL and sexual attitudes.

Our study revealed a significant positive correlation between SHLS and SHAS scores, indicating that participants with greater SHL also held more positive sexual attitudes. Further analysis demonstrated that sexual attitudes explained 19% of the total variance in SHL, establishing the SHAS total score as a significant predictor of the SHLS total score. This finding is particularly salient for understanding the interplay between SHL and sexual attitudes. Similar positive correlations have been reported elsewhere [11], with some researchers emphasizing that sexual knowledge and attitude are vital determinants of SHL [14]. Öztürk Altınkaynak and Özkan further underscored this, observing high SHL, positive sexual attitudes, and lower risky sexual behavior among young women, with greater SHL correlating with a reduced likelihood of engaging in risky sexual behaviors [29]. In light of this, healthcare professionals should develop interventions aimed at fostering SHL and cultivating positive sexual attitudes among young people.

Our participants accessed various sources for sexual health information. The majority (81.1%) utilized online platforms, followed by doctors/specialists (45.9%), friends/acquaintances (41.6%), personal experiences (38.8%), and nurses/midwives (37.0%). The reliability of informal sources like the internet, friends, and personal experiences remains a point of contention. Prior research indicates that most college students primarily consult the internet/media, newspapers/magazines, television, professors, and friends for sexual health information, with far fewer turning to health institutions/professionals or parents [20,31]. Indeed, young people often avoid discussing sex with parents, particularly fathers [31]. Adamu et al. similarly documented that most young individuals rely on friends/peers, media platforms, and teachers, while very few consult health professionals/institutions and parents [4]. Our findings are consistent with this existing literature. Therefore, sexual health counselors must assess whether young adults have access to and utilize reliable sources of sexual health information.

Young adults' propensity for risky sexual behavior [4,31] underscores the importance of SHL competence [4] and sexual attitudes [29]. Kaplan Doğan demonstrated that young women with higher SHL and more positive sexual attitudes engaged in less risky sexual behavior [12]. It is imperative to provide young people with comprehensive, age-appropriate SRH education before they become sexually active. Such education can not only encourage the postponement of first sexual experiences but also promote less risky sexual behaviors. Amanu, Birhanu, and Godesso highlighted that adolescents who receive SRH education from healthcare professionals benefit more than those who acquire information from other sources [4]. Shahrahmani et al. further emphasized that both reliable and unreliable sources of sexual health information predict SHL [14].

Our study found that participants who learned about sexual health from doctors/specialists, nurses/midwives, books, and personal experiences had higher SHLS scores. Similarly, those who accessed reliable information sources also exhibited higher SHLS scores. However, participants who learned about sexual health from friends/acquaintances, siblings, and personal experiences showed higher SHAS scores. This suggests that young people with more positive attitudes toward sexual health may be more comfortable discussing sex with friends and siblings. The role of personal experience is particularly noteworthy for both SHL and sexual health attitudes. Sexual health counselors working with young people who rely heavily on personal experiences should be mindful of potential risky behaviors and consider tailored education to address these.

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High schools and colleges should integrate curricula that promote SHL. Furthermore, governments ought to develop and implement policies to ensure young people acquire SHL [4]. Effective collaboration among policymakers, healthcare professionals, educational institutions, media platforms, religious leaders, parents, and other stakeholders is crucial to comprehensively support young adults in acquiring SHL [4,20].

Limitations

This study has some limitations. These limitations are particularly related to the study being conducted on social media platforms. The first limitation was that the sample consisted only of young adults with social media accounts. Also, probability sampling was not used for all young adults who use social media. This is the second limitation of this study. Therefore, the findings cannot be generalized to both social media users and non-users.

CONCLUSION

Education, gender, sexual health attitudes, and prior sexual health education are significant determinants of SHLS. Young adults who access reliable sources of sexual health information tend to have higher SHLS scores. Ultimately, greater SHL among young adults correlates with higher SRH levels. Therefore, developing effective, tailored interventions to meet young adults' specific needs is essential. Healthcare professionals, especially nurses, must understand the factors influencing SHL and SRH when providing sexual health counseling and planning educational programs.

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Ethics Committee Approval: The study was approved by the Health Sciences Ethics Committee of Ankara Yıldırım Beyazıt University (Date: 01.07.2024, No.: 06/801).

Informed Consent: The data were collected using an online survey, which briefed all young adults on the research purpose, procedure, and confidentiality. Those who agreed to participate clicked on the "Agree" button and then completed the data collection tools. The research adhered to the principles of the Helsinki Declaration for ethical conduct.

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Isolation and characterization of Kumquat-derived exosome-like nanovesicles and their cytotoxic effects on HCT 116 colon cancer cells

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MAIN POINTS

- Uniform and reproducible KNVs were successfully isolated and purified using sucrose density gradient ultracentrifugation method.
- The NTA results showed that the purified KNVs appeared as homogeneous vesicles with an approximate diameter of 153.1±1.0 nm and a concentration of $6.67x10^{12}$ particles particles/mL.
- KNVs showed strong cytotoxic activity against HCT 116 cells in a concentration- and time-dependent manner

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ABSTRACT

Aim: We aimed to isolate and characterize Kumquat-derived exosome-like nanovesicles (KNVs) and evaluate their potential therapeutic effects on colon cancer.

Materials and Methods: KNVs were obtained by the ultracentrifugation method, and their purification was carried out by the sucrose density gradient ultracentrifugation method. The NTA method was used to measure the size distribution and particle concentration of KNVs. The BCA assay was utilized to determine the total protein concentrations of KNVs. MTT analysis was performed to examine the cytotoxic effects of KNVs against HCT 116 colon cancer cells.

Results: Uniform and reproducible KNVs were successfully isolated. High yield and pure KNVs were obtained in the 30% sucrose layer after sucrose density gradient ultracentrifugation. NTA results showed that KNVs sizes were 153.1±1.0 nm and particle concentrations were 6.67 × 10^{12} particles/mL. The total protein concentration of KNVs were determined as 1,79 μg/μL. Cell viability results revealed that KNVs showed strong cytotoxic activity against HCT 116 cells in a concentration- and time-dependent manner. Furthermore, at a concentration of 20 μg/mL KNVs, HCT 116 cells showed a 50% reduction in cell viability in 48 hours.

Conclusion: Consequently, our study shows that KNVs may hold promise as therapeutic candidates for the treatment of colon cancer in the future, and the research serves as a valuable resource for further research.

Keywords: Kumquat, Exosome-like nanovesicles, HCT 116, Colon cancer

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INTRODUCTION

Cancer stands out as one of the most significant health issues of the 21st century. Globally, one in every six deaths (16.8%) and one in every four deaths due to non-communicable diseases (22.8%) are attributed to cancer [1]. Colorectal cancer or colon cancer (CC) is the third most common type of cancer and the second leading cause of cancer-related deaths [2]. CC is a cancer characterized by a high mutation burden, resulting from the accumulation of somatic mutations, and it possesses a genetically complex structure. This cancer type typically progresses through a process known as the adenoma-carcinoma sequence and is closely associated with environmental and biological factors such as inflammation [3]. Standard treatment strategies for CC include surgical intervention, chemotherapy, and targeted therapies. While surgical intervention may be applied in early-stage patients, approximately 20% of patients are diagnosed with metastases, which often makes surgery unfeasible. Chemotherapy serves as the primary treatment for metastatic CC. Depending on RAS gene mutations and tumor location, adding targeted agents such as bevacizumab or cetuximab enhances the efficacy of chemotherapy. Despite these treatments, the prognosis of the

disease typically remains unfavorable [4]. Additionally, the chemotherapeutic drugs used in CC treatment often lead to severe side effects, including gastrointestinal and neurological toxicities, anemia, and dermatological issues [5,6]. These challenges emphasize the urgent need for the development of innovative treatment strategies to achieve more effective outcomes in CC therapy. Today, one of the most notable and promising approaches for treating cancer is the therapeutic use of exosomes [7].

Various cell types secrete exosomes, which are extracellular vesicles (EVs) with a size range of 30 to 150 nm. Upon their initial discovery, these vesicles were thought to be structures solely responsible for eliminating cellular waste [8]. However, it is now known that exosomes carry bioactive components such as proteins, RNA molecules and lipids, and play significant roles in various biological processes, including signal transduction, antigen presentation, and the regulation of immune responses [8-10]. These versatile functions of exosomes have enabled their exploration as a therapeutic tool in specific fields, such as CC treatment. Studies on the efficacy of mammalian exosomes in CC therapy have indicated that these exosomes may enhance treatment efficacy by targeting cancer cells [11-13]. However, the high risk of contamination during the isolation and purification processes of mammalian exosomes [14], as well as their potential to cause high levels of toxicity and immunogenicity [15,16], complicate their therapeutic use. Furthermore, it has been emphasized that the production of mammalian exosomes is limited, with variations in exosome production capacity between different cell types, and the amount of exosomes released from certain beneficial cells may be insufficient to achieve a clinical effect [17]. Additionally, the high costs, safety risks, and ethical issues associated with mammalian exosomes are among the main factors hindering their use in clinical applications. To overcome these limitations and risks, alternative sources of exosomes must be explored. Plant-derived exosome-like vesicles (PELNVs) are one of these alternatives. The ability of PELNVs' miRNAs to target mammalian genes and facilitate both intercellular and interspecies communication has drawn the attention of researchers [18]. PELNVs exhibit characteristics similar to mammalian exosomes in terms of molecular content [19] and offer significant advantages over mammalian exosomes when compared to synthetic carriers. These advantages include being non-toxic, having low immunogenicity, the ability to cross the blood-brain barrier, having good biocompatibility, and stability in the gastrointestinal tract [18, 19]. These features provide significant advantages over current drug delivery systems. A significant advantage of PELNVs, distinct from mammalian exosomes, is their possession of secondary metabolites. Flavonoids, anthocyanidins, and phenolic acids present in PELNVs endow them with strong antioxidant properties [20]. Flavonoids such as hesperidin and quercetin, abundantly found in the peel and fruit of citrus fruits, stand out not only for their antioxidant properties but also for their anti-inflammatory effects [21]. According to a study by Raimondo et al., lemon-derived EVs were found to suppress the ERK/NF-κB signaling pathways, which decreased the expression of pro-inflammatory cytokines (IL-6 and TNF-α) in macrophages [20]. Furthermore, it has been shown in the literature that PELNVs may inhibit tumor growth and induce cancer cells to undergo apoptosis. In a study conducted by Takakura et al., Citrus limon L.-derived nanovesicles were reported to exert inhibitory effects on the growth of three different CC cell lines carrying K-Ras activation mutations [22]. In another study, nanovesicles isolated from mandarin juice were loaded with siRNA molecules targeting the DDHD domain-containing protein 1 (DDHD1) gene with an efficiency of 13%, resulting in a 60% suppression of DDHD1 gene expression in SW480 cells. Consequently, cell viability in CC cells was reduced by 17-23% [23].

Fortunella margarita Swing, a member of the citrus family (Rutaceae), has recently attracted significant attention. In a recent study conducted by Vrca et al., the essential oils of kumquat were reported to exhibit cytotoxic effects on cancer cell lines (HeLa, HCT 116, U2OS) while demonstrating low toxicity in healthy cells. Additionally, antibacterial activity against S. aureus and E. coli, along with antioxidant properties, was highlighted [24]. Although the anticancer properties of kumquat have been documented in the literature, no data regarding the PELNVs of Kumquat have been reported. In this study, we aimed to isolate and characterize the PELNVs derived from the Kumquat fruits (KNVs) and to evaluate their potential therapeutic effects against the HCT 116 CC cell line by cytotoxicity assay *in vitro*.

MATERIALS AND METHODS

Plant material and isolation of KNVs

Fresh (Kumquat) fruits (1 kg) obtained from a private farmer in Antalya-Türkiye, were kept at -80 C until the isolation procedure. Before the KNVs isolation, kumquat fruits were carefully washed 3 times with sterile distilled water. Subsequently, the exocarp and mesocarp of the kumquat were peeled, the seeds were removed and the endocarp was taken. A blender was used to chop Kumquat endocarp in sterile phosphate buffered saline (PBS) solution at high speed for two minutes. Plant cell wall residues were then removed by filtering the homogenate through a 100 μm nylon mesh. The filtrate was subjected to centrifugation at 300 × g for 15 min, 2.000 × g for 30 min at 4 C to remove large particles and cellular debris and then underwent centrifugation at 20.000 g for 30 min at 4 C to eliminate microparticles. Subsequently, the supernatant was collected and ultracentrifuged at 100.000 × g for 3 h at 4 C using an ultracentrifuge (Optima XPN-100, Beckman Coulter, Brea CA, USA). The obtained pellets were resuspended in 5 mL sterile PBS and filtered at 0.22 μm pore filter. After isolation, KNVs were stored at −80 C. No more than 1 freeze–thaw cycle was used during experiments.

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KNVs purification using sucrose gradient centrifugation

In order to purify KNVs, their suspension was loaded on top of a discontinuously dispersed sucrose gradient solution (8%, 30%, 45%, and 60%, w/v) and then ultracentrifuged at 120.000 × g for 2 h at 4 C using an SW 32 rotor. KNVs were collected from the 30% sucrose layer and diluted with 30 mL PBS. Purified KNVs were ultracentrifuged at 100,000×g for 1 h at 4 C, and then the pellet was resuspended in 1 mL of sterile PBS. Purified KNVs were stored at −80 C until use.

Nanoparticle tracking analysis (NTA)

Quantification and size distributions of KNVs were determined using Nanoparticle Tracking Analysis (NTA) with a 488 nm laser and a high-sensitivity CMOS camera. The isolated KNVs were analyzed utilizing NanoSight NS300 (Malvern Instruments). The NTA method measured the size and concentration of particles in liquid suspension using light scattering and Brownian motion. The tracking algorithm utilized each particle's movement in Brownian motion to estimate the diffusion coefficient (Dt). Using the diffusion coefficient, the Stokes-Einstein equation calculated the particle diameter [25]. The samples were put into the device's vessel after being diluted with sterile-filtered PBS, and the size dispersion was determined using the NTA method. The test was carried out using the NTA 3.3.301 software, and the video recorded the nanoparticles at least five times at 60-second intervals.

Bicinchoninic acid assay (BCA)

The BCA protein assay kit (Thermo Fisher Scientific, 23225) was used to measure the total protein concentrations of KNVs. Briefly, 25 µl of standards were added to the 96-well plate at increasing concentrations. KNVs were diluted in ddH_2O and 25 µl of exosome mixture was added to each well. Solutions A and B in the kit were mixed at a ratio of 1:50 and 175 µl mixture was added to the standards and KNV samples. After that, the plate was incubated for half an hour at room temperature (RT). Multiskan GO spectrophotometer (Thermo Fisher Scientific) was used to make measurements at a wavelength of 562 nm. The standard curve graph was used to calculate the total protein concentrations of the obtained KNVs. Each experiment was performed in 3 replicates.

Cell culture

The human HCT 116 CC cell line was purchased from the American Type Culture Collection (ATCC, CCL-247) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biosera, USA). Medium was supplemented with 10% fetal bovine serum (FBS, Gibco),100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) and 2 mM L-Glutamine (Hyclone). The cells' morphology and growth were assessed every day under an inverted microscope. HCT 116 CC cells were cultivated at 37°C with 5% CO_2 in a humidified incubator and were routinely checked for mycoplasma contamination [26]. For the KNV treatment, DMEM was supplemented with 10% exosome-depleted FBS.

Cell viability assay

The cytotoxic effects of KNVs on HCT 116 cell line was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, AR1156, Boster) assay. Cells were seeded at $1x10^4$ cells/well density into 96-well cell culture plates. Following overnight incubation, HCT 116 CC cells were washed and treated with different concentrations of KNVs (0 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL) for 24 h or 48 h. Then, 100 µL of fresh culture media with 10 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Subsequently, the supernatants were discarded and the remaining formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO, P60-36720100, PAN-Biotech). MTT analysis was carried out three times in triplicate. Optical density (OD) values were measured at a wavelength of 570 nm using a Multiskan GO spectrophotometer, and the percentage of viable cells was calculated as: Type% % viable cells (OD treatments/OD untreated control) × 100.

Statistical analysis

The data were statistically compared by PRISM v7 (GraphPad, w) software. First, the Shapiro-Wilk test was used to determine the normalization of data. Two-way RM ANOVA and Sidak's multiple comparisons test were used for statistical analysis. Data are given as mean ± standart deviation (SD) and $p<0.05$ values considered statistically significant. Each analysis was carried out three times in triplicate.

RESULTS

Isolation of KNVs

KNVs were isolated from fresh Kumquat fruits (Figure 1A) using a differential centrifugation method. Large particles and debris were disposed of following a series of low-velocity centrifugation processes. Subsequently, the collected supernatants were ultracentrifuged at 100.000 × g. KNVs were obtained in the form of orange pellets found at the bottom of the ultracentrifuge tubes (Figure 1B). The KNV pellets were dissolved in sterile PBS and subsequently filtered with a 0.22 µm pore filter. KNVs were purified with sucrose density gradient ultracentrifugation. KNVs were obtained in the 30% sucrose layer (Figure 1C).

Characterization of KNVs

Characterization of KNVs, including size distribution and particle concentration, was performed using NTA via particle-by-particle inspection. As a result of NTA, it was determined that KNVs purified by the sucrose density gradient ultracentrifugation method appeared as homogeneous vesicles, had a diameter of approximately 153.1±1.0 nm, and had a concentration of 6.67×10^{12} particles/mL (Figure

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Table 1. Statistical analysis of MTT cell viability assay results. Data are given as mean±SD.

	0 μg/mL/Control	2.5 μg/mL	5 μg/mL	10 μg/mL	20 μg/mL	40 μg/mL
24 h	99.69±1.65	95.01±1.32 p=0.76	85.65±4.29* p<0.001	72.45±2.12* p<0.001	60.92±3.08* p<0.001	34.15±4.53* p<0.001
48 h	99.40±2.76	89.30±2.72* p<0.001	77.83±3.10* p<0.001	60.40±4.66* p<0.001	46.51±3.38* p<0.001	15.68±2.31* p<0.001

**p values <0.05 were considered significant compared with 0 μg/mL.*

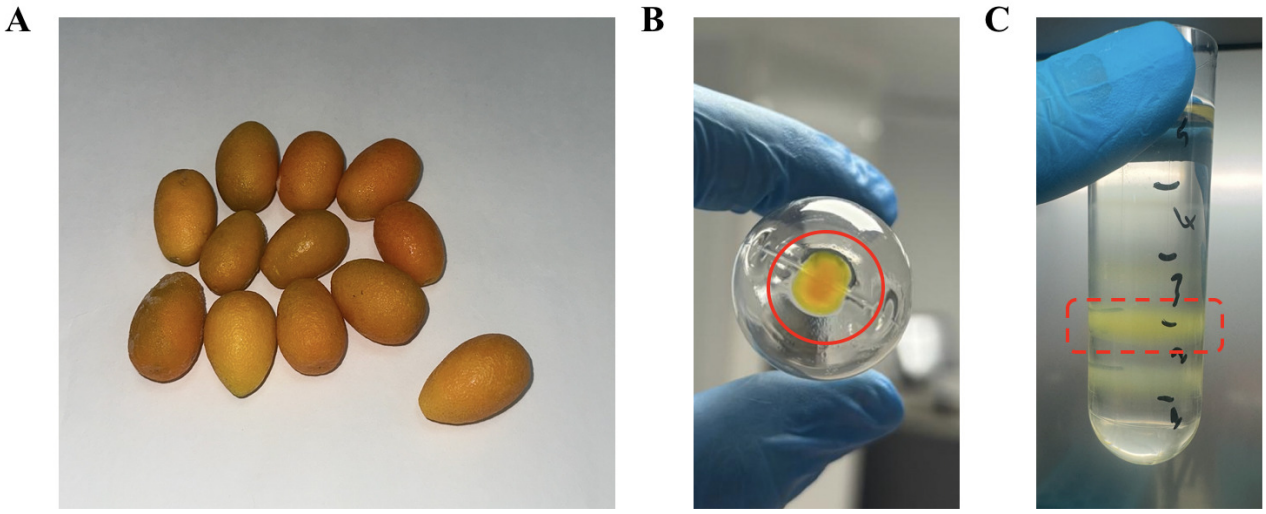

Figure 1. Isolation of KNVs. Kumquat fruits (A); KNVs pellet after 100.000 × g ultracentrifugation (B); Purified KNVs after sucrose gradient (8/30/45/60%, w/v) ultracentrifugation (C).

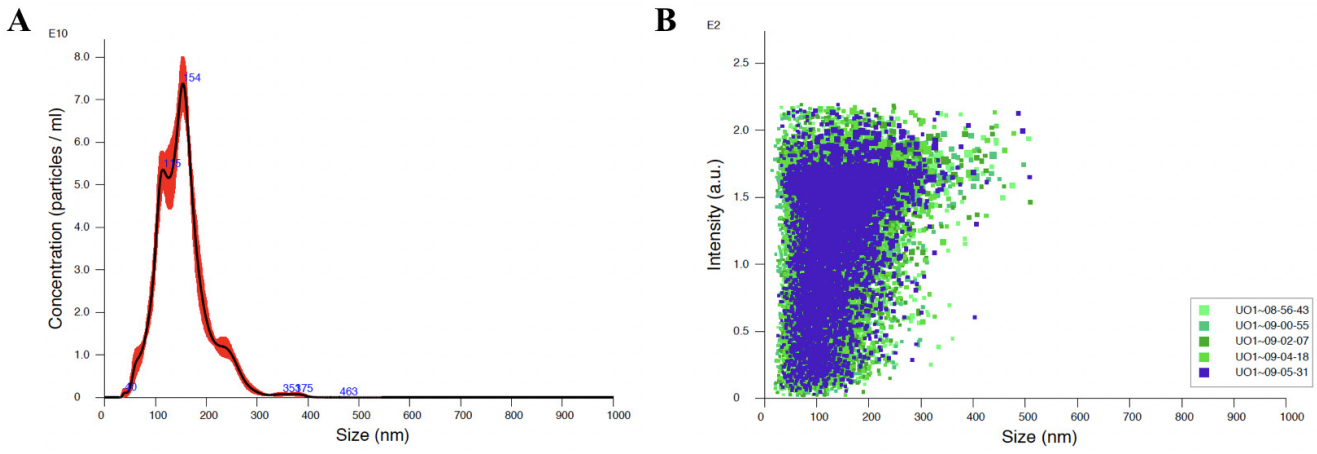

Figure 2. Characterization of KNVs. NTA results showing the size distribution (A) and intensity (B) of KNVs.

2A and 2B). The quantity of KNVs was estimated based on protein concentration using the BCA protein analysis kit. The total protein concentration of KNVs were determined as 1.79 μg/μL.

Cell viability results

We performed an MTT assay to detect the effects of KVNs on the viability and proliferation of HCT 116 cells. For this purpose, we treated HCT 116 CC cells with increasing concentrations of KNVs (2.5, 5, 10, 20, and 40 μg/mL) for durations of 24 and 48 hours, followed by the administration of MTT reagent. According to our findings, KNV's treatment caused concentration- and time-dependent cytotoxicity in HCT 116 cells. It was found that 40 μg/mL KNVs concentration reduced HCT 116 cell viability to 34% and 15%

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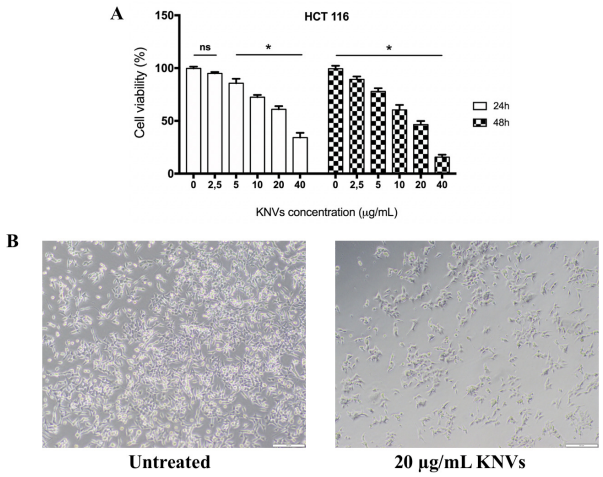

Figure 3. The effect of KNVs on the viability and proliferation of HCT 116 cells. The cell viability was detected using the MTT assay (A), Microscopic images of untreated and 20 µg/mL KNV treated groups (B). The data represent mean ± standard deviation (n = 3). * indicates statistical significance of differences in cell viability treated with KNVs compared to untreated cell viability ($p < 0.05$, Two-way RM ANOVA).

at 24 and 48 hours, respectively, while 20 µg/mL KNVs concentration reduced cell viability to 61% and 46% compared to the untreated group (Figure 3A, Table 1). Therefore, when HCT 116 cells were co-cultured for 48 hours with a concentration of 20 µg/mL KNVs, which caused approximately 50% cell viability, increased cytotoxic and cytopathic activity was observed in the cells under an inverted microscope (Figure 3B).

DISCUSSION

Exosomes are a subpopulation of EVs, which are nanoscale lipid bilayer particles secreted from living cells into the surrounding environment, playing critical roles in intercellular communication, tumor metastasis and signal transduction [27]. Exosomes derived from mammalian cells have recently gained widespread use in various biomedical fields, including tissue reconstruction, drug delivery, and diagnosis [28]. Despite the great therapeutic nanoplatform potential of these exosomes, some major problems limit their clinical applications, including low production efficiency, time-consuming and laborious manufacturing procedures, and difficulties in obtaining high-quality and homogeneous exosomes [28-30]. It's exciting to note that plant cells also release exosome-like vesicles, which are mass-produced, eco-friendly, economical, and biosafe nanoplatforms [28, 30, 31]. PELNVs are identical in morphology and functionality to their mammalian analogues, and their inability to be detected by the immune system prolongs their circulation time in the blood. Additionally, unlike their mammalian counterparts, they do not harbor zoonotic or human pathogens [32, 33].

Ultracentrifugation is the most widely used technique for purifying exosomes and has long been considered the gold standard for isolating exosomes of relatively homogeneous sizes [33, 34]. Recent studies on the biological activities of PELNVs have largely used the 30%–45% interphase of sucrose layers [33]. In our study, relatively homogeneous and stable exosome-like nanovesicles were successfully isolated from the 30% sucrose layer and used in the study.

NTA is a real-time imaging technique that can quickly detect the size and concentration of exosomes and is frequently used for exosome characterization [35]. The spectrum of the size of exosome-like nanovesicles obtained from plants varies between 50 and 500 nm among plant species and even within the same species [30, 36]. NTA results showed that the mean size of KNVs was 153.1±1.0 nm, and the nanoparticle concentration was measured as 6.67×10^{12} particles/mL. According to the BCA results of KNVs, the total protein concentration was determined as 1.79 µg/µL. When the total protein amounts obtained with BCA were compared with the particle concentrations measured with NTA, it was determined that purified and high-yield KNVs were obtained, and these amounts were sufficient for our study.

There is increasing evidence that reveals the regulatory roles of PELNVs in critical processes of the organisms, such as metabolism, inflammation, homeostasis, and tumorigenesis [28]. Significantly, these platforms have been used to treat a variety of inflammatory and malignant diseases because they contain versatile bioactive substances (polyphenols, functional proteins, and flavones). Over the last decade, a large number of natural and green nanovesicles have been successfully isolated from edible plants [28, 30]. PELNVs obtained from ginger, lemon, grapefruit, grape, and Chinese bamboo shoots are reported to have anticancer properties and have anti-proliferative effects both in vivo and in vitro. Tea flower-derived PELNVs have been reported to accumulate in breast tumors and their lung metastatic sites after intravenous injection or oral administration, inhibit the growth and spread of breast cancer, and regulate the gut microbiome [37]. You et al. reported that PELNVs derived from cabbage and red cabbage loaded with the chemotherapy drug doxorubicin (DOX) killed SW480 CC cells. They also showed that by loading miR-184 into cabbage PELNVs, nucleic acids can be efficiently transported by PELNVs [38]. Raimondo et al. reported that PELNVs derived from lemon juice inhibited Acetyl-CoA Carboxylase 1, thereby suppressing the growth of CC cells [39]. Furthermore, in another study, lemon exosomes were shown to increase the mRNA levels of the pro-apoptotic molecules Bad and Bax while decreasing the levels of the pro-survival molecules Survivin and Bcl-XL. It was also noted that lemon exosomes exhibited anticancer properties by inducing TRAIL-mediated apoptosis in various tumor cell lines [40]. With this study, we aimed to investigate, for the first time in literature, the cytotoxic effects of kumquat-derived nanovesicles on HCT 116 CC cell line. According to the MTT results, 20 µg/mL KNVs concentration was found to reduce HCT 116 cell viability to 61% and 46% at 24 and 48 hours, respectively. Researchers have shown in numerous

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studies that PELNVs trigger the anticancer mechanism, particularly by inducing apoptosis in tumor cells in vitro. The results of our cytotoxicity analysis are also consistent with the literature and suggest that KNVs may exert the cytotoxic effect most likely through apoptosis.

Plants have been used for thousands of years to treat many diseases due to their therapeutic properties. The fact that plants produce thousands of different secondary compounds with therapeutic properties in their leaves, roots, seeds, and flower buds is the reason why plants have been used for therapeutic purposes for so long. For this reason, PELNVs obtained from different plant species are being tested in cancer treatment, and the number of studies in this field is increasing day by day. In our study, uniform and reproducible KNVs were successfully isolated with high yield and purity by sucrose density gradient ultracentrifugation. Our preliminary results indicate that KNV treatment induces concentration- and time-dependent cytotoxicity in HCT 116 CC cells.

Limitations

There are limitations of our study. We used a single cell line in our research to test our KNVs. Using different colon cancer cell lines will be better representative of the groups and will hence provide more accurate results. Also, the inclusion of normal colon cells will reveal the level of safety and biocompatibility of KNVs.

CONCLUSION

In conclusion, in terms of therapeutic applications, we consider KNVs as a potential nanomedical drug with potential in CC treatment as a single agent or in combination with other drugs. Our study is a preliminary step for further detailed analyses and sheds light on the mechanism of action of KNVs and their biosafety and biodistribution analyses in both in vitro and in vivo models.

Ethics Committee Approval: It is a study that does not require ethics committee approval.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there are no financial or personal relationships that could be perceived as potential conflicts of interest influencing the research reported in this article.

Author Contributions: M.Ö: Conception, Design, Supervision, Materials, Analysis and/or Interpretation, Critical Review; H.B.Ö: Conception, Materials, Critical Review; B.Ş.H: Conception, Writing, Critical Review; H.Ö: Conception, Design, Materials, Data Collection and/or Processing, Literature Review, Writing, Critical Review; R.B.Ç. Data Collection and/or Processing, Analysis and/or Interpretation, Critical Review; S.G: Data Collection and/or Processing, Analysis and/or Interpretation, Critical Review.

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Comparison of fetal thyroid measurements between treated subclinical hypothyroidism and euthyroid pregnancies: A prospective observational study

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MAIN POINTS

- Adequately treated maternal subclinical hypothyroidism does not significantly affect fetal thyroid dimensions, suggesting that appropriate levothyroxine therapy normalizes potential developmental impacts on fetal thyroid morphology.
- No significant correlations were found between maternal TSH levels, levothyroxine dosage, and fetal thyroid measurements, indicating that fetal thyroid development progresses independently when maternal thyroid function is appropriately managed.
- Amniotic fluid index remained significantly elevated in the subclinical hypothyroidism group despite treatment, revealing a potential subclinical manifestation of maternal thyroid dysfunction that persists even with adequate levothyroxine therapy.
- Standardized ultrasound measurement protocols with high reproducibility (ICC: 0.89, kappa: 0.85) demonstrated the feasibility of reliable fetal thyroid assessment during routine prenatal care.
- Early diagnosis and treatment of subclinical hypothyroidism are crucial for ensuring normal fetal thyroid development, supporting current guidelines for levothyroxine therapy in pregnancy.

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ABSTRACT

Aim: To investigate the impact of maternal subclinical hypothyroidism (SCH) treated with levothyroxine on fetal thyroid dimensions during pregnancy.

Materials and Methods: In this prospective observational study, 50 pregnant women with treated SCH and 52 euthyroid controls underwent ultrasonographic evaluation between 20 and 39 gestational weeks were recruited. Fetal thyroid circumference (FTC) and fetal thyroid area (FTA) were measured, and correlations with maternal TSH levels and levothyroxine dosage were analyzed.

Results: No significant differences were found between the in fetal thyroid measurements in SCH and control groups before and after adjustment for gestational age. Correlation analyses revealed negligible associations between maternal thyroid function parameters and fetal thyroid size. Although the levothyroxine dose showed a weak negative trend with fetal thyroid measurements, the difference was not statistically significant. The amniotic fluid index (AFI) was significantly higher in the SCH group despite treatment.

Conclusion: Adequately treated maternal SCH does not appear to affect fetal thyroid development. These findings support the importance of early diagnosis and levothyroxine therapy for normalizing maternal thyroid function and potentially protecting fetal outcomes.

Keywords: Hypothyroidism, Pregnancy, Prenatal ultrasonography, Thyroxine

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INTRODUCTION

Thyroid hormones (THs), primarily thyroxine (T4) and triiodothyronine (T3), are indispensable regulators of human development, metabolism, and homeostasis, beginning from the earliest stages of embryogenesis. They play crucial roles in cell differentiation, growth, neurogenesis, and energy metabolism, and are particularly essential for fetal brain development and thermoregulation [1]. The physiological demands of pregnancy induce significant alterations in thyroid hormone production, transport, and metabolism to accommodate maternal and fetal needs. These changes, in turn, increase the vulnerability of women to thyroid dysfunction during gestation.

Thyroid dysfunction, defined as excessive or insufficient pro-

duction of hormones by the thyroid gland, is one of the most common endocrine disorders affecting women of reproductive age, including during pregnancy [2]. Throughout the gestational period, a range of thyroid dysfunctions may occur, including overt hypothyroidism, subclinical hypothyroidism (SCH), and hyperthyroidism [3]. Overt hypothyroidism is defined as the presence of serum thyroid-stimulating hormone (TSH) levels greater than 10 mIU/L and decreased fT4 concentrations. SCH is characterized by elevated TSH levels, whereas fT4 concentrations are normal [4]. Epidemiological data indicate that SCH affects approximately 10% of the adult population and has a prevalence of 3.47% among pregnant women [5]. The major etiology of hypothyroidism is iodine deficiency in developing countries, whereas autoimmune thyroiditis is the primary factor in [6].

In 2011, the American Thyroid Association (ATA) published standardized guidelines for the management of thyroid dysfunction in pregnancy. This recommendation recommends upper limits of 2.5 mIU/L and 3.0 mIU/L for the first and second trimesters, respectively, for the diagnosis of subclinical hypothyroidism in pregnancy. Consequently, after thorough examination of population samples from many ethnic groups, the ATA updated these guidelines in 2017, establishing the maximum limit for normal blood TSH levels in early pregnancy at 4 mIU/L [7].

The fetal thyroid gland achieves functional maturity between 18 and 20 weeks of gestation, and during early pregnancy, the fetus predominantly relies on maternal circulating fT4 for growth and development [8]. Consequently, maternal fT4 serves as the sole supply of thyroid hormone for the developing fetus throughout this period. During early gestation, the fetus requires maternal THs for neuronal proliferation and migration [9]. Maternal thyroid hormone deficiency can result in various complications during pregnancy. Neurological deficits in infancy and adolescence, including reduced intelligence quotients, delays in neurocognitive functioning, and underdeveloped psychomotor skills, are major complications of maternal hypothyroidism in early gestation [10].

In addition to long-term neurodevelopmental risks, maternal hypothyroidism has also been linked to numerous obstetric complications. These include miscarriage, preterm birth, preeclampsia, placental abruption, gestational diabetes mellitus (GDM), intrauterine growth restriction (IUGR), and increased perinatal mortality [11–13].

Despite the established association between maternal thyroid dysfunction and adverse outcomes, the precise mechanisms underlying these effects are still not fully understood. Several pathophysiological pathways have been proposed. The initial mechanism involves the direct influence of maternal T4 on the developing fetal neurological system through specific thyroid hormone receptors, which has been corroborated by animal studies identifying receptor pathways [14]. Another hypothesis suggests that alterations in maternal thyroid status may interfere with the maturation and regulatory capacity of the fetal hypothalamic-pituitary-thyroid (HPT) axis, which governs the fetal endocrine response. A less frequently explored but potentially significant mechanism proposed is that maternal thyroid dysfunction may directly influence the development and morphology of the fetal thyroid gland itself. This could occur via disruptions in maternal–fetal thyroid hormone transfer or through immunological and metabolic influences, potentially resulting in long-lasting structural or functional alterations.

The third hypothesis, which has received insufficient attention in the literature, proposes that maternal thyroid dysfunction may directly affect fetal thyroid tissue development, potentially leading to structural changes that persist postnatally. This hypothesis is particularly significant as it offers a quantifiable parameter through prenatal ultrasound measurement of fetal thyroid size, yet there remains a critical gap in research specifically examining the relationship between maternal SCH, levothyroxine treatment, and fetal thyroid dimensions. Understanding these mechanisms is crucial because they may represent different pathways through which maternal thyroid status ultimately influences fetal cognitive and physiological development.

In this study, we aimed to investigate the relationship between maternal thyroid function and fetal thyroid development by comparing fetal thyroid gland measurements obtained via ultrasonography in pregnant women diagnosed with and treated for SCH and those in euthyroid pregnant women. Through this comparison, we sought to provide insights into the mechanisms by which maternal thyroid status may influence fetal endocrine organogenesis and overall development, with potential implications for prenatal surveillance and therapeutic strategies.

MATERIALS AND METHODS

Study design and setting

This prospective observational study was performed in the Department of Perinatology, Haseki Training and Research Hospital, University of Health Sciences, Istanbul, Turkey, between January and December 2024. The study protocol received approval from the Institutional Ethics Committee (Registration Number: 23-2024 dated October 9, 2024) and was executed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all participants following the provision of detailed information regarding the study protocols.

Participants

Initially, 140 pregnant women were assessed for eligibility. Among them, 38 were excluded due to chronic maternal conditions (n=5), fetal chromosomal abnormalities (n=5), incomplete laboratory data (n=18), and inadequate ultrasound imaging (n=10). As a result, 102 participants met the inclusion criteria and were enrolled in the study. The participants were subsequently divided into two groups: 50 preg-

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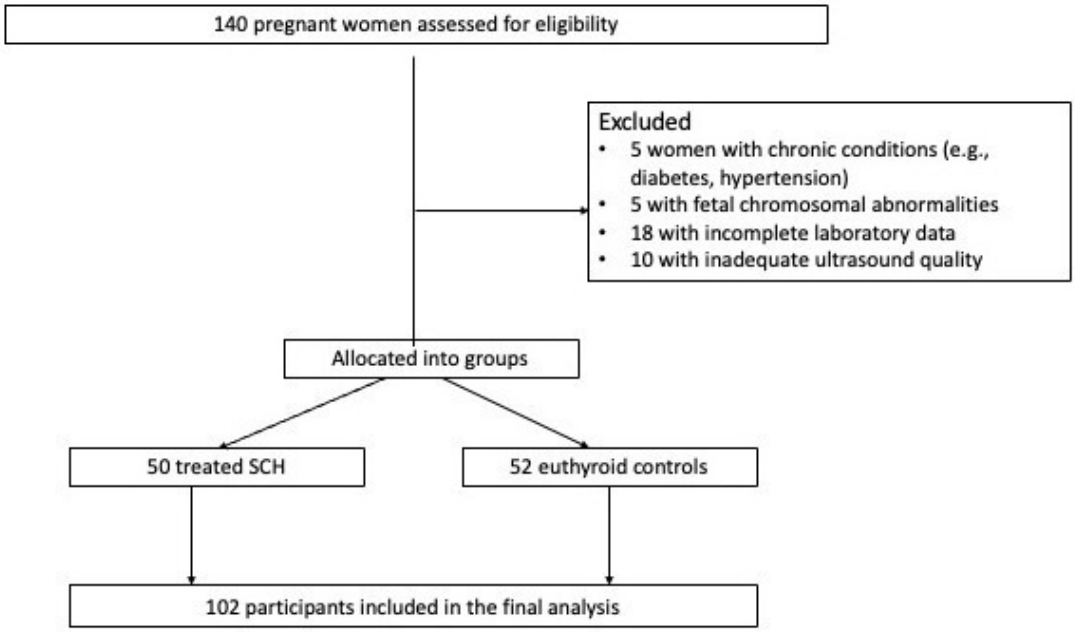

Figure 1. Flowchart of participant enrollment, exclusion, group allocation, and inclusion in final analysis.

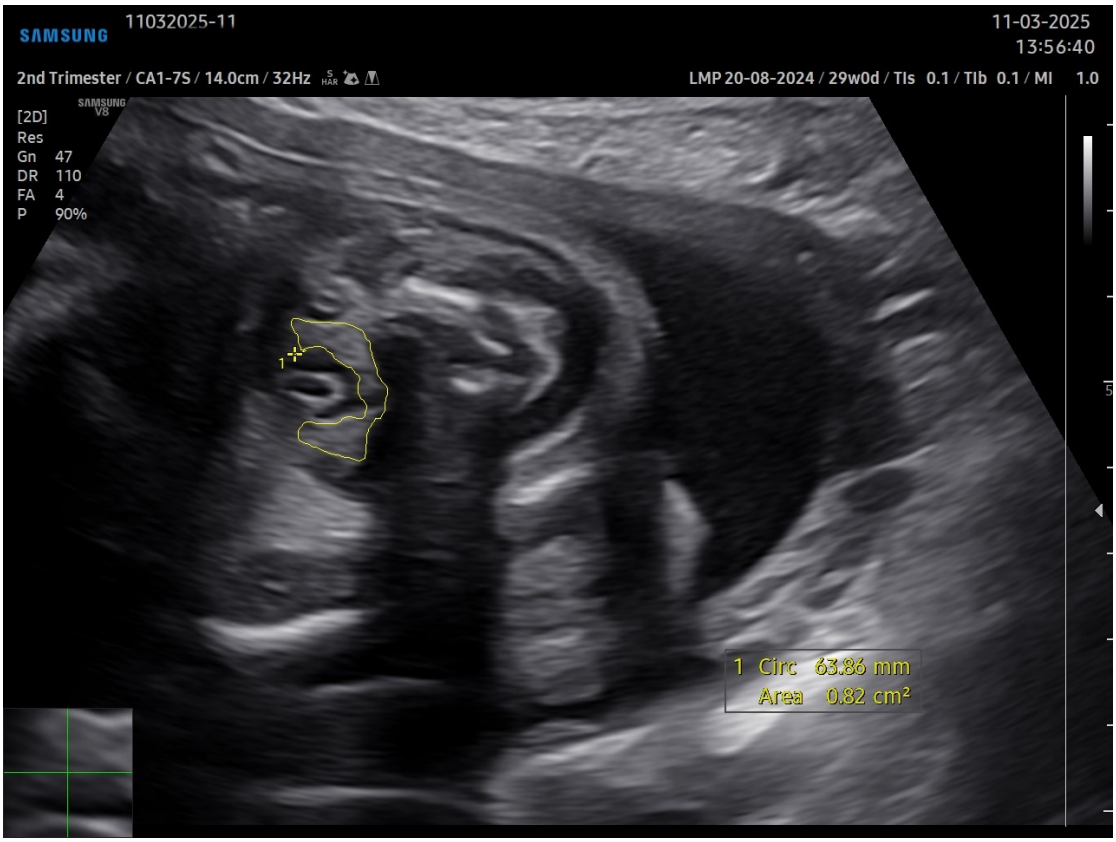

Figure 2. Axial view of the fetal neck showing thyroid gland measurement with circumference and area obtained using caliper and field measurement tools.

nant women with treated SCH and 52 euthyroid controls. The participant selection process and study flow are illustrated in Figure 1.

The study included patients diagnosed with SCH either before pregnancy or during the first trimester (defined by TSH levels exceeding 4 mIU/L with normal fT4 values; normal reference range for free T4 was 8.9–17.1 ng/L and for free T3 was 2.0–4.4 ng/L, measured by chemiluminescent immunoassay). Additionally, euthyroid pregnant women were included as a control group to enable a comparative analysis

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between those with treated SCH and those with normal thyroid function. Iodine intake was not systematically assessed or standardized among participants. Therefore, variations in iodine status constitute a potential confounding factor, and this limitation is acknowledged in the Discussion section. Gestational age was precisely established by confirming the last menstrual period on the first trimester CRL measurement.

The subsequent circumstances were excluded from the study: multiple gestations; pre-existing maternal conditions (including chronic hypertension, renal disease, hepatic disorders, other endocrine disorders); major neurological or psychiatric disorders; fetal structural or chromosomal abnormalities; known placental pathologies; cases with incomplete laboratory assessments or inadequate ultrasound examinations; and those who did not provide written informed consent. The sample size calculation was performed using G*Power software (release 3.1.9.7, from Heinrich Heine University in Düsseldorf, Germany). Drawing on earlier research that examined fetal measurements in pregnancies complicated by thyroid disorders, we selected a moderate effect size (d=0.6) for our comparative analysis between the two separate groups. With a type I error (α) of 0.05 and type II error (β) of 0.20 (corresponding to 80% power), the minimum required sample size was calculated as 45 participants per group. Considering potential dropouts and technical difficulties during ultrasound measurements, we aimed to include at least 50 participants in each group. The final study population comprised 102 participants (50 in the SCH group and 52 in the control group), exceeding the minimum requisite sample size.

Ultrasound imaging methods

The fetal thyroid gland can be reliably assessed after 14 weeks by transvaginal ultrasonography and after 18 weeks by transabdominal ultrasonography [15]. Despite its clinical importance, few studies in the literature have established normative data on fetal thyroid size across gestational ages [16,17].

In our study, all ultrasonographic examinations were performed by a single experienced operator using a high-resolution ultrasound device (SAMSUNG V8, Samsung Medical Systems, Potenza, Italy) equipped with a convex array transducer (frequency range: 2–8 MHz).

A standardized imaging protocol was strictly followed to minimize interindividual variability in fetal thyroid measurements. Imaging was performed in the axial plane of the fetal neck at the level of the transverse view of the trachea, where the trachea appears centrally located between the two carotid arteries. Both lobes of the thyroid gland were identified based on their hyperechoic contours and homogeneous internal echotextures. The circumference of each lobe was manually traced using the ultrasound machine's caliper tool, and the area (cm^2) was calculated automatically using the integrated field measurement function (Figure 2). Special care was taken to avoid compression artifacts, and all measurements were obtained with the fetal neck in a neutral position (neither flexed nor extended) to prevent distortion. To ensure the reliability of the measurements, intraobserver reproducibility was evaluated by repeating measurements in 20 randomly selected fetuses after a 1-week interval by the same operator, yielding an intraclass correlation coefficient (ICC) of 0.89 (95% CI: 0.872–0.918). Interobserver reproducibility was assessed by a second independent sonographer who performed measurements on another 20 fetuses, resulting in a Cohen's kappa coefficient of 0.85, indicating excellent agreement.

Statistical analysis

The distribution of continuous variables was assessed using both the Kolmogorov-Smirnov and Shapiro-Wilk tests. Variables that exhibited a normal distribution were expressed as mean ± standard deviation (SD) and compared between groups using Student's t-test, whereas non-normally distributed variables were expressed as median (interquartile range, IQR) and compared using the Mann-Whitney U test. Correlation analyses were performed using Pearson's correlation coefficient for normally distributed variables and Spearman's correlation coefficient for nonnormally distributed variables. For categorical variables, statistical comparisons between groups were performed using appropriate tests based on the distribution of expected frequencies. Specifically, the Chi-square test was used when the expected frequencies met standard assumptions (expected count $\geq$5 in at least 80% of cells), and Fisher's exact test was applied when these assumptions were not satisfied. This approach ensured the validity of the statistical inferences for all the categorical data analyses. Statistical significance was defined as a p-value less than 0.05. To adjust for variations in gestational age, z-scores were calculated for fetal ultrasound measurements based on the entire study population (n=102), and group comparisons were made using Student's t-test. The correlation strength was classified as negligible ($|r| < 0.1$), weak ($0.1 \leq |r| < 0.3$), moderate ($0.3 \leq |r| < 0.5$), strong ($0.5 \leq |r| < 0.7$), or very strong ($|r| \geq 0.7$). For subgroup analyses, SCH patients were divided into low-dose ($\leq$50 mcg/day) and high-dose (>50 mcg/day) levothyroxine groups, and linear regression analyses were conducted to explore the relationship between levothyroxine dose and fetal thyroid dimensions, with coefficients of determination (R^2) reported.

RESULTS

The study population comprised 102 pregnant women: 50 with treated SCH and 52 euthyroid controls. Table 1 shows the demographic and clinical attributes of pregnant women with SCH (n=50) compared to the control group (n=52). Maternal age was similar between groups (p=0.124), whereas women with SCH had significantly higher weight (77.02 ± 14.65 kg vs. 70.60 ± 13.29 kg, p=0.022). Obstetric history parameters exhibited no significant differences between the groups (p>0.05).

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Table 1. Demographic and clinical characteristics of pregnant women with and without SCH.

Variable	SCH Group (n=50)	Control Group (n=52)	p-value
Maternal Characteristics			
Age (years)	30.84 ± 6.11	29.12 ± 5.13	0.124
Weight (kg)	77.02 ± 14.65	70.60 ± 13.29	0.022*
Height (cm)	160.44 ± 5.63	160.92 ± 5.31	0.655
Gravidity	3.00 (2.00-4.00)	2.00 (3.00)	0.083
Parity	1.34 ± 1.14	1.27 ± 1.12	0.752
Abortion	0.00 (1.00)	0.00 (0.00)	0.690
Laboratory Values			
TSH (mIU/L)	6.53 ± 1.88	2.07 ± 0.50	<0.001*
T3 (ng/L)	3.05 ± 0.52	3.02 ± 0.56	0.758
T4 (ng/L)	10.82 (3.40)	10.73 (1.69)	0.324
Fetal Ultrasound Measurements			
Gestational Week	24.00 (7.00)	26.00 (7.00)	0.098
BPD (mm)	60.96 ± 15.18	67.62 ± 11.90	0.015*
HC (mm)	233.97 ± 52.16	248.86 ± 43.41	0.120
AC (mm)	216.09 ± 61.36	232.89 ± 51.91	0.139
FL (mm)	43.49 (18.97)	47.14 (15.96)	0.246
EFW (g)	1068.62 ± 877.88	1194.03 ± 717.16	0.429
AFI	55.16 ± 8.84	47.83 ± 10.86	0.001*
Fetal Thyroid Measurements			
FTC (cm)	4.99 ± 1.74	5.12 ± 1.36	0.671
FTA (cm²)	0.75 ± 0.49	0.82 ± 0.42	0.427

*Statistically significant (p<0.05). Data with normal distribution are presented as mean ± standard deviation, while data with non-normal distribution are presented as median (IQR). The IQR (interquartile range) represents the difference between Q3 and Q1 as a single value.
SCH: Subclinical Hypothyroidism, TSH: Thyroid Stimulating Hormone, BPD: Biparietal Diameter, HC: Head Circumference, AC: Abdominal Circumference, FL: Femur Length, EFW: Estimated Fetal Weight, AFI: Amniotic Fluid Index, FTC: Fetal Thyroid Circumference, FTA: Fetal Thyroid Area.

Table 2. Z-score comparison of fetal ultrasound and thyroid measurements.

Variable	SCH Group (n=50)	Control Group (n=52)	t-value	p-value
Fetal Biometry Z-scores				
BPD (mm)	-0.15 ± 1.07	0.14 ± 0.84	-1.58	0.118
HC (mm)	-0.13 ± 1.09	0.12 ± 0.90	-1.26	0.211
AC (mm)	-0.14 ± 1.13	0.13 ± 0.85	-1.40	0.165
FL (mm)	-0.17 ± 0.92	0.16 ± 1.05	-1.74	0.085
EFW (g)	-0.08 ± 1.10	0.08 ± 0.90	-0.83	0.409
AFI	0.34 ± 0.81	-0.33 ± 1.00	3.73	<0.001*
Fetal Thyroid Z-scores				
FTC (cm)	-0.07 ± 1.14	0.07 ± 0.85	-0.72	0.471
FTA (cm²)	-0.08 ± 1.07	0.08 ± 0.93	-0.81	0.419

*Statistically significant (p<0.05). BPD: Biparietal Diameter, HC: Head Circumference, AC: Abdominal Circumference, FL: Femur Length, EFW: Estimated Fetal Weight, AFI: Amniotic Fluid Index, FTC: Fetal Thyroid Circumference, FTA: Fetal Thyroid Area.

TSH levels were significantly elevated in the SCH group (6.53 ± 1.88 mIU/L vs. 2.07 ± 0.50 mIU/L, p<0.001), with T3 and T4 levels remaining similar between groups. Fetal ultrasound measurements showed significantly smaller BPD in fetuses of SCH mothers (p=0.015) and higher AFI in the SCH group (p=0.001). Other fetal biometric parameters and gestational age showed no significant differences between groups. Fetal thyroid circumference (FTC) and fetal thyroid area (FTA) were comparable between groups (p=0.671 and p=0.427, respectively).

To standardize and compare fetal ultrasound measurements between groups, we calculated Z-scores using the entire study population (n=102) as reference. Table 2 presents the z-score comparison of fetal ultrasound and thyroid measurements between the SCH and control groups. Upon adjustment for gestational age using z-scores, most fetal biometry measurements exhibited no statistically significant variations between the groups. The z-scores for fetal biometric measures showed no significant differences between the SCH and control groups (all p>0.05). The AFI z-score was considerably elevated in the SCH group (0.34 ± 0.81) relative to the control group (-0.33 ± 1.00, p<0.001). Fetal thyroid measurements expressed as z-

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Table 3. Correlation analysis between maternal tsh, levothyroxine dose and fetal thyroid measurements.

Parameters	Coefficient	p-value	Strength	n
All Participants				
TSH - FTC	-0.013	0.896	Negligible	102
TSH - FTA	-0.027	0.788	Negligible	102
Hypothyroidism Group				
TSH - FTC	0.062	0.668	Negligible	50
TSH - FTA	0.080	0.581	Negligible	50
Levothyroxine dose - FTC	-0.156	0.279	Weak	50
Levothyroxine dose - FTA	-0.207	0.149	Weak	50

TSH: Thyroid Stimulating Hormone, FTC: Fetal Thyroid Circumference, FTA: Fetal Thyroid Area. Correlation strength interpretation: $|r| < 0.1$: Negligible; $0.1 \leq |r| < 0.3$: Weak; $0.3 \leq |r| < 0.5$: Moderate; $0.5 \leq |r| < 0.7$: Strong; $|r| \geq 0.7$: Very strong.

Table 4. Comparison of fetal thyroid measurements by levothyroxine dose groups and regression analysis.

Analysis	FTC (cm)	FTA (cm²)	p-value
Dose Group Comparison			
Low dose (≤50 mcg, n=32)	5.190 ± 1.609	0.803 ± 0.455	-
High dose (>50 mcg, n=18)	5.104 ± 1.702	0.721 ± 0.502	-
p-value	0.855	0.557	-
Linear Regression Analysis			
Regression equation	FTC = 5.570 - 0.007 × Dose	FTA = 0.910 - 0.002 × Dose	-
R^2	0.028	0.043	-
p-value	0.279	0.149	-

FTC: Fetal Thyroid Circumference; FTA: Fetal Thyroid Area; R^2: coefficient of determination.

scores also showed no significant differences between groups, with FTC z-scores of -0.07 ± 1.14 vs. 0.07 ± 0.85 (p=0.471) and FTA z-scores of -0.08 ± 1.07 vs. 0.08 ± 0.93 (p=0.419) for the SCH and control groups, respectively.

Correlation studies were conducted to investigate the associations among maternal TSH levels, levothyroxine dosage, and fetal thyroid measurements (Table 3). In the overall study population (n=102), no significant correlations were found between maternal TSH and FTC (r=-0.013, p=0.896) or FTA (r=-0.027, p=0.788), with both showing negligible correlation strength. Similarly, within the SCH group (n=50), maternal TSH levels showed negligible correlations with FTC (r=0.062, p=0.668) and FTA (r=0.080, p=0.581). The analysis of levothyroxine treatment dose in the SCH group revealed weak negative correlations with both FTC (r=-0.156, p=0.279) and FTA (r=-0.207, p=0.149), but these associations did not reach statistical significance.

To evaluate the potential effects of levothyroxine dosage on fetal thyroid development, we conducted both categorical and continuous analyses (Table 4). We divided patients in the SCH group into low-dose (≤50 mcg/day, n=32) and high-dose (>50 mcg/day, n=18) subgroups. No statistically significant differences were observed in FTC (5.190 ± 1.609 cm vs. 5.104 ± 1.702 cm, p=0.855) or FTA (0.803 ± 0.455 cm^2 vs. 0.721 ± 0.502 cm^2, p=0.557) between the two dose groups.

Linear regression analysis was performed to further investigate the relationship between levothyroxine dose and fetal thyroid measurements as continuous variables. The regression equations (FTC = 5.570 - 0.007 × Dose; FTA = 0.910 - 0.002 × Dose) indicated a mild negative relationship between dose and thyroid measurements, suggesting a slight decrease in fetal thyroid size with increasing levothyroxine dose. However, these relationships were not statistically significant (p=0.279 for FTC and p=0.149 for FTA), and the coefficients of determination (R^2 = 0.028 for FTC and R^2 = 0.043 for FTA) indicated that the levothyroxine dose explained only 2.8-4.3% of the variation in fetal thyroid measurements. These findings suggest that the levothyroxine dose does not significantly influence fetal thyroid development in pregnant women treated for subclinical hypothyroidism.

DISCUSSION

This prospective observational study investigated the relationship between maternal SCH, levothyroxine treatment, and fetal thyroid dimensions in pregnant women. Our findings revealed no significant differences in fetal thyroid measurements between pregnant women with treated SCH and euthyroid controls, suggesting that appropriate levothyroxine treatment may normalize any potential effects of maternal SCH on fetal thyroid development.

Although raw measurements had shown a significantly smaller BPD in fetuses of mothers with SCH (Table 1), standardization using z-scores eliminated this difference (p=0.118), suggesting that the apparent discrepancy was likely

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attributable to minor variations in gestational age distribution between groups rather than a true biological effect of maternal thyroid status.

Our correlation analysis revealed negligible associations between maternal TSH levels and fetal thyroid dimensions (both FTC and FTA), suggesting that maternal thyroid function has no significant effect on fetal thyroid size when appropriately managed with levothyroxine. These findings support the idea that the fetal thyroid gland may develop independently when maternal thyroid dysfunction is effectively controlled.

Notably, our findings revealed a persistently higher AFI in the SCH group even after z-score standardization (0.34 ± 0.81 vs. -0.33 ± 1.00, p<0.001). This observation suggests that maternal thyroid dysfunction may influence fetal fluid homeostasis independently of its effects on structural growth. Several mechanisms could explain this association, including altered renal function due to subtle changes in thyroid hormone levels reaching the fetus, modified placental function affecting fluid exchange, and changes in fetal swallowing and urine production. This finding is particularly interesting because it highlights a potential subclinical manifestation of maternal SCH that persists despite adequate levothyroxine treatment. Previous studies by Mukherjee et al. [18] and Idris et al. [19] reported similar findings regarding amniotic fluid dynamics in pregnancies complicated by thyroid dysfunction, although they primarily focused on overt rather than subclinical hypothyroidism.

Although the correlations between levothyroxine dose and fetal thyroid dimensions did not reach statistical significance, a consistent negative trend was observed. This suggests that higher levothyroxine doses could exert a subtle protective effect against subclinical fetal thyroid enlargement (goiter). While preliminary and hypothesis-generating, this observation warrants further investigation in larger, prospective studies.

Importantly, early detection and treatment of SCH before conception or in the first trimester are critical to ensure optimal thyroid hormone support during the crucial phases of fetal development. However, since our study exclusively included treated SCH cases without an untreated comparator group, definitive conclusions regarding the protective effects of levothyroxine on fetal thyroid organogenesis cannot be reached. Further studies with untreated cohorts are needed to validate this hypothesis.

Early detection and treatment of maternal SCH before conception or during the first trimester are considered essential for supporting normal fetal development, particularly during the early phases of thyroid gland organogenesis. In our study, all patients with SCH had already initiated levothyroxine therapy before or during early pregnancy, potentially mitigating any adverse effects of maternal thyroid dysfunction on fetal thyroid morphology. The absence of significant differences in fetal thyroid dimensions between treated SCH cases and euthyroid controls may reflect the protective influence of timely intervention. However, given that our study did not include an untreated SCH group, we cannot definitively determine whether delayed or absent treatment would have resulted in impaired fetal thyroid development. Therefore, while our findings are encouraging, they should be interpreted with caution. Nevertheless, this approach aligns with prior studies [20–24], which showed that untreated maternal hypothyroidism—whether overt or subclinical—is consistently associated with increased risks of adverse perinatal and neurodevelopmental outcomes, including miscarriage, preterm delivery, fetal growth restriction, and impaired neurocognitive development in offspring.

Our study's findings align with those of Feng et al. [25], who prospectively assessed the impact of maternal hypothyroidism on fetal thyroid development and found no significant differences in fetal thyroid volumes between pregnant women with hypothyroidism and euthyroid controls. Similarly, our study demonstrated that adequately treated SCH did not significantly alter fetal thyroid dimensions. The consistency between these results reinforces the hypothesis that well-managed maternal thyroid dysfunction does not adversely affect fetal thyroid morphology, suggesting that current treatment protocols are effective in ensuring normal fetal thyroid development.

These findings have relevant clinical implications. They suggest that when maternal SCH is appropriately diagnosed and treated early in pregnancy, fetal thyroid development appears unaffected, reinforcing current guideline recommendations for levothyroxine therapy. The observed negative trend between levothyroxine dose and fetal thyroid size, although not statistically significant, raises the possibility that higher doses may contribute to more normalized fetal thyroid morphology. This hypothesis should be explored in future prospective studies with larger sample sizes. Moreover, the consistently elevated AFI observed in the SCH group despite treatment warrants further investigation into the subclinical effects of maternal thyroid dysfunction on fetal fluid regulation. Longitudinal studies incorporating thyroid antibody status, iodine levels, and functional fetal thyroid hormone measurements will provide a comprehensive understanding of maternal-fetal thyroid interactions and guide individualized treatment approaches.

In contrast to Feng et al, our study also explored the correlation between maternal levothyroxine dose and fetal thyroid dimensions, revealing a weak but nonsignificant negative correlation. This suggests that higher levothyroxine doses may have subtle effects on fetal thyroid growth, a hypothesis requiring further validation in larger studies. Although our findings support the safety of levothyroxine therapy in pregnancy, future research should investigate whether prolonged exposure to higher doses affects fetal thyroid function in a clinically meaningful way.

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Beyond maternal hypothyroidism, Luton et al. [26] highlighted the importance of fetal thyroid ultrasonography in pregnancies complicated by Graves' disease and demonstrated that fetal thyroid size, when assessed via ultrasound, can serve as an important marker of fetal thyroid dysfunction. Their results underscore the clinical value of fetal thyroid ultrasound as a diagnostic tool in high-risk pregnancies, reinforcing its potential role in monitoring maternal thyroid disorders during pregnancy.

Despite the clinical significance of this topic, a comprehensive review of the literature revealed a notable paucity of studies evaluating the effects of maternal thyroid dysfunction on fetal thyroid gland morphology using ultrasonographic assessment. Apart from the aforementioned investigations by Feng et al. [25] and Luton et al. [26], there is a substantial gap in research directly examining the relationship between maternal thyroid parameters and fetal thyroid dimensions through imaging modalities. This scarcity of evidence highlights the unique contribution of our study to the existing knowledge base and underscores the need for further investigation in this critical area of maternal-fetal thyroid physiology.

Several methodological strengths enhance the reliability of our findings. First, our prospective observational design with standardized measurement protocols minimized potential measurement bias. Second, the calculation of z-scores to standardize measurements across different gestational ages allowed for more accurate comparisons between groups. Third, the comprehensive assessment of maternal characteristics and detailed evaluation of fetal biometry provided a context for interpreting the thyroid measurements. Fourth, the high interobserver and intraobserver reproducibility (kappa coefficient of 0.85 and ICC of 0.89, respectively) demonstrate the reliability of our ultrasound measurement technique.

Limitations

Nevertheless, our research does include specific constraints that should be considered. First, although our sample size exceeded the minimum required based on power calculations, larger cohorts might detect subtle differences that our study may have missed. Second, we did not administer pretreatment thyroid function tests to all participants, limiting our ability to analyze the impact of pretreatment TSH severity on fetal outcomes. Third, longitudinal measurements throughout pregnancy could have provided comprehensive information about the developmental trajectory of the fetal thyroid gland.

An important limitation of our study was the lack of thyroid antibody (TPOAb/TgAb) data, which could have influenced both maternal thyroid function and fetal thyroid physiology independently. As the 2017 ATA guidelines stratify treatment recommendations based on antibody positivity, future studies should include antibody status to more precisely evaluate maternal-fetal thyroid dynamics.

CONCLUSION

In conclusion, our prospective observational study demonstrated that maternal SCH, when adequately treated with levothyroxine, does not significantly affect fetal thyroid dimensions. The negligible correlations observed between maternal TSH levels, levothyroxine dose, and fetal thyroid measurements suggest that fetal thyroid development progresses independently once maternal thyroid function is normalized. These findings underscore the importance of early diagnosis and appropriate treatment of SCH, ideally before conception or in the first trimester. Our study contributes valuable data to the limited body of evidence on the ultrasonographic assessment of fetal thyroid development in the context of maternal thyroid dysfunction and provides a foundation for future longitudinal studies exploring the long-term outcomes of these associations.

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Informed Consent: The purpose and nature of all procedures performed were properly explained to each pregnant woman and she was asked to sign a written informed consent form to participate in this study.

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Sonographic evaluation of diaphragm thickness in pediatric patients with steatotic liver disease

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MAIN POINTS

- There is a positive correlation between anthropometric z-scores and diaphragm thickness.
- Diaphragm thickness increases in children with hepatosteatosis.
- Diaphragm thickness increases in obese children.

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ABSTRACT

Aim: The most common liver disease in children is metabolic dysfunction-associated steatotic liver disease. We expect increased diaphragm thickness in pediatric patients with hepatosteatosis. We aimed to compare diaphragm thickness in children with hepatosteatosis with that in the control group and to discuss the results in light of the current literature.

Materials and Methods: The study included 56 patients with metabolic dysfunction-associated steatotic liver disease and 78 healthy controls. The diaphragm measurement of the patient and control groups was performed from the anterior caudal part of the diaphragm at the end of expiration from the right and left sides.

Results: Diaphragm thickness, BMI, weight, and height Z scores were significantly greater in the group with fatty liver. We found a significant positive correlation between the stages of fatty liver and anthropometric measurement Z score values with diaphragm thickness.

Conclusion: The current study found that diaphragm thickness was thicker in pediatric patients with hepatosteatosis than that in healthy individuals and was positively correlated with anthropometric measurements. However, further studies are needed to evaluate diaphragmatic muscle function.

Keywords: Hepatosteatosis, Ultrasonography, Diaphragm thickness, Children

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INTRODUCTION

The most common liver disease in children is metabolic dysfunction-associated steatotic liver disease (MASLD). It occurs in patients with visceral fat, dyslipidemia, and insulin resistance. The natural history of MASLD in children has not been fully defined [1-4]. It is suggested that versican, hsCRP and IL-6 levels are higher in children with obesity than in their healthy peers and that fatty liver disease is more common as a result [5]. Han and colleagues [6] studied the molecules versican released from adipose tissue and biglycan released from macrophages. In their study on mice, the authors showed that targeted deletion of adipose tissue-derived versican resulted in decreased chemotaxis and consequently decreased hepatic inflammation. They showed that deletion of macrophage-derived biglycan decreased macrophage accumulation and chemokine/cytokine release. Inflammatory processes occurring in adipose tissue cause the early development of insulin resistance, dyslipidemia, and hepatosteatosis in obesity [5].

Respiratory function and capacity are affected by obesity, but its pathophysiology cannot be clearly explained. The decrease in the thickness of the diaphragm, which is the main respiratory muscle that performs respiratory function, deteriorates respiratory function [7,8]. It has been shown that children with obese hepatosteatosis have higher intima-media thickness and are more prone to atherosclerosis compared with obese children without hepatosteatosis and healthy controls, and that the thickness of epicardial fat tissue increases further in obese patients with metabolic syndrome [9,10]. Given the increased frequency of hepatosteatosis in obese individuals [11], we expect increased diaphragm thickness in pediatric patients with hepatosteatosis. In order to realize this theory, which we found to be lacking in the literature, we aimed to examine the difference in diaphragm thickness in the pediatric

patient group with hepatosteatosis compared with the healthy control group and to discuss the results in light of the current literature.

MATERIALS AND METHODS

Study group: Patients with steatotic liver detected by ultrasound (US) and at least one risk factor for cardiometabolic dysfunction, such as overweight/obesity/visceral adiposity, dysglycemia, hypertension, or dyslipidemia, were diagnosed with steatotic liver disease associated with metabolic dysfunction [1]. These patients constituted the patient group of our study.

Control group: The study was conducted with healthy children of the same age group without any chronic diseases.

Patients with malnutrition, chronic liver disease, chronic lung disease, congenital heart failure, chest deformity, and myopathy were excluded from the study.

The study was approved by the University Hospital Non-Vascular Clinical Research Ethics Committee. (Approval date: 19.04.2021, Session No: 2021/15, Decision No: 05).

Our study complied with the principles of the Declaration of Helsinki. A consent form was obtained from the patients before the study began.

Selection of patient group

All patients included in this group had fatty liver disease associated with metabolic dysfunction. There was no accompanying chronic liver disease. Patients were selected consecutively from those who visited the pediatric gastroenterology clinic and were diagnosed with MASLD.

Metabolic dysfunction

MASLD was diagnosed in the presence of one of the following criteria [12-15].

- Overweight or Obesity [16],

  - Those with a body mass index between the 85th and 95th percentiles were considered overweight.

  - Those with a body mass index ≥95th percentile were considered obese.

- Type 2 diabetes mellitus

- ≥2 metabolic disorders (increase in waist circumference according to age and gender, high arterial blood pressure, high triglyceride, low high-density lipoprotein level, presence of prediabetic findings (fasting glucose 100-125 mg/dL, postprandial glucose 140-199 mg/dL or HbA1c 5.7-6.4%), high insulin resistance homeostasis model assessment [HOMA-IR] score and increased plasma high-sensitivity C-reactive protein [hs-CRP]) levels.

Radiological evaluation

The ultrasound assessment of the cases was randomly performed by a radiologist with 12 years of experience in ultrasound. An E9-LOGIQ XDclear 2.0 device (USA 2017) and a linear low-frequency sensor (Matrix clear/6-15 MHz xd) were used for ultrasound evaluation. The diaphragm was measured bilaterally at the end of expiration and from the anterior caudal part of the diaphragm. An age-appropriate convex abdominal probe was used. The distance between the peritoneal and parietal lines in the longitudinal plane was calculated in mm (Figure 1) [17,18].

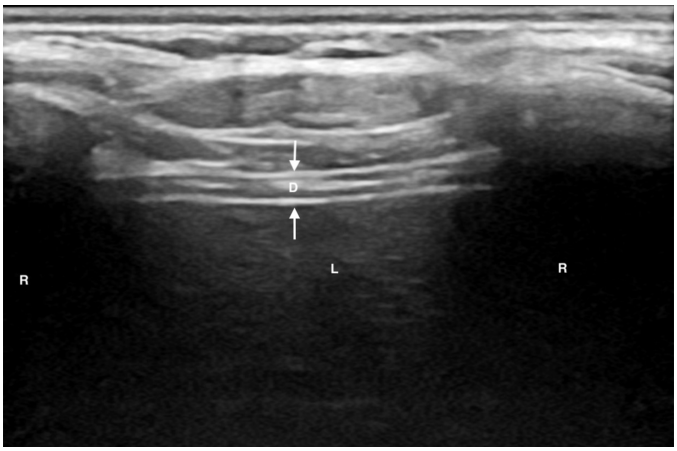

Figure 1. Diaphragm on longitudinal-plane ultrasonography. (D: diaphragm, L: liver, R: rib).

Power analysis

We could not find any study in the literature that compared the diaphragm thickness of pediatric patients with hepatosteatosis and metabolic syndrome–related liver disease with the healthy group. Therefore, assuming an effect size of 0.5, alpha: 0.12, power: 0.85, critical t-value: 1.53, 53 cases were detected in each group.

Statistical analysis

Statistical analyzes were performed using the Statistical Package for the Social Sciences (SPSS version 22.0 software (Chicago/USA). The normality of data distribution was tested using visual (histogram and probability charts) and analytical methods (Kolmogorov-Smirnov and Shapiro-Wilk tests). Descriptive analyzes were presented as percentile, mean, and standard deviation. Normally distributed numerical data were compared using the independent samples t-test, and non-normally distributed numerical data were compared using the Mann-Whitney U test. The chi-square test was used to compare the frequency rates of categorical variables.

A one-way ANOVA test was used to determine the arithmetic mean of the dependent variable between more than two independent groups. Posthoc analysis and the Scheffe test were used to determine the differences between groups with respect

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to this independent variable. Correlation analysis was performed to determine whether there was a linear relationship between two numerical variables and, if so, the direction and intensity of this relationship. If these numerical data showed a normal distribution, Pearson's correlation was preferred; otherwise, Spearman's rank correlation was preferred. The p-value accepted as statistically significant was <0.05.

A one-way ANOVA test was used to evaluate the arithmetic means of diaphragm thickness according to the degree of hepatosteatosis and the differences between the groups.

The independent Student's t-test was used to compare diaphragm thickness between groups. A Pearson correlation test was performed to determine the relationship between diaphragm thickness and anthropometric measurements.

RESULTS

Of the subjects included in the study, 78 were healthy controls and 56 were pediatric patients with MASLD. All patients with MASLD had hepatosteatosis. Seven patients (12.5%) were overweight and 29 patients (52%) were obese. 17 of these 36 patients (47.2%) had at least one accompanying metabolic dysfunction. Twenty patients (35.7%) were not overweight or obese and had two or more metabolic risk abnormalities. Of these, 12 patients had hypertriglyceridemia, 10 patients had hypertension, 8 patients had low HDL, 6 patients had insulin resistance, and 6 had prediabetes.

Of the cases included in the study, 56 had MASLD and 78 were healthy. The mean age of the cases was 10.17±4.79 (0.1-17) years. 72 (53.7%) of the patients were male. The groups were not statistically different by age and sex (p: 376, p: 122).

When bilateral diaphragmatic thickness was evaluated in relation to age and gender, there was no significant difference (p<0.05) (Table 1). Bilateral diaphragm thickness was significantly greater in the hepatosteatosis group. At the same time, height-weight measurement z scores were found to be significantly higher in the hepatosteatosis group (Table 1).

When the diaphragm thicknesses of the obese and non-obese hepatosteatosis patient groups were evaluated, the right and left diaphragms were found to be significantly thicker in the obese group (p: 0.043, p: 0.048, respectively)

When the diaphragm thickness and anthropometric measurement Z scores of the patients were compared with the healthy control group according to the stage of hepatosteatosis, the anthropometric measurement Z scores and diaphragm thickness were found to be significantly higher in the patient group with hepatosteatosis compared with the healthy cases in all stages (Table 2).

When the relationship between diaphragm thickness and anthropometric measurement scores of the patients was evaluated, moderate positive correlations were noted between diaphragm thickness and weight and body mass index z-scores. We found a weak positive correlation between height z-scores and diaphragm thickness. We found that bilateral diaphragm thickness increased with increasing degree of hepatosteatosis (moderately positive correlation) (Table 3).

DISCUSSION

The diaphragm is an important respiratory muscle that plays an active role during inspiration and expiration. Ultrasonography is a safe examination method that can evaluate the function and structure of the diaphragm when performed by a competent practitioner. It provides for interpretation depending on the experience of the operator [19]. Low cost, bedside applicability, ease of application, and the ability to obtain dynamic and high-resolution images are the main advantages of US [18,20,21]. Diagnosis and monitoring of diaphragm muscle problems in intensive care patients with muscle disease are performed using routine ultrasound scanning [22]. In the intensive care unit, the use of mechanical ventilation results in diaphragmatic deformation [23]. Diaphragm ultrasound has been used as a predictive tool to identify the likelihood of extubation failure while weaning from mechanical ventilation [24]. It may also be useful for assessing diaphragm function in patients with neuromuscular disease and for demonstrating diaphragm thinning in patients undergoing mechanical ventilation [19,25].

We could not find a study in the literature evaluating the diaphragm thickness of pediatric cases with hepatosteatosis. However, there are studies reporting on the relationship between the diaphragm muscle, which is both a skeletal and respiratory muscle, and nutritional status . It has been documented that the diaphragm thickness of malnourished children is lower than that of their healthy counterparts, exhibiting a positive correlation with z scores derived from weight-height measurements [8]. In addition, epicardial adipose tissue thickness has been shown to increase in obese children compared to healthy group [26]. Our study showed that weight-height z-scores and diaphragm thickness were higher in the group of patients with hepatosteatosis than that in the healthy cases. In addition, a significant positive correlation was found between diaphragm thickness and anthropometric z-scores, which is consistent with the literature.

It has been emphasized that the increase in weight and blood pressure in adults may cause changes in epicardial fat tissue in conjunction with anthropometric markers. In a study of Spanish children, a relationship was found between body mass index, anthropometric parameters, and epicardial fat tissue. These measurements were associated with increased epicardial fat tissue thickness, which does not indicate early pathology but carries a risk of developing cardiovascular disease [26,27]. The endocardial adipose tissue of overweight children has been found to show significant positive correlations with BMI and anthropometric measurements, similar to those determined in adults [28-30]. In this study, unlike the literature, diaphragm thickness was evaluated. In patients with hepatosteatosis, weight-height measurement z-scores were significantly higher than those of the healthy con-

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Table 1. Evaluation of differences in diaphragm thickness between groups.

	Female (68)	Male (66)	p
Right diaphragm thickness	1.60±0.40	1.55±0.42	0.484
Left diaphragm thickness	1.56±0.39	1.52±0.42	0.584
	Healthy control (78)	MASH (56)	p
Age	9.96±5.11	10.49±4.28	0.521
Weight Z score	-0.22±1.13	1.83±1.58	<0.001
Height Z score	-0.019±1.02	0.813±2.08	0.003
BMI Z score	-0.26±1.07	1.69±1.26	<0.001
Right diaphragm thickness	1.37±0.28	1.87±0.38	<0.001
Left diaphragm thickness	1.33±0.27	1.84±0.36	<0.001

Statistics: Independent Student's t-test. Abbreviations; MASH: Steatotic liver disease associated with metabolic dysfunction, BMI: Body mass index.

Table 2. Evaluation of diaphragm thickness according to hepatosteatosis degree.

	Healthy control (78)	Hepatosteatosis (56)			
		Grade 1 (29)	Grade 2 (21)	Grade 3 (6)	p
Right diaphragm thickness	1.367±0.284[a]	1.837±0.407[b]	1.876±0.360[b]	2.033±0.377[b]	<0.001
Left diaphragm thickness	1.334±0.272[a]	1.817±0.414[b]	1.846±0.331[b]	1.950±0.320[b]	<0.001
Weight Z-score	-0.220±1.133[a]	1.638±1.548[b]	2.095±1.689[b]	1.906±1.501[b]	<0.001
Height Z-score	-0.194±1.020[a]	0.620±1.226[b]	1.085±3.060[b]	0.796±1.121[b]	0.018
BMI Z score	-0.261±1.073[a]	1.520±1.372[b]	1.892±1.076[b]	1.802±1.432[b]	<0.001

Statistics: One-way ANOVA Post Hoc, Scheffe Tests, The difference between the mean values of a and barn is statistically significant (p<0.05).

Table 3. Evaluation of the correlation between weight and height Z scores and diaphragm thickness.

		Weight Z-score (134)	Height Z-score (134)	BMI Z-score (134)	Hepatosteatosis Grade (56)
Right diaphragm thickness	r	0.510	0.176	0.515	0.569
	p	<0.001	0.042	<0.001	<0.001
Left diaphragm thickness	r	0.539	0.220	0.554	0.572
	p	<0.001	0.011	<0.001	<0.001

Statistics: Pearson's correlation. Abbreviation; BMI: Body mass index.

trol group. In the hepatosteatosis group, 29 (52%) pediatric patients were obese. The results of the study showed a positive correlation between weight-height measurement z-scores and diaphragm thickness, which is consistent with the literature. We attributed the reason for the thickening of the diaphragm muscle in the MASLD group to the fact that the patients in this group were larger than the healthy group and that the diaphragm muscle was more hypertrophied due to the increased workload stemmed from this excess weight.

Diaphragm ultrasonography provides qualitative information about the shape, movement, and changes in muscle size. The nature of this information is dependent on the practitioner's training. It also cannot provide quantitative information about diaphragm muscle function.

Limitations

These are the limitations of our study. In addition, this study is the only one to report diaphragm thickness in children patients with hepatosteatosis, making the study valuable.

CONCLUSION

In conclusion, this study showed that diaphragm thickness was thicker in pediatric patients with hepatosteatosis than in healthy individuals and was positively correlated with anthropometric measurements. This suggests that the diaphragm muscle is a useful tool for nutritional assessment. However, comprehensive studies are needed to evaluate the function of the diaphragm muscle.

Ethics Committee Approval: Kahramanmaraş Sütçü İmam University Non-Interventional Clinical Research Ethics Committee (Approval date: 19.04.2021, Session No: 2021/15, Decision No: 05).

Informed Consent: Our study complied with the principles of the Declaration of Helsinki. A consent form was obtained from the patients before the study began.

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Coexistence of hypertension and antinuclear antibodies: High blood pressure as a potential risk factor for autoimmunity

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MAIN POINTS

- HT may contribute to vascular stress and low-grade inflammation, which could potentially facilitate the development of autoantibodies.
- Approximately 50% of hypertensive patients were found significantly positive for ANAs (anti-dsDNA, anti-ENA, and anti-Hep-2 nucleus).
- This finding confirms previous reports of an association between HT and autoantibodies, and further suggests that hypertensive patients should be monitored for potential autoimmune conditions.

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ABSTRACT

Aim: Hypertension (HT) is characterized by endothelial damage, vascular wall stress, and inflammation, potentially fostering autoantibody production. The prevalence of antinuclear antibodies (ANAs), common autoantibodies associated with systemic autoimmune diseases, remains unclear in non-autoimmune conditions like HT. This study aimed to investigate the presence of ANAs (anti-dsDNA, anti-ENA, anti-Hep-2 nucleus) in HT patients and compare these findings with healthy individuals.

Materials and Methods: This experimental case-control study included 32 hypertensive patients (7 men, 25 women; age 48.9 ± 6.6) and 32 age- and gender-matched healthy controls (7 men, 25 women; age 48.0 ± 5.2). HT status was self-reported based on prior diagnoses. ANAs, including anti-dsDNA, anti-ENA, and anti-Hep-2 nucleus antibodies, were measured using validated ELISA kits.

Results: Body mass index (BMI) and ages were comparable between groups (p>.05). Median ANA index values and positivity rates (%) for hypertensive and healthy groups were: anti-dsDNA [1.25 (59.4%) vs. 0.8 (28.1%)], anti-ENA [0.92 (46.9%) vs. 0.64 (21.9%)], and anti-Hep-2 nucleus [0.93 (43.8%) vs. 0.84 (18.8%)]. All three ANA tests showed significantly higher ANA levels and positivity rates in the hypertensive group compared to controls (p<0.05).

Conclusion: Our findings indicate higher ANA levels and positivity rates in individuals with HT compared to healthy controls, suggesting a potential link between HT and autoantibody production. Further long-term prospective studies are needed to determine the clinical significance of this elevated ANA frequency and the potential role of these antibodies in the development of autoimmune diseases.

Keywords: Hypertension, Antinuclear antibody, dsDNA, ENA

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INTRODUCTION

Hypertension (HT), characterized by persistently high blood pressure, is a major global health issue significantly contributing to the burden of cardiovascular diseases, stroke, and kidney failure. Approximately 3.5 billion adults worldwide are at risk of HT, making it a leading risk factor for global disease burden and mortality [1]. This condition accounts for 9.4 million deaths and 212 million disability-adjusted life years (DALYs) lost annually, representing 8.5% of the global disease burden [2]. The pathogenesis of HT is complex and multifactorial, involving genetic predisposition, age, obesity, sedentary lifestyle, and high-sodium diets [3-5].

Autoantibodies, produced by the immune system to target the body's own tissues, play a key role in autoimmune disorders. Around 5-7% of the global population is affected by autoimmune diseases linked to these autoantibodies, accounting for 0.5-2% of all deaths [6]. Among them, antinuclear antibodies (ANAs) bind to intracellular components such as the nucleus, DNA, RNA, and centromeres, contributing to cellular dysfunction, inflammation, and tissue damage [7,8]. ANAs are broadly classified into two main subgroups: anti-dsDNA, which targets genetic material, and anti-ENA (extractable nuclear antigens), which targets various intracellular components.

While commonly associated with autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome,

and rheumatoid arthritis, ANAs have also been detected in individuals without a diagnosed autoimmune condition [9]. Notably, ANA positivity has been reported in various chronic disorders, including type 2 diabetes, chronic kidney disease, and cardiovascular diseases [10-12]. These conditions often share common features such as persistent low-grade inflammation and tissue injury, which may trigger non-specific immune activation and lead to the production of autoantibodies [13].

Similarly, HT is characterized by chronic vascular wall stress, endothelial damage, and sterile inflammation—conditions that may create a microenvironment conducive to immune activation [14,15]. These processes could expose normally sequestered nuclear antigens to the immune system, potentially triggering ANA production in susceptible individuals. In this context, we hypothesized that ANA levels might be elevated in individuals with HT compared to normotensive individuals. The aim of our study was to determine the prevalence of ANA positivity in patients with HT and to compare it with healthy controls, in order to explore potential immunological features associated with HT.

MATERIALS AND METHODS

This study for ethical approval was obtained from the Bingöl University Health Sciences Scientific Research and Publication Ethics Committee (2025-25/1).

Sample size determination

The sample size was determined using the G*Power software program [16]. A power analysis for two independent groups (independent t-test) indicated that with a Type I error (α) of 0.05, a Type II error (β) of 0.20 (Power = 0.80), a large effect size (f = 0.75), and equal group sizes, a minimum of 29 participants per group would be required [17,18].

Study design and participants

This study was designed as a case-control study, involving 32 hypertensive individuals (7 men, 25 women; mean age 48.9 ± 6.6 years) and 32 age- and gender-matched healthy controls (7 men, 25 women; mean age 48.0 ± 5.2 years). The presence of HT in patients was determined based on previous diagnoses and self-reports. Control group participants were selected based on self-reported healthy status, including individuals who stated no known chronic diseases or previous medical diagnoses.

Among the 32 individuals in the HT group, 16 had additional chronic conditions such as DM, cardiovascular diseases (e.g., venous insufficiency, arrhythmia), asthma, thyroid disorders, chronic lung disease, hepatitis B, endometrial cancer, or polycystic ovary syndrome (PCOS). To allow for clearer interpretation of the autoantibody results, individuals with HT but without any additional chronic diseases were also evaluated separately.

Determination of ANA positivity

ANA levels were measured using three different validated ELISA test kits: anti-dsDNA, anti-ENA, and anti-Hep-2 nucleus [19,20].

- The anti-dsDNA assay utilized purified double-stranded calf thymus DNA as the antigen.
- The anti-ENA kit included multiple specific antigens (Sm, nRNP, La/SS-B, and Jo-1).
- The anti-Hep-2 nucleus kit was based on whole-cell nuclear extracts derived from the HEp-2 cell line (ATCC, CCL-23).

The sensitivities for the anti-dsDNA, anti-ENA, and anti-Hep-2 nucleus tests were 93.8%, 83.3%, and 90%, respectively, while their specificities were 91.7%, 83.3%, and 87.5%, respectively [19]. The intra- and inter-assay coefficients of variation for these tests were 7.8%, 7.5%, and 9.9%, respectively [19].

All procedures followed the kit protocols. Serum samples were diluted 1/100 and added to the wells alongside negative and positive control samples. Subsequently, anti-human IgG conjugated with biotin and streptavidin peroxidase was added. Plates were washed three times with 0.05% Tween-20 before each solution addition. Finally, a chromogenic substrate (tetramethylbenzidine, TMB) was added, and the reaction was halted with 11% H_2SO_4. Plates were read using a spectrophotometer at 450 nm.

Calculation of ANA results

The cut-off value for positivity was determined using the cut-off control, as described in the kit protocol. It was calculated using the formula: average OD of negative controls + 3 standard deviations (SD). Sample OD values were converted to an antibody index (Ab index) using the formula: Ab index = Sample OD / Cut-off OD. Values <1.0 were classified as ANA IgG negative, while values $\geq$1.0 were classified as ANA IgG positive. The test was deemed valid if the Ab index of the positive control was >1.1 and the negative control was <0.9.

Statistical analysis

The normality of the data was assessed using the Shapiro–Wilk test. Age and BMI, which were normally distributed, were compared between groups using the independent samples t-test. ANA levels did not follow a normal distribution; thus, they were compared between the hypertensive and control groups using the Mann-Whitney U test. The Mann-Whitney U test was also employed for the comparison of ANA positivity in hypertensive patients without comorbid chronic diseases. For categorical variables, comparisons were made using the chi-square (χ^2) test. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

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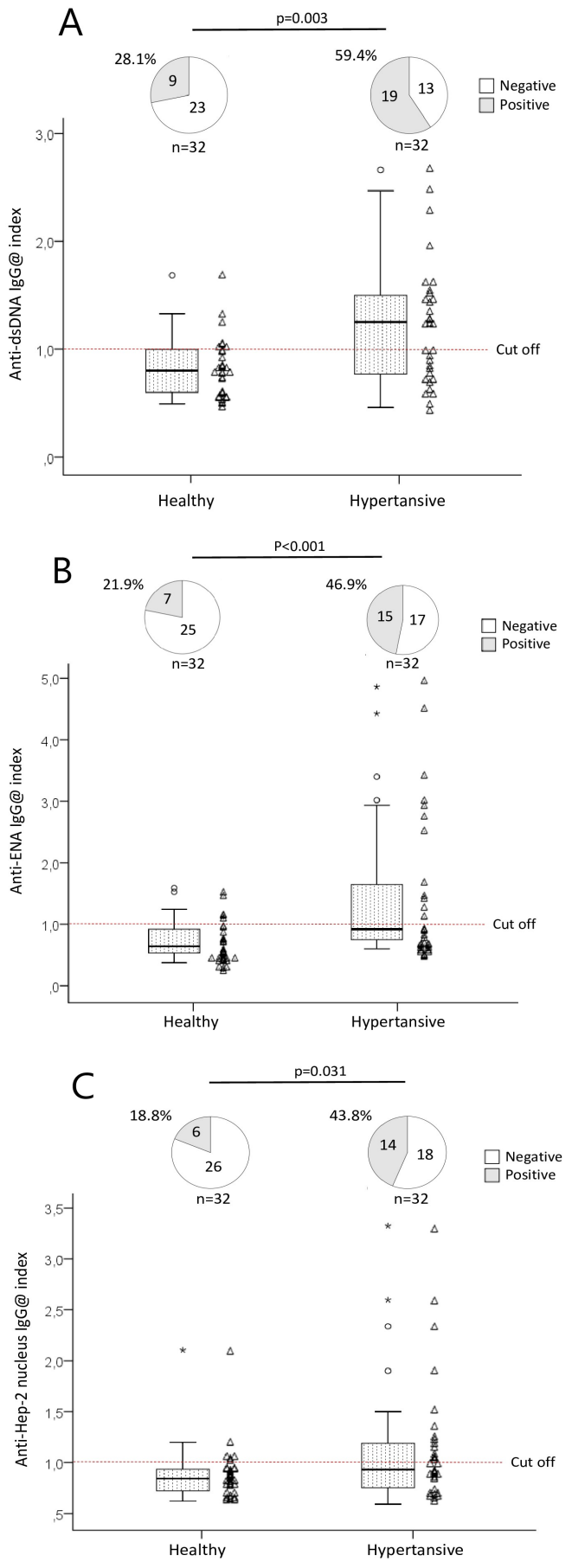

Figure 1. The anti-dsDNA (A), anti-ENA (B), and anti-Hep-2 nucleus (C) antibody levels and positivity percentages in hypertensive and healthy individuals. In all three assays, ANA levels in hypertansive individuals were found to be significantly higher compared to healthy individuals.

Table 1. Age of the BMI of the participants.

	HT (n=32)	Healthy (n=32)	p value
Age	48.0 ± 5.2	48.9 ± 6.6	0.419
BMI	29.1 ± 4.2	27.5 ± 4.2	0.206

RESULTS

The age and body mass index (BMI) distributions were comparable between the healthy and hypertensive groups (p>0.05) (Table 1). Among the 32 individuals with HT, 16 had isolated HT, while the remaining 16 had additional chronic conditions. These included 8 with DM, 6 with cardiovascular problems (e.g., venous insufficiency, arrhythmia), 2 with asthma, 2 with hypothyroidism, 2 with hyperthyroidism, 1 with hepatitis B, 1 with chronic lung disease, 1 with endometrial cancer, and 1 with PCOS.

Comparison of ANA levels between hypertensive and healthy individuals

In the anti-dsDNA test, 19 samples (59.4%) from the hypertensive group and 9 samples (28.1%) from the healthy group were positive (p = .003) (Figure 1A). For the anti-ENA test, 15 samples (46.9%) from the hypertensive group and 7 samples (21.9%) from the healthy group were positive (p<0.001) (Figure 1B). In the anti-Hep-2 nucleus test, 14 samples (43.8%) from the hypertensive group and 6 samples (18.8%) from the healthy group were positive (p = 0.031) (Figure 1C).

ANA positivity in hypertensive patients without comorbid chronic diseases

To mitigate potential confounding effects from other chronic diseases known to trigger ANA formation, we re-evaluated ANA positivity in the subset of 16 individuals with isolated HT. Among these patients, 10 (62.5%) tested positive for anti-dsDNA, 11 (68.8%) for anti-ENA, and 10 (62.5%) for anti-Hep-2 nuclear antibodies (Fig. 2). The frequency of these positive results was significantly higher compared to healthy individuals (p<0.05).

DISCUSSION

Our study, which investigated the relationship between HT and ANAs, revealed that nearly half of the hypertensive patients tested positive for ANAs. While ANAs are also found in healthy individuals [9], their elevated prevalence in our hypertensive cohort suggests a close relationship between HT and ANA positivity. This relationship is likely bidirectional, meaning HT could contribute to ANA development, and ANAs might potentially trigger HT [14, 21]. Although not yet fully understood, the possible link between HT and autoantibody production has been associated with mechanisms such as chronic inflammation, endothelial stress, oxidative injury, and genetic predisposition [22–25].

HT can lead to vascular wall damage and endothelial cell dysfunction, potentially initiating inflammatory responses and

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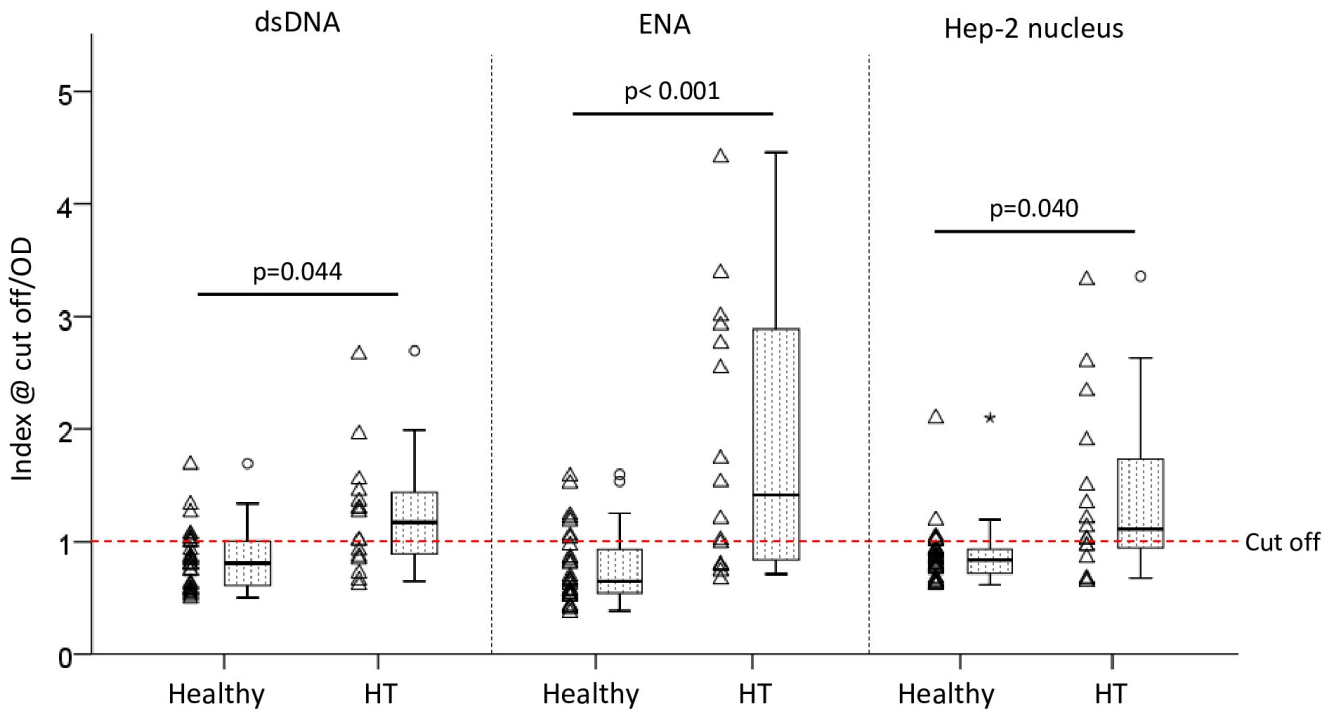

Figure 2. Comparison of ANA levels between hypertensive individuals without additional chronic conditions (n=16) and healthy controls (n=32). Among the hypertensive group, ANA positivity was observed in 62.5% for anti-dsDNA, 68.8% for anti-ENA, and 62.5% for Hep-2 nuclear antibodies. These rates were significantly higher compared to the healthy control group (p<0.05).

fostering chronic inflammation. Sung et al. observed elevated levels of C-reactive protein (CRP), a key inflammatory marker, in individuals with HT [23]. Similarly, Bautista et al. demonstrated that patients with higher levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were more likely to have HT [26]. During this inflammatory process, the immune system may begin to target the body's own structures as it continuously clears cellular debris from damaged cells and tissues, potentially leading to the production of autoantibodies like ANAs. Osmori et al. [27] suggested that chemically modified self-proteins, often found in inflamed tissues, are potential candidates for autoantibody production. Their study also noted that epitope spreading—the diversification of immune responses from the initial epitope to other epitopes on the same or different antigens—which can lead to autoantibody production, is frequently observed in the inflamed tissues of patients with rheumatoid arthritis [27]. This interplay between HT, inflammation, and autoantibodies may help explain the high ANA prevalence we observed in our hypertensive patients.

In our study, the high ANA production in hypertensive patients may also be linked to oxidative stress, a key mechanism in HT development [24]. Reactive oxygen species (ROS) are crucial for vascular wall homeostasis, but their increased production can contribute to HT pathophysiology [28-30]. This often coincides with reduced bioavailability of nitric oxide (NO) and antioxidants, a phenomenon observed in both experimental models and human HT. Free radicals can disrupt normal cellular functions, causing damage to DNA, proteins, and lipids. This oxidative damage and subsequent protein modifications may be linked to autoantibody pathogenesis. Kuruen et al. [31] demonstrated that oxidative modifications of proteins can trigger antibody production in various diseases, including SLE, alcoholic liver disease, DM, and rheumatoid arthritis (RA). Additionally, Ramani et al. [32] stated that immune responses against tissues and organs increase with oxidative stress, further exacerbating the pathobiology of autoimmune diseases. In this context, the interaction between oxidative stress, immune responses, and autoantibody production suggests that the high prevalence of ANAs in hypertensive patients could be related to oxidative stress.

Genetic factors have long been recognized as contributors to HT pathogenesis. Research indicates that several genes involved in vascular tone regulation, sodium balance, and the renin-angiotensin system are associated with an increased risk of HT development [33]. Furthermore, polymorphisms in genes encoding proteins involved in oxidative stress pathways, such as NADPH oxidase and superoxide dismutase, may predispose individuals to HT by exacerbating oxidative damage within the vasculature [34]. This heightened oxidative stress can lead to endothelial dysfunction, a hallmark of HT, which may in turn trigger inflammatory responses and autoantibody

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production.

Familial aggregation studies further support a shared genetic vulnerability, as HT and autoimmune diseases often co-occur in families [35]. This suggests common genetic factors might contribute to both conditions, either through a direct predisposition to immune dysregulation or via the inflammatory effects of HT on the immune system. Similarly, genetic susceptibility to autoimmune diseases, particularly through polymorphisms in immune-regulatory genes, has been linked to autoantibody production [36]. Emerging research highlights a genetic overlap between HT and autoimmune diseases, with certain genetic variants associated with both conditions [37]. This overlap may help explain the higher prevalence of autoantibodies, such as ANAs, observed in hypertensive patients.

Limitations

Our study has several limitations. Participants' HT status was based on self-reports of prior diagnoses rather than direct clinical measurements. While we specifically asked about medically diagnosed conditions to minimize recall bias, this method inherently carries some degree of subjectivity.

Furthermore, among the 32 individuals in the HT group, 16 had additional chronic conditions such as DM, cardiovascular diseases (e.g., venous insufficiency, arrhythmia), asthma, thyroid disorders, chronic lung disease, hepatitis B, endometrial cancer, or PCOS. These comorbidities could potentially influence immune-related parameters, including ANA levels, and were therefore considered a limitation. To mitigate this, subgroup analyses were performed, and ANA measurements were evaluated separately in hypertensive individuals with and without these additional chronic conditions.

Finally, due to limited information in patient files, our study could not include data on the duration of HT diagnosis, the healthcare provider responsible for the initial diagnosis, or whether the condition was being managed with medication.

CONCLUSION

The high prevalence of ANAs observed in hypertensive patients in our study may indicate an increased risk of autoimmunity. However, long-term prospective studies are essential to confirm this link and to determine the clinical significance of this finding. Chronic inflammation, oxidative stress, endothelial dysfunction, and genetic predisposition are among the potential mechanisms contributing to HT-associated ANA production. Further molecular and longitudinal studies are critically needed to clarify the underlying immunological pathways linking HT with autoantibody formation.

Ethics Committee Approval: Ethical approval was obtained from the Bingöl University Health Sciences Scientific Research and Publication Ethics Committee (2025-25/1).

Informed Consent: As this study was designed retrospectively, obtaining patient informed consent was not required.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there are no conflicts of interest to disclose.

Author Contributions: FD: Conceptualization, Methodology, Validation, Formal analysis, Writing - Original Draft; SY: Formal analysis, Supervision, Project administration, Writing - Review & Editing

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Factors affecting mortality in cases of perinatal asphyxia treated with hypothermia; A 10-year experience: A retrospective cohort study

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MAIN POINTS

- Lower rectal temperature at admission was identified as the only independent predictor of mortality in multivariate analysis.
- Acute renal failure and the need for multiple inotropic agents were significantly associated with higher mortality rates.
- Lower 5-minute Apgar scores and severe abnormalities on aEEG were strongly linked to poor outcomes.
- Cesarean delivery and lower gestational age were more common among non-survivors, indicating higher risk.
- Initial hypothermia severity had a critical impact on survival, emphasizing the importance of early and precise thermal management.

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ABSTRACT

Aim: Birth asphyxia and intrapartum brain injury can cause death or irreversible brain damage. The only known treatment that improves prognosis is therapeutic hypothermia. Our goal was to assess the parameters associated with death in newborns receiving therapeutic hypothermia.

Materials and Methods: This retrospective cohort study was conducted in a neonatal intensive care unit (NICU) and included neonates who underwent therapeutic hypothermia due to perinatal asphyxia over 10 years. Patients were categorized into survivors and nonsurvivors based on their in-hospital outcomes. Demographic characteristics, clinical parameters, laboratory findings, and complications were retrieved from the patients' medical records. Prognostic factors were compared between groups using appropriate statistical methods, and multivariate logistic regression was performed to identify independent predictors of mortality.

Results: A total of 114 newborns treated with therapeutic hypothermia were analyzed, of whom 11 (9.6%) died due to perinatal asphyxia (PA). Non-survivors had significantly lower gestational ages (37±2 vs. 38.4±1.6 weeks, p=0.042), lower 5-minute Apgar scores (5 [1--5] vs. 5.5 [4--7], p=0.048), and higher cesarean delivery rates (81.8% vs. 41.7%, p=0.011). Severe findings on aEEG and Sarnat Stage III were more common among nonsurvivors (p<0.001 for both). Non-survivors also exhibited significantly lower admission rectal temperatures [33.8°C (32--36) vs. 35.8°C (34.5--36), p=0.023], higher rates of thrombocytopenia (81.8% vs. 42.7%, p=0.013), acute renal failure (90.9% vs. 39.8%, p=0.001), and cranial ultrasound abnormalities (27.3% vs. 3.9%, p=0.019). In the multivariate logistic regression analysis, only a lower rectal temperature at admission remained an independent predictor of mortality (OR: 1.520, 95% CI: 1.061--2.178, p = 0.023).

Conclusion: Factors such as lower Apgar scores, acute renal failure, and hypothermia severity on admission are strongly associated with mortality. The early identification and management of these risk factors are critical for improving outcomes in neonates treated with therapeutic hypothermia.

Keywords: Hypoxic-ischemic encephalopathy, Morbidity, Mortality, Perinatal asphyxia, Therapeutic hypothermia

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INTRODUCTION

High rates of morbidity and mortality are associated with perinatal asphyxia (PA), a dangerous disease exacerbated by oxygen deprivation and organ malfunction [1]. Compared to high-income countries, low- and middle-income countries (LMICs) have a significantly higher incidence of the disease. Hypoxic-ischemic encephalopathy (HIE), which is caused by PA, affects up to 20 per 1000 live births in LMICs and leads to approximately one million deaths annually [2]. Long-term neurologic disabilities include cerebral palsy, different degrees of vision or hearing impairment, cognitive and developmental delay or learning challenges, behavioral, coordination, and gross motor issues, and epilepsy [2]. At least one of the following requirements must be met to define neonatal asphyxia, even though there is no conclusive test available to make the diagnosis: Ten minutes Apgar score ≤5, metabolic acidosis

(pH ≤7.0 or BE ≤-12 mMol/L in the umbilical artery or within 1 hour of life), and requirement for resuscitation lasting longer than 10 minutes [3].

Little is known about neuroprotective techniques. Therapeutic hypothermia (TH), which is the established neuroprotective strategy for term and near-term newborns with moderate or severe hypoxic-ischemic encephalopathy (HIE), is initiated within six hours of birth [2, 4-6]. Treatment reduces moderate-to-severe disabilities from 62% to 41% by the age of 18–22 months in these patients [7]. Mechanisms for this neuroprotection are multifactorial, including slowing down cerebral oxygen demand and inflammatory cascade after reperfusion and limiting apoptosis, endothelial dysfunction, and free radical release [5,6]. TH mainly aims to slow down brain metabolism to minimize damage [8]. Whole-body metabolic demands can be reduced by up to 10% when core body temperature is lowered by 1℃ [9]. Common hypothermia techniques include selective head and whole-body cooling [2]. However, the effect of this treatment on outcomes can vary depending on different factors [10]. Therefore, recent researchers are interested in identifying the factors that influence mortality in patients with perinatal asphyxia undergoing TH [11]. We aimed to analyze the factors associated with mortality in neonates who were treated with therapeutic hypothermia over 10 years in our center.

MATERIALS AND METHODS

This retrospective cohort study was conducted at the Neonatal Intensive Care Unit (NICU) of Kocaeli University Hospital and included neonates treated with therapeutic hypothermia (TH) for perinatal asphyxia (PA) between March 2013 and December 2023. The study protocol received approval from the Non-interventional clinical research ethics committee of Kocaeli University (Date: 27.05.2024, No: E.594842) and was carried out according to the Declaration of Helsinki.

Inclusion and Exclusion criteria

Eligible neonates were born at ≥36 weeks of gestation, admitted within 12 hours of birth, and diagnosed with moderate to severe hypoxic-ischemic encephalopathy (HIE) based on clinical evaluation with the Modified Sarnat and Sarnat Scale and/or abnormal amplitude-integrated EEG (aEEG) findings. The key diagnostic criteria for perinatal asphyxia included low Apgar scores (≤5 at 10 minutes), metabolic acidosis (arterial pH ≤7.00 or base excess ≤−12 mmol/L within the first hour of life), and the need for resuscitation lasting more than 10 minutes. The exclusion criteria were as follows: major congenital anomalies, chromosomal disorders, severe intrauterine growth restriction (<3rd percentile), and conditions incompatible with TH (e.g., severe cranial hemorrhage, life-threatening coagulopathy, known metabolic disorders).

Therapeutic hypothermia protocol

Therapeutic hypothermia was initiated within six hours of birth using either whole-body or selective head cooling, depending on patient characteristics and equipment availability. The target core temperature was maintained between 33.0°C and 34.0°C for 72 hours, followed by gradual rewarming at 0.5°C/hour. This protocol was primarily adapted from the Turkish Neonatal Society Guideline on Neonatal Encephalopathy, which aligns with international standards. Therapeutic hypothermia is also applicable in certain chromosomal anomalies, such as trisomy 21, according to the national guidelines, whereas it is not recommended in cases with severe intrauterine growth restriction (birth weight <3rd percentile) [12].

Data collection

Data were extracted from hospital records, including demographic variables (birth weight, gestational age, sex, delivery mode), clinical data (Apgar scores, Sarnat stage, aEEG findings), and laboratory values (pH, base excess, lactate, troponin, platelet counts). The body temperature at admission and time to TH initiation were also recorded.

Clinical complications during hospitalization included thrombocytopenia (platelet count <150,000/mm^3), acute renal failure (defined as serum creatinine >1.5 mg/dL or urine output <1 mL/kg/hour for 24 hours), and necrotizing enterocolitis (NEC). NEC was diagnosed and staged based on the modified Bell's criteria, incorporating clinical findings (abdominal distension, bloody stools), radiological evidence (pneumatosis intestinalis, portal venous gas, pneumoperitoneum), and laboratory abnormalities (e.g., metabolic acidosis, thrombocytopenia). Additional outcomes, such as convulsions, hypotension, need for inotropic support, and cranial ultrasound findings, were also documented.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 29.0 (IBM Corp., Armonk, NY, USA). Continuous variables were assessed for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Since most continuous data were not normally distributed, they were summarized using medians and interquartile ranges (IQR), and group comparisons were performed using the nonparametric Mann–Whitney U test. Categorical variables are summarized as frequencies and percentages. Pearson's Chi-square test was used for comparisons of categorical variables when the expected cell count was ≥5, and Fisher's Exact Test was applied when the expected count was <5. Relative risk (RR) with 95% confidence intervals (CI) was calculated for key clinical outcome measures.

To identify the independent predictors of mortality, univariate logistic regression analyses were conducted. Variables with a p-value <0.10 in the univariate analyses were included in a multivariate binary logistic regression model using the standard "Enter" method. No stepwise variable selection or machine learning–based model optimization was performed.

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Table 1. Characteristics of the patients.

Characteristic		Survivor (n=103)	Non-survivor (n=11)	p-value
Birth weight (g)		3248 ± 563	2997 ± 532	0.165[a]
Gestational week		38.4 ± 1.6	37 ± 2	0.042[a]
Sex				
Male		64 (62.1)	8 (72.7)	0.489[b]
Female		39 (37.9)	3 (27.3)	
Mode of Delivery				
Cesarean Delivery		43 (41.7)	9 (81.8)	0.011[b]
Vaginal		60 (58.3)	2 (18.2)	
Apgar (5th min)	Median (IQR)	5.5 (4--7)	5 (1--5)	0.048[c]

a: Independent Sapmples T-Test, b: Pearson Chi-square test, c: Mann-Whitney U test.

Table 2. Therapeutic hypothermia parameters and neurological assessment findings.

Characteristic		Survivor (n=103)	Non-survivor (n=11)	p-value
pH	Median (IQR)	6.99 (6.87--7.1)	6.93 (6.76--7)	0.236[c]
BE (mmol/L)	Median (IQR)	-17 (19.6--13.7)	-16.5 (22.8--13.7)	0.796[c]
Lactate (mg/dl)	Median (IQR)	12 (8--17)	13.5 (7--19.2)	0.585[c]
aEEG, n (%)				<0.001[b]
Normal		16 (15.5)	0 (0)	
Moderate findings		82 (79.6)	4 (36.4)	
Severe findings		5 (4.9)	7 (63.6)	
Cooling method				0.922[b]
Selective Head		39 (37.9)	4 (36.4)	
Whole body		64 (62.1)	7 (63.6)	
Sarnat Stage				<0.001[b]
Stage 1		50 (48.5)	1 (9.1)	
Stage 2		49 (47.6)	1 (9.1)	
Stage 3		4 (3.9)	9 (81.8)	
Hypothermia onset time	Median (IQR)	5 (3--6)	6 (4--8)	0.106[c]
Rectal temperature at admission	Median (IQR)	35.8 (34.5--36)	33.8 (32--36)	0.023[c]

c: Mann-Whitney U test, b: Pearson Chi-square test.

Because binary logistic regression is a classical statistical approach, no additional model optimization was performed.

Model performance was evaluated by examining the Hosmer-Lemeshow goodness-of-fit test, the Nagelkerke R^2 statistic, and the overall classification accuracy. The odds ratios (OR) and 95% confidence intervals (CI) were reported. A two-sided p-value <0.05 was considered statistically significant.

RESULTS

A total of 114 newborns treated with therapeutic hypothermia were included in the study. Among them, 103 (90.4%) survived and 11 (9.6%) died during hospitalization.

Table 1 summarizes the baseline characteristics of the survivor and non-survivor groups. There were no significant differences between the groups regarding birth weight (3248±563 g vs. 2997±532 g, p=0.165) and sex distribution (male: 62.1% vs. 72.7%, p=0.489). However, nonsurvivors had a significantly lower gestational week at birth than survivors (38.4±1.6 vs. 37±2 weeks, p=0.042). cesarean delivery was more common among nonsurvivors than among survivors (81.8% vs. 41.7%, p=0.011). The 5-minute Apgar score was significantly lower in non-survivors than in survivors [median (IQR): 5 (1–5) vs. 5.5 (4–7); p=0.048].

Table 2 presents the therapeutic hypothermia parameters and neurological findings. No significant differences were observed between survivors and nonsurvivors regarding initial pH (p=0.236), base excess (p=0.796), or lactate levels (p=0.585). However, severe findings on aEEG were significantly more frequent among nonsurvivors (63.6% vs. 4.9%, p<0.001). Most survivors had moderate aEEG findings (79.6%). The cooling method (selective head vs whole body) did not significantly differ between groups (p=0.922). Severe Sarnat Stage (Stage 3) was observed in 81.8% of non-survivors versus 3.9% of survivors (p<0.001). Non-survivors were more hypothermic on admission [median (IQR): 33.8°C (32–36) vs. 35.8°C (34.5–36); p=0.023].

Table 3 summarizes the clinical complications and organ dysfunctions during hospitalization. Thrombocytopenia (81.8%

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Table 3. Clinical complications, organ dysfunctions, and supportive interventions during hospitalisation.

Complication		Survivor (n=103)	Non-survivor (n=11)	p-value
Thrombocytopenia				0.013[b]
Yes		44 (42.7)	9 (81.8)	
No		59 (57.3)	2 (18.2)	
Bleeding disorder				0.995[b]
Yes		28 (27.2)	3 (27.3)	
No		75 (72.8)	8 (72.7)	
Acute Renal Failure				0.001[b]
Yes		41 (39.8)	10 (90.9)	
No		62 (60.2)	1 (9.1)	
Elevated Troponin I				0.264[b]
Yes		30 (36%)	5 (83%)	
No		73 (70.9)	6 (54.5)	
Hypotension				0.001[b]
Single inotropic agent		41 (40%)	1 (9%)	
Multiple inotropic agents		36 (35)	10 (90.9)	
No		26 (25.2)	0 (0)	
Pulmonary Hypertension				0.969
Yes		9 (8.7)	1 (9.1)	
No		94 (91.3)	10 (90.9)	
Respiratory Support				0.790[b]
Hood		3 (2.9)	0 (0)	
Non-invasive ventilation		15 (14.6)	1 (9.1)	
Invasive ventilation		84 (81.6)	10 (90.9)	
Liver involvement				0.192[b]
Yes		89 (86.4)	11 (100)	
No		14 (13.6)	0 (0)	
Necrotizing enterocolitis				0.103[d]
Yes		4 (3.9)	2 (18.2)	
No		99 (96.1)	9 (81.8)	
Convulsion				0.171[d]
Yes		31 (30.1)	6 (54.5)	
No		72 (69.9)	5 (45.5)	
Cranial US abnormalities				0.01[9d]
Yes		4 (3.9)	3 (27.3)	
No		99 (96.1)	8 (72.7)	
Duration of hospitalisation	Median (IQR)	13 (2–38)	7 (5–14)	0.667[c]

b:Pearson Chi-Square Test, d: Fisher's Exact Test, c: Mann-Whitney U Test.

Table 4. Therapeutic hypothermia parameters and neurological assessment findings.

Variable	Univariate p-value	Univariate OR (95% CI)	Multivariate p-value	Multivariate OR (95% CI)
Gestational week	0.009	1.581 (1.113–2.247)	0.324	1.266 (0.793–2.022)
Mode of Delivery (Cesarean)	0.011	0.159 (0.036–0.699)	0.126	0.211 (0.029–1.545)
Apgar score (5^{th} min)	0.014	1.417 (1.061–1.892)	0.746	0.937 (0.632–1.389)
Sarnat Stage (Stage 3)	0.012	0.106 (0.021–0.524)	0.150	0.150 (0.011–1.987)
Admission Rectal Temp (°C)	<0.001	1.589 (1.167–2.163)	0.023	1.520 (1.061–2.178)
Thrombocytopenia	0.013	0.166 (0.038–0.726)	0.459	0.437 (0.049–3.914)
Acute Renal Failure (ARF)	0.001	0.066 (0.012–0.361)	0.348	0.298 (0.024–3.733)
Cranial US Abnormalities (TFUS)	0.002 (TFUS(1))	0.109 (0.019–0.621)	0.706	0.430 (0.059–3.123)

vs. 42.7%, p=0.013) and acute renal failure (90.9% vs. 39.8%, p=0.001) were significantly more frequent in non-survivors than in survivors. Although elevated troponin levels were observed more frequently in nonsurvivors (83% vs. 36%), this difference did not reach statistical significance (p=0.264). The requirement for multiple inotropic agents was significantly higher in non-survivors than in survivors (90.9% vs. 35%, p=0.001). Cranial ultrasound abnormalities were signif-

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icantly more common among nonsurvivors than among survivors (27.3% vs. 3.9%, p=0.019). There were no significant differences between groups regarding bleeding disorders, pulmonary hypertension, respiratory support methods, liver involvement, necrotizing enterocolitis, or convulsions.

The results of the logistic regression analysis are shown in Table 4. In the univariate analysis, gestational week, mode of delivery, 5-minute Apgar score, Sarnat Stage, admission rectal temperature, thrombocytopenia, acute renal failure, and cranial ultrasound abnormalities were significantly associated with mortality. However, in the multivariate analysis, only a lower rectal temperature at admission remained an independent predictor of mortality (OR: 1.520, 95% CI: 1.061–2.178, p = 0.023). The other variables did not retain statistical significance after adjustment.

DISCUSSION

Our study determined that various factors, such as demographic factors, factors related to the decision to start TH, factors related to the administration of TH treatment, and complications during HT, are important in affecting mortality in asphyxiated neonates treated with hypothermia.

TH is widely used to reduce neurological damage in newborns with asphyxia [11,13-15]. A recent study found that TH is neither safe nor effective in low-income countries, although several randomized controlled studies have demonstrated that it lowers the mortality rates in neonates with moderate and severe HIE [12,16]. We think that this conclusion needs serious thought. As a result, it is essential for identifying the variables influencing mortality in the HIE group that received hypothermia treatment. We anticipate that this research will significantly advance our understanding of PA management and outcomes in NICUs.

Significant independent predictors of mortality in neonates receiving hypothermia treatment were found using regression analysis. The chance of death increased by 3.2 times after cesarean delivery. This result is in line with research that suggests emergency cesarean procedures could not directly affect outcomes but rather signal serious perinatal impairment [17,18]. Acute renal failure emerged as the strongest predictor, increasing mortality risk by 6.5 times, underscoring the importance of early detection and management of renal dysfunction in asphyxiated neonates [19,20]. Additionally, the need for multiple inotropic agents was linked to a 7.9-fold increased mortality risk, indicating the severity of hemodynamic instability in non-survivors.

The guidelines emphasize that TH has positive results when initiated within 6 hours of the diagnosis of hypoxia. All patients, except four, were referred from other hospitals because our NICU is the region's reference facility. Since we also take patients from different parts of the nation, the start time for TH was extended to the 12th hour following the determination of asphyxia. In our investigation, the time to begin hypothermia treatment did not differ significantly between survivors and nonsurvivors.

Mortality was higher among male neonates, although the difference was not statistically significant. The incidence of mortality in neonates with HIE who underwent TH was 9.6%. This rate is generally lower than that reported in the literature. This pleasant result may be the effect of using standard national guidelines during the resuscitation and management of patients with perinatal asphyxia [7].

Our study found that gestational age significantly affects mortality, with survivors having a higher median gestational age than nonsurvivors. This finding is consistent with the literature, which indicates that premature birth increases the risk of mortality after PA [21,22].

The increased prevalence of cesarean birth among nonsurvivors raises the possibility that the delivery method may affect the prognosis of asphyxia-affected neonates. Apgar scores are essential in determining neonatal prognosis. Our study found that lower 5th-minute Apgar scores were associated with higher mortality (p=0.048), which is consistent with previous studies [23,24].

Our study showed that extremely low initial body temperature significantly increases mortality (p=0.023), highlighting the importance of maintaining optimal body temperature [25]. Our analysis revealed no appreciable difference between the effects of selective head cooling and whole-body cooling on mortality. Previous studies corroborate this result [25,26].

In our investigation, mortality was substantially correlated with both acute renal failure and high troponin levels. These results are in line with earlier research suggesting that cardiac and renal dysfunctions are important variables influencing the outcome of neonates who have asphyxia [27,28]. However, we did not determine the influence of thrombocytopenia, convulsion, liver failure, or necrotizing enterocolitis on mortality in our cohort. Although complications such as thrombocytopenia, liver involvement, necrotizing enterocolitis, and convulsions were evaluated, they did not demonstrate statistically significant associations with mortality in this cohort. The limited sample size may have affected the ability to detect significant differences.

The need for respiratory support was not significantly different between survived and nonsurvived neonates.

Our findings on the need for multiple inotropic agents as a strong predictor of mortality highlight the importance of comprehensive cardiovascular monitoring in NICUs. The association between hemodynamic instability and poor outcomes has been demonstrated in prior studies, and our results further underscore this critical aspect of care.

CONCLUSION

In conclusion, our study with ten-year experience identifies gestational age, male gender, cesarean section, initial low body temperature, low Apgar scores, acute renal failure, high

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troponin levels, and the requirement of multiple inotropic agents as significant factors affecting mortality in PA treated with hypothermia. To improve outcomes in neonatal care, doctors treating patients should have a better understanding of key predictive markers related to HIE.

Ethics Committee Approval: The study protocol received approval from the Non-interventional clinical research ethics committee of Kocaeli University (Date: 27.05.2024, No: E.594842) and was carried out according to the Declaration of Helsinki.

Informed Consent: Patients were not required to give their informed consent for inclusion in this retrospective study, because we used anonymous clinical data and individual cannot be identified according to the data present.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there is no conlflict of interest

Author Contributions: T.D: Conceptualization; Methodology; Supervision; Writing – original draft, O.S.P: Data curation; Formal analysis; Visualization; Writing – review & editing, A.G: Investigation; Resources; Validation; Writing – review & editing.

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Effects of anti-TNF-α treatment on lipid profile in inflammatory bowel disease

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MAIN POINTS

- This study evaluates the effect of anti-TNF treatment on lipid profile in pateints with IBD.
- No significant alterations were demonstrated in total cholesterol, LDL and triglyceride levels over 24 weeks. A significant increase in HDL levels was observed from baseline to 24 weeks.
- There are studies in the literature with conflicting results on this issue. Therefore, prospective studies with larger sample sizes, including long-term follow-up of patients and dietary factors, are warranted.

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ABSTRACT

Aim: Tumor necrosis factor-alpha (TNF-α) plays a significant role in the pathogenesis of inflammatory bowel disease (IBD) and is associated with atherosclerosis and dyslipidemia. Despite the established use of anti-TNF-α antagonists in the treatment of IBD, the impact of these drugs on lipid profiles remains unclear, with conflicting evidence in the literature. Our study aims to assess the effect of anti-TNF treatment on lipid profile in pateints with IBD.

Materials and Methods: Lipid profiles, including total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and the atherogenic index, were measured in 103 patients (66 patients with CD, 37 with UC) at baseline and at 12 and 24 weeks of TNF-α inhibitor treatment, and the results were compared between the groups.

Results: No significant change in cholesterol levels was observed over the course of 24 weeks (p=0.349). However, a noteworthy increase in HDL levels was observed from baseline to 24 weeks (p=0.016). No significant alterations in LDL and triglyceride levels were noticed over 24 weeks. The atherogenic index demonstrated no significant changes over the treatment period (p=0.462).

Conclusion: Anti-TNF-α therapy, either with infliximab or adalimumab, among patients with IBD does not lead to a considerable alteration in lipid profiles after 3 and 6 months of treatment, with the exception of a significant increase in HDL.

Keywords: Inflammatory bowel diseases, Tumor necrosis factor-alpha, Dyslipidemia, Chronic inflammation, Atherosclerosis

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of inflammatory bowel disease (IBD), the common feature of which is chronic inflammation of the gastrointestinal tract. Chronic inflammation is believed to contribute to the development of cardiovascular complications by contributing to all atherosclerotic processes [1]. Despite a systematic review assessing 11 studies concluding that IBD is not linked to elevated cardiovascular mortality [2, 3] and a meta-analysis results showing that mortality from cardiovascular disease was decreased [4], there were also studies indicating an increase in the risk of cardiovascular complications, even if not associated with an increase in mortality [3, 5-9]. Therefore, it is essential to evaluate and treat cardiovascular risk factors, including dyslipidemia, in patients with IBD.

Tumor necrosis factor-alpha (TNF-α) has been demonstrated to play a key role as a pleiotropic proinflammatory factor in the pathogenesis of IBD, and preclinical and clinical evidence supports the role of TNF-α in atherosclerosis and dyslipidemia [10-13]. For more than two decades, anti-TNF-α inhibitors have been the primary cornerstone in the treatment of IBDs. These drugs regulate the excessive immune response in the body, thereby reducing inflammation and helping control symptoms. As a strong modulator of inflammation, TNF-α inhibitors should be expected to improve lipid profiles in patients, but there are conflicting studies in the lit-

erature. The mechanisms by which anti-TNF treatment affects lipid profiles are not fully understood, but potential explanations include reduced inflammation, improved insulin sensitivity, modulation of adipokines, direct effects on lipid metabolism, and indirect effects through changes in disease activity [14-17].

Understanding the mechanisms by which these drugs might impact lipid metabolism is crucial. Therefore, the main goal of the current study was to evaluate the impact of TNF-α inhibitors on the lipid profiles of individuals diagnosed with IBD.

MATERIALS AND METHODS

This retrospective observational study aimed to evaluate the demographic characteristics of patients with inflammatory bowel disease (IBD) and assess the effects of anti-TNF-α treatment on lipid profiles. The study was conducted at Ankara City Hospital, Ankara, Turkey, from January 2020 to October 2023. Ethical approval of the research was obtained from the Institutional Review Committee (No: E1/4326/2023).

Study population

Subjects diagnosed with either CD or UC were eligible for inclusion. Participants were required to be currently undergoing treatment with anti-TNF-α medication, either adalimumab or infliximab. Patients with other chronic inflammatory conditions, contraindications to TNF-α inhibitors, treatment with anti-TNF-α inhibitors less than 6 months, history of any malignancy, younger than 18 years old, pregnant, or unwilling to participate were excluded from the study. In addition, subjects on antilipidemic agents were not included in the study.

Data collection

During the study, printed and electronic medical records were searched for data collection. Demographic data include age, sex, history of abdominal surgery, and body mass index (BMI). Regarding CD; disease duration, behavior, current treatment, and presence of perianal pathologies were recorded for each patient. In addition, regarding UC, extension of the disease was recorded. Patients undergoing anti-TNF-α inhibitor treatment were assessed for lipid profiles, including total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and the atherogenic index at baseline (start of treatment), 12 weeks, and 24 weeks of the TNF-α inhibitor treatment.

Statistical analysis

Statistical analyses were carried out by using The Statistical Package for Social Sciences (SPSS, version 24.0) for Windows (IBM Corp.; Armonk, NY, USA). The Kolmogorov–Smirnov test was used to evaluate the normality of the continuous variable distribution. Normal distributed continuous variables were represented as mean ± standard deviation and were compared using the repeated measures ANOVA with a post hoc Bonferroni test. Non-normally distributed continuous variables were described as medians (minimum and maximum) and were compared using the Friedman test with the post hoc Wilcoxon test. Categorical variables are described as frequency (percentage). A p-value of <0.05 was set statistically significant. Bonferroni correction was applied to control for Type I error rate, and the adjusted significance level was set at p <0.017 for the three time point comparisons. Power analysis was performed using G*Power version 3.1 (Heinrich Heine Universität, Dusseldorf, Germany) using repeated measures F-tests. The predicted power is 0.96 with a 0.05 type 1 error.

RESULTS

The demographic characteristics of the study cohort, which consists of the CD and UC groups, are given in Table 1. In the CD group (n=66), patients exhibited a mean age of 41.0 ± 13.1 years, with a male predominance of 62.1%. The median disease duration was 68.0 months (range: 12.0 – 276.0), and the mean BMI was 23.7 ± 4.8. Notably, 51.5% of CD cases localized to the terminal ileum (L1), while the majority presented with inflammatory behavior (B1, 59.1%). Perianal disease was evident in 33.3%, and 47.1% had undergone prior major abdominal surgery. Adalimumab and infliximab were the predominant medications (62.1% and 37.9%, respectively. In the UC group (n=37), patients had a mean age of 44.9 ± 13.4 years, with 67.6% being male. The median disease duration was 84.0 months (range: 12.0-300.0), and the mean BMI was 23.7 ± 4.6. The majority of UC cases exhibited extensive disease (73.0%).

Table 2 demonstrates the impact of TNF-α inhibitor treatment on lipid profiles over 12 and 24 weeks. The results revealed no significant change cholesterol levels over the course of 24 weeks (p=0.349). However, a noteworthy increase in HDL levels was observed from baseline to 24 weeks (p=0.016). No significant alterations in LDL and triglyceride levels were noticed over 24 weeks. The atherogenic index demonstrated no significant changes over the treatment period (p=0.462). These findings suggest a substantial influence of TNF-α inhibitor treatment on HDL levels in our cohort of patients with IBD.

DISCUSSION

This study demonstrated that undergoing anti-TNF-α therapy, with adalimumab or infliximab, among patients with IBD does not lead to a considerable alteration in lipid profiles after 3 and 6 months of therapy, except a significant increase in HDL.

In the current literature, the effect of anti-TNF-α therapy on lipid profile is mostly studied in rheumatological diseases like systemic lupus erythematosus and rheumatoid arthritis, with inconsistent results. Our results regarding the increase

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Table 1. Demographic characteristics of all patients with IBD.

	Crohn (n=66)	Ulcerative colitis (n=37)
Age, years, mean ± SD	41.0 ± 13.1	44.9 ± 13.4
Sex (Male), n (%)	41 (62.1)	25 (67.6)
Disease duration, months, median (min-max)	68.0 (12.0 -- 276.0)	84.0 (12.0 - 300.0)
BMI, kg/m^2, mean ± SD	23.7 ± 4.8	23.7 ± 4.6
UC Extension, n (%)		
Left Sided		10 (27.0)
Extensive		27 (73.0)
CD localization, n (%)		
Ileal (L1)	34 (51.5)	
Colonic (L2)	8 (12.1)	
Ileo-colonic (L3)	24 (36.4)	
CD Behavior, n (%)		
Inflammatory disease (B1)	39 (59.1)	
Stenosing (B2)	11 (16.7)	
Penetrating (B3)	16 (24.2)	
P (Perianal disease), n (%)	22 (33.3)	
Prior major abdominal surgery, n (%)	31 (47.1)	0 (0.0)
Current medication, n (%)		
Adalimumab	41 (62.1)	23 (62.2)
Infliximab	25 (37.9)	14 (37.8)

BMI: body mass index, Montreal classification of Crohn's disease (CD); Disease location (L): L1 terminal ileum, L2 colon, L3 ileocolon; Disease behavior (B): B1 non stricturing non penetrating; B2 stricturing, B3 penetrating, UC: ulcerative colitis.

Table 2. Effects of anti-TNF-α inhibitor treatment on lipid profile.

	Baseline (1)	12 weeks (2)	24 weeks (3)	p value	p value (1-2)	p value (1-3)	p-value (2-3)
Total cholesterol (mg/dl)	162.98 ± 42.99	167.15 ± 43.26	165.57 ± 40.1	0.349	--	--	--
HDL (mg/dl)	42 (36-51)	45 (36-54)	45 (38-54)	**0.016**	0.137	0.003	0.120
LDL (mg/dl)	90.28 ± 35.56	90.39 ± 37.11	90.77 ± 35.89	0.976	--	--	--
Triglycerides (mg/dl)	128 (93-170)	132 (99-190)	134 (98-177)	0.648	--	--	--
Atherogenic index	0.1 ± 0.26	0.13 ± 0.27	0.11 ± 0.25	0.462	--	--	--

HDL: high-density lipoprotein, LDL: low-density lipoprotein.

in HDL levels are consistent with those of most of these studies [17-20]. However, data regarding the comparable effects of anti-TNF-α therapy in individuals with IBD are rather limited, and conflicting results are also present. For instance, in a study that included 128 patients with IBD who received infliximab or adalimumab, no significant changes in lipid profile were observed after 1 and 3 years of treatment [21]. Although our study had a shorter follow-up period than this study, we did not observe any changes except for HDL levels. In another systematic review, the use of anti-TNF-α agents showed no association with changes in total cholesterol following both induction and maintenance therapy, and no long-term alterations in HDL, LDL, or triglyceride levels were observed, but short-term changes in HDL, LDL, and triglyceride were observed in one study [22]. A different cohort consisting of 22 patients with IBD who received infliximab was prospectively monitored for 14 weeks. There was no alteration in triglyceride or LDL levels, but there were significant increases cholesterol and HDL levels compared with baseline [23]. In another prospective study involving 111 patients with CD treated with infliximab, it was noted that plasma levels of total cholesterol and HDL showed an increase during the first 3 months of treatment, followed by a relatively stable pattern thereafter [24]. When we compare the findings of these studies with our own study, we believe that an increase in HDL levels can be seen in the short-term follow-up, but long-term follow-up studies should also be emphasized. In contrast with the results of some studies with long follow-up periods, an outcome from the 3-year monitoring of 56 patients with IBD undergoing anti-TNF-α therapy revealed significant increases in the AI index, total cholesterol, and LDL levels, while HDL levels remained unaffected [25].

As evident from studies published in the literature have shown inconsistent findings regarding the correlation between anti-TNF-α therapy and lipid alterations in IBD. The lipid profiles seen in IBD are influenced by a complicated interplay of inflammatory cytokines, acute phase reactants, and severe inflammation or surgical intervention that disrupts intestinal integrity [26]. Moreover, medications such as statins or corticosteroids can affect lipid metabolism. Therefore, we

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are likely to attribute these inconsistencies across studies to factors such as sample size differences, varying study durations, and inadequate adjustment for covariates such as comorbidities, levels of inflammation control with treatment, disease severity, response to treatment, and other medications used.

Limitations

The major limitation of this study was its retrospective design. In addition, dietary factors that may affect lipid levels could not be evaluated. Lastly, this was a single-center study with a relatively small sample size. Therefore, multicenter prospective studies with larger sample sizes, including long-term follow-up of patients and dietary factors, are needed.

CONCLUSION

While some evidence suggests the potential benefits of TNF-α inhibitors on lipid profiles, further large-scale, longer-duration, and more uniform studies are needed to better understand their effects in different patient populations and to clarify the underlying mechanisms. Therefore, it is essential for health care providers to monitor lipid levels regularly in patients receiving TNF-α inhibitors and to individualize treatment based on their specific lipid profile and overall cardiovascular risk.

Ethics Committee Approval: The ethics committee approval for the study was obtained from Ankara City Hospital Clinical Research Ethics Committee (No: E1/4326/2023).

Informed Consent: Given the retrospective nature of the study, informed consent was not required from patients.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors have no competing interests to declare.

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Blau syndrome: A case report with unusual symptoms and literature review

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ABSTRACT

Blau syndrome (BS) is an autosomal dominant disease that is brought on by changes in the gene that codes for the NOD-like receptor (NLR) protein (*NOD2*). It is characterized by the trinity of granulomatous polyarthritis, rash, and uveitis One-third to one-half of BS patients were found to have atypical symptoms. This case report presents the clinical experience of a BS patient with unusual findings at a tertiary care center in Istanbul.

A 7-year-old male patient admitted to the outpatient clinic with a complaint of abdominal pain. There were rash attacks with fever for 2 years. He has had abdominal pain that has been going on for years and was previously operated on due to intussusception. There were no hereditary familial diseases found in the patient's family history. In the superficial tissue ultrasonography, intussusception was observed in an intestinal segment. Pathological examination of segmental resection of ileum, cecum, and appendix showed that there were findings of acute appendicitis, peritonitis with surface fissuration, and ulcer. The skin biopsy showed noncaseating, granulomatous infiltration with epithelioid cells and lymphocytes. In the genetic test, the *NOD2* c.1835C>T heterozygous mutation was detected. Based on this, BS was diagnosed. The patient was started on adalimumab and additional colchicine treatment.

This syndrome can mimic other systemic inflammatory diseases in the early stages. This case report shows that we need to consider the diagnosis of BS in more detail in cases who raise clinical suspicion.

Keywords: Blau syndrome, Invagination, NOD2 mutation

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INTRODUCTION

Blau syndrome (BS, OMIM #186580) is a rare, autosomal dominant inflammatory disorder caused by mutations in the nucleotide-binding oligomerization domain-containing 2 (*NOD2*) gene (OMIM 605956), which encodes a NOD-like receptor (NLR) protein. The classical clinical triad of BS includes granulomatous polyarthritis, dermatitis, and uveitis. A skin rash typically appears within the first year of life, although exceptions have been reported. Polyarthritis commonly develops between the ages of two and four, often presenting as "boggy" synovitis and tenosynovitis—distinctive features of Blau arthritis. According to data from the Blau International Registry, 96% of patients with polyarticular arthritis at onset exhibited a boggy or exuberant joint appearance [1].

The proximal interphalangeal joints of the hands, along with the knees, ankles, and wrists, are the commonly affected peripheral joints. In contrast, the axial skeleton and temporomandibular joint are typically spared, and other peripheral joints are less frequently involved. Tenosynovitis is another hallmark of the disease, characterized by visibly swollen tendon sheaths. The commonly affected tendons include the tibialis posterior, pes anserinus, peroneal tendons, and wrist extensors. Despite the chronic and exuberant nature of the arthritis, range of motion is generally well preserved, especially in larger joints, and joint damage is rare. Finally, uveitis develops in approximately 60–80% of patients by the age of 48 months [1].

Here we present a 7-year-old male patient with unusual findings including abdominal pain and intussusception.

CASE REPORT

A 7-year-old male patient was admitted to the outpatient clinic with a chronic abdominal pain that had persisted for several years. He had previously undergone cataract surgery at the age of three and later he was operated on for intussusception. Over the past two years, he has experienced recurrent episodes of fever accompanied by rash. There were no complaints such as arthritis, oral or genital aphthae, Raynaud's phenomenon, dry mouth, or dry eyes. The parents were non-consanguineous, and no hereditary familial diseases were reported in the family history.

Superficial soft tissue ultrasonography was performed in the region of the abdominal complaint and showed that a 3.5 cm palpable mass (measuring approximately 33x34 mm in transverse axis) was detected in the right lower quadrant. Ultrasonographic characteristics of the mass suggested an intussusception in the bowel segment with surrounding mesenteric inflammation and multiple adjacent millimeter lymph nodes. A clear distinction between ileo-ileal and ileocecal regions was noted.

Segmental resection and appendectomy were performed and histopathological examination showed that cecum, and appendix showed findings consistent with acute appendicitis, surface fissuring, ulceration, and peritonitis. Subepithelial non-caseating granulomas were identified within lymphoid follicles, along with perineural and perivascular granulomatous inflammation. Some areas demonstrated inflammatory infiltration involving vessel walls and nerve plexuses. The small intestine also exhibited mucosal inflammation with focal fissures, cryptitis, and reactive lymphoid hyperplasia of Peyer's patches. There was non-granulomatous perivascular inflammation around medium-sized vessels.

Given the presence of granulomatous appendicitis, further evaluation for systemic granulomatous diseases (e.g., Crohn's disease, infections, immunodeficiencies, and vasculitis) was recommended. Inflammatory involvement of the appendix and small intestinal vessels suggested possible vasculitic changes, either secondary to inflammation or as part of a primary vasculitis process. While some granulomas exhibited necrotic changes, necrosis was not identified in most.

Colonoscopy revealed skip lesions with ulceration and hyperemia in the terminal ileum. The cecal mucosal vascular pattern was normal, with no erosions, ulcers, or polypoid lesions. The ascending colon showed normal mucosal vasculature and no ulcerative lesions. However, the descending colon and rectum were hyperemic with ulceration and membranous lesions. The rectosigmoid junction appeared particularly hyperemic.

Comprehensive laboratory and imaging evaluations were performed. ANA profile, ANCA, ACE levels, spot urine protein/creatinine, calcium/creatinine ratios, and immunoglobulin levels were tested. Uveitis screening, renal Doppler ultrasound, abdominal ultrasonography, high-resolution computed tomography (HRCT), and Interferon-γ Release Assay (IGRA) were also performed. Thoracic CT, renal Doppler, and abdominal US findings were within normal limits. Our laboratory findings showed a negative ANA and antiphospholipid antibody profiles, normal complement levels (C3 and C4), elevated liver enzymes (AST 52 U/L, ALT 63 U/L), positive stool occult blood, negative IGRA test. There were no evidence of uveitis on ophthalmologic examination.

Skin biopsy revealed non-caseating granulomatous inflammation with epithelioid cells and lymphocytes. Genetic testing identified a heterozygous *NOD2* c.1835C>T mutation. Based on clinical, pathological, and genetic findings, a diagnosis of Blau syndrome was established. The patient was started on adalimumab 40 mg subcutaneously once weekly under off-label approval. Colchicine was also added to the treatment regimen.

A signed consent form was obtained from the patient on 29/04/2025.

DISCUSSION

BS, a granulomatous autoinflammatory disease, was first described by Blau in 1985 [2]. Although symptoms of BS typically appear before the age of five, a proper diagnosis may not be made until later in life, especially if the presentation does not include the typical triad of clinical manifestations or if the symptoms appear one after the other rather than all at once [3]. Even though it is primarily recorded among Caucasians, there have been reports of BS in East Asia, including 34 instances in Japan [4], 19 cases in China [5], and 4 cases in South Korea [6]. In BS and sarcoidosis, there are many similar features, such as noncaseating granuloma, skin rash, and eye involvement. Early-onset sarcoidosis (EOS, MIM No. 609464) was previously thought to be a form of childhood sarcoidosis that began at a young age and had a progressive course. Since it was eventually shown that both BS and EOS have mutations in the *NOD2* gene, they are now regarded as one and the same disease [7-9].

A member of the NOD-like receptor family, the *NOD2* protein is mostly produced by antigen-presenting cells like macrophages and monocytes. In order to bind muramyl dipeptide (MDP), a breakdown product of the bacterial peptidoglycan, *NOD2* has a tripartite structure that includes two N-terminal caspase recruitment domains, one centrally located NTPase triphosphatase domain (NACHT domain), and a C-terminal domain with several leucine rich repeat motifs. Inflammation and apoptosis result from the MDP's activation of *NOD2*, which in turn promotes nuclear factor kappa light chain enhancer of activated B cells (NF-κB) [7]. Variants in the *NOD2* gene (OMIM *605956), which codes for the protein known as nucleotide-binding oligomerization domain 2, were known to cause BS in 2001. The 334 residue is regarded as the mutational hot point since the bulk of BS cases are caused by two known *NOD2* pathogenic variants, p.(Arg334Trp) and p.(Arg334Gln), which are found in exon

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4 [10]. The parents of the patient were healthy, despite the fact that BS is inherited from autosomal dominant. We believe the disease was caused by a de novo mutation in our case, and BS stemming from de novo mutations may manifest sporadically.

BS can have a variety of differential diagnosis. BS is frequently confused with other inflammatory disorders that are more prevalent or well-known since the symptoms are typically nonspecific and do not manifest at the same time. Prior to being identified with BS, the main diagnoses in the study by Matsuda et al. [11], were juvenile idiopathic arthritis, Behçet's diseases, Takayasu's arteritis, and Kawasaki disease. In our patient, unusually, abdominal pain was at the forefront. The patient underwent intussusception surgery for the second time

There are many therapeutic modalities that are available with a varying degree of efficacies. A modest dose of corticosteroids can be administered as a maintenance treatment after high-dose corticosteroids have been used to manage the acute inflammatory phase of BS.

As steroid-sparing agents, immunosuppressants such as methotrexate and azathioprine are frequently added. Biologic agents may be utilized if these treatments are unable to control the illness. Since the overproduction of TNF by macrophages is believed to be a major factor in the autoinflammation associated with BS, tumor necrosis factor-alpha (TNF-α) inhibitors are the commonly utilized therapeutic agents. While limited examples exist, inhibitors of interleukin (IL)-1β and IL-6 have demonstrated efficacy in specific clinical scenarios [12]. We started the patient on adalimumab, a fully human recombinant monoclonal antibody with high affinity. This drug is used as a TNF-α inhibitor in various autoimmune conditions.

CONCLUSION

In this case report, we present a confirmed case of BS. It shows that BS mimics other systemic inflammatory diseases in the early stages, leading to diagnostic difficulties. The diagnosis depends on clinical suspicion.

Informed Consent: It was conducted in compliance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. A signed consent form was obtained from the patient on 29/04/2025.

Peer-review: Externally peer-reviewed.

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ERRATUM

In the article, *"Doganay D. Punctoplasty surgery combined with 22-gauge intracath intubation in punctal stenosis: A practical, cost-effective, and efficient method."* Ann Med Res. 2025;32(4):169-172, published in the April 2025 issue (Vol. 32, Issue 4), the following correction has been made:

The informed consent statement for Figure 1, which includes the publication of the patient's image and related clinical information, has been properly included at the end of the article under the "Informed Consent" section in the PDF version.

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