Gülhane Medical Journal

ISSN: 1302-0471
e-ISSN: 2146-8052

Gulhane Med J September 2022 Volume 64 Issue 3

www.gulhanemedj.org

64/3

Gülhane
Medical Journal
Gülhane Tıp Dergisi

Gülhane Medical Journal is the official scientific publication of the Gülhane Faculty of Medicine, University of Health Sciences Türkiye.

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Publisher Certificate Number: 14521

Printing at: Son Sürat Daktilo Dijital Baskı Merkezi Sanayi ve Ticaret Ltd. Şti.
Gayrettepe Mahallesi, Yıldızposta Caddesi, Evren Sitesi A Blok No: 32 D: 1 - D: 3 34349 Beşiktaş, İstanbul, Türkiye
Phone: +90 (212) 288 45 75 **E-mail:** info@sonsuratdaktilo.com
Printing Date: September 2022
ISSN: 1302-0471 **E-ISSN:** 2146-8052
International scientific journal published quarterly.

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Gülhane Medical Journal (GMJ) is an international, multidisciplinary and peer reviewed journal for researchers and health-care providers. It is published four times a year, and accepts submissions in English. It is the official journal of the Gülhane Faculty of Medicine, University of Health Sciences Türkiye.

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Message from the Editor-in-Chief

Message from the Editor-in-Chief,

While the COVID-19 pandemic and the threat of new variants follow a lower pressure during this summer, the world has been dealing with growing concerns about food and energy shortage in the richest global regions. Its potential public health consequences are predictable in the long term.

Concerning COVID-19, yet there is no cure, and much has been learned about the transmission and potential prevention measures. Ongoing research has shown that the pandemic has been a continuous threat, even greater than what has been predicted, to the biological and mental health of the populations.

A significant proportion of our readers and authors have been involved in the fight against COVID-19. We understand from the increasing number of COVID-19 submissions that they have also kept their research works active, taking directions to pandemic-related variables and outcomes.

In the third issue of GMJ in 2022, we have selected highly interesting original articles, reviews, and case reports. As the journal's publishing team, we try to keep covering a wide range of articles from different disciplines.

We thank all contributors to GMJ.

M. Ali Gülçelik, M.D., Prof.
Editor-in-Chief

DOI: 10.4274/gulhane.galenos.2021.25733
Gulhane Med J 2022;64:208-16

A systematic review and meta-analysis on blood levels of cytokines/chemokines in COVID-19 cases

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Date submitted:
10.02.2021
Date accepted:
05.07.2021
Online publication date:
08.09.2022

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Keywords: Chemokine, cytokine, COVID-19, blood, meta-analyses

ABSTRACT

We assessed the blood levels of the most important factors such as cytokines/chemokines in Coronavirus disease-2019 (COVID-19). PubMed/Medline and Scopus as two important databases were searched up to March 26, 2020. To analyze the data, we used Review Manager 5.3 software. Out of forty-two records retrieved from two databases, 10 studies were involved in the analysis. Thirty-three cytokines/chemokines were checked. The levels of 27 cytokines/chemokines in COVID-19 patients were higher than the healthy controls, and among 20 cytokines/chemokines; the levels of 10 cytokines/chemokines in severe COVID-19 patients were higher than non-severe COVID-19 patients. Also, out of three cytokines, one had a higher level in the intensive care unit (ICU) patients compared to the non-ICU patients. The findings showed the cytokine storm syndrome in COVID-19 patients, especially in patients with severe disease.

Introduction

In January 2020, severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) extend to various cities in China, and cases with novel Coronavirus disease-19 (nCoV-19) or Coronavirus disease-2019 (COVID-19) have now been confirmed in several countries (1-5). Patients with poor prognostic characteristics at the time of hospitalization often experience significant mortality complications, particularly acute respiratory distress syndrome (ARDS) with various conditions such as multiple organ failure and blood clots (6). Human-to-human transmission is highly correlated with the SARS-CoV-2, and respiratory droplets and human-to-human contact can be the main routes of transmission (7,8). At present, no specific drug is available to treat patients with COVID-19 infection. Hence, there is an immediate need for safe and effective treatment for COVID-19, particularly in severe patients (9). Excessive production of proinflammatory cytokines/chemokines or even hypercytokinemia (cytokine storms) occurs in the Middle East respiratory syndrome-Coronavirus (MERS-CoV)

and SARS-CoV infections (10-12) and is associated with acute lung damage and ARDS development (9,11). Storm cytokines have been described as a systemic inflammatory response to infections and drugs, leading to overactive immune cells and the production of proinflammatory cytokines (13). Recent studies have reported a reduction in the number of peripheral blood lymphocytes and an increase in the level of inflammatory cytokines in COVID-19 patients (14,15). However, it is largely obscure how various subtypes of lymphocytes, as well as the kinetics of inflammatory cytokines in peripheral blood, alter during COVID-19 (16). One research also found that COVID-19 mortality might be due to "cytokine storm syndrome" activated by the virus or fulminant myocarditis (17). Another study (18) reported high morbidity and mortality due to increased levels of interleukin (IL)-6, IL-8, IL-2R, IL-10, and tumor necrosis factor-alpha (TNF-α). The cytokine level may even be used as a prognostic factor for critically ill patients (18). In the study of Takahashi et al. (19), the levels of both IL-8 and IL-18 in the male patients were higher than in female ones; however, a worse prognosis was seen in females with an increased cytokine level. They suggested a bias for gender while interpreting the result. Here, this meta-analysis evaluated the blood levels of cytokines/chemokines in the patients with COVID-19 for better clarification of some aspects of this disease.

Methods

The meta-analysis was performed according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) protocols (20).

Search strategy

Two PubMed/Medline and Scopus databases were comprehensively searched by an author (M.S) to retrieve all relevant references published until March 26, 2020, without restrictions. The searched queries were ("COVID-19" or "nCoV-19" or "Coronavirus disease-19" or "SARS-CoV-2") and ("cytokine*" or "interleukin*" or "interferon*" or "chemokine*"). The citations (all types of studies) correlating with our topic were manually searched, as well.

Eligibility criteria

The inclusion criteria were (1) studies including two separation groups; (2) studies assessing the relationship between blood cytokine levels and COVID-19; (3) the presence of SARS-CoV-2 found by the quantitative polymerase chain reaction method; (4) studies that the data to estimate the mean difference (MD) and 95% confidence interval (CI) in COVID-19 patients and healthy controls.

The exclusion criteria were as (1) studies with insufficient and irrelevant data; (2) conference papers, review articles, book chapters, and meeting abstracts.

The severe type of COVID-19 is defined by the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (21) in the studies as follows: 1. Respiratory distress with a respiratory rate greater than 30 per minute; 2. Oxygen saturation ≤93% in the resting state; 3. Arterial blood oxygen partial pressure (PaO_2)/oxygen concentration (FiO_2) ≤300 mmHg. Additionally, intensive care unit (ICU) patients were the patients who had been admitted to the ICU because they required high-flow nasal cannula or higher-level oxygen support measures to correct the hypoxemia.

Study selection

The titles and abstracts of the retrieved studies were independently checked by two authors (M.R and M.S). Then, both authors selected the relevant articles, while the full-text articles were retrieved by another author (H.N) and he excluded several full-texts.

Data extraction

The data from each study included in the analysis were independently extracted by two authors (M.R and M.S). If there was a discrepancy between the two authors, another author (H.N) helped make the last decision. All the authors endorsed the quality of the articles and reviewed the manuscript.

Statistical Analyses

The crude MD and 95% CI were estimated using Review Manager 5.3 software. Heterogeneity was evaluated across the studies applying both Cochran Q (22) and I^2 metrics (23). Additionally, $P_{heterogeneity}$ or P_h <0.1 and I^2 >50% identified a statistically significant heterogeneity; hence, the analysis of the random-effects model was used to estimate the values of the pooled MD (95% CI). Otherwise, we used the fixed-effects model. The publication bias across the studies with Egger and Begg's tests for the analyses of more than three studies was analyzed by comprehensive meta-analysis version 2.0 software with a $p<0.05$ as statistically significant.

Some studies presented the values of cytokines/chemokines in standard errors and medians (interquartile), which were changed into standard deviation (SD) and mean SD, respectively (24). The blood cytokine/chemokine levels are presented in "pg/mL." The levels of cytokines/chemokines in some studies were estimated based on the graphs by GetData Graph Digitizer 2.26 software. The quality score of each study was performed based on the Newcastle-Ottawa Quality Assessment Scale (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp).

Results

Forty-two records were retrieved from both databases and after removing the duplicate and irrelevant records, seventeen articles met the criteria (Figure 1). Seven full-text articles were excluded for other reasons (one case report, two reviews, and

four articles without healthy control groups). Finally, ten studies (7,16,25-32) were involved in the meta-analysis.

The characteristics of the studies included in the analysis are identified in Table 1. Eight studies (7,16,26-31) compared the cytokine levels in both severe and non-severe COVID-19 patients, two studies (25,29) compared the cytokine levels between non-ICU and ICU patients, and three studies (29,30,32) included healthy control groups. The quality score of each study is shown in Table 1.

The funnel plots are shown in Supplementary Appendix 1 and a summary of the main results is shown in Tables 1, 2, and 3. Table 2 shows the comparison of the levels of cytokines/chemokines in COVID-19 patients versus healthy controls in serum and plasma. The pooled results showed a significant difference between the two groups (COVID-19 patients versus healthy controls) evaluating the levels of IL-1β (MD: 1.02; p<0.00001), IL-1Rα (MD: 692.22; p=0.002), IL-2 (MD: 5.02; p=0.0001), IL-2Rα (MD: 35.84; p=0.02), IL-4 (MD: 1.12; p<0.00001), IL-5 (MD: 5.58; p=0.007), IL-6 (MD: 10.54; p=0.009), IL-7 (MD: 14.21; p<0.0001), IL-8 (MD: 12.27; p=0.008), IL-9 (MD: 28.45; p<0.00001), IL-10 (MD: 9.31; p=0.0003), IL-12 (p70) (MD: 2.65; p=0.0006), IL-13 (MD: 3.32; p<0.00001), IL-15 (MD: 70.15; p=0.01), IL-17 (MD: 1.02; p<0.00001), TNF-α (MD: 18.94; p=0.0002), IFN-γ (MD: 12.42; p=0.0002), IP-10 (MD: 1725.35; p=0.003), G-CSF (MD: 86.33; p=0.0002), MIP-1α (MD: 1.60; p<0.0001), M-CSF (MD: 19.10; p=0.01), CTACK (MD: 325.52; p<0.00001), GM-CSF (MD: 1.22; p<0.001), MCP-1 (MD: 26.88; p=0.003), FGF basic (MD: 10.37; p<0.00001), RANTES (MD: 3010.06; p=0.03), and Eotaxin (MD: 9.57; p=0.02), not for HGF (MD: 546.77; p=0.05), MCP-3 (MD: 3.37; p=0.05), MIG (MD: 636.66; p= 0.05), MIP-1β (MD: 14.42; p=0.08), VEGF (MD: 105.15; p=0.05), and PDGF-BB (MD: 857.47; p=0.26).

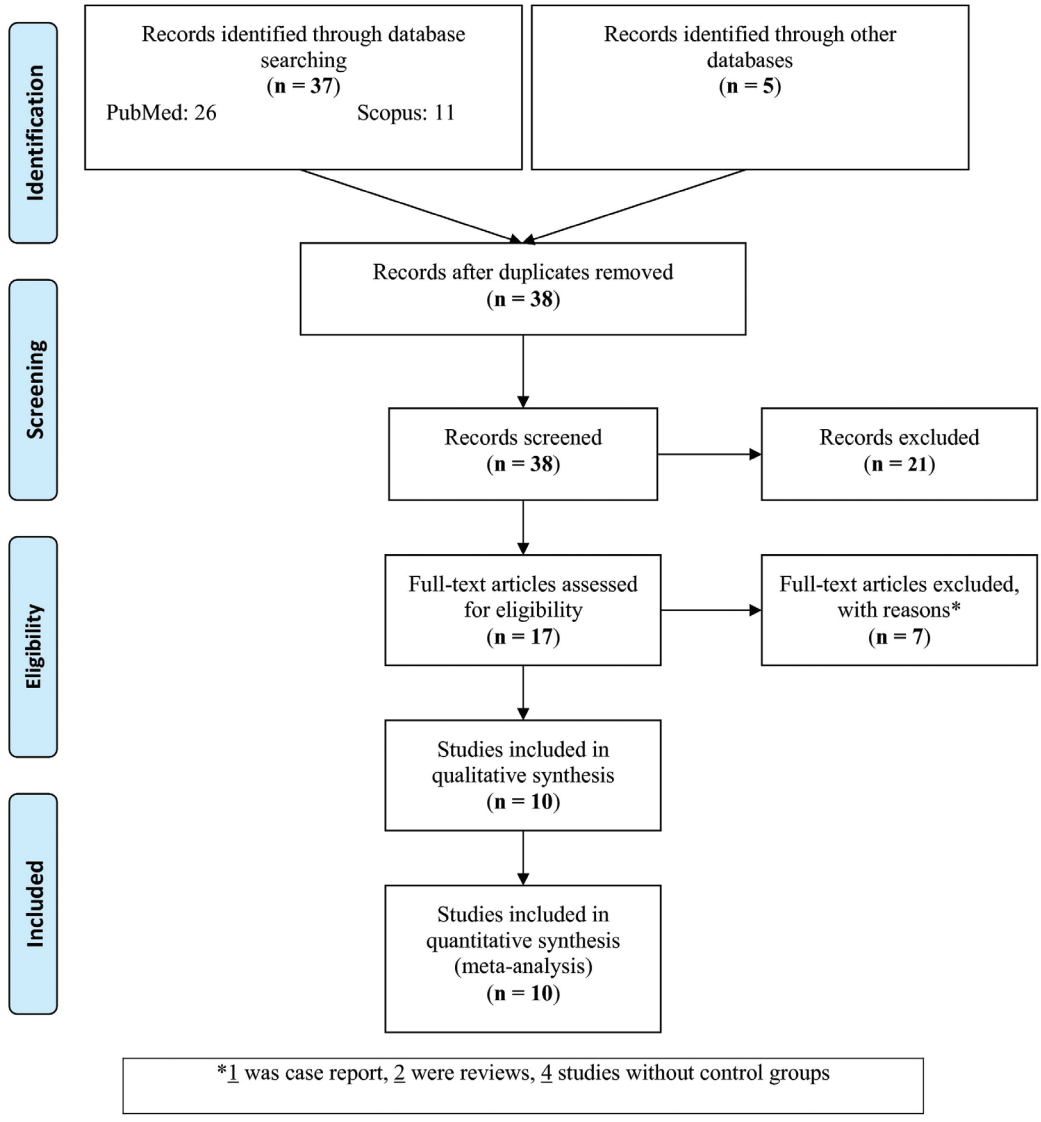

Figure 1. Flowchart of study selection

Table 1. Characteristics of studies included in the meta-analysis (n=10)

Study, year	No of cases	No of controls	Median age of cases, year	Median age of controls, year	Male % of cases	Male % of controls	Quality score	Marker
Cai et al. (25) 2020	240 (no ICU care) & 58 (ICU care)	NA	40 & 64	NA	46.3 & 56.9	NA	6	Il-6
Diao et al. (27) 2020	249 (non-severe) & 20 (severe)	NA	Range: 5-97	NA	NA	NA	6	Il-10, TNF-α, Il-6
Gao et al. (28) 2020	28 (non-severe) & 15 (severe)	NA	43 & 45.2	NA	57 & 60	NA	7	Il-6
Huang et al. (29) 2020	28 (no ICU care) & 13 (ICU care)	4	49 & 49	Adult	68 & 85	NA	6	IL-1b, IL-1RA, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-17, IFN-γ, TNF-α, PDGF-BB, IP-10, G-CSF, IL-13, IL-9, Eotaxin, VEGF MIP-1β, RANTES, MIP-1α, FGF basic, IL-12 (p70), IL-5, IL-15, MCP-1
Chen et al. (26) 2020	15 (non-severe) & 9 (severe)	NA	56	NA	72	NA	6	IL-1b, IL-2R, IL-6, IL-10, IL-8, TNF-α
Liu et al. (30) 2020	4 (non-severe) & 8 (severe)	8	62.5	28	66.7	50	6	M-CSF, IL-10, IL-17, IL-4, IP-10, IL-7, IL-1RA, G-CSF, IFN-γ, IL-2, PDGF-BB, HGF, MCP-3, MIG, MIP-1α, MIP-1b, TNF-α, IL-8, IL-13, IL-9, Eotaxin, VEGF, RANTES, MIP-1α, FGF basic, IL-12 (p70), IL-5, IL-15, MCP-1
Liu et al. (16) 2020	27 (non-severe) & 13 (severe)	NA	43.2 & 59.7	NA	29.6 & 53.8	NA	6	IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ
Qin et al. (31) 2020	166 (non-severe) & 286 (severe)	NA	53 & 61	NA	54.2 & 48.2	NA	7	TNF-α, Il-1β, Il-2R, Il-6, Il-8, Il-10
Wan et al. (7) 2020	45 (non-severe) & 18 (severe) for IL-6, 97 (non-severe) & 21 (severe) for IFN-γ, 102 (non-severe) & 21 (severe) for others	NA	43.0 & 61.3	NA	53.9 & 52.4	NA	6	IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ
Yang et al. (32) 2020	19 (non-severe) & 34 (severe)	8	51 & 63.5	NA	47.3 & 64.7	NA	6	FN-γ, IL-1RA, IL-2RA, IL-6, IL-10, IL-18, HGF, MCP-3, MIG, M-CSF, G-CSF, MIG-1a, CTACK, IP-10

NA: Not available, ICU: Intensive care unit, CI: Confidence interval, IL: Interleukin, TNF-α: Tumor necrosis factor-alpha

Table 3 shows the comparison of the blood levels of cytokines/chemokines in severe versus non-severe COVID-19 patients. The pooled results illustrated a significant difference between severe and non-severe COVID-19 patients in IL-1Rα (MD: 1427.24; p=0.008), IL-2R (MD: 504.98; p=0.02), IL-2Rα (MD: 33.14; p<0.00001), IL-6 (MD: 20.52; p<0.00001), IL-8 (MD: 7.27; p<0.01), IL-10 (MD: 2.13; p<0.00001), G-CSF (MD: 80.58; p<0.00001), HGF (MD: 700.67; p=0.005), MCP-3 (MD: 6.48; p<0.00001), and MIG (MD: 1039.43; p=0.0005) levels, not for the IL-1β (MD: 0.15; p=0.84), IL-2 (MD: 2.12; p=0.29), IL-4 (MD: 0.88; p=0.06), IL-17 (MD: 2.50; p=0.33), IFN-γ (MD: 4.66; p=0.09), TNF-α (MD: 0.47; p=0.28), IP-10 (MD: 7991.86; p=0.21), CTACK (MD: 82.97; p=0.42), MIP-1α (MD: 0.82; p=0.54), and M-CSF (MD: 34.62; p=0.18) levels.

The comparison of the blood levels of three cytokines (IL-6, TNF-α, and IL-10) in ICU versus non-ICU patients is shown in Table 4. The pooled results indicated a significant difference between the two mentioned groups just in the TNF-α (MD: 14.46; p=0.004) level, not IL-6 (MD: 12.88; p=0.10) and IL-10 (MD: 4.41; p=0.06) levels.

Table 2. Comparison of blood levels of cytokines/chemokines between COVID-19 patients and healthy controls

Cytokine, pg/mL	No of studies	MD	95% CI		p-value	Chi²	Z	I²	P_h
			Min	Max					
IL-6	3	10.54	2.62	18.47	0.009	25.07	2.61	80%	0.0001
IL-10	3	9.31	4.25	14.37	0.0003	50.65	3.61	90%	<0.00001
TNF-α	2	18.94	8.94	28.95	0.0002	8.46	3.71	65%	0.04
IFN-γ	3	12.42	5.80	19.04	0.0002	37.24	3.68	87%	<0.00001
IL-1b	2	1.02	0.57	1.47	<0.00001	6.70	4.48	55%	0.08
IL-1Rα	3	692.22	257.31	1127.12	0.002	30.98	3.12	84%	<0.00001
IL-2	2	5.02	2.47	7.57	0.0001	9.14	3.86	67%	0.03
IL-4	2	1.12	0.67	1.57	<0.00001	16.99	4.87	82%	0.0007
IL-17	2	4.98	3.00	6.96	<0.00001	4.80	4.94	38%	0.19
IL-8	2	12.27	3.23	21.30	0.008	31.70	2.66	91%	<0.00001
IL-2Rα	2	35.84	4.77	66.91	0.02	36.93	2.26	92%	<0.00001
IP-10	3	1725.35	579.14	2871.57	0.003	45.17	2.95	89%	<0.00001
G-CSF	3	86.33	41.20	131.45	0.0002	26.21	3.75	81%	<0.0001
MIP-1α	3	1.60	0.83	2.38	<0.0001	15.23	4.05	67%	0.009
M-CSF	2	19.10	4.13	34.06	0.01	21.12	2.50	86%	<0.0001
HGF	2	546.77	-10.01	1103.55	0.05	15.59	1.92	81%	0.001
MCP-3	2	3.37	-0.00	6.75	0.05	25.10	1.96	88%	<0.0001
MIG	2	636.66	13.96	1259.37	0.05	16.87	2.00	82%	0.0008
CTACK	2	325.52	186.28	464.75	<0.00001	2.83	4.58	0%	0.42
MIP-1b	2	15.42	-2.11	32.95	0.08	6.26	1.72	52%	0.10
GM-CSF	2	1.22	0.62	1.83	<0.0001	2.20	3.95	0%	0.53
MCP-1	2	26.88	9.07	44.70	0.003	18.39	2.96	84%	0.0004
IL-15	2	70.15	13.96	126.33	0.01	2.71	2.45	0%	0.44
IL-5	2	5.58	1.53	9.63	0.007	0.12	2.70	0%	0.73
IL-12 (p70)	2	2.65	1.14	4.16	0.0006	3.32	3.45	10%	0.34
FGF basic	2	10.37	6.65	14.09	<0.00001	4.52	5.46	34%	0.21
RANTES	2	3010.06	226.48	5793.67	0.03	10.73	2.12	72%	0.01
VEGF	2	105.15	-1.64	211.95	0.05	41.23	1.93	93%	<0.00001
Eotaxin	2	9.57	1.84	17.30	0.02	0.70	2.43	0%	0.87
IL-9	2	28.45	23.50	33.41	<0.00001	1.34	11.25	0%	0.72
IL-13	2	3.32	1.86	4.77	<0.00001	8.04	4.47	63%	0.05
PDGF-BB	2	857.47	-655.68	2406.63	0.26	31.24	1.12	90%	<0.00001
IL-7	2	14.21	7.89	20.52	<0.0001	13.41	4.41	78%	0.004

COVID-19: Coronavirus disease-2019, MD: Mean difference, CI: Confidence interval, P_h: $P_{heterogeneity}$, IL: Interleukin, TNF-α: Tumor necrosis factor-alpha, Min: Minimum, Max: Maximum

Both Egger and Begg's tests on the analyses with more than three studies were used (IL-6, IL-10, TNF-α, and IFN-γ levels in severe COVID-19 compared to non-severe COVID-19 patients) (Figure 2). The results didn't show any publication bias across the studies (p>0.05).

Discussion

Studies have shown that cytokines and chemokines can play a significant role in the immunity and immune system of patients with viral infections (9). The nCoV-19 can lead to severe and even fatal respiratory illnesses such as ARDS (14) and COVID-19 treatment depends primarily on the patient's immune system. When the overactive immune system kills the virus, it produces many inflammatory agents, which lead to severe cytokine storms (33). The virus can activate immune cells (such as T-cells, B-cells, macrophages, dendritic cells, neutrophils, and monocytes) and living tissue cells, which produce a large number of inflammatory cytokines (34). This meta-analysis of cytokines reported that the blood levels of most cytokines were higher in the COVID-19 patients than in the healthy controls. Moreover, several cytokines (IL-6, IL-8, G-CSF, IL-10, IL-1Rα, IL-2, HGF, IL-2Rα, MIG, and MCP-3) had higher levels in more severe than non-severe COVID-19 patients. Additionally, the blood levels of TNF-α in the ICU patients were higher than in the non-ICU patients. Finally, generalizations cannot be made for other cytokines.

SARS-CoV-2 is a novel beta-Coronavirus dependent on the Sarbecovirus subgenus of the Coronaviridae family (2). Inflammatory responses due to viral infections play a significant role in the severity of pulmonary pathology (35,36). The virus

Table 3. Comparison of blood levels of cytokines/chemokines between severe COVID-19 and non-severe COVID-19 patients

Cytokine, pg/mL	No of studies	MD	95% CI		p-value	Chi²	Z	I²	P_h
			Min	Max					
IL-6	8	20.52	13.83	27.21	<0.00001	18.61	6.01	62%	0.010
IL-10	6	2.13	1.69	2.57	<0.00001	6.08	9.57	18%	0.30
TNF-α	5	0.47	-0.38	1.32	0.28	11.96	1.08	67%	0.02
IFN-γ	4	4.66	-0.70	10.02	0.09	19.85	1.71	85%	0.0002
IL-1b	3	0.15	-1.31	1.62	0.84	2.16	2.16	54%	0.14
IL-1Rα	2	1427.24	372.97	2481.51	0.008	1.38	2.65	27%	0.24
IL-2	2	2.12	-1.82	6.07	0.29	17.48	1.06	94%	<0.0001
IL-4	3	0.88	-0.05	1.82	0.06	17.77	1.85	89%	0.0001
IL-17	2	2.50	-2.50	7.50	0.33	20.03	0.98	95%	<0.0001
IL-8	3	7.27	1.68	12.87	0.01	6.06	2.55	67%	0.05
IL-2R	2	504.98	44.66	965.31	0.03	138.73	2.15	99%	<0.00001
IL-2Rα	2	33.14	23.49	42.79	<0.00001	0.07	6.37	0%	0.79
IP-10	2	7991.86	-4397.72	20381.45	0.21	4.46	1.26	78%	0.03
G-CSF	2	80.58	45.78	115.38	<0.00001	0.01	4.54	0%	0.94
MIP-1α	2	0.82	-1.79	3.42	0.54	4.95	0.61	80%	0.03
M-CSF	2	34.62	-16.34	85.57	0.18	2.22	1.33	55%	0.14
HGF	2	700.67	210.5	1190.78	0.005	0.00	2.80	0%	0.96
MCP-3	2	6.48	4.13	8.84	<0.00001	1.34	5.39	26%	0.25
MIG	2	1039.43	455.75	16.23.11	0.0005	0.60	3.49	0%	0.44
CTACK	2	82.97	-120.31	286.24	0.42	0.23	0.80	0%	0.63

COVID-19: Coronavirus disease-2019, MD: Mean difference, CI: Confidence interval, P_h: $P_{heterogeneity}$, IL: Interleukin, Min: Minimum, Max: Maximum, TNF-α: Tumor necrosis factor-alpha

Table 4. Comparison of blood levels of cytokines/chemokines in ICU versus non-ICU patients with COVID-19

Cytokine, pg/mL	No of studies	MD	95% CI		p-value	Chi²	Z	I²	P_h
			Min	Max					
IL-6	2	12.88	-2.28	28.04	0.10	1.36	1.67	26%	0.24
IL-10	2	4.41	-0.12	8.94	0.06	0.00	1.91	0%	0.95
TNF-α	2	14.46	4.71	24.21	0.004	0.47	2.91	0%	0.49

MD: Mean difference, CI: Confidence interval, P_h: $P_{heterogeneity}$, IL: Interleukin, COVID-19: Coronavirus disease-2019, Min: Minimum, Max: Maximum, ICU: Intensive care unit, TNF-α: Tumor necrosis factor-alpha

particles extend via the respiratory mucosa and infect other cells, inducing a cytokine storm in the body, triggering many immune responses, and altering peripheral leukocytes and lymphocytes (28), like SARS-CoV-2 (14,31). One study (28) reported that IL-6 levels could be applied to estimate and diagnose the adult COVID-19 severity. SARS-CoV-2 infection increases the secretion of IL-4 and IL-10 and inflammation, which makes a difference with the SARS-CoV infection (37). Due to the high levels of cytokines caused by 2019-nCoV-19 infections, corticosteroids have been continuously applied to remedy patients with severe diseases to get the potential benefits by decreasing inflammatory lung injury (29). One study (30) confirmed that the levels of cytokines could increase in COVID-19 patients because several cytokines/chemokines (IL-1RA, IL-2, IL-4, IL-7, IFN-α2, IFN-γ, IL-10, IL-12, IL-17, IP-10, M-CSF, and G-CSF) were linearly related to lung injury and would be potential biological markers for COVID-19 severity. Studies have shown serum elevated levels of IP-10, MIP-1α, IL-6, IL-8, and MCP1 in the SARS-CoV-infected patients (38,39), and IFN-α2, IFN-γ, IL-10, IL-15, and IL-17 in the plasma levels of the patients with MERS-CoV (40). A study suggested that a subgroup of severe COVID-19 patients have cytokine storm syndrome (41).

The pathophysiology of the above unusual pathogenicity for SARS-CoV or MERS-CoV is not fully understood. Preliminary studies have shown that elevated serum proinflammatory cytokines (e.g., IFN-γ, IP10, MCP1, IL-1β, IL-6, and IL-12) are associated with pulmonary inflammation and extensive lung damage in SARS patients (36). Patients infected with coronavirus had high levels of IL-1β, IP10, IFN-γ, and MCP1, which may lead to the activation of T-helper-1 cell responses. Additionally, patients who required ICU admission had higher concentrations of TNF-α, IP10, G-CSF, MCP1, and MIP-1α, than patients who did not require ICU admission, indicating that cytokine storms were associated with disease severity (29). Lymphocyte subsets play an important role in regulating the body's immune system, with each cell restricting and regulating each other. One study showed that among nCoV-19 pneumonia patients, the reduction in CD4 + T-cells was 52.90% and 95.24% in the mild and severe groups, respectively. The reduction in CD8 + T-cells was 28.40% and 61.90% in the mild and severe groups, respectively, which indicates that T lymphocytes are inhibited in severe patients when the body is resistant to nCoV-19 infection (7).

Although the mechanism of cytokines is largely unknown, efforts to use them or their inhibitors for treating diseases have been successful and acceptable (42,43). Despite their unpleasant side effects, IFN-α and IFN-γ are often used clinically (42,44,45), based on which, IFN-γ is an official drug that is recommended for the diagnosis and treatment of COVID-19-infected pneumonia (46,47). Additionally, one study showed that IL-6 was an early index of the cytokine release syndrome in this pneumonia (48). Therefore, we should consider the potential therapeutic role of extracorporeal cytokine removal in treating COVID-19-associated cytokine storms (49-51) in the future.

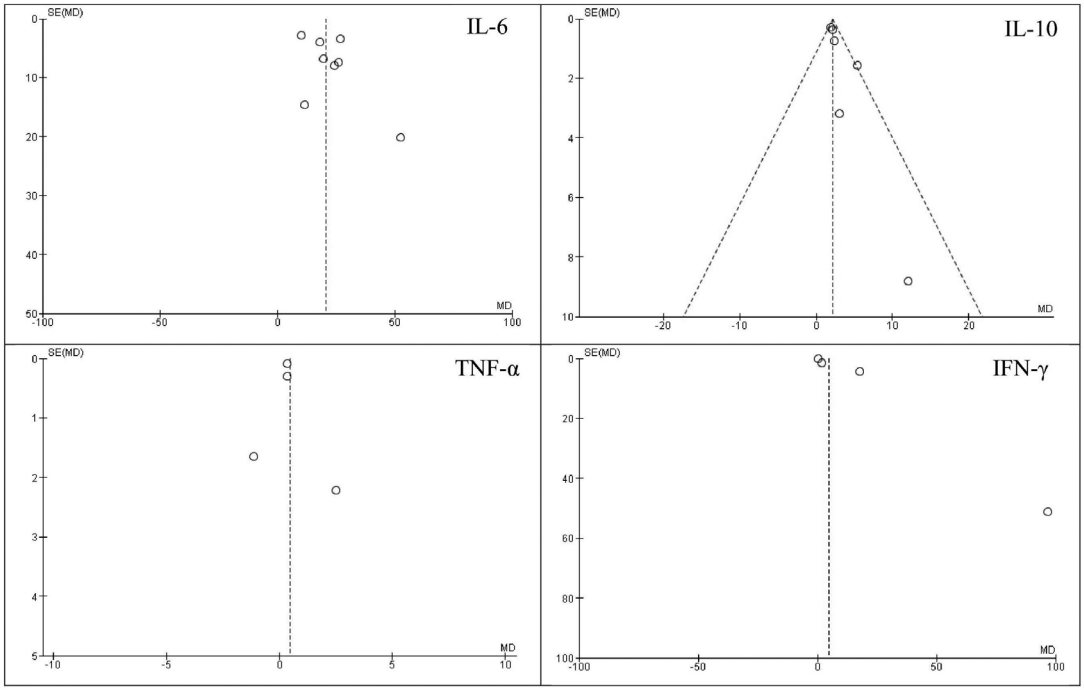

Figure 2. Funnel plots of IL-6, IL-10, TNF-α, and IFN-γ levels in severe COVID-19 compared with non-severe COVID-19 patients
TNF-α: Tumor necrosis factor-alpha, COVID-19: Coronavirus disease-2019, IL: Interleukin

Study Limitations

Limitations of the study were 1) the disease is new and not much research has been done raising concerns of bias across studies, 2) additional analyses were not available (e.g., subgroup and meta-regression analyses), 3) several studies reported their data on the graphs and we had to estimate them based on the software, 4) several studies did not report the mean (±SD) and we had to estimate them based on the formula. The strength of the study was the inclusion of all studies with English and non-English full-texts and preprint studies.

Conclusion

The results confirmed the cytokine storm syndrome in COVID-19 patients, particularly the severe cases. Therefore, the treatment of this syndrome in this disease in the future is recommended as a new treatment to reduce the possible side effects, and studies with more samples and different regions are needed to confirm the results of this meta-analysis. However, generalizations cannot be made for cytokines, which were evaluated in only two studies.

Ethics

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: M.R., M.S., Design: M.R., M.S., Data Collection or Processing: H.N., F.N., B.S., Analysis or Interpretation: M.R., M.S., Literature Search: M.S., Writing: M.R., H.N., F.N., B.S., M.S.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors gratefully acknowledge the Research Council of Kermanshah University of Medical Sciences (Grant number: 4010091) for the financial support.

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DOI: 10.4274/gulhane.galenos.2021.40427
Gulhane Med J 2022;64:217-21

Changes in neutrophil-to-lymphocyte ratio following treatment with dapagliflozin in patients with type 2 diabetes mellitus

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Date submitted:
05.07.2021
Date accepted:
21.12.2021
Online publication date:
08.09.2022

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Keywords: Dapagliflozin, neutrophil-to-lymphocyte ratio, sodium-glucose cotransporter-2 inhibitor, type 2 diabetes mellitus

ABSTRACT

Aims: Dapagliflozin, a sodium-glucose cotransporter-2 inhibitor, is indicated for glycemic control in type 2 diabetes mellitus (T2DM). In this study, we investigated the changes in neutrophil-to-lymphocyte ratio (NLR), a marker of inflammation, in patients with T2DM after the initiation of dapagliflozin.

Methods: This retrospective study included patients (aged 18 to 75 years) with T2DM who were prescribed dapagliflozin 10 mg once daily additional to their existing diabetic treatment. Patients with a history of chronic liver disease, chronic renal failure, infection, inflammatory disease, and the use of drugs affecting bone marrow were included. The duration of treatment was set at 12 weeks.

Results: The study included 98 patients with a mean age of 54.3±8.0 years, with a female predominance of 61.2%. At 12th week, there was a statistically significant decrease in fasting glucose (199.6 mg/dL vs. 164.3 mg/dL, p<0.001), glycated hemoglobin (HbA1c) (8.92% vs. 8.01%, p<0.001), leukocyte count (7.79 10^3/mm^3 vs. 8.36 10^3/mm^3, p=0.018) and neutrophil count (4.44 10^3/mm^3 vs. 4.84 10^3/mm^3, p=0.027). Lymphocyte count (2.56 10^3/mm^3 vs. 2.72 10^3/mm^3, p=0.150) and NLR (1.86 vs. 1.89, p=0.758) also showed some increase but the difference was not statistically significant.

Conclusions: This study showed significant increases in leukocyte and neutrophil counts in T2DM patients taking dapagliflozin, but lymphocyte count and NLR remained unaltered.

Introduction

Diabetes mellitus (DM) is a chronic disease characterized by the inability of the body to use insulin effectively or the inability of the pancreas to produce sufficient insulin (1).

Chronic inflammation plays an important role in the development and progression of DM and its complications. Some studies show that there is an increase in the levels of inflammatory cytokines such as C-reactive protein, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in patients with diabetes (2,3).

The ratio of neutrophil count to lymphocyte count (neutrophil to lymphocyte ratio, NLR) is accepted as an indicator of systemic inflammation. NLR has been increased in inflammatory conditions such as hepatosteatosis, malignant conditions, thyroiditis, cardiac conditions, and inflammatory bowel disease (4-10). NLR is considered a marker of inflammation in diabetes and its microvascular and macrovascular complications (11-13).

Additionally, studies have described the relationship between NLR and glycemic control in patients with diabetes. Studies have shown that mean NLR values are higher in patients with poor glycemic control compared to patients with good glycemic control (14,15).

Dapagliflozin is a sodium-glucose cotransporter-2 (SGLT-2) inhibitor used for treating type 2 DM (T2DM). SGLT-2 inhibitors cause SGLT-2 inhibition in the renal proximal tubules, reducing the glucose reabsorption from the kidney and increasing glucose excretion through the urinary tract (1). Since they act independently of insulin, they can be used in any stage of diabetes (1).

Dapagliflozin has positive effects on the heart and kidneys (16-18). While it shows these effects, its effects on inflammation are not exactly known. In our study, we investigated the effect of dapagliflozin on inflammation by looking at NLR levels before and after dapagliflozin.

Methods

This retrospective study included patients with T2DM who were started treatment with dapagliflozin 10 mg once daily at the Internal Medicine Outpatient Clinics of the University of Health Sciences Türkiye, Haydarpaşa Numune Training and Research Hospital between July 2017 through December 2018. The main enrollment criterion was the use of dapagliflozin in addition to the existing treatment for at least 12 weeks. Additional inclusion criteria were age between 18 and 75 years and estimated glomerular filtration >60 mL/min/1.73 m^2. Patients with chronic liver disease and/or transaminase levels above 5 times the upper limit of normal, hematological diseases, on drugs affecting the bone marrow, splenic disease, active infectious diseases, acute or chronic inflammatory diseases, and malignancy were excluded. Patients on glucagon-like peptide 1 analogs were excluded. Patients who were on statins for more than 24 weeks at the time of dapagliflozin treatment were included.

The demographic characteristics, chronic diseases, fasting glucose, glycated hemoglobin (HbA1c), blood urea nitrogen (BUN), creatinine, hematocrit, thrombocyte count, leukocyte count, neutrophil count, and lymphocyte count were recorded. NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count from the complete blood count. The same variables were recorded in the next visit, at least after 12 weeks of dapagliflozin treatment.

The study was approved by the University of Health Sciences Türkiye, Haydarpasa Numune Training and Research Hospital (22.04.2019/5386). All human studies were conducted under the rules of the Declaration of Helsinki.

Statistical Analysis

Statistical analysis for the Statistical Package for the Social Sciences Statistics 23 (IBM Corp., Armonk, NY, USA) software was used to perform statistical calculations. The normality of the distribution was evaluated by the Kolmogorov-Smirnov test. Descriptive statistical methods, including percentage and mean±standard deviation or median (interquartile range), were used to display the basic characteristics. Associations between glucose, HbA1c and NLR variables were assessed using Pearson's correlation analysis. A paired t-test was used to compare biochemical and hematological parameters before and after dapagliflozin in patients with T2DM. The statistical significance level was set as $p<0.05$.

Results

A total of 98 patients, including 60 females (61.2%) and 38 males (38.8%), were included in the study. The mean age of the patients was 54.3 years. The median diabetes duration of the patients was 7 years (Table 1). Thirty-eight patients had hypertension, 41 patients had dyslipidemia, 10 patients had coronary artery disease and 2 patients had cerebrovascular disease. Four patients had diabetic retinopathy and 22 patients had diabetic neuropathy.

Mean basal glucose was 199.6±73.2 mg/dL, HbA1c was 8.9±1.7%, BUN was 13.9±3.5 mg/dL, creatinine was 0.79±0.08 mg/dL, leukocyte count was 7.79±1.35 $10^3/mm^3$, neutrophil count was 4.44±1.08 $10^3/mm^3$, lymphocyte count was 2.56±0.67 $10^3/mm^3$, and NLR was 1.86±0.76 (Table 2).

A statistically significant positive correlation was observed between glucose and NLR values (r=0.206, p=0.043). There was no significant correlation between HbA1c and NLR values (r=0.140, p=0.170) (Table 3).

A significant decrease was observed in glucose and HbA1c levels after dapagliflozin treatment (p<0.001, p<0.001). Although there was an increase in BUN and creatinine levels, this change was not statistically significant (p=0.07, p=0.614). A statistically significant increase was detected in leukocyte and neutrophil levels (p=0.018, p=0.027). A statistically insignificant

Table 1. Demographic characteristics and comorbid diseases (n=98)

Age (years), mean±SD (range)	54.3±8.0 (31-69)
Gender, n (%)	
Female	60 (61.2)
Male	38 (38.8)
Diabetes duration (years), median (IQR)	7 (4.75-9)
Diabetic retinopathy, n (%)	4 (4.1)
Diabetic neuropathy, n (%)	22 (22.4)
Hypertension, n (%)	38 (38.8)
Dyslipidemia, n (%)	41 (41.8)
Coronary artery disease, n (%)	10 (10.2)
Cerebrovascular disease, n (%)	2 (2)

SD: Standard deviation, IQR: Interquartile range

increase was detected in lymphocyte and NLR levels (p=0.150, p=0.758). Significant increases were detected in HbA1c and hematocrit levels (p=0.028, p=0.028). A nonsignificant increase was documented in the platelet level (p=0.573).

Discussion

In this study, we observed no significant change in the NLR values in patients with diabetes after dapagliflozin treatment. On the other hand, there was a significant relationship between glucose and NLR, and significant increases in leukocyte, neutrophil, HbA1c, and hematocrit levels. A significant decrease was also observed in glucose and HbA1c levels. There was no significant change in lymphocyte or platelet levels.

DM is not only a metabolic disease but also inflammatory mechanisms play an important role from its development to its complications (19). NLR has been used as an inflammatory marker (20). Concerning potential correlations between NLR, glucose, and HbA1c, different results can be encountered in the literature. Buyukkaya et al. (21) showed a positive association between serum glucose and NLR elevation. Although Verdoia et al. (8) reported a statistically significant positive correlation between NLR and serum glucose, they did not observe any relationship between NLR and HbA1c. However, Sefil et al. (22) showed a positive association between NLR and HbA1c levels. Kim et al. (23) also reported a positive correlation between NLR and fasting plasma glucose in T2DM patients, but they could not find a relationship between NLR and HA1c. In our study, a positive statistically significant correlation was noted between NLR and glucose in T2DM patients, and no significant relationship was found between NLR and HbA1c. NLR values may differ according to age (24). The different ages of the patients included in the studies may have been effective in obtaining the different results between NLR and HbA1c in the studies. The patient ages in our study were similar to those in the study by Kim et al. (23).

Several authors have shown the effects of SGLT-2 inhibitors on inflammation. Maayah et al. (25) demonstrated the positive effects of empagliflozin on lipopolysaccharide-induced septic shock in mice. It has also been shown that empagliflozin reduces systemic and renal inflammation that leads to the development of sepsis-related kidney injury. Heerspink et al. (26) showed that canagliflozin decreases the levels of TNF receptor-1, IL-6, matrix metalloproteinase-7, and fibronectin-1 in patients with T2DM. Kohlmorgen et al. (27) reported that dapagliflozin did not affect leukocyte, neutrophil, and lymphocyte counts. Although there was a significant increase in leukocyte and neutrophil levels in our study, there was no change in NLR values. Wortmann et al. (28) reported that empagliflozin increased neutrophil levels and improved neutrophil functions by decreasing the intracellular 1,5-anhydroglucitol-6-phosphate (1,5-AG-6-P) level in patients with glycogen storage disease type 1b (GSD1b) (28). Resaz et al. (29) showed improvement in neutrophil functions by reducing the accumulation of 1,5-AG-6-P in myeloid cells in their study

Table 2. Biochemical and hematological parameters before and after dapagliflozin in patients with T2DM

	Before dapagliflozin treatment	After dapagliflozin treatment	p
Glucose (mg/dL), mean±SD	199.63±73.24	164.30±53.07	<0.001*
HbA1c (%), mean±SD	8.92±1.70	8.01±1.63	<0.001*
BUN (mg/dL), mean±SD	13.90±3.50	14.50±3.46	0.07
Creatinine (mg/dL), mean±SD	0.79±0.08	0.80±0.14	0.614
Leukocyte (10^3/mm^3), mean±SD	7.79±1.35	8.36±1.92	0.018*
Neutrophil (10^3/mm^3), mean±SD	4.44±1.08	4.84±1.37	0.027*
Lymphocyte (10^3/mm^3), mean±SD	2.56±0.67	2.72±0.81	0.150
NLR, mean±SD	1.86±0.76	1.89±0.68	0.758
HbA1c (g/dL), mean±SD	13.12±1.76	13.69±1.83	0.028*
Hematocrit (%), mean±SD	40.05±4.94	41.64±5.10	0.028*
Platelet (10^3/mm^3), mean±SD	267.24±66.72	261.64±71.53	0.573

*p<0.05.
NLR: Neutrophil lymphocyte ratio, HbA1c: Glycated hemoglobin, BUN: Blood urea nitrogen, T2DM: Type 2 diatebes mellitus

Table 3. Pearson correlation analysis between glucose and HbA1c and NLR before dapagliflozin treatment in patients with T2DM

	NLR	
	r*	p
Glucose	0.206	0.043**
HbA1c	0.140	0.170

*r: Pearson's correlation coefficient, **p<0.05.
HbA1c: Glycated hemoglobin, NLR: Neutrophil lymphocyte ratio, T2DM: Type 2 diatebes mellitus

on induced GSD1b mouse models. In our study, the increase in neutrophil count may be due to the effect of dapagliflozin on the intracellular 1,5-AG-6-P level.

Hematocrit levels increase during SGLT-2 inhibitor therapy (30). Since this increase in hematocrit during SGLT-2 inhibitor treatment was accompanied by an increase in the BUN creatinine ratio, it was interpreted that it could be related to hemoconcentration due to the diuretic effect of the SGLT-2 inhibitor (31). Recent studies show that this increase in hematocrit is not solely due to hemoconcentration. It is estimated that erythropoiesis increases with treatment with SGLT-2 inhibitor treatment (32). In patients with diabetes, the erythropoietin level increases after the initiation of SGLT-2 inhibitors (33). After an increase in the number of reticulocytes, HbA1c and hematocrit levels increase (33). Ghanim et al. (34) showed that another mechanism may be effective in the increase in hematocrit values. They suggested that the increase in hematocrit values was related to increased erythropoiesis due to decreased hepcidin levels after dapagliflozin treatment (34).

In our study, a statistically significant increase was observed in HbA1c and hematocrit levels before and after dapagliflozin treatment. This increase in HbA1c and hematocrit values was not accompanied by a significant increase in BUN and creatinine values, which we can count as hemoconcentration indicators. As mentioned in the literature (32,34), this increase may be due to the improvement of possible tubulointerstitial damage in our patients, as well as to the increase in erythropoiesis due to the decrease in hepcidin level.

SGLT-2 inhibitors are recommended in the ADA 2021 diabetes guideline as the second choice after lifestyle modification and metformin therapy in patients with atherosclerotic cardiovascular disease and chronic kidney disease (35). Ferrannini et al. (36) reported that dapagliflozin monotherapy provided a statistically significant reduction of 0.66% in HbA1c after 24 weeks of treatment compared to placebo. In our study, a statistically significant decrease of 0.91% in mean HbA1c was detected after 12 weeks of treatment with dapagliflozin 10 mg once daily.

This study has some limitations. This study was a retrospective study. The most important limitation is the lack of detailed information about the use of drugs and/or the diseases affecting the biochemical and hematological parameters of the patients. The absence of a control group is also among the limitations of the study.

Conclusion

This study showed that treatment of patients with T2DM with dapagliflozin beyond 12 weeks was not associated with a significant change in NLR, despite alterations observed in some other hematological indices. Changes in hematological parameters in patients using dapagliflozin suggest that leukocytes, neutrophils, lymphocytes, and NLR cannot be used as inflammatory markers in these patients.

Ethics

Ethics Committee Approval: Approval for this study was given by the University of Health Sciences Türkiye, Haydarpasa Numune Training and Research Hospital (protocol no: 5386, date: 22.04.2019).

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: S.B, F.M.T., Design: S.B, F.M.T., Data Collection or Processing: S.B., Analysis or Interpretation: S.B., Literature Search: S.B, F.M.T., Writing: S.B.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2021.85057
Gulhane Med J 2022;64:222-8

Characteristics of fetal conotruncal heart anomalies

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Date submitted:
29.11.2021
Date accepted:
24.12.2021
Online publication date:
08.09.2022

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Keywords: Conotruncal heart anomalies, fetal echocardiography, prenatal diagnosis, ultrasonography

ABSTRACT

Aims: We investigated the clinical characteristics and pregnancy outcomes of conotruncal heart anomalies (CTAs), which constitute a significant part of congenital heart diseases in the prenatal period.

Methods: This study analyzed patients with CTA diagnosed between 2015 and 2020. The incidence of tetralogy of Fallot (TOF), transposition of the great arteries (TGA), double outlet right ventricle (DORV), truncus arteriosus (TA), and interrupted aortic arch type B (IAA-B) was identified. The time of diagnosis and accuracy of CTAs, concomitant anomalies (cardiac and extracardiac), and chromosomal disorders observed together were examined. Pregnancy outcomes and neonatal survival rates were evaluated.

Results: A total of 396 congenital heart diseases were examined. CTA was diagnosed in 102 (25.8%) of the patients. Ninety-nine patients with available data were analyzed. A total of 33 (33.3%) patients were diagnosed with TOF, 30 (30.3%) with DORV, 16 (16.2%) with TA, 16 (16.2%) with TGA, and 4 (4%) with IIA-B. CTA was an isolated anomaly in 28 (28.3%) of the patients. The other patients had one or more accompanying cardiac/extracardiac anomalies. The prenatal invasive diagnostic was examined in 40 (40.4%) of the patients, and chromosomal anomalies were detected in 16 (40%) of them. In 18 (18.4%) of the patients, pregnancy was terminated at the request of the parents. Intrauterine demise occurred in 4 (4.9%) pregnancies that were not terminated, and neonatal death occurred in 26 (32.1%) of the newborns.

Conclusions: The presence of additional cardiac, extracardiac, and genetic abnormalities are common in CTAs and are associated with adverse outcomes.

Introduction

During the embryological development of the heart, abnormalities in the division and rotation of the primordial heart tube lead to the formation of conotruncal heart anomalies (CTAs), which are characterized by impaired ventriculoarterial connection (1,2). CTAs are a type of congenital heart disease that includes anomalies such as tetralogy of Fallot (TOF), transposition of the great arteries (TGA), double outlet right ventricle (DORV), truncus arteriosus (TA), and interrupted aortic arch type B (IAA-B) (3,4).

CTAs constitute 10-12% of congenital heart diseases in the postnatal series (5). Their prevalence in the prenatal period ranges between 16% and 30%, with variable diagnostic accuracy (4,6,7). Because each defect has more than one variation, making a definitive diagnosis in the prenatal period is challenging (4,8). The diagnosis rates and diagnostic accuracy have gradually increased in parallel with advances in cardiac screening programs and techniques (4).

The course of pregnancies in patients with CTAs is associated with concomitant structural and genetic abnormalities. In the absence of concomitant comorbidities, the prognosis

depends primarily on the anatomy of the lesion (9). In cases where postnatal cardiac adaptation is impaired, the need for intervention in the early stages of life makes prenatal diagnosis a crucial factor (2,10).

This study primarily evaluated data from fetuses with CTAs diagnosed in the perinatology and pediatric cardiology units, and examined the accompanying cardiac/extracardiac structural anomalies, genetic disorders, and outcomes of pregnancy. In this way, a reference that can be used in prenatal counseling during pregnancies diagnosed with fetal CTA will be created.

Methods

Study design

This single-center and retrospective study analyzed data from the patients followed up after a diagnosis of fetal CTA in the perinatology and pediatric cardiology units of a tertiary hospital, between 2015 and 2020. The study was approved by the University of Health Sciences Türkiye, Etlik Zubeyde Hanim Gynecological Diseases Training and Research Hospital Local Ethics Committee (decision no: 03, date: 14.02.2020). Owing to the retrospective nature of the study, the need for informed consent was waived. Data were obtained from the hospital's electronic record system and patient files. The demographic characteristics of the patients, history of diabetes, diagnosis of congenital heart disease in a previous pregnancy, gestational week at diagnosis, CTA type, accompanying cardiac and extracardiac anomalies, chromosomal disorders, and pregnancy outcomes were analyzed.

Fetal echocardiography

The study included subjects who underwent fetal echocardiography between the abovementioned dates. In the routine clinical protocol, fetal echocardiography is performed by an experienced perinatology specialist and a pediatric cardiologist. The final diagnosis is made, and follow-up procedures are planned by a shared decision. The same device and 4C probe were used for all patients in the analysis (Voluson E6 convex volumetric probe, GE Healthcare, Milwaukee, WI, USA; Vivid S6 ultrasound, GE Medical Systems, Horten, Norway). The clinical protocol during the study period agreed with the ISUOG guidelines. In the routine, following the examination of the upper abdominal image, four-chamber view of the heart, ventricular outflow tracts, three vessels, three vessels and tracheal sections, pulmonary and systemic venous connections, ductal and aortic arch images, and Doppler flow patterns in the heart and veins (11,12), CTA is diagnosed by assessing the cardiac examination plans, particularly ventricular outflow tracts, three-vessel view (3VV), three-vessel trachea view, ductal and aortic arch images, and blood flow patterns.

Working protocol

Patients with a prenatal diagnosis of TOF, TGA, TA, DORV, and IAA type B were included in the study (Figures 1 and 2). In routine practice, patients with a suspected cardiac anomaly are screened for fetal anatomy by an experienced perinatology specialist. Patients should are informed of the detected CTA and any other abnormalities, and a prenatal invasive testing procedure appropriate for the week of gestation is recommended to detect concomitant genetic disorders. Karyotype analysis is recommended in line with standard indications such as advanced maternal age, increased risk of aneuploidy in the first or second trimester screening test, and the presence of malformations on ultrasound. Chromosomal microarray (A-CGH) and deletion of 22q11 study are recommended along with karyotype analysis for all patients with CTA.

Parents are given detailed advice on the characteristics, treatment options, and prognosis of the anomaly by a team consisting of a maternal-fetal medicine specialist and a

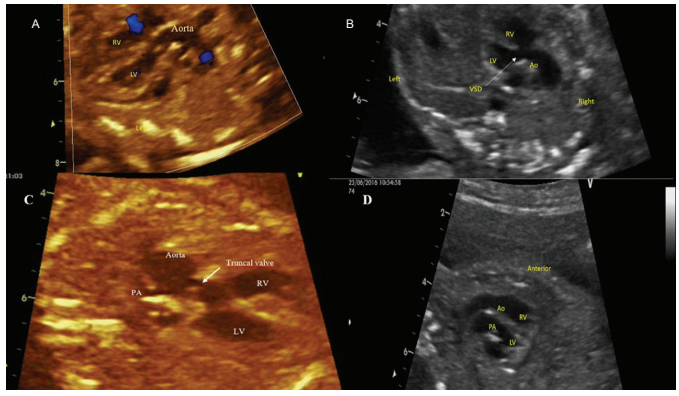

Figure 1. A) Normal left ventricle outflow tract, B) tetralogy of Fallot, overriding aorta and subaortic ventricular septal defect, C) truncus arteriozus with common truncal valve, D) transposition of great arteries, parallel course of great arteries on outlet section

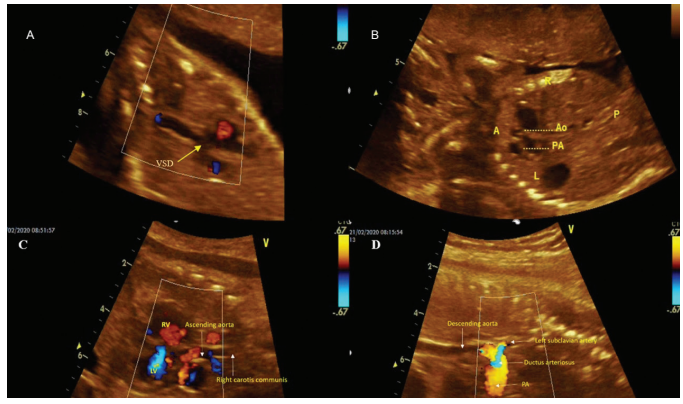

Figure 2. A, B) Double outlet right ventricle and aorta lie side by side on the right of the pulmonary artery, C, D) interrupted aortic arch tip B, ascending aorta connects to the right common carotid artery; the left subclavian artery exits from the distal aorta

pediatric cardiologist. The final decision regarding the follow-up or termination of pregnancy is made following the decision of the parents. In cases of intrauterine death or termination of pregnancy, an autopsy is recommended. If patients enrolled in the study according to the above-described algorithm did not have a prenatal invasive genetic diagnosis and had no karyotyping in the postnatal period, their karyotype was considered normal according to the pediatric clinical examination. Finally, patients with a confirmed diagnosis of CTA in the postnatal period were included in the study.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences 26 (SPSS) (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test was used to assess whether the data were distributed normally. Non-normally distributed numerical data were compared using the Kruskal-Wallis test and were expressed as medians (interquartile range). Categorical data are expressed as numbers (percentages). A p-value of <0.05 was considered statistically significant.

Results

During the analyzed period, 396 patients were diagnosed with congenital heart disease. CTA was detected in 102 (25.8%) fetuses. Three patients whose postnatal results could not be acquired were excluded from the study. Thus, data from 99 patients were analyzed. A total of 33 (33.3%) patients were diagnosed with TOF, 30 (30.3%) with DORV, 16 (16.2%) with TA, 16 (16.2%) with TGA, and 4 (4%) with IAA-B. Except for the cases of pregnancy termination and intrauterine demise, the diagnosis was confirmed during postnatal echocardiography, cardiac catheterization, or surgery in all patients. The median maternal age of the patients included in the study was 27 (18-44) years. The median gestational age at the time of diagnosis was 22 (17-37) weeks. No significant difference was observed between the CTA subgroups in terms of maternal age or gestational week at diagnosis (p=0.127 and p=0.781, respectively). Pregestational diabetes was detected in 10% of the patients with TOF. Of the entire cohort, 2% of patients had a history of congenital heart disease in their previous pregnancy. Table 1 presents the subtypes, clinical features, and pregnancy outcomes of CTAs in patients.

Karyotype analysis

The prenatal invasive diagnostic was examined in 40 (40.4%) of the study population. A-CGH was performed on 20 (50%) of these pregnant women who underwent prenatal diagnostic testing. The prevalence of the chromosomal anomaly was 16 (16.2%) in the entire cohort. The most chromosomal anomaly was detected in the DORV subgroup with a rate of 4%. Aneuploidies were identified as the most common group of chromosomal abnormalities. Table 1 presents pregnancy outcomes and chromosomal disorders of the entire study population by subtypes of CTA. These outcomes appeared worse in cases of DORV and IAA-B.

Additional anomalies

Overall, 28.3% of the cohort had isolated CTA. Among the CTAs, TOF was the most common isolated anomaly. TGA was the subgroup most frequently accompanied by an additional cardiac anomaly [except the ventricular septal defect (VSD)]. The additional extracardiac anomaly was most common in the IAA-B subgroup. Among the subgroups, TA was the group in which the association of both additional cardiac and extracardiac anomalies was most common. Tables 2 and 3 present the associated cardiac and extracardiac anomalies with CTAs.

Discussion

CTAs are a type of congenital heart disease with a high incidence of accompanying structural anomalies and chromosomal disorders. It is characterized by severely adverse fetal and neonatal outcomes. The data obtained in this study serve as a reference for prenatal counseling to parents for fetal CTA in the antenatal period.

Congenital heart diseases are the most common cause of infant death due to congenital anomalies. Their prevalence in live births is 6-10 per 1,000 neonates (13,14). CTAs are a subgroup of congenital heart diseases that may need immediate postnatal intervention (2). Diagnosis of CTAs is made by visualizing the ventricular outflow tracts and major vascular structures and examining their association with echocardiography. The literature emphasizes that cases with CTA in the prenatal period constitute 16-30% of patients with congenital heart disease (4,6,7,15). In our study, patients with CTAs constituted 25.8% of all patients with congenital heart disease cases, which agreed with the literature.

The accuracy of prenatal diagnosis of CTAs is low because of the difficulties in identifying the association between the ventricles and great arteries and the great arteries themselves. The diagnosis rates have increased steadily with the inclusion of cardiac outflow tract and 3VV in prenatal fetal cardiac screening programs (4,16,17). The postnatal confirmation rate of prenatal diagnosis was 88% in our study. The most common misdiagnosis was noted in the DORV group. This finding was consistent with the literature (4,7,18). The diagnostic accuracy rates of CTA increase with sequential echocardiographic examinations performed during the prenatal period. In our study, the median number of fetal echocardiography performed in the cases was two. The median gestational age at diagnosis was 22 weeks in our study, which was earlier than that reported in some recent studies (7,19). In this study, the high rate of extracardiac abnormalities led to CTA being diagnosed at early weeks of gestation, as in the study by Lin et al. (20).

Table 1. Basic characteristics of the study population and pregnancy outcomes

	TOF	DORV	TA	TGA	IAA-B	p*	Total
Number of cases, n (%)[a]	33 (33.3)	30 (30.3)	16 (16.2)	16 (16.2)	4 (4.0)		99 (100)
Maternal age, median (IQR)[b]	24 (9)	29 (8)	28 (11)	29 (7)	24 (8)	0.127	28 (8.0)
Gestational weeks at diagnosis, median (IQR)[b]	21.5 (7)	22 (7)	22 (3)	21 (7)	21 (12)	0.781	22 (7.0)
Congenital heart disease in past pregnancy, n (%)[a]	1 (3.0)	1 (3.3)	-	-	-		2 (2.0)
Diabetes mellitus, n (%)[a]	2 (6.1)	3 (10)	1 (6.3)	1 (6.3)	-		6 (6.1)
Additional anomalies, n (%)[a]							
None	14 (42.4)	8 (26.7)	2 (12.5)	3 (18.8)	1 (25)		28 (28.3)
Cardiac	4 (12.1)	10 (33.3)	5 (31.3)	9 (56.3)	1 (25)		29 (29.3)
Extracardiac	7 (21.2)	4 (13.3)	4 (25)	1 (6.3)	2 (50)		18 (18.2)
Cardiac+extracardiac	8 (24.2)	8 (26.7)	5 (31.3)	3 (18.8)	-		24 (24.2)
Karyotype analysis, n (%)[a]							
Normal	28 (84.8)	22 (73.3)	14 (87.5)	16 (100)	3 (75)		83 (83.8)
Abnormal	5 (15.2)	8 (26.7)	2 (12.5)	-	1 (25)		16 (16.2)
Trisomy 21	3 (9.1)	1 (3.3)	-	-	-		
Trisomy 18	-	3 (10.0)	-	-	-		
Trisomy 13	-	3 (10.0)	1 (6.3)	-	-		
Trisomy 9	-	1 (3.3)	-	-	-		
22q11 del	2 (6.1)	-	1 (6.3)	-	1 (25)		
Pregnancy outcomes, n (%)[a]							
Termination	4 (12.9)	8 (26.7)	3 (18.8)	1 (6.3)	2 (40.0)		18 (18.4)
IUFD	-	33 (10.0)	-	1 (6.3)	-		4 (4.1)
Neonatal death	4 (12.9)	10 (33.3)	2 (12.5)	7 (43.8)	3 (60.0)		26 (26.5)

[a]Number (percentage); [b]Median (interquartile range); *Kruskal-Wallis test. p <0.05 shows statistical significance.
DORV: Double outlet right ventricle, IAA-B: Interrupted aortic arch type B, IUFD: Intrauterine fetal demise, TA: Truncus arteriosus, TGA: Transposition of the great arteries, TOF: Tetralogy of Fallot, 22q11 del: 22q11 microdeletion

Table 2. Cardiac anomalies accompanying CTAs

TOF (33)	DORV (30)	TA (16)	TGA (16)	AA-B (4)
ASD	ASD	PLSVC	PLSVC	VSD
AVSD	AVSD	ARSA	Mitral atresia	PLSVC
PLSVC	PLSVC	Partial APVC	Tricuspid atresia	ARSA
Right aortic arch	ARSA	Tricuspid regurgitation	Tricuspid regurgitation	Aortic hypoplasia
Sinus bradycardia	Aortic stenosis		Pulmonary stenosis	Heterotaxia
Ductus arteriosus agenesis	Mitral atresia		Pulmonary hypoplasia	
Heterotaxia	Aortic hypoplasia		Aortic hypoplasia	
Absent pulmonary valve	Tricuspid regurgitation		Left ventricle hypoplasia	
Double inlet left ventricle	Tricuspid atresia		Right ventricle hypoplasia	
	Pulmonary stenosis		Single ventricle	
	Pulmonary hypoplasia		Heterotaxia	
	Left ventricle hypoplasia			
	Total APVC			
	Sinus bradycardia			
	Heterotaxia			

APVC: Anomalous pulmonary venous connection, ARSA: Aberrant right subclavian artery, ASD: Atrial septal defect, AVSD: Atrioventricular septal defect, CTA: Conotruncal heart anomalies, DORV: Double outlet right ventricle, IAA-B: Interrupted aortic arch type B, PLSVC: Persistent left superior vena cava, TA: Truncus arteriosus, TGA: Transposition of the great arteries, TOF: Tetralogy of Fallot, VSD: Ventricular septal defect

Table 3. Extracardiac anomalies accompanying CTAs

TOF (33)	DORV (30)	TA (16)	TGA (16)	IAA-B (4)
Cystic hygroma	Cystic hygroma	Cystic hygroma	Hydrocephalus	Cystic hygroma
Ventriculomegaly	Ventriculomegaly	Ventriculomegaly	Hydronephrosis	Thymus hypoplasia
CCA	CCA	Mega cisterna magna	SUA	Micrognathia
Unilateral renal agenesis	Cerebellum hypoplasia	Cephalocele		Cleft palate
Bilateral renal agenesis	Encephalocele	Cerebellum hypoplasia		Hydrops fetalis
Unilateral MDK	Hydrocephalus	Micrognathia		
PRUV	Anencephaly	Renal agenesis		
Intestinal obstruction	Diaphragmatic hernia	Omphalocele		
Anal atresia	Omphalocele	Bilateral MDK		
Bronchogenic cyst	Uroenteric fistula	Pes equinovarus		
Limb anomalies	Hydrops fetalis	SUA		
Sirenomelia type 1	Limb anomaly			
SUA	SUA			

CCA: Corpus callosum agenesis, CTA: Conotruncal heart anomalies, DORV: Double-outlet right ventricle, IAA-B: interrupted aortic arch type B, MDK: Multicystic dysplastic kidney, PRUV: Persistent right umbilical vein, SUA: Single umbilical artery, TA: Truncus arteriosus, TGA: Transposition of the great arteries, TOF: Tetralogy of Fallot

The most common types of CTA observed in the entire cohort were TOF and DORV cases. Cases of TOF comprised the highest percentage (33.3%) of patients with CTAs, consistent with the literature (7,6,9). TOF, which is the most common CTA type, is also the most common cyanotic congenital heart disease. The absence of pulmonary valve syndrome, which is one subgroup of TOF, was noted in 3% of patients, which agreed with the literature (21). VSD + pulmonary atresia (TOF with pulmonary atresia), previously designated as a severe case of TOF, was observed in one patient (22). Cases with DORV accounted for 30% of the study population and 7.6% of all congenital heart diseases. The frequency of DORV cases among CTAs was higher than that reported in the literature but was similar to a recent study (9). Consistent with previous studies, the IAA-B group was the least common type of CTA (3,8).

The complex pathophysiology underlying the CTA explains the excess of accompanying anomalies. In the study cohort, 42.4% CTAs had extracardiac anomalies. Prior studies of fetal CTAs reported rates of extracardiac anomalies ranging from 25-37% (4,23). Consistent with the literature, the most common additional extracardiac anomalies in our study were associated with the central nervous system, genitourinary system, limbs, and cystic hygroma (4). Additional cardiac anomalies were found in 47.5% of the cases. The most common additional cardiac anomalies associated with CTA in the study population were septal defects and heterotaxy syndromes. Galindo et al. (7) reported an increase in nuchal translucency (NT) thickness in the first trimester in 72% of fetuses with CTA. In this study, there are not enough data about NT measurements of fetuses. However, notably, 10% of the patients have a single umbilical artery, which is a soft marker. The rate and distribution of associated cardiac/extracardiac anomalies in our study are similar to those reported by Lin et al. (20), who analyzed 129 fetuses with CTA. Key components of current management strategies for CTAs include the identification of required postpartum intervention and delivery at a tertiary care center with expertise in neonatal care. Therefore, fetal anatomy should be carefully examined in terms of concomitant anomalies in fetuses with CTA detected during the prenatal period (24,25).

CTAs are multifactorial anomalies and genetic and environmental factors play a role in their etiopathogenesis. Genetic anomalies are observed with different characteristics and frequencies in CTA subtypes. In our study, the rate of genetic disorders was 16.2%, which agreed with the findings of a study by Sivanandam et al. (4). The most common genetic abnormalities were aneuploidies and 22q11 microdeletion. Consistent with the literature, these genetic anomalies were observed more frequently in the presence of accompanying structural anomalies such as right aortic arch, abnormal right subclavian artery, and thymic hypoplasia/aplasia (26,27). The 22q11 microdeletion detected in this cohort was observed in patients with TA, IAA-B, TOF, and absence of pulmonary valve syndrome; this finding was consistent with the literature (28,29). Consistent with the literature, patients with TGA did not have chromosomal abnormalities, and genetic disorders were more common in the DORV cases (8,30). In a recent study, the genetic imbalance was found in 38% of cases with CTA using the A-CGH method (31). In our study, the genetic anomalies were detected at a lower rate, possibly because not all patients underwent an A-CGH examination.

CTAs are a group of cardiac anomalies that are characterized by poor clinical outcomes (4,7). The neonatal mortality rate in

our study was higher than that reported in two recent studies (9,23). It is possible to explain this situation with an excess of accompanying structural anomalies. In our study, survival after the neonatal period was higher in patients with isolated anomalies. This finding was more prominent in patients with TOF.

The limitations of our study lie in its retrospective design, lack of chromosome analysis in the whole sample of patients, and lack of autopsy in cases of pregnancy termination and intrauterine fetal demise. A low number of subjects in the IIA-B subgroup is considered another limitation.

Conclusion

In conclusion, this study displays the structural (cardiac and extracardiac) anomalies and genetic disorders accompanying CTAs and their impact on adverse outcomes.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Türkiye, Etlik Zubeyde Hanim Gynecological Diseases Training and Research Hospital Local Ethics Committee (decision no: 03, date: 14.02.2020).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: O.Y., D.Ş., A.Y., Design: A.K., O.Y., D.Ş., Data Collection, or Processing: A.K., Ö.Y.Ç., G.D., Analysis, or Interpretation: A.K., Ö.Y.Ç., G.A.Y., Literature search: A.K., G.D., G.A.Y., Writing: A.K., O.Y.

Conflict of Interest: The authors declare they have nothing to disclose regarding conflict of interest concerning this manuscript.

Financial Disclosure: The authors declare that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.92905
Gulhane Med J 2022;64:229-34

Efficacy of ifosfamide, carboplatin and etoposide chemotherapy protocol in relapsed refractory germ cell tumors

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Date submitted:
02.11.2021
Date accepted:
04.01.2022
Online publication date:
08.09.2022

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Keywords: Germ cell tumor testicular cancer, ifosfamide, carboplatin, etoposide

ABSTRACT

Aims: This study aimed to evaluate the overall survival (OS) after the ifosfamide carboplatin plus etoposide (ICE) protocol for patients with relapsed refractory germ cell tumors (GCT). Differences in the contribution of the ICE protocol to patient survival according to the number of the treatment lines (four or more lines vs. three or fewer lines) were also evaluated.

Methods: This retrospective cross-sectional study included patients with relapsed refractory GCT who had previously received multiple-line chemotherapy. Gender, age, clinical stage at diagnosis, tumor marker levels, visceral metastasis status, previous treatment protocols, response rates to ICE, follow-up time, and hematologic side effects were recorded. The primary endpoint was OS after the ICE. The secondary endpoint was the difference in OS between patients who received the ICE on the fourth and subsequent lines vs. those who received the third and previous lines.

Results: The final sample included 15 patients (median age 26; males: 93.3%). At diagnosis, 80% of the patients had stage IIIC disease. The median OS in the whole group was 14.0 [interquartile range (IQR) 15.4] months. The median (IQR) OS after the ICE protocol in patients with three or fewer lines was significantly higher than in those who received four or more lines [21.6 (34.5) vs 10.8 (11.6), p=0.034]. Grade 3 neutropenia (46.6%), anemia (40%), and thrombocytopenia (40%) were frequently observed.

Conclusions: For heavily pretreated relapsed/refractory GCT, the ICE may show the potential to provide a significant survival. However, more severe hematological side effects may be encountered

Introduction

Germ cell tumors (GCT) are among the most common solid malignancies in the male population, especially in the second and third decades (1). Especially with the platinum-based treatment approach, a good response can be obtained even in advanced GCT patients. The 5-year overall survival (OS) for advanced disease is 80-90% (2).

There is an established systemic treatment approach in the first, second and third lines of advanced GCT. However, chemotherapy protocols that can be preferred in the fourth and next lines in patients with good performance status despite having a resistant disease are lacking (3-6). In this sense, it may be reasonable to evaluate potential treatment options in patients who are few but still need therapy.

The ifosfamide, carboplatin plus etoposide (ICE) protocol has been frequently preferred as a high-dose chemotherapy approach (7,8). However, this approach is usually used in the early lines (7). To the best of our knowledge, there is little information on the use of the ICE protocol in later lines and there is no recent up-to-date data. Considering the abovementioned information, we showed the patient characteristics, posttreatment survival time, and side effects of the ICE protocol in all relapsed refractory GCT patients. As a general oncological concept, the

survival benefit provided by the transition to each advanced line therapy is considered to decrease. Therefore, we also aimed in the current study to show whether the use of the ICE protocol in patients receiving four or more lines of chemotherapy shows the same impact on survival compared to those receiving three or fewer chemotherapy lines.

Methods

Study design and population

This retrospective, single-center study was performed using the medical records of outpatients and inpatients with relapsed/refractory GCT from a tertiary clinic from January 2017 to June 2021. The inclusion criteria were age greater than or equal to 18 years, those with histologically confirmed advanced stage testicular cancer, imaging-proven metastases at diagnosis, or recurrent disease. The exclusion criteria were age <18 years and insufficient clinical data. This study was approved by the Gülhane Training and Research Hospital Local Ethics Committee (protocol number: 2021/58, date: 29.09.2021). Gender, age, localization, the histology of the primary malignancy, and stage at the time of diagnosis were recorded. Lung, liver, bone, and lymph node metastasis status before the ICE protocol, serum tumor marker status, and International Germ Cell Cancer Collaboration Group (IGCCCG) risk group and previous systemic treatments (BEP: Bleomycin, Cisplatin, Etoposide. TIP: Paclitaxel, Ifosfamide, and Cisplatin. HDC: High Dose Chemotherapy. GEMPOX: Gemcitabine, Paclitaxel, and Oxaliplatin) were evaluated within the scope of the study (9). Response to treatment and observed side effects were recorded after the ICE protocol. Survival after the ICE protocol, survival times from the first diagnosis, and survival status (alive/died) were evaluated. The patients were divided into two according to the systemic treatment lines they received before the ICE protocol (four or more lines vs. three or fewer lines). The interval between the first diagnosis and the ICE protocol is defined as the time between the first diagnosis of the patient and the date of starting the ICE protocol. OS after ICE protocol was calculated as the time from the start of the ICE protocol to the last seen date or the patient's death date. The interval between the first diagnosis and last visit is defined as the time elapsed from the diagnosis to the last follow-up visit or death. The ICE protocol was as follows: ifosfamide 1667 mg/m^2/day for 3 days, mesna 1667 mg/m^2/day for 3 days, carboplatin AUC 5 for a single day, etoposide 100 mg/m^2/day for 3 days. Granulocyte colony-stimulating factor (G-CSF) administration is recommended routinely after the ICE protocol.

Study endpoints

The primary endpoint of the study was to demonstrate OS in the entire group after the ICE protocol. Additionally, any difference between the survival times according to the systemic treatment lines they received before the ICE protocol (four or more lines vs. three or fewer lines) was also studied.

Definition of complete response, partial response, progressive disease, and stable disease

Complete remission was defined as the disappearance of all clinically and radiologically detectable lesions and the normalization of tumor markers. A More than 20% reduction in tumor burden was defined as partial response (PR). A tumor growth greater than 20% was defined as Progressive Disease. Any other response was classified as a stable disease (10).

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Descriptive data are presented as a percentage of the total. The normality of the continuous variables was examined using the Kolmogorov-Smirnov test. Normally distributed continuous data were expressed as mean±standard deviation and non-normally distributed were expressed as median [interquartile range (IQR)]. Between-group differences were tested using the chi-square test, Student's t-test, or Mann-Whitney U test, as appropriate. A p-value less than 0.05 was considered statistically significant.

Results

Main characteristics

The final sample included 15 patients (median age 26; males: 93.3%). The median age was 26 (IQR: 14). Eighty percent of the sample had stage IIIC disease at the time of initial diagnosis. All GCTs were in the non-seminomatous histological type. In 73.3% of patients, the serum tumor marker levels were S3 [S3: lactate dehydrogenase (LDH) >10 × ULN or hCG (mIU/mL) >50,000 or AFP (ng/mL) >10,000]. According to the IGCCCG risk classification, 66.6% of the patients were in the poor risk category. In both groups, lung and liver metastases were frequently detected before the ICE protocol (53.3% for lung metastases and 80% for liver metastases, respectively). Patients who received ≥4 lines of treatment before the ICE protocol received the BEP, TIP, HDC, and GEMPOX protocols, respectively. The characteristics of the patients are presented in Table 1.

Response rates and side effects

The response rate to the ICE protocol (sum of complete response rate and PR rate) was 53.2%. The median OS in the general group after ICE was 13.9 (IQR: 15.3) months. The patients were divided into two groups according to the order in which they received the ICE protocol (four or more lines vs. three or fewer lines). The median OS (IQR) after the ICE protocol in patients with three or fewer lines was significantly higher than

Table 1. Demographic and clinical characteristics of the patients

	Total (n=15)	Before ≤3 lines of systemic therapy (n=6)	Before ≥4 lines of systemic therapy (n=9)
Gender, male, n (%)	14 (93.3)	6 (100)	8 (88.8)
Age, years, median (IQR)	26 (14)	23.5 (11.8)	31 (14)
Clinical stage (AJCC 8th), n (%)			
I	2 (13.3)	1 (16.6)	1 (11.1)
IIIB	1 (6.6)	-	1 (11.1)
IIIC	12 (80.0)	5 (83.3)	7 (77.7)
Serum tumor markers, n (%)			
S0	3 (20.0)	1 (16.6)	2 (22.2)
S1	1 (6.6)	-	1 (11.1)
S3	11 (73.3)	5 (83.3)	6 (66.6)
IGCCCG risk groups, n (%)			
Good risk	3 (20.0)	1 (16.6)	2 (22.2)
Intermediate risk	1 (13.3)	-	3 (22.2)
Poor risk	10 (66.6)	5 (83.3)	5 (55.5)
Visceral metastasis, n (%)			
Lung	8 (53.3)	3 (50.0)	5 (55.5)
Liver	12 (80.0)	5 (83.3)	7 (77.7)
Bone	3 (20.0)	2 (33.3)	1 (11.1)
Systemic treatments before ICE protocol, n (%)			
BEP+TIP+HDC+GEMPOX	9 (60.0)	-	9 (100)
BEP+TIP	4 (26.6)	4 (66.6)	-
BEP+TIP+VIP	2 (13.3)	2 (33.3)	-

AJCC 8th: The eighth edition American Joint Committee on Cancer, S0: Marker's blood level within normal limits, S1: LDH <1.5 × ULN, hCG (mIU/mL) <5000 and AFP (ng/mL) <1000, S2: LDH: 1.5 to 10 × ULN or hCG (mIU/mL) 5000 to 50,000 or AFP (ng/mL) 1000 to 10,000, S3: LDH >10 × ULN or hCG (mIU/mL) >50,000 or AFP (ng/mL) >10,000, IGCCCG: International Germ Cell Cancer Collaborative Group, ICE: Ifosfamide, carboplatin, etoposide chemotherapy protocol, BEP: Bleomycin, cisplatin, etoposide, TIP: Paclitaxel, ifosfamide and cisplatin, HDC: High dose chemotherapy, GEMPOX: Gemcitabine, paclitaxel and oxaliplatin, VIP: Ifosfamide, etoposide, cisplatin, RBPC: Red blood packed cells, PAS: Platelet additive solution, IQR: Interquartile range

in those who received four or more lines [21.6 (34.5) vs 10.8 (11.6), p=0.034]. Grade 3 neutropenia (46.6%), anemia (40%), and thrombocytopenia (40%) were frequently observed in all patients. Treatment side effects and responses to treatment are presented in Table 2.

Discussion

GCT is considered among the chemosensitive malignancies like lymphomas. However, the therapeutic management of patients with relapsed/refractory GCT is still challenging. Despite repeated chemotherapy lines and even HDC treatment, there are still patients with residual tumor burden and good performance status. Although the number of patients reaching this clinical condition is generally low, chemotherapy protocols that are expected to be effective in patients who are still relapsed or refractory despite receiving multi-line therapy are needed. The ICE protocol is an approach that comes to the fore at this point. In this study, the use of the ICE protocol was evaluated in patients with relapsed/refractory GCT who received multi-line therapy. There was a significant difference in the time to the last visit after ICE in patients who received ≤3 lines of treatment before compared to those who received ≥4 lines of treatment. The explanation for such a difference may be the higher fragility and lower performance of patients receiving more chemotherapy. Thanks to the use of chemotherapy protocols containing cisplatin, especially in patients with extensive visceral metastases and different negative prognostic features, advanced GCT can be successfully treated initially (5,6). All patients included in our study had been treated with combination chemotherapy protocols containing at least two lines of cisplatin in the past. The majority of the patients in the current study had liver metastases before the ICE protocol. The liver is the most common site of extrapulmonary organ metastases in patients with advanced GCT (11). In contrast to lung metastases, liver, brain, and bone metastases represent a poor prognostic feature in GCT (12-14). Given that the patients in the current analysis received multi-line therapy, a high frequency of liver metastases may be predictable. Lung metastases were also detected in more than half of our patients.

For men with good-risk advanced testicular GCTs, relapse-

Table 2. Treatment-related characteristics of the patients

	Total (n=15)	Prior to ≤3 lines of systemic therapy (n=6)	Prior to ≥4 lines of systemic therapy (n=9)	p
Best objective response after ICE protocol, n (%)				
Complete response	1 (6.6)	1 (16.6)	-	-
Partial response	7 (46.6)	2 (33.3)	5 (55.5)	-
Stable disease	3 (20.0)	1 (16.6)	2 (22.2)	-
Progressive disease	4 (26.6)	2 (33.3)	2 (22.2)	-
Interval between first diagnosis and ICE protocol, mean (SD), months	51.06 (35.04)	62.16 (41.61)	43.66 (30.20)	0.335
Overall survival after ICE protocol, median (IQR), months	13.95 (15.36)	21.62 (34.54)	10.80 (11.63)	0.034
Interval between first diagnosis and last visit, mean (SD), months	73.77 (43.21)	94.09 (37.25)	60.23 (43.41)	0.143
Haematologic side effects after ICE protocol				
Neutropenia, n (%)				
None	3 (20.0)	1 (16.6)	1 (22.2)	-
Grade 1	4 (26.6)	-	2 (44.4)	-
Grade 2	1 (6.6)	1 (16.6)	-	-
Grade 3	7 (46.6)	4 (66.6)	3 (33.3)	-
Anemia, n (%)				
None	3 (20.0)	1 (16.6)	1 (22.2)	-
Grade 1	2 (13.3)	-	1 (22.2)	-
Grade 2	4 (26.6)	2 (33.3)	2 (22.2)	-
Grade 3	6 (40.0)	3 (50.0)	3 (33.3)	-
Thrombocytopenia, n (%)				
None	3 (20.0)	1 (16.6)	2 (22.2)	-
Grade 1	3 (20.0)	1 (16.6)	2 (22.2)	-
Grade 2	3 (20.0)	1 (16.6)	2 (22.2)	-
Grade 3	6 (40.0)	3 (50.0)	3 (33.3)	-
Febrile neutropenia, n (%)	7 (46.6)	4 (66.6)	3 (33.3)	-
RBPC infusion counts, median (IQR)	2 (6)	3 (7.75)	2 (4)	-
PAS infusion counts, median (IQR)	1.5 (3)	2 (6)	2 (5.67)	-

ICE: Ifosfamide, carboplatin, etoposide chemotherapy protocol, SD: Standard deviation, IQR: Interquartile range, RBPC: Red blood packed cells, PAS: Platelet additive solution

free survival has been reported to be above 70% following first-line chemotherapy. Up to 50% of men with intermediate or poor-risk disease traits with the relapsed disease following first-line chemotherapy require additional treatment (15-18). In our study, most patients had IGCCCG moderate or poor-risk disease, most patients received ≥3 lines of chemotherapy before the ICE protocol. One of the important indicators of prognosis in GCT is the high course of serum tumor markers (12). In our study, serum tumor markers in were in the highest category in most patients, namely, S3 [S3: LDH >10 × ULN or hCG (mIU/mL) >50,000 or AFP (ng/mL) >10,000] before the ICE protocol. This finding may be explained by the resistant clinical course and poor prognosis characteristics of the patients in the current analysis. Patients who show relapse after the second-line chemotherapy and patients who progress within one month of completing the first cisplatin-based chemotherapy or during the treatment are considered platinum-resistant diseases. This group is often treated with HDC therapy. HDC achieves successful endpoints for treating patients with relapsed or treatment refractory GCT. HDC is also a treatment approach that achieves a favorable outcome when used after the second line (19-21). Erturk et al. (22) reported a 1-year progression-free survival (PFS) rate of 57.8% and a 1-year OS rate of 77.5% after a single course of HDC in patients with relapsed/refractory GCT. Additionally, median OS and PFS were 21.5±1.8 and 20±2 months, respectively. In our study, more than half of the patients

had received HDC treatment before. Patients who still require treatment despite receiving this dose-dense treatment are one of the main groups for which survival outcomes are to be evaluated in this study. The treatments available for cases with relapsed or still a residual disease, even after dose-dense therapy such as HDC, are very limited. In these difficult-to-manage clinical situations, gemcitabine-based therapies such as gemcitabine plus oxaliplatin, gemcitabine, oxaliplatin, and paclitaxel (GEMPOX) can be used. Surgery may be recommended if the patient has a residual tumor suitable for surgery. A second HDC treatment may also be recommended if the patient's performance and tumor burden are suitable. GEMPOX therapy has shown a positive impact on PFS and OS endpoints in cases of testicular cancer that relapsed or remained refractory after cisplatin-based chemotherapy (23-25). In a previous study, successful real-life data of the combination of GEMPOX in patients with relapsed or refractory GCTs were reported (26). One-year OS, PFS, and overall response rate were reported to be significantly higher in favor of the clinical benefit. In our study, many patients received the GEMPOX protocol before the ICE protocol. In these patients, we can state that there was a significant median time from ICE to the last visit. This gives us an idea that the ICE protocol is useful in the post-GEMPOX period. Hematological side effects were frequently observed after the ICE protocol in the current analysis, which may be considered acceptable as most patients received multiple-line chemotherapy, including HDC. Although G-CSF was used routinely in our cases, neutropenia was frequently observed (27). However, the frequency of febrile neutropenia did not differ between those who received three or fewer systemic treatments and those who received four or more systemic treatments. Also, the frequency of anemia and thrombocytopenia were similar between the groups. Hence, hematological side effects of the ICE chemotherapy protocol are prominent features beyond the past chemotherapy regimens and bone marrow reserve.

This paper has several limitations. First, the number of patients was low, limiting the generalizability of the findings to different populations. Second, the retrospective design of the study raises the possibility of errors in data quality. Third, since the analysis was cross-sectional, the results cannot be assumed to be causal. Finally, follow-up times and intervals cannot be controlled in retrospective analyses.

Conclusion

In conclusion, the current study that was conducted on a less common, chemotherapy-resistant malignancy with residual tumor requiring advanced-line chemotherapy despite initial multiple-line chemotherapy showed that the ICE protocol was associated with a favorable outcome profile including OS. Nevertheless, hematological side-effects were quite common.

Ethics

Ethics Committee Approval: This study was approved by the Gülhane Training and Research Hospital Local Ethics Committee (protocol number: 2021/58, date: 29.09.2021).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.B.A., İ.E., R.A., G.S.K., N.K., Concept: M.B.A., İ.E., N.K., Design: M.B.A., R.A., N.K., Data Collection or Processing: M.B.A., E.Ö., G.S.K., Analysis or Interpretation: M.B.A., İ.E., N.K., Literature Search: M.B.A., G.S.K., Writing: M.B.A., E.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declare that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.59454
Gulhane Med J 2022;64:235-9

The impact of oocyte denudation without a pre-incubation on intracytoplasmic sperm injection outcomes

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Date submitted:
30.11.2021
Date accepted:
10.01.2022
Online publication date:
08.09.2022

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Keywords: ICSI, multinucleation, denudation, embryo quality, fertilization, pregnancy

ABSTRACT

Aims: Debate over the optimal timing of oocyte denudation following oocyte retrieval continues. Multinucleation has been associated with lower embryo quality and implantation rate. Defining oocyte characteristics might increase the chances of better embryo selection. This study aimed to investigate the laboratory and clinical outcomes of intracytoplasmic sperm injection when the oocytes were denuded immediately after oocyte retrieval.

Methods: A prospective randomized sibling-oocyte study was performed. The participants were under 40 years of age with more than 5 oocytes. Patients with male factors were excluded. The study and control groups were formed by simple randomization. The study group underwent oocyte denudation immediately. In the control group, oocytes were routinely incubated in an equilibration solution for 2 h until the removal of the cumulus cells. Outcome measures were normal fertilization (two pronuclei, 2PN), multinucleation rate, the proportion of good quality embryos, blastocyst formation, and pregnancy rate.

Results: A total of 792 oocytes were collected from 54 participants (mean age: 30.6±3.7 years). The fertilization rate was higher in the study group (n=209 in 376) compared with the controls (n=201 in 416) (55.6% vs. 48.3%, p=0.041). The multinucleation rate (6.6% vs. 4.3%, p=0.150), proportion of grade 1 embryos on day 3 (48.7% vs. 43%, p=0.112) and day 5 (27.7% vs. 23.8%, p=0.214), the proportion of embryos reaching blastocyst stage (34.0% vs. 28.1%, p=0.072), and the pregnancy rates (78.6% vs. 71.4%, p=1.000) were similar.

Conclusions: Our results demonstrated that immediate removal of cumulus cells does not have any negative impact on intracytoplasmic sperm injection outcomes.

Introduction

The clinical absence of pregnancy after 12 months of regular and unprotected sex is defined as infertility (1), an issue studied by researchers for years. Since the first successful induction of ovulation followed by pregnancy in 1963, assisted reproductive technologies (ART) have developed rapidly, causing a drastic change to find suitable treatment options for infertile couples (2). Today, intracytoplasmic sperm injection (ICSI) has become a widely employed treatment option in every ART unit. ICSI outcome highly relies on oocyte quality (3), and the evaluation of oocyte characteristics and oocyte behavior during ICSI might assist in enhancing cycle outcome rates (4).

Corona cumulus cells originate from granulosa cells and are located around the oocyte. They are implicated in oocyte growth and maturation, ovulation, and fertilization in mammals (5). These cells are removed in a process called oocyte denudation

to allow the evaluation of oocyte maturation and microinjection during ICSI. Routinely, the oocytes undergo a pre-incubation period for several hours following oocyte retrieval (6). However, no consensus on the timing of oocyte denudation has been established (6-10). Recent studies using oocytes from mice reported that long-term oocyte culture with the surrounding cumulus cells promotes oocyte aging and induces apoptotic changes (11-13). Also, Zhou et al. (10) demonstrated that 4-hour co-incubation of gametes and early removal of cumulus cells improved the clinical outcomes in patients with initial complete fertilization failure. However, abnormal fertilization with more than two visible pronuclei in couples with good-fertilizing capabilities has also increased significantly increased.

Multinucleation corresponds to the presence of more than one nucleus in a blastomere. Multinucleated blastomeres are frequently observed in ART. The incidence positively correlates with factors such as shorter stimulations, a higher number of oocytes retrieved, and a higher follicle-stimulating hormone dose for stimulation (14). Desch et al. (15) showed that embryo multinucleation at the two-cell stage affects birth potential negatively. Xiong et al. (16) compared the effects of denudation of oocytes after 6 h or 20 h post-insemination on the outcomes of in vitro fertilization (IVF) and reported similar results in terms of multinucleation (16). To our knowledge, the effect of early oocyte denudation on multinucleation in patients undergoing ICSI has not been assessed before.

Defining oocyte characteristics might allow the ART facilities to predict its developmental potential and increase the chances of better embryo selection for transfer (17). This study aimed to investigate the laboratory and clinical outcomes of ICSI when the oocytes were denuded at time points 0 or 2 h after oocyte retrieval.

Methods

Patients

This prospective randomized sibling-oocyte study included participants from Acibadem Altunizade Hospital, Unit of ART (Istanbul, Türkiye) between 01.11.2020 and 30.08.2021. The inclusion criteria were as follows: Females under 40 years of age, at least 5 oocytes retrieved, and no male factor. The local clinical ethics board of Maltepe University Faculty of Medicine, İstanbul approved this study with an approval number of 2020/900/82 on 21.10.2020. A written consent form was obtained from the patient. The study was conducted in accordance with the principles of the revised Declaration of Helsinki (18).

Ovarian stimulation, oocyte pick up, and denudation

Pituitary downregulation was performed either by a gonadotropin-releasing hormone agonist (GnRH-a) or a GnRH antagonist. GnRHa leuprolide acetate (Lucrin 0.5 mg/mL, Abbott, Madrid, Spain) was injected in the late luteal phase before the treatment cycle. The GnRH antagonist cetrorelix acetate (Cetrotide, Baxter Oncology GmbH, Halle, Germany) injections started on the fifth day of the treatment cycle. Both were applied daily until the trigger ovulation. Before initiating the IVF cycle, baseline ultrasounds were performed. Gonadotropin injections were started in the absence of more than 2 cm cysts on cycle days 2 or 3 and the dose used ranged from 150 international units (IU) to 300 IU.

A human chorionic gonadotropin (hCG) injection was administered when at least 3 follicles equal to or more than 17 mm in diameter were observed. Follicular aspiration was performed 35-36 h after ovulation was triggered.

The oocytes of the patients were divided into two groups by simple randomization using a closed envelope method: (i) control group: cumulus-cell removal was performed 2 h after oocyte pick up (OPU) (time point: 2 h, as in the routine applications), (ii): a study group: cumulus-cell removal was performed immediately (time point: 0). Opaque sealed envelopes with serial numbers contained random allocation cards with the names of the control or study group prepared by an independent laboratory technician. The envelopes were opened sequentially to randomly allocate the sibling oocytes to either group.

The 2-hour incubation of the oocytes of the control group until denudation was performed in equilibration solution (G-IVF, Vitrolife-Swemed/Sweden) in a culture incubator with 5-6% CO_2, 5% O_2, 37 °C and 90% humidity. The bonds of the corona cells were loosened using the hyaluronidase enzyme (HYASE, Vitrolife-Swemed/Sweden) and the cells were cleaned using a denudation pipette. After the decellularization of the oocyte, its maturation was evaluated under a light microscope and the morphological evaluation of the oocytes was performed. ICSI was performed following cumulus-cell removal.

Assessment of fertilization and multinucleation

Fertilization was determined 16-18 hours following ICSI. 2PN and two polar bodies were required for normal fertilization.

To determine the multinucleation status, blastomeres were assessed for the presence of more than one nucleus. Each blastomere was evaluated for multinucleation 42 h, 46 h and 50 h after fertilization and multinucleated embryos were noted.

Evaluation of the embryos on days 3 and 5, and assessment of pregnancy

Embryo quality on days 3 and 5 was determined based on the morphological criteria and on the grading system described by Gardner and Schoolcraft (19) in 1999, respectively. Maternal serum β-hCG ≥10 mIU/mL 10-12 days after embryo transfer was used to define pregnancy.

Statistics Analysis

Statistical analyses were performed using Number Cruncher Statistical System (NCSS) (NCSS LLC, Kaysville, Utah, USA). Comparisons of fertilization rates, multinucleation rates, embryo development on days 3 and 5 and the rates of reaching the blastocyst stage were performed using Pearson's chi-square test. Fisher's exact test was employed to compare the pregnancy rates of the patients. Statistical significance was set with a p-value <0.05.

Results

Clinical characteristics of the patients

This study was conducted with 54 patients. The mean age of the patients was 30.6±3.7 (19-36) and the mean number of retrieved oocytes was 14.7±6.4 (5-39). Two groups of oocytes were formed by simple randomization using a closed envelop method according to the denudation timings o and 2 h, corresponding to the study and control groups, respectively. Three hundred and seventy-six oocytes (47.5%) in the study group and 416 oocytes (52.5%) in the control group were observed as suitable for ICSI with a total number of 792.

The association of early oocyte denudation on fertilization, embryo quality and blastocyst formation

As provided in Table 1, the fertilization rate significantly increased when the cumulus cells were removed immediately after OPU without a pre-incubation than the 2 h incubation (55.6% vs. 48.3%, p=0.041). No significant differences were obtained between the study and control groups regarding the multinucleation rate (6.6% vs. 4.3%, p=0.150), the proportions of Grade 1 day 3 (48.7% vs. 43%, p=0.112), and day 5 embryos (27.7% vs. 23.8%, p=0.214) and the proportion of embryos reaching the blastocyst stage (34.0% vs. 28.1%, p=0.072).

The effect of early oocyte denudation on pregnancy

Embryos of 23 patients were frozen and 10 patients received embryos from both groups during transfer. Therefore, among the 54 patients, 21 patients were included to compare their pregnancy rates (Table 2). Pregnancy was detected in 78.6% of the cases in the study group and 71.4% cases in the control group. There was no statistically significant difference between the pregnancy rates in the groups (p=1.000).

Discussion

Cumulus cells are supporting cells that surround the oocyte and protect it from the microenvironment. Bi-directional communication is responsible for oocyte growth, maturation in the follicles, and early embryonic developmental competence (5). The complex regulation between the oocyte and the cumulus cells results in a coordinated function (20). Oocyte denudation should be performed before ICSI as the cumulus cells affect the microinjection process and a pre-ICSI evaluation of oocyte maturity is required (21). However, the debate over the optimal oocyte denudation timing has been going on. Several studies on mice oocytes reported that apoptotic changes and oocyte aging are induced following a long-term oocyte culture with cumulus cells (11-13). Aged oocytes have a reduced developmental competence (22). However, a systematic review by Wang et al. (21) found that the time from OPU to oocyte denudation did not change ICSI outcomes in most of the studies, though some suggested a short pre-incubation following oocyte retrieval.

Multinucleation is a frequently observed phenomenon in ART. In the study by Xiong et al. (16), a pre-incubation of 6 or 20 h was performed in classical IVF and no difference was reported in terms of multinucleation when the two groups were compared similarly to our findings. Multinucleation is one factor compromising the embryo quality and implantation

Table 1. Fertilization rates, multinucleation rates, proportion of grade 1 embryos on day 3 and on day 5, and blastocyst development in the study group and control group

	Study group (n=376)	Control group (n=416)	[a]p
Fertilization (2PN), n (%)	209 (55.6)	201 (48.3)	0.041*
Multinucleation, n (%)	25 (6.6)	18 (4.3)	0.150
3rd day G1, n (%)	183 (48.7)	179 (43.0)	0.112
5th day G1, n (%)	104 (27.7)	99 (23.8)	0.214
Blastocyst development, n (%)	128 (34.0)	117 (28.1)	0.072

[a]Pearson's chi-square test, 2PN: Two pronuclei, G1: Grade 1

Table 2. Pregnancy rates depending on the timing of oocyte denudation (pregnancy + serum β-human chorionic gonadotropin ≥10 mIU/mL)

	Study group (n=14)	Control group (n=7)	[b]p
Pregnancy (+), n (%)	11 (78.6)	5 (71.4)	1.000
Pregnancy (-), n (%)	3 (21.4)	2 (28.6)	

[b]Fisher's exact test

rate. Therefore, the evaluation of embryo multinucleation is considered an important parameter for embryo transfer (23).

In this prospective randomized sibling-oocyte study, we observed similar multinucleation rates between the control and study groups. Moreover, no statistically significant difference was found in embryo quality on days 3 and 5, blastocyst formation rates, and pregnancy rates when the cumulus cells were removed at time points 0 or 2 h. However, a higher fertilization rate was observed in the study group consisting of the oocytes denuded without a pre-incubation.

Patrat et al. (8) determined that a pre-incubation of 2 h may not increase the proportion of mature oocytes, but lead to the optimal combination of fertilization and implantation rates. However, the inclusion criteria of this study were different from those in our study: attempt rank 1 or 2 of ICSI; female age more than 36 years old; patients with male factor infertility. The cytoplasmic maturity process is not well known. The authors suggest that retrieved oocytes from stimulated cycles are cytoplasmically immature although they reach the Metaphase 2 stage, and still require cumulus cells for cytoplasmic maturation (8,24,25). However, several other studies reported no influence of the timing of oocyte denudation after OPU on the fertilization rate (6,26,27).

One of the most important processes in ART is the selection of the best embryo for transfer. The purpose of embryo grading is to select the embryo with the highest implantation potential (28). In our study, we hypothesized that oocytes that were denuded immediately after oocyte retrieval would have a higher development capacity based on the relation between cumulus cells and oocyte aging (11-13). However, no difference was observed between the study group and the control group in terms of embryo quality on days 3 and 5. However, Mizuno et al. (6) obtained a significantly higher percentage of good-quality blastocysts when the oocytes were denuded 2 h after OPU compared to those denuded at time point 0. Wang et al. (21) reviewed the studies focusing on the relationship between OPU-oocyte denudation time and embryo quality. The researchers reported that most studies did not demonstrate any effect on embryo quality, while some suggested an extended pre-incubation to increase the rate of good-quality embryos. Few studies also evaluated the blastocyst formation rates. While Hassan (29) reported a higher blastocyst formation rate after a pre-incubation with intact cumulus cells, Ishikawa et al. (30), Mizuno et al. (6) and Naji et al. (27) demonstrated no significant differences related to OPU-oocyte denudation time. The differences in methodologies, such as hCG-OPU time, inclusion criteria, or sample size might account for the different effects obtained in the studies.

We also included pregnancy rates in our study. There was no significant difference between the two groups in terms of pregnancy rates. Although the number of patients was limited, our result is in accordance with the studies with a higher sample size (6,10,27,31,32). Bárcena et al. (32) explained this result with high-quality oocytes, which may withstand aging *in vitro.* Others obtained higher pregnancy rates when the oocyte culturing with cumulus cells was performed and proposed that cumulus cells should be maintained during a pre-incubation period (8,26).

Study Limitations

To our knowledge, this is the first report in the literature regarding a relationship between the timing of oocyte denudation and multinucleation in patients undergoing ICSI. Therefore, evaluation of the association between early oocyte denudation and multinucleation in patients undergoing ICSI is the major strength of our study. However, the small number of participants to evaluate the pregnancy outcome is a major limitation. Additionally, the effect of longer periods of culture between OPU and denudation was not assessed. Further multi-center randomized controlled studies with a larger sample size are necessary to confirm the higher fertilization rates obtained with the oocytes denuded immediately after oocyte retrieval and better understand the effects of cumulus removal on embryo quality and developmental fate.

Conclusion

In conclusion, we demonstrated a higher fertilization rate when the oocyte denudation was performed immediately after the oocyte retrieval compared to an incubation period of 2 h. However, other parameters, including multinucleation rates, embryo quality on days 3 and 5, blastocyst formation rates, and pregnancy rates were similar between the groups. Our results indicate that the removal of cumulus cells immediately after OPU does not have any negative impact on ICSI outcome.

Ethics

Ethics Committee Approval: Ethical approval was obtained by the Maltepe University Clinical Research Ethics Committee (approval number: 2020/900/82, date: 21.10.2020).

Informed Consent: All patients were informed about the study and the consent document received.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: Z.B.D., M.C., Y.D.Ç., B.S., Design: Z.B.D., M.C., Y.D.Ç., B.S., Data Collection, or Processing: Z.B.D., Ş.K., Analysis, or Interpretation: Z.B.D., Ş.K., M.C., Y.D.Ç., B.S., Literature Search: Z.B.D., Y.D.Ç., Writing: Z.B.D., M.C., Y.D.Ç., B.S.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declare that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.82905
Gulhane Med J 2022;64:240-7

The relationship of atherogenic index of plasma with endothelial dysfunction biomarkers in patients with metabolic associated fatty liver disease

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Date submitted:
19.10.2021
Date accepted:
18.01.2022
Online publication date:
08.09.2022

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Keywords: Metabolic associated fatty liver disease, atherogenic index of plasma, endothelial dysfunction, atherosclerosis

ABSTRACT

Aims: The atherogenic index of plasma (AIP) is a marker used to predict atherosclerosis and cardiovascular disease (CVD). In this study, we examined the relationship of AIP with markers of endothelial dysfunction (ED) [asymmetric dimethylarginine (ADMA) and adiponectin] and early atherosclerosis [high-sensitivity C-reactive protein (hs-CRP)] in patients with metabolic associated fatty liver disease (MAFLD).

Methods: This was a cross-sectional study with retrospective enrollment. AIP was defined as the logarithmically transformed ratio of triglyceride to high-density lipoprotein cholesterol. All patients were divided into two groups according to whether they had steatohepatitis or fibrosis and were compared. Mean differences between two independent groups were assessed using the independent Student's t-test and Mann-Whitney U test as appropriate.

Results: A total of 129 male subjects with biopsy-proven MAFLD were enrolled. There were no significant differences regarding AIP (0.64±0.039 vs. 0.62±0.033, p=0.773) between patients with steatohepatitis (n=54) and without steatohepatitis (n=75). Additionally, similar findings were observed among subjects with fibrosis (n=84) and without fibrosis (n=45). However, there was no association of AIP with ADMA, adiponectin, hs-CRP, insulin and HOMA-IR levels (p=0.176, p=0.636, p=0.810, p=0.068, and p=0.126, respectively).

Conclusion: The lack of association between AIP and the biomarkers of ED or early atherosclerosis implies that this index may not be a significant predictor of CVD in MAFLD.

Introduction

Non-alcoholic fatty liver (FL) disease (NAFLD) is the most common chronic liver disease both in Türkiye and in the world. Recently, it was recommended that the disease be named and defined as metabolic associated FL disease (MAFLD) (1). The pathogenesis of MAFLD starts with hepatic fat accumulation, in association with peripheral insulin resistance (2,3). MAFLD is considered the liver component of metabolic syndrome (MetS) and is strongly associated with obesity, hypertension, type 2 diabetes mellitus (T2DM), and dyslipidemia (4,5). Overall, the combination of metabolic disorders leads to a significant increase in cardiovascular disease (CVD) risk. Ultimately, it has been reported that MAFLD confers an independent risk of CVD, apart from MetS and traditional cardiovascular risk factors (6-8).

Endothelial dysfunction (ED) plays an important role in the pathogenesis of the initial stage of atherosclerosis and is also a key factor in predicting future CVD (9,10). Multiple factors take part in the pathogenesis of ED, including increased oxidative stress, elevated asymmetric dimethylarginine (ADMA), angiotensin 2 and homocysteine, and decreased adiponectin levels (11-13). ADMA, an analog of L-arginine is a biomarker that decreases nitric oxide synthesis and therefore is associated with ED and CVD (14). Simultaneously, adiponectin is an important peptide secreted by adipocytes and is measured at low levels in obesity, MetS, T2DM, and CVD (15). Both ADMA and adiponectin are well-known biomarkers of ED and atherosclerosis (14,16). However, high-sensitivity C-reactive protein (hs-CRP), a systemic inflammatory marker connected with ED and atherosclerosis, is directly related to the risk of CVD (17).

The atherogenic index of plasma (AIP), a logarithmically transformed ratio of molar concentrations of triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C), is positively and strongly associated with obesity, T2DM, and MetS (18). Therefore, it has been reported to be associated with atherosclerosis and suggested as a novel biomarker for future risk of atherosclerosis and CVD (19). There are few studies in the current literature investigating the role of AIP in patients with MAFLD (20,21). However, the role of AIP in the prediction of increased CVD in MAFLD is unclear. To the best of our knowledge, the association of AIP with ED and atherosclerosis biomarkers has not been previously investigated in subjects with MAFLD. Therefore, this study examined the associations of AIP with cardiometabolic risk factors, and especially the relationship of AIP with ED or atherosclerosis in patients with MAFLD.

Methods

Study design and population

This retrospective study was performed using a previously obtained dataset of routine patient follow-up at the Gastroenterology Department, Gülhane Faculty of Medicine, Ankara, Türkiye (22,23). The participants were asymptomatic men who had undergone evaluation for elevated transaminases. Blood tests and liver biopsy were performed as part of the clinical algorithm. Patients with hypertension, T2DM, or those on medications that may affect the glucose or lipid metabolism (e.g., fibrates, statins) were excluded. The current study was approved by the Local Ethics Committee of Balikesir University Faculty of Medicine (approval no: 2020/164, date: 23.09.2020) and the study protocol conforms to the Helsinki Declaration.

MAFLD was diagnosed by the presence of one of the specific clinical conditions (overweight, obesity, T2DM, or evidence of metabolic dysregulation) in NAFLD patients. The metabolic dysregulation was defined by the presence of two of the criteria (24); 1) waist circumference (WC) ≥102 cm for men; 2) TG ≥150 mg/dL; 3) blood pressure ≥130/85 mmHg; 4) HDL-C <40 mg/dL for men; 5) prediabetes [e.g., glycated hemoglobin 5.7-6.4%, fasting plasma glucose (FPG) 100 to 125 mg/dL, or 2 h glucose levels 140 to 199 mg/dL]; 6) hs-CRP >2 mg/L; and 7) homeostasis model assessment of insulin resistance (HOMA-IR) index ≥2.5.

Anthropometric measurements

Clinical and laboratory data were collected at the time of the liver biopsy. Height, weight, and WC of all patients were measured after 8 h of fasting. WC was measured as the midway between the lowest rib and the level of the anterior superior iliac crests. Body mass index (BMI) was calculated as body weight (kg)/height^2 (m^2). Blood pressure was measured in a seated position three times, and mean blood pressure was determined. The diagnostic criteria for hypertension were systolic and diastolic blood pressure ≥140/90 mmHg.

Biochemical analyses

FPG, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, uric acid, bilirubin, gamma-glutamyl transpeptidase (GGT), total cholesterol (TC), TG, and HDL-C levels were evaluated by the enzymatic colorimetric method (Olympus Diagnostics Hamburg, Germany). Low-density lipoprotein cholesterol was calculated by following Friedewald's formula [TC-(TG/5+HDL-C)] (25). Basal insulin levels of the patients were measured by the chemiluminescence method (Roche Diagnostics GmbH, Mannheim, Germany). Insulin resistance [HOMA-IR=Fasting insulin (μU/mL) × FPG (mg/dL)/405] was measured using a formula correlated with the euglycemic-hyperinsulinemic clamp method (26).

Plasma ADMA levels were measured by ELISA (ADMA direct ELISA kit, Immunodiagnostic AG, Bensheim, Germany) (detection limit of ADMA assay=0.04 μmol/L). Intra-assay CV ranged from 5.8% to 7.9%, while inter-assay CV ranged from 7.6% to 10.8% for the ADMA assay. Measurements were

performed using an ELISA BioTek Synergy HT plate reader (BioTek Instruments Inc., Winooski, VT, USA). Serum levels of adiponectin were also measured using the ELISA (Human Adiponectin ELISA Kit, Cat. No: E09; Reutlingen, Germany). Intra-assay CV ranged from 2.35% to 4.66%, while inter-assay CV ranged from 5.7% to 6.72% for adiponectin. The minimum detectable concentration of adiponectin was 0.6 ng/mL. Measurements were implemented using an ELISA BioTek Synergy HT plate reader (BioTek Instruments Inc., Winooski, VT, USA). Serum hs-CRP levels were measured using the immune turbidimetric-fixed rate method with a biochemical auto-analyzer (Olympus AU 2700, Olympus Diagnostics, Hamburg, Germany). Intra-assay CV and inter-assay CV were 5.8% and 3.1%, respectively. The minimum detectable concentration for hs-CRP was 0.07 mg/L.

Assessment of AIP

The AIP was calculated as the logarithmic transformation of TG to HDL-C ratio [AIP=Log (TG/HDL-C)].

Liver histology

An experienced hepatopathologist blinded to subjects' details reviewed the histology slides to search for inflammation and/or fibrosis using the classification of Kleiner et al. (27). NAFLD activity score (NAS) was calculated as the unweighted sum of steatosis [none, mild, moderate, and severe (0-3)], lobular inflammation [inflammatory foci per 200× field (0 is no foci; 1 is <2 foci per 200× field, 2 is 2-4 foci per 200× field, 3 is >4 foci per 200× field)], and hepatocellular ballooning [none, few balloon cells, and many cells/prominent ballooning (0-2)] scores. As a result, the subjects were classified into three groups namely simple steatosis [SS, (NAS=0-2)], borderline steatohepatitis [BSH, (NAS=3-4)] and definite steatohepatitis (DSH) [DSH, (NAS ≥5)]. The fibrosis score was assessed using a 6-point scale [1a, b=mild (1a) / moderate (1b) zone 3 perisinusoidal fibrosis; 1c=portal fibrosis only; 2=zone 3 and portal/periportal fibrosis; 3=bridging fibrosis; 4=cirrhosis].

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 22.0 package program (IBM, Corp., Armonk, NY, USA). Normality assumptions were tested via Shapiro-Wilk analysis. Variables were expressed as mean±standard error (SE) when normally distributed and as median (25^{th}-75^{th} percentiles) when non-normally distributed. Mean differences between two independent groups were assessed using the independent samples t-test and Mann-Whitney U test as appropriate. We performed a one-way ANOVA test to determine mean differences between more than two groups. The correlation between numerical parameters was tested by the Pearson or Spearman methods. Differences and correlations were considered significant at $p<0.05$.

Results

The study included 129 patients with MAFLD (age, mean±SE: 32.1±0.5 years). Anthropometric, clinical, and laboratory data are shown in Table 1. Most patients were in the range of overweight or obesity. The distribution of histopathological parameters is shown in Table 2. Patients with SS and BSH were combined in one group described as non-DSH (n=75), with a mean±SE age of 32.7±0.7 years and DSH (n=54), with mean±SE age of 31.2±0.8 years. Fibrosis (F1-F3) was observed in 84 of 129 patients (65.1%). ALT and AST values were significantly higher in patients with fibrosis or steatohepatitis ($p<0.05$, for both). However, there was no significant difference in other parameters such as BMI, WC, glucose, insulin, HOMA-IR index, and lipid parameters. Additionally, there were also no significant differences in AIP, ADMA, adiponectin, and hs-CRP levels between the two groups. However, similar findings were observed among subjects with (n=84) and without fibrosis (n=45) except for insulin (p=0.007) and HOMA-IR (p=0.008) (Tables 3, 4).

Table 1. Anthropometric and laboratory data of the study population

	Values
Age (year)	32.08±0.53*
BMI (kg/m²)	28.2 (26.45-30.25)**
WC (cm)	100 (96-104)**
FPG (mg/dL)	93.67±0.95*
TC (mg/dL)	204±3.89*
TG (mg/dL)	166 (117-256)**
HDL-C (mg/dL)	40 (35-45)**
LDL-C (mg/dL)	124.76±3.07*
AST (U/L)	48 (37.5-58.5)**
ALT (U/L)	101 (74.5-130.5)**
GGT (U/L)	56 (44-76.75)**
UA (mg/dL)	6.6 (5.75-7.16)**
DBil (mg/dL)	0.16 (0.11-0.21)**
IBil (mg/dL)	0.6 (0.41-0.84)**
Insulin (mIU/L)	13.82 (10.11-19.79)**
Adiponectin (µg/mL)	3.94 (2.93-5.26)**
ADMA (µmol/L)	0.4 (0.33-0.49)**
hs-CRP (mg/L)	2.04 (1.19-3.26)**
HOMA-IR	2.98 (2.14-4.73)**
AIP	0.63±0.03*

*Mean±S.E., **Median (25^{th}-75^{th} percentiles). BMI: Body mass index, WC: Waist circumference, FPG: Fasting plasma glucose, TC: Total cholesterol, TG: Triglyceride, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma-glutamyl transferase, UA: Uric acid, DBil: Direct bilirubin, IBil: Indirect bilirubin, ADMA: asymmetric dimethylarginine, hs-CRP: High-sensitive C-reactive protein, HOMA-IR: Homeostatic model assessment of insulin resistance, AIP: Atherogenic index of plasma

Table 2. Histopathological findings in the study population		
	n	%
Fibrosis		
0	45	34.9
1	76	58.9
2	6	4.7
3	2	1.6
Steatosis		
0	8	6.2
1	42	32.6
2	45	34.9
3	34	26.4
Lobular inflammation		
0	11	8.5
1	81	62.8
2	37	28.7
Hepatocellular ballooning		
0	28	21.7
1	83	64.3
2	18	14.0

AIP was positively correlated with GGT level (r=0.366, p<0.001) and, negatively correlated with direct bilirubin level (r=-0.487, p<0.001). However, there was no association of AIP with ADMA, adiponectin, hs-CRP, insulin and HOMA-IR levels (p=0.176, p=0.636, p=0.810, p=0.068, and p=0.126, respectively). Moreover, analysis of the AIP with the histological findings (including steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis scores) also showed no association between these parameters (p=0.505, p=0.388, p=0.599 and p=0.849, respectively).

Discussion

The results of this study show that the AIP calculated in patients with MAFLD was not related to inflammation or fibrosis nor the surrogate markers of ED and atherosclerosis. To the best of our knowledge, this is the first study searching for the relationship between AIP and inflammation or ED or liver histology in patients with MAFLD (below, the implications of these findings will be discussed in detail).

However, there are limited data regarding the relationship of AIP with NAFLD. Wang et al. (20) evaluated 538 subjects with ultrasonographically diagnosed NAFLD. They found a strong association of AIP with NAFLD in the multivariable logistic

Table 3. Demographic and laboratory characteristics of patients with DSH and non-DSH			
Variable	**Non-DSH (n=75)**	**DSH (n=54)**	**p-value**
Age (year)	32.71±0.732	31.2±0.76	0.165
BMI (kg/m²)	28.4 (27-31)	28.15 (26.15-29.5)	0.238
WC (cm)	99 (96-104.08)	100 (97-103.88)	0.764
FPG (mg/dL)	93.18±1.257	94.35±1.443	0.541
TC (mg/dL)	206.33±5.142	201.26±5.979	0.522
TG (mg/dL)	167 (110-255)	149.5 (122-258.5)	0.854
HDL-C (mg/dL)	41 (36-46)	38.5 (35-44.25)	0.211
LDL-C (mg/dL)	128.27±4.088	119.87±4.584	0.178
AST (U/L)	42 (34-53)	56 (41.75-65.25)	**<0.001**
ALT (U/L)	89 (63-112)	120 (93.75-163.25)	**<0.001**
GGT (U/L)	56 (41-83)	58 (45-76.5)	0.740
UA (mg/dL)	6.58 (5.69-7.1)	6.67 (5.92-7.26)	0.344
DBil (mg/dL)	0.15 (0.11-0.2)	0.16 (0.12-0.25)	0.292
IBil (mg/dL)	0.57 (0.4-0.77)	0.7 (0.46-0.9)	0.059
Insulin (mIU/L)	12.16 (9.56-19.78)	14.45 (10.36-20.71)	0.287
Adiponectin (µg/mL)	4 (2.85-5.61)	3.79 (3.04-4.84)	0.635
ADMA (µmol/L)	0.41 (0.34-0.48)	0.4 (0.33-0.56)	0.630
hs-CRP (mg/L)	2.04 (1.18-3.47)	2.06 (1.17-3.06)	0.660
HOMA-IR	2.78 (2.09-4.62)	3.39 (2.34-4.81)	0.351
AIP	0.62±0.033	0.64±0.039	0.773

Data are expressed as the mean±SE, and median (25^{th}-75^{th} interquartile range).
BMI: Body mass index, WC: Waist circumference, FPG: Fasting plasma glucose, TC: Total cholesterol, TG: Triglyceride, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma-glutamyl transferase, UA: Uric acid, DBil: Direct bilirubin, IBil: Indirect bilirubin, ADMA: Asymmetric dimethylarginine, hs-CRP: High-sensitive C-reactive protein, HOMA-IR: Homeostatic model assessment of insulin resistance, AIP: Atherogenic index of plasma, DSH: Definite steatohepatitis

Table 4. Demographic and laboratory characteristics of patients with and without fibrosis

Variable	Without fibrosis (F0) (n=45)	With fibrosis (F1-F3) (n=84)	p-value
Age (year)	31.4±0.8	32.4±0.7	0.354
BMI (kg/m²)	28.2 (26.9-30.0)	28.3 (26.3-30.5)	0.884
WC (cm)	98.0 (97.0-103.4)	100.0 (96.0-104.8)	0.680
FPG (mg/dL)	92.7±1.8	94.2±1.1	0.451
TC (mg/dL)	206.3±5.7	203.1±5.2	0.700
TG (mg/dL)	169.0 (122.5-264.0)	166.0 (115.3-255.5)	0.729
HDL-C (mg/dL)	40 (34.5-43.5)	40.5 (36.0-46.8)	0.198
LDL-C (mg/dL)	127.1±4.1	123.5±4.2	0.580
AST (U/L)	40 (34-51.5)	51.5 (39.3-64.0)	**0.001**
ALT (U/L)	84 (66.5-114.0)	108.5 (85-140.8)	**0.012**
GGT (U/L)	56 (42.5-86.5)	56 (44.0-76.0)	0.855
UA (mg/dL)	6.4 (5.6-7.2)	6.6 (6.07-7.2)	0.243
DBil (mg/dL)	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.735
IBil (mg/dL)	0.6 (0.4-0.8)	0.6 (0.4-0.9)	0.349
Insulin (mIU/L)	11.2 (9.29-15.6)	15.38 (10.28-23.55)	**0.007**
Adiponectin (µg/mL)	3.72 (2.77-5.01)	3.96 (3.04-5.38)	0.577
ADMA (µmol/L)	0.41 (0.33-0.51)	0.4 (0.33-0.48)	0.780
hs-CRP (mg/L)	1.75 (1.07-2.99)	2.11 (1.30-3.37)	0.249
HOMA-IR	2.45 (2.07-3.70)	3.66 (2.36-5.36)	**0.008**
AIP	0.64±0.044	0.62±0.031	0.596

Data are expressed as the mean±SE, and median (25^{th}-75^{th} interquartile range). p values were calculated using Student's t-test and Mann-Whitney U test as appropriate. BMI: Body mass index, WC: Waist circumference, FPG: Fasting plasma glucose, TC: Total cholesterol, TG: Triglyceride, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma-glutamyl transferase, UA: Uric acid, DBil: Direct bilirubin, IBil: Indirect bilirubin, ADMA: Asymmetric dimethylarginine, hs-CRP: High-sensitive C-reactive protein, HOMA-IR: Homeostatic model assessment of insulin resistance, AIP: Atherogenic index of plasma

regression analysis. Additionally, among the other metabolic factors, such as BMI, WC, and lipid profile, AIP was the best predictor of NAFLD in this study. In another study, Dong et al. (21) analyzed the relationship between AIP and ultrasonographically diagnosed NAFLD in non-obese subjects. AIP was significantly and positively correlated with NAFLD. Moreover, in univariate and multivariate regression analysis, AIP is an independent risk factor for NAFLD. In another cross-sectional study conducted by Xie et al. (28), 7,838 subjects were involved in evaluating the association between AIP and FL and assessing the predictive ability of AIP for FL. AIP was significantly higher in the FL group than in the non-FL group. Additionally, a significantly elevated risk of FL was observed in the higher quartile of AIP compared with that in the lowest quartile following adjustment of gender and age. As far as we know, this is the first study in the literature to investigate the role of AIP in patients with MAFLD. However, no significant difference was observed between the groups with and without DSH in terms of the AIP levels and between patients with and without fibrosis. In correlation analysis, AIP was related to direct bilirubin and GGT levels. However, all histological findings, especially fibrosis, were not significantly associated with AIP levels in subjects with MAFLD. As mentioned above, all the studies that investigated the association of AIP with NAFLD were conducted in subjects with ultrasonographically diagnosed FL. Although it is widely used in the evaluation of FL in clinical practice, liver ultrasonography lacks sufficient sensitivity and specificity to detect liver inflammation and fibrosis. Therefore, the major strength of our study was the use of liver biopsy to diagnose NAFLD, the gold standard method for evaluating liver histology. Considering our findings, we suggest that AIP is not a useful index for predicting MAFLD in routine clinical practice.

A large body of evidence suggests that NAFLD is associated with an increased risk of atherosclerosis and CVD, independently of classical risk factors and components of the MetS (29,30). Several key clinical paradigms are relevant concerning atherosclerosis and CVD formation in patients with NAFLD. For example, atherogenic dyslipidemia, oxidative stress, chronic subclinical inflammation, and dysregulation of adipokines, especially insulin resistance, are the main ones of these parameters (31,32). Recently, a growing body of evidence has indicated that AIP is a good predictor of atherosclerosis and a highly sensitive marker for predicting the risk of future CVD (33,34). Hence, it has been reported that AIP is significantly and positively associated with carotid artery intima-media

thickness, a surrogate marker of early atherosclerosis (35). Additionally, a large case-control study reported that elevated AIP was significantly associated with coronary artery disease (36). However, to the best of our knowledge, no study has investigated the role of AIP in the prediction of ED or atherosclerosis in subjects with NAFLD. In our work, we did not find any significant association between AIP and surrogate biomarkers of ED and early atherosclerosis, namely, ADMA, adiponectin, and hs-CRP levels. It has been reported that AIP is strongly associated with insulin resistance, obesity, and the risk of T2DM (37-40). Additionally, other studies revealed that elevated AIP is a risk factor for developing MetS independent of any components of MetS (41). As mentioned above, due to the small number of subjects with MetS, we couldn't perform an analysis to investigate the association of AIP with MetS in our study population. Because of the well-known relationship between ED with hypertension and T2DM, patients with these metabolic diseases were excluded from this study. Moreover, circulating markers of ED and atherosclerosis are affected by these metabolic confounders (42). Therefore, we believe that the study design by excluding confounding factors is important in terms of its results. After all, we think that the lack of relationship between AIP and ED observed in our study might be related to the absence of MetS in the study population. Considering these data, we suggest that AIP may not be a good predictor of ED in NAFLD and it contributes to the prediction of CVD by acting in concert with other metabolic abnormalities. Hence, there are conflicting reports in the literature regarding the relationship of AIP with CVD. In a cross-sectional study conducted among postmenopausal women, elevated AIP was not associated with the risk of CVD (43). Otherwise, in a prospective cohort study, a low AIP level in contrast with a high AIP level was an independent predictor of all-cause mortality in patients with acute coronary syndrome (44).

To our knowledge, this is the first study to investigate the role of AIP in the prediction of ED or atherosclerosis in patients with MAFLD. However, our study has several limitations. Firstly, the cross-sectional nature of the study precluded any determination of the role of AIP in the prediction of ED or atherosclerosis. For this reason, further prospective studies should be conducted to evaluate the significance of AIP in clinical practice. Secondly, the number of patients decreased because of the strict inclusion criteria. However, we believe that the study design was necessary to achieve the main objective. Thirdly, since the patient population consists of males, these results need to be studied and confirmed in women as well. Lastly, although it is widely used for estimating beta cell function and IR, the HOMA-IR index cannot be as accurate as the euglycemic hyperinsulinemic clamp method, which is the gold standard for assessing insulin sensitivity in humans.

Conclusion

In conclusion, AIP was not associated with either liver histopathology (hepatic inflammation or fibrosis) or surrogate markers of ED and atherosclerosis in the MAFLD patients. Further research is needed to better understand the role of AIP in predicting the clinical severity of MAFLD and the risk of CVD in this clinically relevant condition.

Ethics

Ethics Committee Approval: The study was approved by the Local Ethics Committee of Balikesir University Faculty of Medicine (approval no: 2020/164, date: 23.09.2020) and the study protocol conforms to the Helsinki Declaration.

Informed Consent: Retrospective study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: H.Gü., H.G., G.Ç., S.T., A.F.Ç., Concept: A.K., A.S., T.D., Design: A.S., C.N.E., Data Collection or Processing: H.Gü., H.G., G.Ç., S.T., A.F.Ç., Analysis or Interpretation: A.C.Y., S.T., Literature Search: A.K., A.S., T.D., C.N.E., Writing: A.K., T.D.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.96158
Gulhane Med J 2022;64:248-54

Association between diabetes mellitus and disability in hand osteoarthritis

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Date submitted:
16.06.2021

Date accepted:
23.02.2022

Online publication date:
08.09.2022

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Keywords: Diabetes mellitus, osteoarthritis, hand disability, grip strength

ABSTRACT

Aims: Osteoarthritis (OA) and diabetes mellitus (DM) may coexist frequently. The increase in overall OA incidence is correlated with poor glycemic control and disease duration in patients with DM. However, the association between a DM diagnosis and specifically hand OA has not been explicitly determined. We assessed the association between DM and severity of disability in hand OA.

Methods: This single-center, case-control study prospectively enrolled outpatients with hand OA who visited a physical therapy and rehabilitation clinic. The patients were grouped according to the presence of DM diagnosis. Pain, hand function, grip strength, and quality of life were evaluated and compared between the two groups.

Results: The study included 100 participants [Age: 62.7±10.7 years (33-92); female: 78%]. The mean Australian/Canadian OA hand index of patients with OA with (n=50) and without DM (n=50) was 30.7±10.1 vs. 19.9±9.6, respectively (p<0.001). The mean lateral grip strength of the dominant hand of patients with and without DM was 4.5±2.1 vs. 5.9±2.1, respectively (p=0.002). Lateral grip strength of the non-dominant hand showed a negative correlation with DM duration among women (r=-0.387, p=0.018) and a positive correlation with hemoglobin A1c level among men (r=0.609, p=0.027).

Conclusions: This study showed an association between DM diagnosis and severity of hand disability in patients with hand OA, with different patterns among women and men. Nevertheless, the results were unadjusted for relevant confounders.

Introduction

A greater incidence of soft-tissue and musculoskeletal system pathologies is observed in patients with diabetes mellitus (DM) (1). Many pathological changes in soft tissues have been documented in DM, but the underlying mechanism of these musculoskeletal system disorders has not been understood (2). Bone, cartilage, and soft tissue diseases are commonly encountered in patients with DM (2), and it has been shown that DM is a risk factor for developing several rheumatic diseases (3). Besides, there is a high incidence of adhesive capsulitis, tendinitis, carpal tunnel syndrome, and Dupuytren's contracture in patients with DM (2). Connective tissue activating peptide (CTAP) is a CXC-type platelet chemokine with immunomodulatory and angiogenic activity with effects on the metabolism of connective tissue (4). CTAP is elevated in inflammatory diseases and can delay the completion of the repair phase of inflammatory damage, thereby decreasing collagen formation. CTAP-3 has been detected in diabetics (5).

The incidence of osteoarthritis (OA) was correlated with poor glycemic control and length of time since DM diagnosis (3). Moreover, it was reported that people with diabetic peripheral neuropathy might have a greater risk of the aggressive form of OA (6). However, the mechanism behind the association between DM and OA has not been determined (7).

Limited joint mobility (LJM) in DM is caused by the non-inflammatory thickening and increased stiffness in the peri-

articular structures (8). Although reported in the shoulder, LJM was first observed at hand (9). In the beginning, LJM may be painless and therefore unnoticed; however, LJM may precede severe upper extremity impairments associated with pain or disability. LJM and associated impairments at the hand may significantly impact functionality in patients with DM.

In this study, we compared patients with hand OA according to the presence of DM in terms of hand functionality, quality of life, pain level, and grip strength. We also determined any potential relationship between the DM duration, glycemic control, and hand OA, as well as the impact on the patient's ability to perform daily tasks.

Methods

Study design and participants

This observational and case-control study was conducted in Atatürk Training and Research Hospital after obtaining Ethics Committee Approval (approval number: 59, date: 22.03.2017). The data were collected from the Atatürk Training and Research Hospital Outpatient Clinic between March and June 2017 using a convenience sampling method. Informed consent was obtained from all patients. Inclusion criteria were; (i) patient age between 18 and 90 years, (ii) hand OA diagnosis based on the American Society of Rheumatology (10), and (iii) ability to cooperate and read and write in Turkish. Exclusion criteria were the presence of inflammatory, metabolic, and endocrine diseases other than DM that may lead to secondary OA and any neurological or rheumatological disorder in the hands or upper extremities. The participants were grouped with and without DM. The diagnosis of DM was self-reported and confirmed by the national health database.

Assessment of participants

The demographic characteristics, including gender, age, co-morbidity, and body mass index (BMI) were collected. The duration of DM and hemoglobin A1c (HbA1c) levels were noted.

A hydraulic hand dynamometer (Baseline Hydraulic, Irvington, NY, USA) was used to determine the grip strength. Three measurements were done for each hand while the elbow was at a 90-degree flexion and the forearm and wrist were neutral. The results are reported as means in kg (11). A pinch gauge (Baseline Hydraulic, Irvington, NY, USA) was used to determine finger grip strength, and measurements were done in three different positions-lateral, fingertip, and palmar. For measuring lateral grip, the pinch gauge was pressed by the mid-distal phalanx of the thumb and supported from below by the lateral side of the second phalanx of the index finger. The fingertip grip was determined by squeezing the pinch gauge between the tip of the thumb and the index finger. Palmar measurements were carried out by supporting the pinch gauge laterally with the fingers while pressing on it with the inside of the thumb. Patients were instructed to squeeze the gauge with maximum strength, and each measurement was conducted bilaterally in triplicate, and the means were calculated.

Disability in hand functions was evaluated using three different scales. The Australian/Canadian OA hand index (AUSCAN) evaluates pain, stiffness, and difficulty performing daily activities (12). The validity and reliability of this index have been established, and it has been translated into various languages (12,13). The Duruoz hand index (DHI) measures hand and wrist functionality and consists of eighteen tests with scores ranging from 0-90. The higher the score, the greater the activity limit (14). The grip ability test (GAT) comprises three components: filling a glass with water, putting a paper clip on an envelope, and putting a sock over one hand. A high score indicates impaired hand function (15).

A visual analog scale (VAS) was used to quantify pain levels. A straight line was divided into ten 1-cm sections from 0 to 10, where 0 meant no pain and 10 meant the most severe pain, and patients were asked to indicate the number corresponding to their pain on the scale (16).

The Quality of Life (QoL) was evaluated by the short form-36 (SF-36) questionnaire, which consists of eight sub-groups: vitality, physical function, general health, pain, social function, physical-emotional role limitation, and mental health. It is scored from 0 to 100, with 0 being the worst health status, while 100 indicates the best health. The validity and reliability of the SF-36 have been adequately demonstrated in Türkiye (17).

Outcomes

The primary endpoints were hand grip strength and hand function in diabetic and non-diabetic patients with OA. The secondary endpoint was pain and QoL.

Statistical Analysis

The Statistical Package for the Social Sciences for Windows (version 20.0, IBM.Corp., Armonk, NY, 2011) was used for data analysis, and normal distribution was determined using the Kolmogorov-Smirnov test. Data are expressed as mean ± standard deviation or percentage values. Where appropriate, case-control comparisons were performed by Student's t-test, Mann-Whitney U test, or chi-square test. Pearson's and Spearman's coefficients determined the correlation. Statistical significance was set at $p<0.05$.

Results

The mean age of the study population was 62.7±10.7 (33-92) years, and 78 patients (78%) were female. The mean age of patients with DM (n=50) was 62.2±11.2 years, and those without DM (n=50) were 63.3±10.3 years. The frequency of females in the patients with DM and without the group with diabetes was

74% and 82%. No significant difference was found between the groups regarding sex, age, BMI, and dominant hand (p>0.05). The mean duration of DM was 12.1±7.8 years, and HbA1c averaged 7.7±1.9 in patients with DM. Only two patients had type 1 DM.

The VAS pain scores of the group with DM in motion were significantly higher than those without DM (p=0.045). The VAS pain score measured at rest was numerically higher in the group with DM (p=0.057), but this was insignificant (Table 1).

AUSCAN, DHI, and GAT evaluated hand functionality and severity of the disability, and all were significantly higher in patients with DM than in those without DM. Two components of SF-36, pain, and function, were significantly lower in the group with DM (p<0.05) (Table 2). The dominant hand's lateral grip strength, fingertip grip strength, and palmar grip strength were significantly lower in the group with DM than in those without DM (p<0.05). However, in terms of power grip and palmar grip strength in the non-dominant hand, the groups were not statistically different (p>0.05) (Table 3).

Table 1. Clinical, demographic, and pain characteristics of hand osteoarthritis patients with and without DM

	DM (n=50)	Without DM (n=50)	p
Age, years, mean±SD	62.2±11.2	63.3±10.3	0.717
Female sex, n (%)	37 (74)	41 (82)	0.334
Right hand dominancy, n (%)	48 (96)	48 (96)	0.691
BMI, mean±SD	30.0±4.7	28.4±4.7	0.091
HbA1c, mean±SD	7.7±1.9	-	-
Diagnosis duration, years, mean±SD	12.1±7.8	-	-
VAS at rest, mean±SD	2.1±1.1	1.8±1.2	0.057
VAS in motion, mean±SD	5.4±1.6	4.7±1.6	**0.045**

Chi-square test, Mann-Whitney U test, Fisher's exact test and Student's t-test were used in comparisons according to the distribution characteristics of data. Statistically significant variables are shown in bold.
DM: Diabetes mellitus, SD: Standard deviation, BMI: Body mass index, HbA1c: Hemoglobin A1c, VAS: Visual analog scale

Table 2. Functional level and quality of life in OA patients with DM and those without DM

	DM (n=50)	Without DM (n=50)	p
AUSCAN	30.7±10.1	19.9±9.6	**<0.001**
DHI	45.1±17.5	24.8±17.0	**<0.001**
GAT	70.2±39.3	52.5±35.6	**<0.001**
SF-36	-	-	**-**
1. Pain	33.4±21.1	47.9±24.1	**0.015**
2. Function	21.8±29.7	33.6±29.8	**0.002**

Data are shown as mean±standard deviation. Mann-Whitney U test or Student's t-test were used in comparisons. Statistically significant variables are shown in bold.
OA: Osteoarthritis, DM: Diabetes mellitus, AUSCAN: Australian/Canadian osteoarthritis hand index, GAT: Grip ability test, SF-36: Short form-36, DHI: Duruoz hand index

Table 3. Grip ability comparison between OA patients with and without DM

	DM (n=50)	Without DM (n=50)	p
Dominant hand			
Power grip	18.0±11.0	18.4±8.0	0.301
Lateral grip	4.5±2.1	5.9±2.1	**0.002**
Fingertip grip	3.0±1.7	3.7±1.7	**0.012**
Palmar grip	3.7±1.7	4.4±1.6	**0.007**
Non-dominant hand			
Power grip	17.9±11.2	17.9±7.5	0.406
Lateral grip	4.2±1.9	5.2±1.8	**0.007**
Fingertip grip	2.7±1.6	3.3±1.4	**0.016**
Palmar grip	3.4±1.5	3.9±1.3	0.053

Data are shown as mean±standard deviation. Mann-Whitney U test was used in comparisons. Statistically significant variables are shown in bold.
OA: Osteoarthritis, DM: Diabetes mellitus

The association and correlation of the AUSCAN, DHI, GAT, and two components of the QoL with the different types of grip strength were also evaluated. The functionality component of SF-36 was positively correlated with all indices of grip strength (Table 4).

Among women with DM, disease duration was inversely correlated with all non-dominant hand index strength measures except for the power grip. However, among men with DM, HbA1c levels were positively correlated with lateral grip strength in both dominant and non-dominant hands (Table 5).

Discussion

This study was conducted on patients with hand OA and found that AUSCAN, DHI, VAS pain in motion, and GAT were

Table 4. Correlation of AUSCAN, DHI, GAT, SF-36 with handgrip strength

	AUSCAN		DHI		GAT		SF-36 (pain)		SF-36 (function)	
Grip types	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**
Dominant hand										
Power grip	**<0.001**	-0.446	**<0.001**	-0.336	**0.001**	-0.338	0.232	0.121	**0.001**	0.333
Lateral grip	**<0.001**	-0.577	**<0.001**	-0.500	**<0.001**	-0.362	0.070	0.209	**<0.001**	0.412
Fingertip	**<0.001**	-0.523	**<0.001**	-0.406	**0.001**	-0.330	0.247	0.117	**0.003**	0.297
Palmar grip	**<0.001**	-0.551	**<0.001**	-0.443	**<0.001**	-0.393	0.253	0.115	**<0.001**	0.435
Non-dominant hand)										
Power grip	**<0.001**	-0.460	**<0.001**	-0.374	**0.021**	-0.230	0.168	0.139	**<0.001**	0.364
Lateral grip	**<0.001**	-0.602	**<0.001**	-0.516	**<0.001**	-0.405	0.122	0.156	**<0.001**	0.357
Fingertip	**<0.001**	-0.448	**<0.001**	-0.345	**0.004**	-0.285	0.546	0.061	**0.006**	0.275
Palmar grip	**<0.001**	-0.515	**<0.001**	-0.432	**0.001**	-0.331	0.231	0.121	**<0.001**	0.467

Statistically significant variables are shown in bold.
AUSCAN: Australian/Canadian osteoarthritis hand index, DHI: Duruoz hand index, GAT: Grip ability test, SF-36: Short form-36

Table 5. Correlation of length of DM and hemoglobin A1c level with AUSCAN, DHI, GAT, SF-36, and handgrip strength

	Female (n=37)				Male (n=13)			
	Length of DM		Hemoglobin A1c		Length of DM		Hemoglobin A1c	
	r	p	r	p	r	p	r	p
AUSCAN	0.141	0.404	0.006	0.971	-0.208	0.495	-0.347	0.245
DHI	0.278	0.096	0.167	0.322	-0.094	0.761	-0.533	0.061
GAT	0.086	0.611	-0.036	0.833	0.055	0.858	0.005	0.986
SF-36-function	-0.050	0.767	-0.087	0.607	-0.007	0.982	0.296	0.326
SF-36-pain	0.143	0.399	0.004	0.980	-0.218	0.474	0.078	0.799
Morning stiffness	0.337	**0.041**	0.213	0.205	-0.261	0.390	0.218	0.474
VAS in motion	0.094	0.581	0.125	0.460	-0.297	0.324	-0.273	0.367
VAS in rest	-0.053	0.754	-0.141	0.405	0.019	0.950	-0.140	0.647
Dominant hand								
Power grip	-0.098	0.563	-0.192	0.255	0.129	0.674	0.467	0.108
Lateral grip	-0.127	0.452	-0.076	0.656	0.192	0.529	0.571	**0.041**
Fingertip grip	-0.064	0.705	0.129	0.447	0.017	0.956	0.407	0.167
Palmar grip	-0.280	0.094	-0.100	0.556	0.053	0.864	0.238	0.434
Non-dominant hand								
Power grip	-0.270	0.106	-0.341	**0.039**	0.121	0.694	0.418	0.156
Lateral grip	-0.387	**0.018**	-0.175	0.301	0.376	0.205	0.609	**0.027**
Fingertip grip	-0.392	**0.016**	-0.211	0.211	0.182	0.552	0.464	0.110
Palmar grip	-0.395	**0.016**	-0.305	0.067	0.182	0.552	0.276	0.362

Statistically significant variables are shown in bold.
DM: Diabetes mellitus, AUSCAN: Australian/Canadian osteoarthritis hand index, DHI: Duruoz hand index, GAT: Grip ability test, SF-36: Short form-36, VAS: Visual analog scale

significantly higher, and SF-36 and grip strength were significantly lower in patients with DM compared with the patients without. Grip strength was inversely correlated with AUSCAN, DHI, and GAT. In parallel with the increase in the prevalence of DM and the life expectancy of the patients, DM-related musculoskeletal abnormalities are more commonly observed (18). However, no association between OA and DM has been definitively shown. In this study, we investigated whether DM was linked to impaired hand function and found that the hand function indices were worse, and the two components of the QoL and grip strength were lower in patients with DM.

Turan et al. (19) reported a significant correlation between DHI and dominant handgrip strength in patients with DM, which is consistent with the results of the present study. Also, in agreement with the current study, Savaş et al. (20) determined higher DHI scores in people with diabetes than in healthy controls. The reasons behind these observations may involve the pathological changes including Dupuytren's contracture, trigger finger, and cheiroarthropathy, attributed to DM itself and its duration. These are thought to be associated with microvascular complications (21). Besides, Sayer et al. (22) showed that dysregulation of blood glucose could decrease grip strength in type 2 patients with DM. Thus, these diabetic complications may increase DHI scores. Magnusson et al. (23) found that diabetic patients with OA suffered more severe pain in their hands and showed higher AUSCAN index scores. In another study by this group, long-term type 1 DM (>45 y) was strongly associated with increased pain (high AUSCAN index) and stiffness in the hands and more significant overall disability consistent with a diagnosis of erosive OA (24). Higher AUSCAN index and pain VAS scores than the control group support the findings of our study. Five components of SF-36 were found lower in patients diagnosed with type 2 DM compared to a group of healthy controls (25), which is consistent with our results that the pain and functional components of SF-36 were significantly lower among patients with DM.

Autonomic disorders and sensory neuropathy are common in DM, however, only a few studies have been published on the effect of DM on motor functions. Two studies reported that patients with DM had severe distal muscle weakness (26,27). Also, patients with DM have lower physical functional capacity and hand strength than healthy controls of the same age (28). Li et al. (29) found that although the grip strength of patients with type 2 DM was lower than age-matched controls, their muscle mass was comparable. Thus, handgrip strength can be considered a good indicator of DM that one group has proposed using it as a diagnostic tool in developing countries, along with BMI, age, blood pressure, and other factors to identify patients with DM (30). Additionally, Loprinzi and Loenneke (31) showed that grip strength was a good indicator of the prevalence and severity of type 2 DM in both men and women and that reduced grip strength was associated with higher HbA1c. In another study, de Carvalho e Silva et al. (32) reported that compared to healthy controls, hand function and grip strength were poorer in patients with DM but better than in the subjects diagnosed with hand OA. The current study showed that, compared with the non-diabetic group, hand function and grip strength were reduced in patients with OA with DM.

The mechanisms proposed to explain muscle weakness in the presence of DM is complex. Uncontrolled hyperglycemia can lead to muscle protein breakdown and inadequate energy availability, resulting in poor muscle function (33). Uncontrolled glycemia is also associated with increased production of systemic inflammatory cytokines, C-reactive protein, and fibrinogen, which adversely affect muscle function (34). In addition to the direct cytokine effect on muscle breakdown, neuropathy can be involved in poor muscle function in patients with DM. In an animal study, the relative loss of torque was greater via nerve stimulation (43%) than the force lost indirectly through stimulated muscle (24%), indicating a neural deficit in DM (35). In humans, the severity of diabetic neuropathy is associated with decreased muscle strength (36). Electrophysiological studies also support the findings that the functional neuronal deficit in DM is due to disrupted re-innervation after axonal loss (37).

Type 2 DM and type 1 DM patients have different features of hand OA. Although no relationship was found between type 2 DM severity or duration and hand OA (7), long-term type 1 DM was associated with increased hand pain, disability, and stiffness (24). However, none of the studies examined gender-wide differences. Our results showed that the length of DM duration in women, and HbA1c level in men positively correlated with disability and the severity of hand OA. This novel finding needs to be further evaluated in future studies.

The limitations of our study include the small sample size, which did not allow adjusted analyses, and the lack of information about several comorbid conditions related to hand pain and dysfunction, such as polyneuropathy and carpal tunnel syndrome that are common in patients with DM (21). The lack of radiography evaluations is another limitation.

Conclusion

In conclusion, we showed reduced grip strength, and worse hand function and QoL in patients with hand OA having DM. These findings suggest that OA and type 2 DM have a complex relationship beyond age and BMI. DM may be considered an additional risk factor for OA. More studies are needed to fill in the gaps in our knowledge about how the prevention and control of DM can affect OA progression in humans.

Ethics

Ethics Committee Approval: This observational and case-control study was conducted in Atatürk Training and Research

Hospital after obtaining Ethics Committee Approval (approval number: 59, date: 22.03.2017). The study was conducted under the principles of the Declaration of Helsinki.

Informed Consent: Informed consent was obtained from all patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.A.A., S.K., Design: S.K., Data Collection or Processing: S.A.A., S.K., Analysis or Interpretation: S.A.A., Literature Search: S.A.A., S.K., Writing: S.A.A., S.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.75437
Gulhane Med J 2022;64:255-61

Epithelium-off corneal cross-linking versus transepithelial diluted alcohol and iontophoresis-assisted corneal cross-linking in keratoconus patients with thin corneas

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Date submitted:
27.12.2021
Date accepted:
25.04.2022
Online publication date:
08.09.2022

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Keywords: Keratoconus, thin cornea, transepithelial iontophoresis-assisted cross-linking, epithelium-off cross-linking, hypo-osmolar riboflavin

ABSTRACT

Aims: To evaluate the efficacy and safety of transepithelial diluted alcohol and iontophoresis-assisted corneal cross-linking (DAI-CXL) and compare 24-month visual and topographic outcomes with accelerated CXL using hypo-osmolar riboflavin (A-CXL) in keratoconus patients with thin corneas (below 400 µm with epithelium).

Methods: This retrospective study included keratoconus patients who underwent DAI-CXL or A-CXL. Uncorrected and corrected distance visual acuity (UDVA and CDVA) and data obtained from corneal topography were analyzed at baseline and 12 and 24 months of follow-up. Corneal demarcation line depth (DLD) at 1 month and corneal endothelial cell density (ECD) at 24 months were also evaluated.

Results: The study included 25 eyes of 25 keratoconus patients (mean age: 25.48±6.69 years, male: 52%). DAI-CXL and A-CXL groups consisted of 13 and 12 patients, respectively. In both groups, median UDVA improved significantly at 24 months ($p<0.05$) whereas CDVA was similar despite a trend towards improvement. Median K-max decreased by 2.77 [interquartile range (IQR): 2.67] D and 2.24 (IQR: 4.38) D in DAI-CXL group ($p=0.033$) and A-CXL group ($p=0.060$), respectively. Corneal HOAs showed a significant improvement in only the DAI-CXL group ($p=0.004$). Average DLD was 237±67 µm in DAI-CXL and 242±57 µm in A-CXL ($p=0.346$). No significant changes in ECD were observed in both groups. Median follow-up changes in UDVA, CDVA, K-max, HOAs, and ECD were similar in the groups.

Conclusions: We observed similar efficacy of transepithelial DAI-CXL to A-CXL in slowing down the progression of keratoconus in thin corneas without notable effects during a 24-month follow-up period.

Introduction

Standard (epithelium-off) corneal cross-linking (CXL) has become the gold standard treatment to prevent or slow the progression of corneal ectatic disorders, with satisfactory long-term outcomes (1). After epithelial debridement, the minimum stromal thickness should be at least 400 µm to protect corneal endothelium and other intraocular tissues from irreversible adverse effects of ultraviolet (UV) irradiation based on experimental and clinical research (2,3).

A significant portion of keratoconus patients (25%) are diagnosed at an advanced stage, with a corneal thickness of less than 400 µm, and these patients are at a higher risk of potential progression even after CXL therapy (4,5). To achieve safe and effective stabilization in progressive keratoconus patients with thin corneas (<400 µm), numerous modifications of the standard protocol have been proposed. These modifications include stromal swelling by using hypo-osmolar riboflavin (6), transepithelial and iontophoretic CXL (7,8), pachymetry-guided epithelial debridement (9), contact lens-assisted CXL (10), lenticule-assisted CXL (11), and recently introduced individualized CXL (sub400 protocol) (12).

Transepithelial (epithelium-on) approaches have several advantages over other epithelium-off techniques, including

protection against complications associated with epithelial debridement and preservation of thin corneal thickness before UV irradiation (13). Transepithelial CXL with modified riboflavin solutions including chemical enhancers has been developed to overcome the barrier effect of corneal epithelium against riboflavin. However, when compared with the epithelium-off CXL technique, transepithelial CXL has a reduced efficacy in arresting the progression of ectatic disorders (14,15).

Iontophoresis-assisted CXL (I-CXL), a recently introduced transepithelial treatment, has been found to enhance stromal riboflavin saturation in human cadaveric corneas much more than other transepithelial procedures (16,17). An electric current of 1 mA is used to transmit a negatively charged riboflavin solution with chemical enhancers through the epithelium into the stroma for 5 minutes in standard I-CXL (18). Several clinical trials have shown that the I-CXL is a safe and successful method in the treatment of progressive keratoconus; however, it has a lower therapeutic efficacy compared to epithelium-off CXL (19-22). With these promising outcomes, various changes to the original I-CXL have been proposed to enhance its efficacy (23-28). The total dose of UV-A exposure has been increased in one of these modifications to compensate for the natural barrier effect of epithelium against UV-A that is required for effective CXL (24,25). The iontophoresis cycle (imbibition period) was doubled in the other studies to increase stromal riboflavin saturation (26,28).

Bilgihan et al. (25) have also introduced a new modified I-CXL technique called transepithelial diluted alcohol and iontophoresis-assisted corneal cross-linking (DAI-CXL). Briefly in this technique, two enhancers, which are diluted alcohol (10% ethanol) and iontophoresis (DAI), are utilized to improve the stromal riboflavin saturation while protecting epithelial integrity, and the total UV-A dose was increased to 7.2 J/cm^2 to ensure proper cross-link formation in the stroma (29). The long-term clinical results of this new method have shown similar visual improvement and topographic changes compared to epithelium-off CXL during the 4-year follow-up (30). The present study was performed to compare the longitudinal effects of DAI-CXL and epithelium-off CXL using hypo-osmolar riboflavin on the prevention of the disease progression and visual, refractive and topographic outcomes in keratoconus patients with thin corneas.

Methods

Patient selection

This single-center, retrospective study included patients with thin corneas (<400 μm) who underwent DAI-CXL or accelerated CXL with hypo-osmolar riboflavin solution (A-CXL) for progressive keratoconus between July 2018 and June 2019. After obtaining approval from the Local Ethics Committee of Gazi University Faculty of Medicine (approval number: E.32700, date: 22.02.2021), data were collected from the electronic medical records of the patients.

Patients who had undergone CXL due to keratoconus progression in their clinical follow-up and had preoperative minimum corneal thickness (MCT) between 350 μm and 400 μm (with epithelium) were included in the current study. In addition, patients with a postoperative follow-up of at least 24 months were included in the analysis. Exclusion criteria were previous ocular surgery, the presence of a corneal scar, a history of herpetic keratitis, pregnancy, or lactation at the time of CXL treatment.

CXL procedures

In our clinical approach, based on the outcomes of a previous study (31), A-CXL is performed only in cases with preoperative MCT above 370 μm. If MCT is lower than 370 μm, the DAI-CXL method has been chosen for treatment to preserve the corneal thickness. The key differences between DAI-CXL and A-CXL are summarized in Table 1, and the routine procedures chosen in our clinic were briefly described below.

DAI-CXL protocol

The cornea is soaked in a dextran-free hypotonic riboflavin solution of 0.2% via a corneal iontophoresis device following exposure to 10% ethyl alcohol for 10 seconds. The positive (passive) electrode is placed on the same side of the treated eye's lateral malar area, while the negative (active) electrode, a plastic barrel with a stainless-steel mesh, is placed on the corneal surface. The riboflavin solution is poured into the barrel until the steel mesh is completely coated. Subsequently, the device is set to 1.0 mA for 5 minutes, and iontophoresis is repeated for additional 5 minutes.

Following the double-cycle corneal iontophoresis, the uniformity of riboflavin distribution in the stroma, as well as the presence of riboflavin solution in the anterior chamber is verified with a blue-cobalt filter. The riboflavin that remained on the corneal surface is carefully rinsed off before UV-A irradiation to avoid the attenuation of UV-A transmission through the cornea. The cornea is irradiated by a UV-A light of 365-nm wavelength at 9 mW/cm^2 power for 13 minutes (total energy density: 7.2 J/cm^2). During irradiation, a balanced salt solution is administered to the corneal epithelium every 1 minute to keep it wet and minimize the probability of an epithelial defect. At the end of the procedure, the corneal surface is treated with a topical antibiotic and a therapeutic contact lens was applied.

A-CXL protocol

Corneal epithelium debridement is performed within an 8-mm diameter and corneal stroma is saturated with iso-osmolar riboflavin solution 0.1%, one drop every minute for 30 minutes. Then, hypo-osmolar 0.1% riboflavin solution is applied every 30 seconds until the MCT reaches 400 μm before UV

irradiation. Corneal thickness is measured 3 times by ultrasonic pachymetry in the area corresponding to the thinnest location according to the preoperative pachymetry map. Finally, CXL is performed by exposing the cornea to UV-A light at an irradiance of 9 mW/cm^2 for 10 minutes (total energy density: 5.4 J/cm^2). One drop of hypo-osmolar riboflavin solution is applied over the cornea every 2 minutes during irradiation. A topical antibiotic is applied at the end of the procedure, and a therapeutic CL is placed over the cornea.

Data collection and outcome measures

In our clinical practice, examination of visual acuity including the manifest refractive correction and also corneal topographic evaluation is performed to determine the status of disease progression. Moreover, the demarcation line depth (DLD) at 1 month and annual specular microscopic evaluation are routinely performed in all patients undergoing CXL procedure, and all data obtained are stored in their medical files. Data obtained from preoperative and postoperative 12- and 24-month follow-ups of patients with at least 2 years of postoperative follow-up were analyzed.

The main outcomes in the current analysis were visual, refractive, and topographic parameters. The decimal values of uncorrected distance visual acuity (UDVA) and corrected distance visual acuity (CDVA) measured by the Snellen chart and converted to logarithm of the minimum angle of resolution (logMAR) units were retrieved for statistical analysis. Further, the manifest spherical equivalent (MSE) values were recorded. The parameters obtained from the corneal topography were as follows; maximum keratometry (K-max), mean keratometry (K-mean), cylindrical reading, MCT, and total corneal higher-order aberrations (HOAs). Corneal aberrations are routinely measured for a pupil diameter of 4 mm.

Data from the anterior segment optical coherence tomography (AS-OCT) were analyzed to evaluate the corneal demarcation line at 1 month. The distance from the corneal epithelium to the visible hyperreflective line in the stroma, measured using a flap tool option provided by the device, was defined as the DLD. The results of corneal endothelial cell density (ECD) measurement using specular microscopy were also recorded.

Statistical Analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) 20 (IBM Corp., Armonk, NY, USA). The distribution of the data was analyzed by the Shapiro-Wilk test. Differences between visits within groups were analyzed using paired t-test for normally distributed data and Wilcoxon signed-rank test for non-normally distributed data. Inter-group analyses were performed by using the t-test for normally distributed data and Mann-Whitney U test for non-normally distributed data. Categorical variables were analyzed by using Fisher's exact test. P values less than 0.05 were considered statistically significant.

Results

Electronic medical records of 187 CXL patients were reviewed and 13 patients [27.46±7.52 (19-34) years, 9 female (69.3%)] who underwent DAI-CXL and 12 patients [23.33±5.12 (17-29) years, 9 male (75.0%)] who underwent A-CXL between July 2018 and June 2019 were included in the analysis. Age distribution was similar in the two groups (p=0.152), whereas male sex ratio was more prominent in the A-CXL group (p=0.004). Preoperative and postoperative visual, refractive, and topographic data of the patients were summarized in Table 2. Baseline clinical parameters in the two groups were comparable in terms of visual acuity, K-readings, corneal thickness, and corneal aberrations (p>0.05) (Table 2).

Table 1. CXL methods

	DAI-CXL	A-CXL
Epithelium status	On	Off
Protocol modification	Ethanol 10% for 10 seconds	Accelerated UV application
Chromophore (concentration)	Riboflavin 0.2%	Riboflavin 0.1%
Chromophore carrier	No enhancer, no dextran	Dextran
Chromophore osmolarity	Hypo-osmolar	Iso-osmolar and hypo-osmolar
Iontophoresis current (mA)	10	NA
Saturation time (minutes)	10 (2 cycles of 5 minutes)	30
Intensity of UVA (mW)	9	9
Duration of UVA (minutes)	13	10
Irradiation mode	Continuous	Continuous
UVA source	CXL-Vario; Peschke Meditrade GmbH, Switzerland	CXL-Vario; Peschke Meditrade GmbH, Switzerland
Total fluence (J/cm^2)	7.2	5.4

DAI-CXL: Transepithelial diluted alcohol and iontophoresis assisted corneal cross-linking, A-CXL: Accelerated corneal cross-linking with hypo-osmolar riboflavin solution, UVA: Ultraviolet A

Table 2. Comparison of preoperative and postoperative visual, refractive and topographic outcomes of DAI-CXL (n=13) and A-CXL (n=12) groups

	Group	Baseline	12 month	P‡	24 months	P‡	Median change	P‡
UDVA (logMAR)	**DAI-CXL**	1.30 (0.20)	1.30 (0.52)	0.144	0.70 (0.10)	**0.028**	-0.50 (0.30)	0.387
	A-CXL	0.52 (0.84)	0.52 (0.90)	**0.039**	0.52 (0.60)	**0.046**	0.00 (0.66)	
	P†	0.251	0.456		0.332			
CDVA (logMAR)	**DAI-CXL**	0.39 (0.12)	0.39 (0.17)	0.273	0.30 (0.10)	0.109	-0.12 (0.10)	0.277
	A-CXL	0.30 (0.50)	0.22 (0.51)	**0.050**	0.30 (0.19)	0.068	-0.08 (0.55)	
	P†	0.829	0.601		0.730			
MSE (D)	**DAI-CXL**	-2.50 (3.50)	-2.50 (2.75)	0.102	-2.00 (2.20)	**0.028**	0.50 (0.95)	0.470
	A-CXL	-5.00 (7.00)	-6.25 (4.13)	0.678	-5.00 (7.50)	0.953	-0.50 (2.50)	
	P†	0.360	0.220		0.299			
K-max (D)	**DAI-CXL**	62.64 (7.12)	63.34 (11.38)	0.972	59.29 (7.45)	**0.033**	-2.77 (2.67)	0.936
	A-CXL	60.40 (16.19)	64.74 (18.34)	0.875	64.02 (14.74)	0.060	-2.24 (4.38)	
	P†	0.376	0.295		0.270			
K-mean (D)	**DAI-CXL**	56.81 (7.10)	57.16 (7.76)	0.196	56.58 (8.26)	0.422	-0.23 (1.58)	0.270
	A-CXL	52.03 (6.10)	53.37 (7.75)	0.480	51.76 (7.06)	0.092	0.10 (1.13)	
	P†	0.152	0.152		0.098			
Topographic cylinder (D)	**DAI-CXL**	-8.11 (2.05)	-6.81 (3.02)	0.507	-5.51 (3.50)	0.388	1.19 (2.50)	0.936
	A-CXL	-4.04 (4.60)	-4.42 (4.52)	**0.019**	-4.46 (4.55)	0.347	-0.15 (1.22)	
	P†	0.728	0.689		0.936			
MCT (mm)	**DAI-CXL**	362.00 (64.50)	352.00 (26.00)	0.674	380.00 (41.00)	0.814	-15.00 (50.00)	0.503
	A-CXL	380.00 (41.50)	390.00(56.50)	0.814	372.00 (42.50)	0.754	10.00 (45.00)	
	P†	0.060	0.295		0.810			
Corneal HOAs (mm)	**DAI-CXL**	2.02 (1.59)	1.29 (1.36)	0.098	1.41 (1.73)	**0.004**	-0.27 (0.58)	0.114
	A-CXL	1.82 (1.35)	1.50 (0.86)	0.328	1.50 (0.93)	0.195	-0.27 (0.47)	
	P†	0.406	0.376		1.000			

Data were shown as median (interquartile range). †Wilcoxon signed-rank test, ‡Mann-Whitney U test. Boldface, significant values, p<0.05. Median change was calculated for each parameter as 24 months postop-preop. DAI-CXL: transepithelial diluted alcohol and iontophoresis assisted corneal cross-linking, A-CXL: Accelerated corneal cross-linking with hypo-osmolar riboflavin solution, UDVA: Uncorrected distance visual acuity, CDVA: Corrected distance visual acuity, logMAR: Logarithm of the minimum angle of resolution, MSE: Manifest spherical equivalent, K-max: Maximum keratometry, K-mean: Mean keratometry, D: Diopter, MCT: Minimum corneal thickness, HOAs: Higher-order aberrations

Visual acuity and refractive outcomes

Median UDVA improved significantly in the A-CXL group at 12 and 24 months compared to baseline (p=0.039 and p=0.046, respectively). In the DAI-CXL group, UDVA improved only at 24 months (p=0.028). The improvement in both groups was similar at the 24-month follow-up visit (p=0.387). There was a trend of improvement in CDVA at 24 months in both groups, but not statistically significant. Comparative analysis of the change in CDVA was similar in both groups at the 24-month follow-up visit (p=0.277). Although median MSE decreased significantly only in the DAI-CXL group at 24 months (p=0.028), the changes in MSE values in both groups were similar at 24 months (p=0.470).

Topographic outcomes

Median K-max decreased by -2.77 [interquartile range (IQR): 2.67] D and -2.24 (IQR: 4.38) D in DAI-CXL group (p=0.033) and A-CXL group (p=0.060), respectively at 24 months. These improvements in K-max were similar in both groups (p=0.936). Median K-mean slightly improved in both groups, without statistical significance (p=0.363 and p=0.311, respectively). Similarly, no difference was found in improvement in K-mean between groups at the end of the follow-up (p=0.270). The median topographic cylinder improved statistically only in the A-CXL group at 12 months and inter-group analyses did not show statistical significance (p=0.936). During the follow-up, there was a non-significant decrease in MCT in the A-CXL group and the changes in corneal thickness were similar between the groups at 24 months (p=0.503). Total corneal HOAs analysis showed a significant improvement in only the DAI-CXL group at 24 months (p=0.004), but there was no difference in the median changes in HOAs between groups at the 24-month follow-up visit (p=0.114).

Efficacy of procedures

An evident corneal demarcation line was identified using AS-OCT in all eyes 1 month after the procedures, with an average

DLD of 237.07±67.91 μm (range: 145 to 301 μm) in DAI-CXL and 242.55±57.52 μm (range: 154 to 317 μm) in A-CXL (p=0.346). At the 24-month follow-up visit, the K-max values in the DAI-CXL group remained stable (≤1 D change) or decreased more than 1 D in 12 eyes (92.31%). Similarly, stabilization or improvement in K-max was observed in 11 eyes (91.67%) in the A-CXL group. Disease progression (increase in K-max >1 D) further occurred in 1 eye in each group during the follow-up period (p=1.000).

Safety of procedures

At 24 months, mean ECD changed from 2534.12±163.20 cells/cm^2 to 2418.75±186.42 cells/mm^2 in DAI-CXL group and from 2504.42±126.07 cells/cm^2 to 2425.64±109.31 cells/mm^2 in A-CXL group at 12 months postoperatively (p=0.273 and p=0.099, respectively). The mean change of ECD was not statistically significant between the groups (-113.75±119.93 cells/cm^2 in DAI-CXL vs -76.85±63.12 cells/cm^2 in A-CXL, p=0.927).

The postoperative epithelial defect was not observed in any patient and contact lenses were removed from all patients on the first postoperative day in the DAI-CXL group. After A-CXL treatment, the epithelial defect was closed within 3-4 days, after which the contact lens was removed. None of the treated patients in either group developed vision-threatening haze/scar or infection during the follow-up.

Discussion

Herein, we demonstrate that DAI-CXL is as effective as A-CXL with hypo-osmolar riboflavin in the stabilization of keratoconus in patients with very thin corneas (below 400 μm with epithelium) during the 2-year follow-up. Moreover, visual and corneal topographic changes are similar between groups without any deterioration in ECD at 2 years.

The management of keratoconic corneas thinner than 400 μm is still an important issue to be solved. One of the suggested methods is transepithelial I-CXL which is effective and safe in halting keratoconus progression in thin corneas (8). However, I-CXL still has a less therapeutic effect compared to standard CXL (20,21). To improve the efficacy of the original I-CXL, many modifications have been proposed (23-28). Transepithelial DAI-CXL is one of these techniques which improves the stromal riboflavin concentration while preserving epithelial integrity via 10% ethanol instead of enhancers in riboflavin solution. The preliminary and long-term clinical results of this new method have shown similar visual and topographic improvement compared to standard CXL (25,30).

To overcome the epithelial barrier to riboflavin, dilute alcohol (10% for 10 seconds) was administered to loosen the adhesions of hemidesmosomes to increase epithelial permeability in the DAI-CXL procedure (32). The other enhancer was iontophoresis, which increased riboflavin diffusion. Furthermore, compared to standard I-CXL, the iontophoresis cycle time was doubled (10 minutes). Because the concentration of riboflavin in corneas treated with 5-minute iontophoresis is two-fold lower in conventional I-CXL than that in corneas treated with an epithelium-off protocol (16,33). Although the exact concentration of stromal riboflavin needed for proper corneal cross-link formation is unknown, preclinical and clinical studies have shown that doubling the imbibition time (2 cycles of iontophoresis) enhanced riboflavin saturation to up to 80% of that yielded with the epithelium-off technique, as well as the effectiveness of the I-CXL in halting the disease progression (26-28,34).

The epithelial photo attenuation of UV-A energy during corneal exposure is another aspect that could limit the CXL reaction in the epithelium-on method. This limitation could be overcome by increasing the overall UV-A fluence (29). In DAI-CXL, the total fluence was increased from 5.4 J/cm^2 to 7.2 J/cm^2. Similarly, Mazzotta et al. (24) enhanced the UV-A energy dose to 7 J/cm^2 and utilized pulsed-light UV-A irradiation (18 mW/cm^2 for 6.28 minutes of exposure duration). The 3-year clinical findings of this modified approach, called enhanced fluence pulsed light iontophoresis (EF I-CXL), showed that it was able to stop keratoconus progression with a significant reduction in K-max of 1.4±0.8 D, but no changes in CDVA and corneal thickness. These findings are in line with our 2-year results. We observed that median CDVA and MCT remained stable, while median K-max improved significantly by 2.77 (IQR: 2.67) D in the DAI-CXL group and the median changes in K-max did not differ between groups after 2 years of follow-up.

Cantemir et al. (8) reported the first clinical outcomes of standard I-CXL in keratoconus patients with thin corneas (below 400 μm with epithelium). Fifteen eyes of 15 patients were evaluated and postoperative follow-up was 12 months. The authors have demonstrated improvements in UDVA and CDVA by 0.15 LogMAR which is consistent with our results on UDVA. However, CDVA was not changed after DAI-CXL in our study cohort. There was a significant decrease in K-max by 0.4 D in the previous study but in our case series there was an improvement in K-max by 1.54 D. Moreover, a visible demarcation line was only observed in 5 patients (30%) with an average depth of 184±26 μm in their case series, while there was a prominent demarcation line in all our patients with an average depth of 237±67 μm. Considering the improvement in K-max and DLD values, our results are more favorable compared to the aforementioned study by Cantemir et al. (8), which could be due to changes in the DAI-CXL protocol, such as a combination of extended imbibition time and compensated UV-A irradiation.

According to several authors, the depth of the demarcation line evaluated by AS-OCT or confocal microscopy is an indirect predictor of CXL therapy success (35,36). This measurement could be more beneficial when evaluating the efficacy of a new modified approach, particularly in cases involving I-CXL. In

most standard I-CXL studies, the penetration depth varied from 100 to 240 µm (8,19,37). Wu et al. (34) extended the imbibition period (two cycles of iontophoresis) in EI-CXL and obtained a penetration depth approximately two-fold higher (251 µm) than that achieved with the same iontophoretic device for 5 minutes. After a 5-year follow-up, this 10-minute iontophoretic imbibition improved penetration depth, resulting in comparable keratometric and visual results to standard CXL. Nonetheless, the DLD in the EI-CXL group was still more shallow than in the standard CXL group (34). Furthermore, increasing the total UV-A fluence in EF I-CXL allowed the demarcation line to appear in 80 % of eyes with an average depth of 285 µm (24). In DAI-CXL with the combination of these two modifications, the demarcation line was visible in all patients at an average depth of 237 µm. This is slightly lower than that of the A-CXL group (242 µm), but this difference was not statistically significant.

The protection of the corneal endothelium from UV irradiation is a major safety concern of the CXL technique in thin corneas. Kymionis et al. (3) found a significant decrease in ECD following the standard CXL protocol in keratoconus patients with MCT less than 400 µm after epithelial removal. Permanent endothelial damage caused by CXL could result in a corneal scar and consequent significant visual loss (38). In addition to the efficacy of modified CXL treatment in thin corneas, its safety should be demonstrated by clinical data, including ECD. The safety of this modified method has been also verified by measuring ECD and the maintenance of corneal clarity after the procedure. There was no significant change in ECD 2 years after DAI-CXL. Similarly, Cantemir et al. (8) have also stated the safety of standard I-CXL in thin corneas with no evident changes in ECD.

Cantemir et al. (8) demonstrated an improvement in visual and topographical results in thin corneas following standard I-CXL. However, due to the lack of a control group treated with the epithelium-off approach in the previous study (8), no definite conclusion could be reached regarding the efficacy of the I-CXL method.

This study has several strengths and limitations. The inclusion of a reference intervention is considered the major strength of the present study. However, the single-center and retrospective study design, small sample size, and uneven gender distribution limit the generalizability of the results. We were also not able to perform adjusted analyses due to the low sample size. Finally, although a 24-month follow-up period may be considered sufficient to draw sound conclusions, a longer follow-up duration is required to demonstrate the clinically important outcomes.

Conclusion

In conclusion, DAI-CXL has been proved to be effective in halting keratoconus progression in thin corneas without any side effects during the 2-year follow-up period. This modified I-CXL method could provide a safe and effective alternative epithelium-on CXL treatment option in advanced keratoconus patients.

Ethics

Ethics Committee Approval: The study was approved by the Gazi University Faculty of Medicine of Local Ethics Committee (approval number: E.32700, date: 22.02.2021).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: B.S.U., M.C.Ö., B.A., K.B., Concept: B.S.U., M.C.Ö., K.B., Design: B.S.U., M.C.Ö., B.A., K.B., Data Collection or Processing: M.Y., Analysis or Interpretation: B.S.U., M.C.Ö., B.A., K.B., Literature Search: B.S.U., M.Y., Writing: B.S.U., M.C.Ö., K.B.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.93585
Gulhane Med J 2022;64:262-7

Caffeine intake and bone mineral density in postmenopausal women

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Date submitted:
14.04.2022
Date accepted:
03.08.2022
Online publication date:
08.09.2022

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Keywords: Osteopenia, osteoporosis, caffeine, bone mineral densitometry, dual-energy X-ray absorptiometry, DXA

ABSTRACT

Aims: Osteoporosis is a metabolic bone disease characterized by low bone mass and deteriorated bone tissue. There is ongoing debate about the effect of caffeine intake on bone metabolism due to inconsistent study results. This study aimed to assess the association between caffeine intake and bone mineral density (BMD).

Methods: This single center cross-sectional study, prospectively enrolled postmenopausal women aged between 41 and 65 years who underwent bone mineral density measurement by dual-energy X-ray absorptiometry (DXA) at the lumbar spine and femoral neck. Caffeine intake was estimated using a food frequency questionnaire (FFQ) and a caffeine-specific FFQ. Patients consuming more than 260 mg/day caffeine were classified as high consumers.

Results: The study included 80 subjects (mean age: 57.1±5.6 years). Daily caffeine intake was 229.7±119.5 mg, and 36.3% (n=29) of the patients was classified as high consumers. The mean total T-score was -1.4±0.9 at the lumbar spine and -1.7±0.9 at femoral neck. High and low caffeine consumers had similar mean total T-score at the lumbar spine (-1.4±1.1 vs. -1.4±0.8, p=0.849). However, femoral neck mean T-score was significantly lower in high caffeine consumers (-2.0±0.9 vs. -1.5±0.7, p=0.033). The amount of daily caffeine intake showed a negative, moderate correlation with femoral neck T-score (r=-0.251, p=0.025).

Conclusions: The results of this study suggest that higher caffeine intake may be associated with lower T-scores on DXA of the femoral neck.

Introduction

Osteoporosis is a metabolic bone disease, characterized by low bone mass, deteriorated bone tissue and disrupted bone microarchitecture, which results in increased fracture risk and decreased quality of life. Osteoporosis is an important public health problem, associated with adverse health effects and economic burden. In parallel with the globally increasing aging population, osteoporosis is also increasing worldwide. Osteoporosis is diagnosed either through a history of a fragility fracture without a serious trauma or bone mineral density (BMD) measurement (1) by dual-energy X-ray absorptiometry (DXA).

The risk factors of osteoporosis can be classified as modifiable (low physical activity, inadequate calcium and vitamin D intake, high alcohol and caffeine intake, smoking, and low muscle mass) and non-modifiable factors (hereditariness, Caucasian ethnicity, age, female sex, hormonal status) (2,3). The risk of osteoporosis in women increases after menopause (1,4,5). Inadequate calcium intake and low serum 25 (OH) vitamin D_3 [$25(OH)D_3$] levels are important risk factors for osteoporosis because of increased bone turnover (6).

Caffeine intake may not harm human health unless the amount of consumption exceeds the safe limit (7). However,

high doses of caffeine may lead to damaging effects on bone metabolism, bone healing and osteoblastic activity (8). Caffeine can also reduce calcium absorption and increase its elimination (5). A study on postmenopausal women showed that high caffeine intake can increase lumbar bone loss (9). Another study reported that three or more cups consumption of coffee in a day may be a risk factor for osteoporosis (1). Nevertheless, the effect of caffeine consumption on bone metabolism is still controversial (10). This study aimed to evaluate the association between caffeine intake and bone density health among postmenopausal women with osteopenia or osteoporosis.

Methods

This single-center, cross-sectional study was conducted between May 2019 and December 2021 at Gaziler Hospital Physical Medicine and Rehabilitation Unit. Postmenopausal women aged between 41 and 65 years were included. Patients were excluded if they had secondary osteoporosis, diabetes mellitus, hyperthyroidism, primary hyperparathyroidism, hematologically or gastrointestinal disorders, and autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, or renal diseases. Other exclusion criteria were the history of metabolic bone disease, regular use of vitamin D and/or calcium supplements, anti-acids with calcium; medications that influence bone mass (e.g., corticosteroids, thyroid hormone, lithium, heparin, furosemide and proton pump inhibitors), and smoking. All participants provided signed, informed consent. The study protocol was approved by the Gülhane Training and Research Hospital Ethics Committee (no: 19/197, date: 14.05.2019). All the study procedures followed the Declaration of Helsinki and the Uniform Requirements for articles submitted to biomedical journals.

Demographic and anthropometric data (body weight, height, hip, and waist circumferences) were collected. Body mass index was calculated as weight (kg)/height (m^2). The widest perimeter of the hip was recorded for hip circumference and the waist circumference was measured at 2-cm distal from the umbilicus. Serum $25(OH)D_3$ and serum total calcium levels were retrieved from the hospital registries.

Bone density assessment

Osteopenia or osteoporosis was diagnosed by DXA (Osteosys Primus device, Seoul, Korea). BMD results were classified according to Bone Health and Osteoporosis Foundation Guideline (11). BMD within 1.0 standard deviation (SD) of the mean for a young-adult reference population was classified as 'normal', BMD between 1.0 and 2.5 SD for a young-adult population was classified as 'osteopenia' and BMD 2.5 SD or more below the mean for a young-adult reference population was classified as 'osteoporosis'.

Nutritional evaluation

Two food frequency questionnaires (FFQ) were used in the study to evaluate food intake over the last month: FFQ for nutrient intake (including 42 foods and beverages), and a caffeine-specific FFQ to determine foods and beverages with caffeine content. The adequacy of nutrient intake was assessed according to age and gender using the Turkey Dietary Guidelines (12). The daily intake of macro- and micronutrients, and energy was calculated from the data obtained in the FFQ, using BeBiS software version 7.2 (Bebispro for Windows, 2010). In this study, the caffeine-containing food list used in the article by Işgın et al. (13) was updated and a caffeine-specific FFQ was applied. The consumption of 35 different foods and beverages (coffee, tea, cola, chocolate, etc.) with caffeine content was recorded. The following responses regarding the consumption frequency were used: never, once a month, twice a month, 1-2 times a week, 3-4 times a week, 5-6 times a week and every day. After obtaining data on the caffeine specific FFQ, a national study (14) and US Food Data Central (15) were used to detail how much caffeine each food and beverage contained, and the total caffeine intake was calculated. According to recent data (16), patients consuming more than 260 mg/day were classified as high consumers.

Assessment of physical activity

Physical activity level (PAL) was calculated by dividing the total (durations of activity in minutes multiplied by the physical activity ratio for each activity) by 1,440 min. The calculated PAL values were evaluated as follows: <1.40 sedentary, 1.40-1.69 light, 1.70-1.99 moderate, 2.00-2.40 heavy, >2.40 very heavy activity (17).

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Mac, version 22.0 software (SPSS Inc., Chicago, IL, USA). Categorical variables are presented as numbers and percentages, and the numerical variables as mean and SD. The normality distribution was assessed using the Kolmogorov-Smirnov test. Categorical data were compared using the chi-square test, and continuous data were compared using the Student's t-test. Correlation coefficients were calculated using the Pearson's test. $P<0.05$ was accepted to be statistically significant.

Results

The study included 80 subjects (mean age: 57.1±5.6 years). Mean postmenopausal duration was 8.1±4.4 years. Table 1 shows the basic characteristics of the participants. No patient was underweight, 37.4% (n=30) were overweight and 26.4% (n=21) were obese. Most patients (40.0%, n=32) were graduates of university or higher. The mean PAL value was

1.3±1.1, and most patients were sedentary. When the patients were grouped as low or high consumers of caffeine, there was no statistically significant difference between these groups in terms of main characteristics.

The daily energy, nutrients, and caffeine intake of the participants are shown in Table 2. Mean caffeine intake was 229.7±119.5 mg, with 36.3% (n=29) as high consumers. There was no statistically significant difference between the high consumer and low consumer groups in terms of nutrient intake. Calcium intake was inadequate in 90.0% (n=72) of the patients.

Most patients had osteopenia (61.2% according to the lumbar spine total T-score and 66.3% according to the femoral neck T-score). Classification of the patients according to the lumbar total and femoral neck T-scores showed significant differences concerning the diagnosis of osteopenia or osteoporosis (p=0.013) (Table 3).

The mean total T-score was -1.42±0.89 at the lumbar spine and -1.69±0.87 at femoral neck. High and low caffeine consumers had similar mean total T-score at the lumbar spine (-1.4±1.1 vs. -1.4±0.8, p=0.849). However, femoral neck mean T-score was significantly lower in high caffeine consumers (-2.0±0.9 vs. -1.5±0.7, p=0.033).

While 84.3% of patients (n=43) had osteopenia, 15.7% of patients (n=8) had osteoporosis in low caffeine consumers. In high caffeine consumers, 58.6% of patients (n=17) had osteopenia, and 41.4% of patients (n=12) had osteoporosis. compared to low caffeine consumers, the percentage of patients with osteoporosis was significantly higher in high caffeine consumers (p=0.011).

A negative moderate correlation was determined between caffeine intake and the femoral neck T-score (Table 4). There was no correlation between total caffeine intake and the total T-score.

Table 1. Basic characteristics of the patients

Age, years, mean (SD)	57.1 (5.5)
Serum vitamin D (ng/mL), mean (SD)	26.9 (16.8)
Serum calcium (mg/dL), mean (SD)	10.5 (9.5)
Postmenopausal period (year), mean (SD)	8.1 (4.4)
BMI (kg/m^2), mean (SD)	27.1 (4.4)
Waist circumference (cm), mean (SD)	98.3 (10.8)
Hip circumference (cm), mean (SD)	106.1 (9.7)
Waist/hip ratio, mean (SD)	0.9 (0.05)
BMI classification	
Underweight, n (%)	-
Normal, n (%)	29 (36.3)
Overweight, n (%)	29 (36.3)
Obese, n (%)	21 (26.4)
Education	
Illiterate, n (%)	2 (2.5)
Primary school, n (%)	27 (33.8)
Secondary-high school, n (%)	18 (22.5)
University or Higher, n (%)	32 (40.0)
PAL classification	
Sedentary, n (%)	43 (53.8)
Light activity, n (%)	36 (45.0)
Moderate activity, n (%)	1 (1.2)
Heavy activity, n (%)	0 (0.0)
Very heavy activity, n (%)	0 (0.0)

SD: Standard deviation, BMI: Body mass index, PAL: Physical activity level

Discussion

The results of this study suggest that high caffeine intake deteriorates BMD in postmenopausal women, particularly in the femoral neck. Caffeine intake is widely common worldwide (18).

Table 2. Daily energy and nutrient intake

Caffeine (mg), mean (SD)	229.7 (119.5)
Total Energy intake [kcal], mean (SD)	1661.6 (388.2)
Carbohydrates [g/day], mean (SD)	150.5 (47.4)
Carbohydrates, n (%)	37.2 (7.4)
Protein [g/day], mean (SD)	66.5 (17.9)
Protein, n (%)	16.5 (2.5)
Fat [g/day], mean (SD)	86.2 (23.7)
Fat, n (%)	46.2 (6.6)
Cholesterol [mg/day], mean (SD)	313.8 (115.8)
Fiber [g/day], mean (SD)	20.0 (7.2)
Vitamin A, mean (SD)	1055.7 (867.9)
Vitamin C, mean (SD)	81.0 (40.2)
Vitamin E, mean (SD)	15.4 (6.5)
Calcium, mean (SD)	777.1 (272.0)
Phosphorus, mean (SD)	1189.7 (335.3)
Magnesium, mean (SD)	269.6 (86.8)

SD: Standard deviation

Table 3. The distribution of patients according to the lumbar total and femoral neck T-scores

	Lumbar total T-score		Femoral neck T-score		p*
	n	%	n	%	
Normal	20	25.0	13	16.3	
Osteopenia	49	61.2	53	66.3	0.013
Osteoporosis	11	13.8	14	17.4	

*Chi-square test

Table 4. Correlation between caffeine intake and total lumbar spine and femoral neck T-score

		Lumbar spine	Femoral neck
Caffeine	r	-0.12	-0.251
	p*	0.915	0.025

*Pearson test

Coffee, tea, energy drinks and many carbohydrated drinks, as well as cocoa, chocolate and some dietary supplements contain caffeine (19). Approximately 80% of adults consume at least one caffeine-containing beverage per day (20).

Caffeine consumption is classified as one of the modifiable risk factors for osteoporosis development (2). Reviews of the safe limits of caffeine consumption concluded that 200 mg in a single dose and 400 mg throughout the day should not be exceeded, and that caffeine will not cause harm when the consumption remains in the recommended range (21). In this study, the mean caffeine intake was 229.7±119.5 mg/d that is consistent with the previous data (22).

With respect to the clinical effects of caffeine intake on bones (e.g., BMD loss, fractures), previous research findings are inconsistent. Hallström et al. (5) reported a small reduction in bone density with caffeine intake but no increase in the risk of new fractures. Harter et al. (23) found no association between caffeine intake and calcium intake and bone mass. In contrast, in a meta-analysis by Lee et al. (24), a positive dose-dependent association between caffeine and fracture formation among women was reported. De França et al. (25) investigated the dietary patterns in postmenopausal Brazilian women and observed an inverse relationship between coffee, tea, sweet food consumption and total femoral BMD.

Correlation analysis in the current study showed that the femoral neck T-score but not the lumbar spine T-score was inversely related to the amount of caffeine intake. The differences in T-scores between the BMD measurement sites might have influenced the correlation analyses. Because, differences are common between the BMD measurement sites. Alarkawi et al. (26) reported lower lumbar spine BMD than the femoral neck BMD. Rochmis et al. (27) also reported discordance between the femoral total and femoral neck BMD measurements. Further studies are necessary to explain the exact reason for the site-specific relationship between caffeine intake and BMD.

In this study, 36.3% of the patients who were classified as high consumers, and the femoral total T-score among high consumers was lower than those of the low consumers. This result was in concordance with the correlation analysis, which showed an inverse relationship with caffeine intake. Similarly, Hallström et al. (5) investigated the effect of long-term caffeine consumption on BMD and fracture risk. When the high consumers (≥4 cups) were compared with low consumers (≤1 cup), high coffee intake was associated with 2% lower BMD in proximal femoral BMD and 4% in the lumbar spine.

Concerns related to the correct assessment of caffeine intake have been discussed previously (23). Issues of objectivity occur due to biased methods reliant on patient reports (16). In this study, dietary assessment and caffeine intake were assessed using the FFQ administered by a registered dietician to optimize the method of evaluation of the amount of caffeine intake. In addition to the measurement method of caffeine intake, differences in the metabolism and absorption of caffeine between individuals can change the biological effects of caffeine (28). As can be seen in the results section, the percentage of adequate calcium intake was very low. Calcium is bound to collagen fibers in the form of hydroxyapatite and is essential for bone strength. Low calcium intake has been identified as a risk factor for developing osteoporosis (29). High calcium intake can improve bone mineral content and thus reduce the risk of fractures in menopause (30). However, it should be remembered that taking calcium alone is not enough to reduce the risk of new fractures in the postmenopausal period, and it should be taken with 25(OH)D_3 (31).

There are some limitations of this study, such as the relatively small sample size and lack of longitudinal follow-up. The use of T-scores and the lack of bone density values may also be a limitation as the calculation of T-scores consider additional parameters. Finally, we could not perform adjusted analysis due to small sample size. The major strength of this study was that the food consumption records were taken directly by an experienced dietician.

Conclusion

The results of this study suggest that higher caffeine intake may be associated lower T-scores on DXA of the femoral neck.

Ethics

Ethics Committee Approval: The study protocol was approved by the Gülhane Training and Research Hospital Ethics Committee (no: 19/197, date: 14.05.2019).

Informed Consent: All participants provided signed, informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: K.T.A., Ö.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.38039
Gulhane Med J 2022;64:268-73

Decreased vitamin D levels in children and adolescents with Celiac disease: A nationwide cross-sectional study

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Date submitted:
15.06.2022
Date accepted:
11.08.2022
Online publication date:
08.09.2022

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Keywords: 25-hydroxyvitamin D, vitamin D deficiency, transglutaminase antibody, gluten-free diet

ABSTRACT

Aims: Vitamin D deficiency results from malabsorption in Celiac disease (CD), and it may also be involved in the pathogenesis of CD. There is no clarity regarding vitamin D deficiency in CD. We investigated the frequency of vitamin D deficiency in children with CD compared with controls.

Methods: The database of the Turkish Ministry of Health was used for the cross-sectional descriptive study. Children with CD whose serum tissue transglutaminase (tTG) and serum 25-hydroxyvitamin D [25(OH)D] levels were available in the registry were included. The CD group was further subdivided into tTG (IgG and/or IgA) antibody-negative and positive subgroups. Individuals with CD were classified as compatible and non-compatible with a gluten-free diet (GFD) (GFD-compatible and GFD-non-compatible groups), respectively. Children who had no known malabsorption syndrome formed the control group.

Results: The median serum 25(OH)D level was 18.5 ng/mL in children with CD (n=6717) and 30.7 ng/mL in the control group (n=6717) ($p<0.001$). The vitamin D levels of the GFD-compatible (n=1102) and GFD-non-compatible groups (n=5615) were 19.36 ng/mL and 18.30 ng/mL, respectively ($p<0.001$). The rate of vitamin D deficiency was 56% in the CD group and 12% in the control group ($p<0.001$).

Conclusions: This study found significantly lower serum vitamin D levels in children and adolescents with CD. The results suggest children with CD should be evaluated for vitamin D levels and followed periodically.

Introduction

Celiac disease (CD) is an immune-mediated systemic disease triggered by the intake of gluten in genetically susceptible individuals. The prevalence of CD in the general population is estimated to be 1% in the world (1). Malabsorption caused by villous damage in CD may result in several nutritional deficiencies, including vitamin D (2). Exist different approaches to the routine evaluation of vitamin D levels and treatment of vitamin D deficiency in patients with CD (2).

Vitamin D is a micronutrient that plays an important role in calcium and bone metabolism (3,4). Vitamin D deficiency in the early stages of life may be associated with autoimmune diseases, including CD. Vitamin D might also be a potential protective factor for CD due to its role in immune system regulation (3-5). The variation in the frequency of CD according to the season of birth is partly explained by the seasonal fluctuation in vitamin D levels (6-11). Similarly, a higher frequency of CD in societies living in northern latitudes is partially associated with lower sun exposure (12). Apart from the role of vitamin D in the pathogenesis of CD, vitamin D deficiency observed in patients with CD has been associated with intestinal malabsorption caused (2). A low vitamin D level causes changes in bone metabolism decreasing bone mineral density and an increase in fracture risk (13).

The literature is inconsistent about the frequency of vitamin D deficiency in children with CD. There is also no consensus on the evaluation of vitamin D levels in CD. It has been emphasized that larger studies are required (2). This study evaluated the vitamin D levels in a large series consisting of children and adolescents diagnosed with CD. We investigated the frequency of vitamin D deficiency in children with CD compared with the controls.

Methods

This nationwide study was conducted using the ICD10 registry of the Turkish Ministry of Health National Electronic Database, which covers the public health insurance of more than 95% of the Turkish population, under the supervision of the Ministry of Health. We included child and adolescent (age ≤18 years) patients with CD (ICD: K90.0) who had a serum 25-hydroxyvitamin D [25(OH)D] measurement in the registry and was prescribed gluten-free nutrients provided by the Social Security Institution, between 2014 and 2021. The cases were identified using the ICD10 codes. We excluded patients without a tissue transglutaminase antibody (tTG, IgG and IgA) result. We also excluded patients who were diagnosed with other forms of malabsorption (ICD: K90.1-9). Demographic characteristics (e.g., sex, age), standardized 25(OH)D (ng/mL), and the date of the measurements (for seasonality purposes) were recorded. The CD group was further divided into tTG (IgG and/or IgA) antibody-positive and negative subgroups. Antibody-negative patients were considered compatible with a gluten-free diet (GFD) (GDF-compatible group), and positive patients as GFD-non-compatible (GFD-non-compatible group) (14).

The control group included persons who had none of the above-mentioned malabsorption syndromes, including CD, and who had a 25(OH)D measurement in the registry. Before the analyses, there were 8.317 patients and 47,072 control subjects aged 1-18 years. Since only the subjects with a vitamin D level between 1 and 50 ng/mL were included in the analysis, the sample size was reduced to 8127 in the study and 44,786 in the control group. The propensity score matching was used with the nearest neighbor method and 1:1 allocation ratio yielding 6.717 subjects in each group. Sex, age, and seasonality were considered in the propensity score matching analysis. We excluded patients and controls who were prescribed vitamin D supplements during the previous year. Figure 1 shows a flowchart of the study design.

Serum 25(OH)D levels were analyzed with the immunoassay method or high-pressure liquid chromatography in Turkey. Vitamin D deficiency was defined as <20 ng/mL (50 nmol/L), and vitamin D insufficiency was defined as 20 to 29.9 ng/mL (50-74.9 nmol/L) (15,16). 25(OH)D levels higher than 30 ng/mL were considered normal; vitamin D deficiency was defined as a serum 25(OH)D level less than 20 ng/mL, and insufficiency between 20 and 30 ng/mL.

Statistical Analysis

Statistical analyses were conducted using R software (R Core Team, 2020). The normality of data distribution was investigated using the Kolmogorov-Smirnov test. Continuous variables are presented as medians (quartile deviation). The Mann-Whitney U or Kruskal-Wallis H tests were used to test between-group differences. To prevent any p-hacking problems when studying a large sample, the r effect size was given for Mann-Whitney U tests, and the eta-squared effect size was given for the Kruskal-Wallis H tests. To determine the correlations between age and vitamin Ds, Spearman's correlation coefficients were calculated. Differences in different vitamin D-level categories were compared using Pearson's chi-square test. Dunn-Bonferroni post hoc test was conducted for pairwise comparisons. Receiver operating characteristic (ROC) analysis was used to determine the optimal cut-off value for vitamin D in the patient and control groups using the Youden J index. Two-sided $p<0.05$ was considered statistically significant.

Results

The median age of the CD group (n=6717) and control group (n=6717) was 10 years (4 quartile deviation). The proportion of male sex in the CD group and control group was 60%. The median vitamin D levels in the CD group and the control group

were 18.49 ng/mL and 30.70 ng/mL, respectively, and the difference was statistically significant (p<0.001) (Table 1).

The median vitamin D levels in the GFD-compatible (n=1102) and GFD-non-compatible (n=5615) groups were 19.36 ng/mL and 18.30 ng/mL, respectively, and the difference was statistically significant. In a post hoc analysis, vitamin D levels were significantly different (p<0.001) in all pairwise comparisons (Table 2).

Vitamin D deficiency was detected in 56% of the CD group and 12% of the control group. The percentage of patients with vitamin D insufficiency was 30% in the CD group and 35% in the control group. Vitamin D level was normal at 14% in the CD group and 53% in the control group (p<0.001) (Table 3).

There was an inverse correlation between age and vitamin D levels in the CD and control groups (r=-0.268 and r=-0.473, respectively; p<0.001).

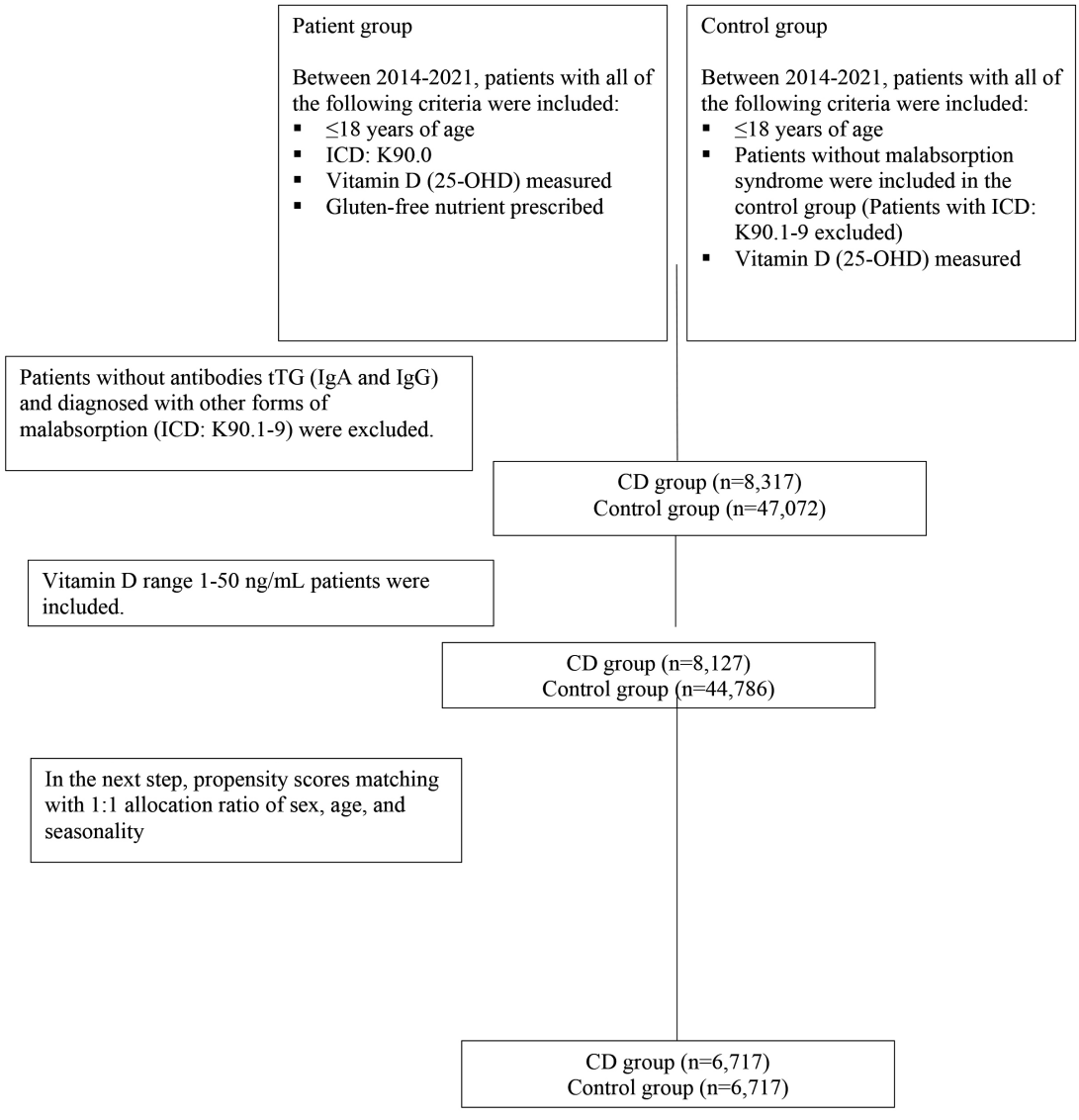

Figure 1. Flow chart of the study population
tTG: Tissue transglutaminase antibody, CD: Celiac disease

Table 1. Serum 25(OH)D levels in the CD group and control group

	Overall (n=13,434)	CD group (n=6,717)	Control group (n=6,717)	p value
Vitamin D, ng/mL, median (quartile deviation)	24.78 (8.18)	18.49 (6.39)	30.70 (7.19)	**<0.001***

*Mann-Whitney U test; means statistically significant. 25(OH)D: 25-hydroxyvitamin D, CD: Celiac disease

Table 2. Serum 25(OH)D levels in the CD group and control group according to gluten-free diet compatibility

	CD group (n=6,717)		Control group (n=6,717)	p value
	Non-compatible group (n=5,615)	Compatible group (n=1,102)		
Vitamin D, ng/mL, median (quartile deviation)	18.30 (6.36)	19.37 (6.50)	30.70 (7.19)	<0.001*

Post-hoc analysis for *Kruskal-Wallis H test; differences in the level of vitamin D significant in all pairwise comparisons (p<0.001). CD: Celiac disease, 25(OH)D: 25-hydroxyvitamin D

Table 3. Comparison of the rates of vitamin D deficiency, insufficiency, and normal vitamin D levels between the CD group control group

	Vitamin D deficiency (vitamin D <20 ng/mL)	Vitamin D insufficiency (vitamin D 20-29.9 ng/mL)	Normal vitamin D (vitamin D >30 ng/mL)	p value
CD group n, (%)	3,774 (56)	1,997 (30)	938 (14)	<0.001*
Control group n, (%)	820 (12)	2,349 (35)	3,528 (53)	

*Pearson chi-square test, CD: Celiac disease

Figure 2 shows the results of the area under the ROC curve analysis. According to Youden's index, the cut-off value of ≤23.195 ng/mL for vitamin D had 68% sensitivity and 80% specificity to predict the classification of an individual as CD.

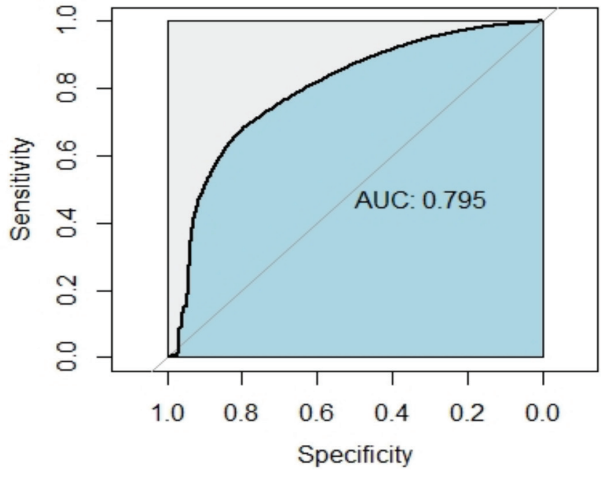

Figure 2. The cut-off value of vitamin D for predicting the CD group vs. control group. For vitamin D AUC=0.795, p<0.001, cut-off value 23.195 ng/mL, 68% sensitivity, 80% specificity
AUC: Area under the curve, CD: Celiac disease

Discussion

This study found lower vitamin D levels among patients with CD than the controls. The proportion of patients with normal vitamin D levels was 12% in the CD group and 56% in the control group. However, in patients with CD, vitamin D levels in patients with good adherence to a GFD were higher than in patients with poor GFD adherence, suggesting still lower vitamin D levels among CD patients with worse GFD compliance.

Several studies have concluded controversial results on serum 25(OH)D levels at the time of CD diagnosis, and while receiving GFD therapy in patients followed up with CD (17,18). Recently, Ahlawat et al. (19) reported that there was no difference between 25(OH)D levels in patients with newly diagnosed CD and controls. Similarly, Villanueva et al. (20) reported that vitamin D levels in patients with CD were not different from the controls. Lerner et al. (21) compared the vitamin D levels in patients with newly diagnosed CD and children with non-specific abdominal pain and observed no difference. In contrast, in the study by Lionetti et al. (2), vitamin D levels in children and adolescents with CD were found to be lower at the time of diagnosis compared with the control group. In this study, the frequency of vitamin D deficiency in patients with CD was 31%, whereas this rate was 12% in the control group. In previous retrospective studies conducted with a limited number of cases, the frequency of vitamin D deficiency in patients with CD ranged from 27% to 70% (22-24).

Consistent with the literature, the frequency of vitamin D deficiency in the current study was 56% in patients with CD and 12% in the control group. Ciacci et al. (17) reported lower vitamin D levels in adult patients with newly diagnosed CD and in adult patients with good adherence to a GFD. In our study, we observed that the vitamin D levels in children and adolescents with CD were lower than in the controls. Similarly, vitamin D levels in children and adolescents with good adherence to GFD were higher than in patients with poor GFD adherence and lower than in the control group. Similar to our study, Lu et al. (25) reported that patients with CD had lower vitamin D levels. Additionally, they found that patients with CD receiving a GFD had vitamin D levels close to those of the control group. The author group hypothesized that vitamin D might play a potential role in the pathogenesis of CD (25). Deora et al. (22) reported that micronutrients other than vitamin D and iron returned to

normal in the 18th month in the follow-up of patients with CD who were started a GFD, but they remained at a suboptimal level even though there was an increase in vitamin D and iron levels. In our study, despite a slightly higher level of vitamin D with the GFD, it was still suboptimal.

There is no clear consensus on the routine evaluation of 25(OH)D level and vitamin D supplementation in patients with CD (2). In 2016, The North American Society of Pediatric Gastroenterology, Hepatology, and Nutrition recommended serum 25(OH)D measurement at the time of diagnosis and annual follow-up after celiac serology returns to normal in children with CD (26). The European Society for Pediatric Gastroenterology, Hepatology, and Nutrition have not published any recommendations in this regard. The results obtained in our study with many patients suggest the necessity for evaluating vitamin D in CD. It is also recommended to ensure the maintenance of vitamin D levels at normal levels in all children and adolescents (2,3). Our analysis also showed that, although the vitamin D levels in patients with CD were higher with GFD, it was still suboptimal. This result points to the importance of vitamin D therapy in patients with CD.

The potential causes of lower levels vitamin D levels in patients with CD are controversial. Traditionally, it is thought to be due to the malabsorption of fat-soluble vitamins (2). Relatively improved vitamin D levels in patients CD with a GFD in our study supports this notion. However, another interesting hypothesis is that vitamin D deficiency observed in the early stages of life may play a role in the pathogenesis of CD rather than its outcome (25,27). Various conditions such as genomic polymorphism and variability in the gut microbiota observed in patients with CD contribute to the low vitamin D levels concerning low vitamin D synthesis (2).

The limitations of our study are that it was designed retrospectively, and the data were obtained through the ICD codes. Another limitation may be the potential differences in the measurement of vitamin D levels in different laboratories. The use of vitamin D supplements was retrieved from the prescription records, limiting the exact knowledge of their use by the patients. Data such as obesity, which may affect vitamin D levels, and geographical features of the region where the patients lived were also not evaluated. Among the strengths of the current study are its large sample size, representation of all regions across the country, and children of all ages and weights.

Conclusion

In conclusion, this study showed lower vitamin D levels in children with CD. Although some improvement was observed in vitamin D levels in patients who were GFD-compatible, it was still lower than in the control group. Due to the close relationship between bone and skeletal health and CD, we suggest that all children with CD be evaluated for vitamin D levels at diagnosis and periodically after that and that children with a deficiency should be managed properly.

Ethics

Ethics Committee Approval and Informed Consent: This nationwide study was conducted with the permission of the Ministry of Health. Ethics committee approval and informed consent were not required for national data studies.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Z.A., M.K., Ş.S.E., O.Ç., M.M.Ü., N.A., Ş.B., Design: Z.A., M.K., Ş.S.E., O.Ç., E.İ., M.Ç., N.A., Ş.B., Data Collection or Processing: Z.A., M.K., Ş.S.E., O.Ç., E.İ., M.M.Ü., N.A., Ş.B., Analysis or Interpretation: Z.A., M.K., Ş.S.E., N.B., E.İ., M.M.Ü., M.Ç., N.A., Ş.B., Literature Search: Z.A., M.K., Ş.S.E., N.B., M.Ç., N.A., Writing: Z.A., M.K., Ş.S.E., N.B., O.Ç., E.İ., M.M.Ü., M.Ç., N.A., Ş.B.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.41033
Gulhane Med J 2022;64:274-80

Knowledge, attitudes, and behaviors of residents and specialists working in tertiary healthcare institutions about drug allergy

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Date submitted:
24.07.2022
Date accepted:
15.08.2022
Online publication date:
08.09.2022

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Keywords: Anaphylaxis, angioedema, drug side effects, hypersensitivity reaction

Presented in: This study was presented as an oral presentation at the 6th International Health Science and Family Medicine Congress, 16-18 April 2021, Izmir, Türkiye.

ABSTRACT

Aims: We evaluated the knowledge, attitudes, and behaviors of residents and specialists working in tertiary healthcare institutions about drug allergy.

Methods: Residents and specialist medical doctors working at a tertiary health institution were included in the study. A questionnaire consisting of questions evaluating occupational and demographic characteristics, knowledge, attitudes, and behaviors about drug allergy was prepared and administered to the participants.

Result: Only 26 (21.3%) of the participants had attended any training on drug allergies. Of the participants, 73 (59.8%) felt competent in taking and interpreting an accurate allergy history for drugs. Of the participants, 107 (87.7%) knew that it is often impossible to reach a definite conclusion about drug allergy based on anamnesis alone. Only half of the participants stated that they could spare enough time for detailed anamnesis about drug allergy in their daily practice. Only 19 (15.6%) of the participants stated that they referred patients with suspected drug allergies to an allergist at a rate of 90-100%. When the answers of the assistant and specialists were compared; the proportion of respondents to the question of the most important drug classes responsible for allergic reactions, including antibiotics and aspirin/other NSAIDs (28.9% vs. 67.7%; $p<0.001$). And the rate of those who responded corticosteroids+antihistamines+adrenaline to the question of the most recommended drug classes to treat drug allergies was found to be higher in specialist physicians (19.8% vs. 71%; $p<0.001$).

Conclusion: This cross-sectional study showed a low level of awareness, knowledge, and competency in the management of drug allergies among residents and specialists from different fields.

Introduction

Drug allergy accounts for less than 10% of drug side effects (1). Drug allergies are seen in 1-2% of hospital admissions and 3-5% of hospitalized patients. However, the actual incidence of drug allergy in children and adults in the community is unknown. Because the diagnosis of allergic drug reactions is often based on history taking, diagnostic tests are not performed, past studies have been mostly conducted with selected patient groups or special reaction types and standardized questionnaires or tests are not used in the evaluation, the data about the true frequency of hypersensitivity reactions due to drugs are limited (2-4).

It is often misleading to reach a diagnosis by only history taking in drug allergies. However, only 5-30% of cases with suspected drug allergies can be confirmed after the tests (5). However, both underdiagnosis (due to underreporting) and overdiagnosis (due to overuse of the term “allergy”) are common, as the diagnosis of drug hypersensitivity is largely based on history (6).

Determining whether the drug is responsible for the allergic condition by applying an algorithmic approach using objective methods is an important practice in patients with suspected drug allergies. In this way, recurrent drug allergies can be prevented in patients, their anxiety about drug use can be reduced, and unnecessary drug restrictions can be avoided (7). Therefore, increasing the awareness of drug allergy among physicians in different specialties, especially among primary care and emergency physicians, is essential (8). Physicians should review the drug allergy history, particularly for the drugs they frequently recommend to their patients, to prevent potential drug reactions (9). Patients with a positive history should be informed to avoid using the related drug and other structurally similar drugs, along with a referral to an allergist for further evaluation (6,10).

Given the lack of sufficient data about the awareness of drug allergies among different specialties in the local context, this study evaluated the knowledge, attitudes, and behaviors of residents and specialists working in tertiary healthcare institutions about drug allergies.

Methods

A cross-sectional survey was designed in accordance with the Declaration of Helsinki and the Good Clinical Practice Guidelines. The study was approved by the Ankara City Hospital Ethics Committee (protocol no: E2-21-270, date: 10.03.2021).

The study population included residents and specialists working in a tertiary healthcare institution, over the age of 18 who agreed to participate in the study. The following questionnaire was used to evaluate the knowledge, attitudes, and behaviors about drug allergy;

Questionnaire

1. **How do you assess the clinical significance of drug allergies in your daily practice?**
   - **a.** Critical
   - **b.** Important
   - **c.** Less important
2. **How often do you encounter patients with drug allergies in your current clinical practice?**
   - **a.** Every day
   - **b.** 1-2 times a week
   - **c.** Every 2-3 months
   - **d.** Almost never
3. **Can you spare enough time for detailed anamnesis about drug allergy in your daily practice?**
   - **a.** Yes
   - **b.** No
4. **Do you feel competent to take and interpret an accurate history of allergy to drugs?**
   - **a.** Yes
   - **b.** No
5. **Do you think it is often possible to reach a definite conclusion about drug allergy based on anamnesis alone?**
   - **a.** Yes
   - **b.** No
6. **Have you attended any training on drug allergies?**
   - **a.** Yes
   - **b.** No
7. **In the presence of which of the following clinical conditions that develop after drug intake, do you suspect drug allergy? (you can tick more than one option)**
   - **a.** Urticaria plaques
   - **b.** Localized/generalized angioedema
   - **c.** Eczematous/maculopapular/bullous skin rashes
   - **d.** Bronchospasm (cough, shortness of breath, wheezing)
   - **e.** Gastrointestinal findings (nausea-vomiting, diarrhea)
   - **f.** Rhinitis, conjunctivitis
   - **g.** Laryngeal edema
   - **h.** Hypotension
   - **i.** Anaphylaxis
8. **What percentage of patients with suspected drug allergies have you referred to an allergist?**
   - **a.** <10%
   - **b.** 20-40%
   - **c.** 50-80%
   - **d.** 90-100%
9. **What is your first advice to a patient with a suspected drug allergy?**
   - **a.** I tell the patients to avoid the drug that causes the allergic reaction
   - **b.** I give information about drug allergy
   - **c.** I have placed a consultation at the allergic diseases department
   - **d.** I refer the patient to the dermatology department
   - **e.** I have suggested another drug that he can use orally without testing
   - **f.** I tell the patients to avoid the drug that causes the allergic reaction and provide information about drug allergy
   - **g.** I tell the patients to avoid the drug that causes the allergic reaction and refer them to the allergic diseases department

10. Is there an allergic disease clinic in your workplace?

a. Yes

b. No

c. I don't know

11. Would you ask a patient describing a drug allergy if he/she has a drug allergy promotion card/tag/necklace?

a. Yes

b. No

12. What are the most important classes of drugs that you consider responsible for allergic reactions?

a. Antibiotics

b. Aspirin/Other NSAIDs

c. Muscle relaxants

d. Flu medications

13. Proton pump inhibitors/H_2 receptor antagonists/Radiocontrast agent

a. Local/General anesthetics

b. Vaccines

14. What are the most recommended classes of drugs for treating drug allergies?

a. Corticosteroids

b. Antihistamines

c. Adrenalin

d. Alternative treatments

e. I do not know

15. How do you evaluate the effectiveness of alternative methods, such as acupuncture and homeopathy, in the diagnosis and treatment of drug allergies?

a. Very effective

b. Effective

c. Ineffective

d. I don't know

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences 18 (SPSS) for Windows 20 (IBM SPSS Inc., Chicago, IL). The normality of the distribution was evaluated with the Kolmogorov-Smirnov test. Numeric variables showing normal distribution were displayed as mean ± standard deviation, and skewed numeric variables were displayed as median (minimum-maximum). Categorical variables are expressed as numbers and percentages. The chi-square test was used to compare the differences between the categorical variables in the residents and specialists. $P<0.05$ was considered significant.

Results

The study population consisted of 122 participants, 91 residents, and 31 specialists. The mean age was 30.3±6.4 years (range: 24-65 years) and there was a 1:1 ratio between women and men. Most participants were medical doctors working in the internal medicine department (82.8%).

The answers given by the participants to the survey questions are shown in Table 1. Only 26 (21.3%) of the participants had attended any training on drug allergies. However, 73 (59.8%) of the participants felt competent/sufficient to take an accurate allergy history for drugs and interpret it. Of the participants, 107 (87.7%) knew that it is often impossible to reach a definite conclusion about drug allergy based on anamnesis alone. Only half of the participants stated that they could spare enough time for detailed anamnesis about drug allergy in their daily practice. Only 19 (15.6%) of the participants stated that they referred patients with suspected drug allergies to an allergist at a rate of 90-100%. When the responses from the residents and specialists were compared; the proportion of respondents to the question of the most important drug classes responsible for allergic reactions was different in the two groups, including antibiotics and aspirin/other NSAIDs (28.9% vs 67.7%; $p<0.001$). The rate of responses to corticosteroids+antihistamines+adrenaline as the most recommended drug classes to treat drug allergies, was also different and higher among specialists (19.8% vs. 71%; $p<0.001$). The proportion of participants who received training on drug allergies (26.4% vs. 6.5%; $p=0.022$) and the proportion of correct responses to the question of which clinical conditions that develop following exposure to a drug may suggest drug allergies were lower among the specialists (49.5% vs. 6.5%; $p<0.001$).

Discussion

We conducted this study to evaluate the level of knowledge about drug allergies among residents and specialists in a tertiary healthcare institution. Drug allergies are not considered direct drug side effects. Moreover, they account for a small proportion of unwanted side effects associated with drugs. Several risk factors take part in the development of drug-induced allergic reactions. These are primarily the drug-related, personal, immunogenic, and pharmacogenetic factors and comorbidities. Considering these risk factors, it is often unpredictable whether a drug-induced allergic reaction may develop or not. Therefore, health teams working in emergency departments, outpatient services, clinics, or intensive care units should be well-equipped about drug allergies.

During the COVID-19 pandemic, when the vaccines, a glimmer of hope at the end of the pandemic, came into use, allergic reactions related to vaccines undoubtedly came to the fore in the whole society. Vaccines were not given due to the fear of an allergic reaction due to the vaccine in many cases.

Table 1. Questionnaire on knowledge, attitudes and behaviors of residents and specialists about drug allergy

Questions	Total (n=122)	Assistants (n=91)	Specialists (n=31)	p value
How do you assess the clinical significance of drug allergies in your daily practice?				
Very important	69 (56.6%)	50 (54.9%)	19 (61.3%)	0.545
Important	48 (39.3%)	36 (39.6%)	12 (38.7%)	
Less important	5 (4.1%)	5 (5.5%)	0 (0)	
How often do you encounter patients with drug allergies in your current clinical practice?				
Every day	0	0	0	0.249
1-2 times a week	19 (15.6%)	16 (17.6%)	3 (9.7%)	
Every 2-3 months	79 (64.8%)	60 (65.9%)	19 (61.3%)	
Almost never	24 (19.7%)	15 (16.5%)	9 (29.0%)	
Can you spare enough time for a detailed anamnesis about drug allergy in your daily practice?				
Yes	61 (50.0%)	48 (52.7%)	13 (41.9%)	0.406
No	61 (50.0%)	43 (47.3%)	18 (58.1%)	
Do you feel competent/competent to take and interpret an accurate history of allergy to drugs?				
Yes	73 (59.8%)	59 (64.8%)	14 (45.2%)	0.060
No	49 (40.2%)	32 (35.2%)	17 (54.8%)	
Do you think it is often possible to reach a definite conclusion about drug allergy based on anamnesis alone?				
Yes	15 (12.3%)	12 (13.2%)	3 (9.7%)	0.758
No	107 (87.7%)	79 (86.8%)	28 (90.3%)	
Have you attended any training on drug allergies?				
Yes	26 (21.3%)	24 (26.4%)	2 (6.5%)	0.022*
No	96 (78.7%)	67 (73.6%)	29 (93.5%)	
Urticaria plaques	113 (92.6%)	84 (92.3%)	29 (93.5%)	0.999
Localized/generalized angioedema	103 (84.4%)	83 (91.2%)	20 (64.5%)	0.001*
Eczematous/maculopapular/bullous skin rashes	83 (68.0%)	73 (80.2%)	10 (32.3%)	<0.001*
Bronchospasm (cough, shortness of breath, wheezing)	119 (97.5%)	89 (97.8%)	30 (96.8%)	0.999
Gastrointestinal findings (nausea-vomiting, diarrhea)	82 (67.2%)	67 (73.6%)	15 (48.4%)	0.018*
Rhinitis, conjunctivitis	55 (45.1%)	48 (52.7%)	7 (22.6%)	0.007*
Laryngeal edema	118 (96.7%)	87 (95.6%)	31 (100.0%)	0.546
Hypotension	108 (88.5%)	79 (86.8%)	29 (93.5%)	0.49
Anaphylaxis	117 (95.9%)	89 (97.8%)	28 (90.3%)	0.197
What percentage of patients with suspected drug allergies have you referred to an allergist?				
<10%	54 (44.3%)	45 (49.5%)	9 (29.0%)	0.036*
20-40%	25 (20.5%)	13 (14.3%)	12 (38.7%)	
50-80%	24 (19.7%)	18 (19.8%)	6 (19.4%)	
90-100%	19 (15.6%)	15 (16.5%)	4 (12.9%)	
Urticaria plaques	113 (92.6%)	84 (92.3%)	29 (93.5%)	0.999
Localized/generalized angioedema	103 (84.4%)	83 (91.2%)	20 (64.5%)	0.001*
Eczematous/maculopapular/bullous skin rashes	83 (68.0%)	73 (80.2%)	10 (32.3%)	<0.001*
Bronchospasm (cough, shortness of breath, wheezing)	119 (97.5%)	89 (97. 8%)	30 (96.8%)	0.999
Gastrointestinal findings (nausea-vomiting, diarrhea)	82 (67.2%)	67 (73.6%)	15 (48.4%)	0.018*
Rhinitis, conjunctivitis	55 (45.1%)	48 (52.7%)	7 (22.6%)	0.007*
Laryngeal edema	118 (96.7%)	87 (95.6%)	31 (100.0%)	0.546
Hypotension	108 (88.5%)	79 (86.8%)	29 (93.5%)	0.49
Anaphylaxis	117 (95.9%)	89 (97.8%)	28 (90.3%)	0.197

Table 1. Continued

Questions	Total (n=122)	Assistants (n=91)	Specialists (n=31)	p value
What percentage of patients with suspected drug allergies have you referred to an allergist?				
<10%	54 (44.3%)	45 (49.5%)	9 (29.0%)	0.036*
20-40%	25 (20.5%)	13 (14.3%)	12 (38.7%)	
50-80%	24 (19.7%)	18 (19.8%)	6 (19.4%)	
90-100%	19 (15.6%)	15 (16.5%)	4 (12.9%)	
What is your first advice to a patient with suspected drug allergy?				
I tell the patient to avoid the drug that causes the allergic reaction	91 (74.6%)	78 (77.2%)	13 (61.9%)	0.173
I give information about drug allergy	12 (9.8%)	10 (9.9%)	2 (9.5%)	
I place a consultation at the allergic diseases department	10 (8.2%)	7 (6.9%)	3 (14.3%)	
I refer the patient to the dermatology department	1 (0.8%)	0	1 (4.8%)	
I suggest another drug that he can use orally without testing	5 (4.1%)	3 (3.0%)	2 (9.5%)	
I tell the patient to avoid the drug that causes the allergic reaction and give information about drug allergy	2 (1.6%)	2 (2.0%)	0	
I tell the patient to avoid the drug that causes the allergic reaction and refer to the allergic diseases department	1 (0.8%)	1 (1.0%)	0	
Is there an allergic diseases clinic in your workplace?				
Yes	92 (75.4%)	69 (75.8%)	23 (74.2%)	<0.001*
No	9 (7.4%)	1 (1.1%)	8 (25.8%)	
I don't know	21 (17.2%)	21 (23.1%)	0 (10%)	
Would you ask the patient describing a drug allergy if he/she has a drug allergy promotion card/tag/necklace?				
Yes	54 (44.3%)	48 (52.7%)	6 (19.4%)	0.001*
No	68 (55.7%)	43 (47.3%)	25 (80.6%)	
What are the most important classes of drugs you consider responsible for allergic reactions?				
Antibiotics	114 (93.4%)	83 (91.2%)	31 (100.0%)	0.198
Aspirin/Other NSAIDs	55 (45.1%)	34 (37.4%)	21 (67.7%)	0.006*
Muscle relaxants	15 (12.3%)	8 (8.8%)	7 (22.6%)	0.089
Flu medications	8 (6.6%)	5 (5.5%)	3 (9.7%)	0.695
Proton pump inhibitors/H_2 receptor antagonists/ Radiocontrast agent	4 (3.3%)	3 (3.3%)	1 (3.2%)	0.999
Local/General anesthetics	68 (55.7%)	40 (44.0%)	28 (90.3%)	<0.001*
Vaccines	27 (22.1%)	16 (17.6%)	11 (35.5%)	0.047*
Antibiotics	35 (28.7%)	26 (28.6%)	9 (29.0%)	0.999
What are the most recommended classes of drugs for treating drug allergies?				
Corticosteroids	68 (55.7%)	51 (50.5%)	17 (81.0%)	0.021*
Antihistamines	93 (76.2%)	73 (72.3%)	20 (95.2%)	0.049*
Adrenalin	57 (46.7%)	44 (43.6%)	13 (61.9%)	0.196
Alternative treatments	3 (2.5%)	3 (3.0%)	0	0.980
I do not know	1 (0.8%)	1 (1.0%)	0	0.999
How do you evaluate the effectiveness of alternative methods such as acupuncture and homeopathy in the diagnosis and treatment of drug allergies?				
Very effective	0	0	0	0.502
Effective	9 (7.4%)	7 (7.7%)	2 (6.5%)	
Ineffective	27 (22.1%)	18 (19.8%)	9 (29.0%)	
I don't know	86 (70.5%)	66 (72.5%)	20 (64.5%)	

NSAIDs: Non-steroidal anti-inflammatory drugs

For this reason, we planned the current study to determine how much knowledge healthcare teams had about drug allergies.

We observed that approximately 20% of our study population had seldom encountered drug allergies. This is a significant finding because drug allergies are rare, contrary to what is known. It was determined that approximately 50% of the population did not have enough time to question their drug allergies while taking anamnesis. However, it was also determined that approximately 40% of the study population did not consider themselves sufficient to take anamnesis about drug allergies and interpret the results. Overall, these results did not differ between residents and specialists, suggesting the lack of anamnesis which is a fundamental practice to identify potential drug allergies in a tertiary healthcare institution. With the help of detailed anamnesis, whether the developing-allergic reaction is related to a drug hypersensitivity reaction and whether it is an immunogenic reaction or a non-immunogenic reaction can be evaluated (11,12). Additionally, information about the severity of the reaction, systemic involvement, and which organs are involved can be obtained through history taking (13-15). Nevertheless, approximately 87% of the study population thought that it was insufficient to draw a firm conclusion about drug allergies based on anamnesis alone. Considering the collected information, there seems an open area in the local context to increase the awareness of relevant history taking to assess past-allergic reactions to estimate potential future events.

We observed that specialists reported a lesser involvement in training in drug allergies compared with residents. Blurring of consciousness, tachycardia, tachypnea, hypotension, arrhythmia, urticaria plaques, general, or local angioedema, rhonchi, wheezing, wheezing, fixed drug eruptions and sweating are the frequently encountered findings in drug allergies. Concerning the responses, signs and symptoms of anaphylaxis, such as urticaria plaques, hypotension, laryngeal edema, and bronchospasm, were similarly marked by the two groups. However, the awareness of residents was higher in findings such as localized/generalized angioedema, gastrointestinal findings, rhinoconjunctivitis, and skin rashes (16,17).

Concerning the behavior of referring a patient to an allergist in case of a suspicious drug allergy, approximately 44% of the study population was identified with the lowest 10% positive responses. The rate of referral of the residents to allergy specialists was slightly higher than that of the specialist doctors. This finding suggests another point for improvement among physicians as such the need for an allergy specialist to determine the type of reaction through diagnostic tests, as well as to recommend safer alternatives and, in some cases, to perform desensitization.

The participants selected recommendations of “stopping the drug that causes an allergic reaction” and “referring to the allergy department” in case of allergic reactions related to a drug. Although this was a satisfactory finding, fewer than half of the participants did prepare a “drug allergy identification card, tag and necklace” for their patients. As such tags can be life-saving in patients presenting with confusion or severe symptoms (14), the information and awareness in this regard need to be improved.

Antibiotics, aspirin and other NSAIDs, and radiocontrast were the most selected agents responsible for allergic reactions, which is consistent with the general practice (10,18-20). The participants responded satisfactorily to the most commonly used medications to treat drug allergies, such as corticosteroids, antihistamines, and adrenaline. The awareness of specialists is higher in this regard. Knowledge and awareness of acupuncture and homeopathy for treating drug allergies were limited in the study group.

The main limitations of our study are its cross-sectional design and small sample size. The major strength is the inclusion of specialists at a high-level care facility. It should also be emphasized that the study provides a comparison between postgraduate specialists and fellows in training, enabling the determination of gaps in experience in early career medical doctors.

Conclusion

In conclusion, the current study showed a low level of awareness and knowledge on the role of history taking about drug allergies, a low level of competency in the interpretation of an accurate drug allergy, and insufficient experience in recognizing the clinical conditions that may develop after taking a drug and guiding a patient with suspected drug allergy.

Ethics

Ethics Committee Approval: The study was approved by the Ankara City Hospital Ethics Committee (protocol no: E2-21-270, date: 10.03.2021).

Informed Consent: A cross-sectional survey study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: H.A., E.S.Ş.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2021.67209
Gulhane Med J 2022;64:281-4

Hidradenitis suppurativa and psoriasis coexistence in patients with Down syndrome: A case series

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Date submitted:
25.08.2021
Date accepted:
23.11.2021
Online publication date:
08.09.2022

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Keywords: Down syndrome, hidradenitis suppurativa, psoriasis

ABSTRACT

Down syndrome is the most common chromosomal disorder associated with skin conditions, including psoriasis and hidradenitis suppurativa. Patients with Down syndrome are more likely to be overweight or obese and diagnosed with metabolic comorbidities such as cardiovascular disorders, inflammatory joint diseases as in psoriasis and hidradenitis suppurativa. Although both hidradenitis suppurativa and psoriasis are associated with Down syndrome, concurrent psoriasis and hidradenitis suppurativa in the same patient with Down syndrome have not been reported in the literature. Here, we describe three patients with Down syndrome having both hidradenitis suppurativa and psoriasis by discussing the common etiopathogenesis considering the current literature.

Introduction

Down syndrome or trisomy 21 is the most common chromosomal abnormality, with a worldwide incidence of 1 in 800 live births. Besides the common phenotypic characteristics such as upslanted palpebral fissures, flattened nasal bridge, nuchal folds, and single palmar flexion crease, individuals with Down syndrome have a higher risk of congenital anomalies and multiorgan comorbidities, including skin disorders (1). Follicular occlusion disorders are frequently observed in patients with Down syndrome, and hidradenitis suppurativa is one of the most common follicular conditions following folliculitis, keratosis pilaris, and acne vulgaris (2). Psoriasis prevalence is also observed higher in patients with Down syndrome compared to controls, even though the exact association between psoriasis and Down syndrome has not been established yet (3). Psoriasis and hidradenitis suppurativa share common immunologic and inflammatory pathways in pathogenesis. In a large-scale population study, it has been shown that there is a significant association between psoriasis and hidradenitis suppurativa (4). However, as far as we know, the coexistence of these disorders has not been previously described in patients with Down syndrome.

Here, we report three patients with Down syndrome being affected by hidradenitis suppurativa and concomitant psoriasis, describe their clinical features, and discuss the common etiopathogenetic pathways.

Case Presentation

The demographic and clinical characteristics of three patients with Down syndrome who had also been diagnosed with both psoriasis and hidradenitis suppurativa are provided in Table 1. All patients were male, obese with a body mass index over 30 kg/m^2, and had congenital thyroid disorders in common. Case 1 had epilepsy and recurrent lower respiratory tract infections. Case 2 had a history of acute lymphoblastic leukemia and familial Mediterranean fever. None of the patients had a family history of hidradenitis suppurativa or psoriasis. The severity of hidradenitis suppurativa was Hurley stage 2 in case 1 and case 3, and Hurley stage 1 in case 2. The type of psoriasis was plaque-type in all patients. Figures 1 and 2 represent the lesions of the hidradenitis suppurativa and psoriasis of Case 1, respectively. Previous treatments of the patients for hidradenitis suppurativa and psoriasis are shown in Table 1. Case 1 was given oral acitretin with a dose of 25 mg/kg per day and the psoriatic lesions improved significantly, but hidradenitis suppurativa lesions did not show sufficient improvement in the three-month period of the treatment. Cases 2 and 3 have been under topical corticosteroid treatment for psoriasis with excellent response. Case 2 benefited from topical clindamycin 2% solution for hidradenitis suppurativa lesions, while oral doxycycline 200 mg BID treatment showed efficacy in case 3.

Discussion

Hidradenitis suppurativa is one of the most common skin disorders in patients with Down syndrome, and the risk of hidradenitis suppurativa is five times higher in patients with Down syndrome than in the normal population (5,6). It has been shown that Down syndrome-related hidradenitis suppurativa appears earlier in life and shows a mild or moderate disease course as in current cases staged as Hurley 1 and 2 (7). The genetic background of hidradenitis suppurativa is based on disrupted notch signaling due to loss-of-function mutations in the genes encoding gamma-secretase transmembranous enzyme complex (8). Blok et al. (9) have suggested that the association between hidradenitis suppurativa and Down syndrome results from the overexpression of the amyloid precursor protein (APP) gene, located on chromosome 21. APP and notch receptors are competitive substrates for the gamma-secretase transmembranous enzyme complex. Thus, increased APP expression may lead to the disruption of notch signaling. Increased expression of APP and consequent notch signaling impairment may stimulate impaired keratinocyte adhesion, migration, and proliferation, causing follicular plugging, which is thought to play a significant role in hidradenitis suppurativa pathogenesis (9). Besides, impaired notch signaling pathways may contribute to the dysregulation of the inflammatory response in patients with hidradenitis suppurativa and Down syndrome.

Table 1. Demographic and clinical characteristics of the patients with Down syndrome having both hidradenitis suppurativa and psoriasis

Characteristics	Case 1	Case 2	Case 3
Age (years)/gender	27/male	24/male	20/male
Body mass index (kg/m^2)	34.2	32	42.6
Family history for hidradenitis suppurativa	No	No	No
Onset of hidradenitis suppurativa	17 years old	15 years old	19 years old
Hidradenitis involvement	Axilla, inguinal folds, abdomen, pubic and intergluteal region	Back	Axilla, intergluteal region
Hurley stage of hidradenitis suppurativa	2	1	2
Family history of psoriasis	No	No	No
Onset of psoriasis	13 years old	20 years old	18 years old
Type of psoriasis	Vulgaris	Vulgaris	Vulgaris
Psoriasis involvement	Dorsum of the hands and feet, bilateral shins, fingernails, and toenails	Dorsum of the hands and fingers, bilateral elbows	Dorsum of the hands, bilateral elbows
PASI score	8	1.6	1.8
Comorbidities	Epilepsy, hypothyroidism, Recurrent lower respiratory tract infections	ALL, FMF, hyperthyroidism	Hypothyroidism
Concomitant treatments	Levothyroxine, levetiracetam, sodium valproate	Allopurinol, colchicine	Levothyroxine

ALL: Acute lymphoblastic leukemia, FMF: Familial Mediterranean Fever, PASI: Psoriasis area severity index

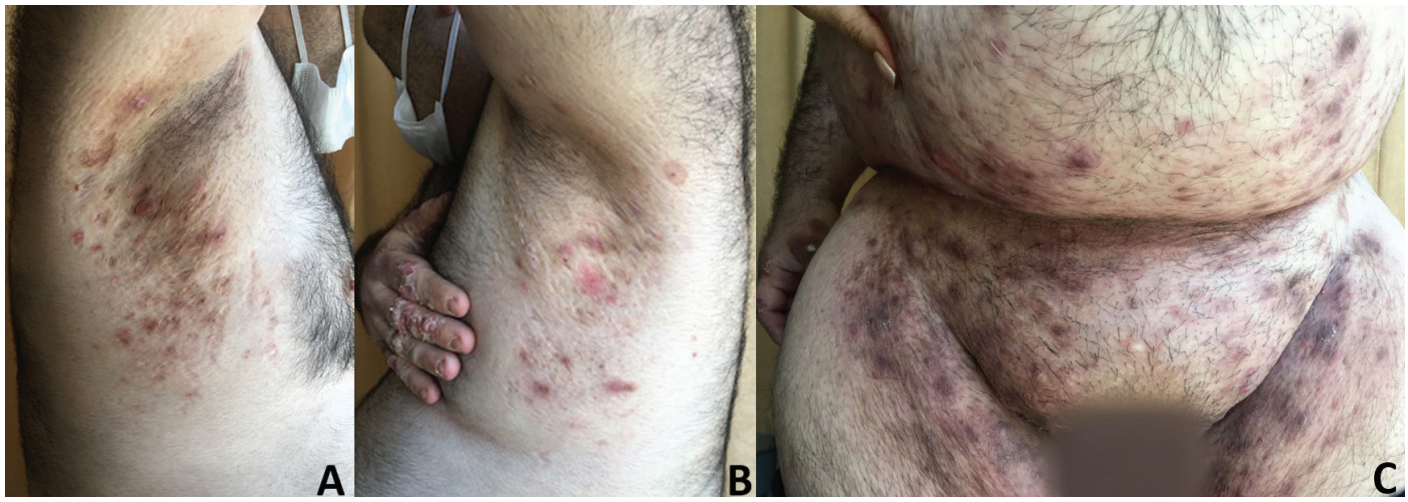

Figure 1. Hidradenitis suppurativa lesions of case 1: Multiple abscesses, postinflammatory hyperpigmented and atrophic scars in axillary (A, B), abdominal, pubic, and inguinal regions (C)

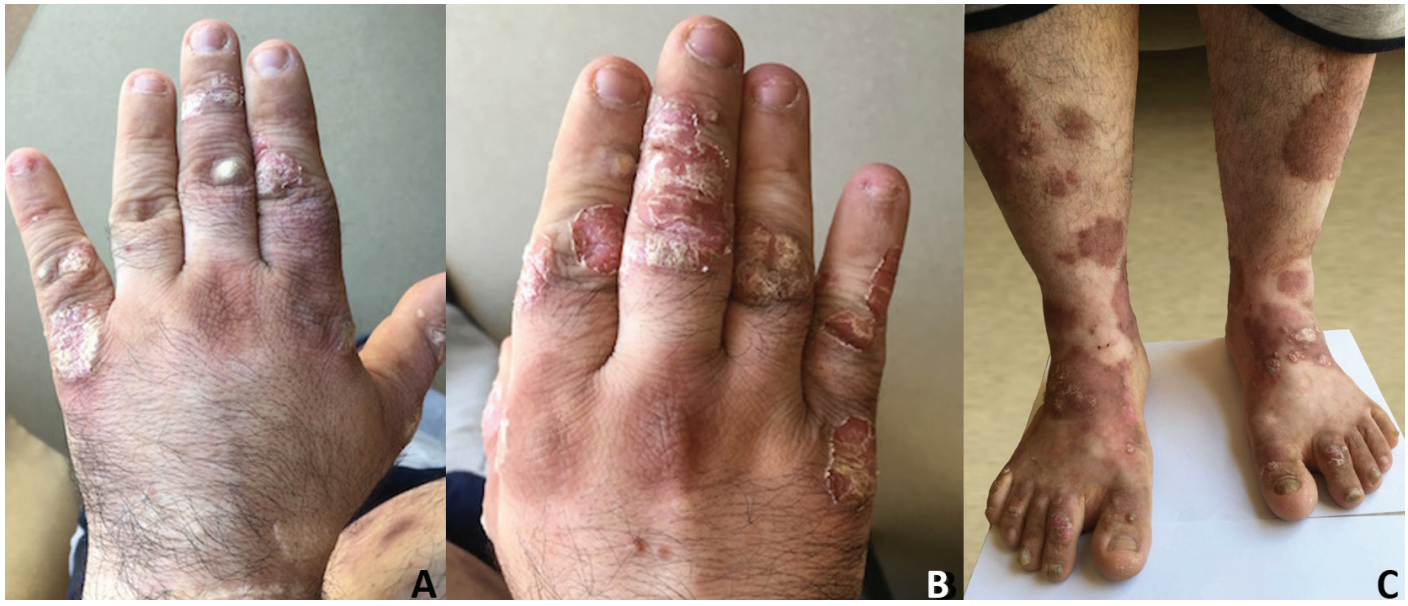

Figure 2. Psoriasis skin and toenail involvement of case 1**:** Erythematous papules and plaques with a silvery scale on the dorsal aspect of the hands (A, B) and the feet, and yellow discoloration and subungual hyperkeratosis on toenails, well-demarcated brownish-red patches on anterior tibial surfaces (C)

Psoriasis is an immune-mediated and chronic inflammatory skin disorder (10). The frequency of psoriasis in patients with Down syndrome has been demonstrated to be approximately 9% (2,6). As these data are based solely on case studies, the relationship between psoriasis and Down syndrome remains to be elucidated. It has been proposed that the high frequency of psoriasis in patients with Down syndrome is related to elevated serum levels of tumor necrosis factor (TNF)-alpha, interleukin (IL)-1 beta, and interferon (IFN)-gamma (4). All of which are thought to be involved in psoriasis pathogenesis and high serum levels of INF-gamma and increased sensitivity to IFN-gamma due to chromosome 21 ploidy in patients with Down syndrome (3).

The IL-23/Th17 and TNF-alpha pathways are common in the molecular pathogenesis of psoriasis and hidradenitis suppurativa. Further, psoriasis and hidradenitis suppurativa share common aggravating factors and comorbidities such as smoking, obesity, and metabolic syndrome (4). Patients with the dual diagnosis of hidradenitis suppurativa and psoriasis have been revealed to have a higher prevalence of obesity (4,11). Obesity occurs in more than 50% of adults with Down syndrome (1). Thus, the association between Down syndrome, hidradenitis suppurativa, and psoriasis may be attributed to the common pathophysiological mechanisms: dysregulated notch signaling and inflammatory mechanisms, and obesity.

Psoriasis and hidradenitis suppurativa share common therapeutic options due to similarities in their pathogenic mechanisms (4). As patients with Down syndrome have a mild severity of hidradenitis suppurativa and psoriasis, treatment with topical corticosteroids and antibiotics may be sufficient to control the diseases (7). Oral acitretin and adalimumab, showing high efficacy on both hidradenitis suppurativa and psoriasis, may be required options in recalcitrant and moderate to severe cases (11,12). However, comorbidities requiring multiple medications and a dysregulated immune system ensuing susceptibility to infections and malignancies in Down syndrome may restrict treatment options (1). Dermatologists should exercise caution, and close follow-up should be recommended when using these treatments in patients with Down syndrome.

Conclusion

In conclusion, the coexistence of hidradenitis suppurativa and psoriasis in patients with Down syndrome is rare but should be kept in mind. Patients with Down syndrome should be carefully examined for accompanying dermatologic disorders. The management of the condition is challenging; hence, patients should be monitored closely after appropriate treatment selection.

Ethics

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: N.D.Ö., P.E., E.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.96729
Gulhane Med J 2022;64:285-6

Comment on "Parents' and healthcare professionals' views and attitudes toward anti-vaccination"

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Date submitted:
10.02.2022
Date accepted:
19.02.2022
Online publication date:
08.09.2022

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Keywords: Vaccination, anti-vaxxer, anti-vaccination

Dear Editor,

I read the valuable article "Parents' and healthcare professionals' views and attitudes toward anti-vaccination" by Karabulut and Zengin (1), focusing on the opinions and experiences of parents and healthcare professionals about childhood vaccination and anti-vaccination, as well as evaluating the ethical aspects of the subject. They clearly state anti-vaccination as a dangerous situation for public health, but also mention it as a sign of "transition from sociality to individuality." I thank them for their meticulous work.

Karabulut and Zengin (1) very delicately point out the importance of ethical values to being respected by people, so that they can make their own choices freely. They also state that "limiting one's actions harm other individuals" is not curtailing the freedom, concluding ethically, one should try to balance and protect individual autonomy and social utility and that, vaccination is vital for protecting children's right to life.

However, for some, "vaccination isn't just an individual choice; it protects those who can't be vaccinated" as mentioned in (2). Since the outbreak of Coronavirus disease-19 (COVID-19) began, there have been many extreme views: Anti-vaccination campaigns hitting record rates, along with the economic difficulties that have caused opportunities for anti-vaccination campaigners to target those who are vulnerable to anti-vaccination sentiment. This article warns that the anti-vaccination community may use anything that would go wrong to create "fear against vaccines" (2).

Exercising autonomy and refusing vaccination may be valid for sensitive personal issues, but if a certain percentage of the population does not get vaccines causing the immunization rate to fall below the herd immunity threshold- varying in every disease, this would harm the population in question (3). Vaccination of a critical number of individuals in a certain population reducing the risk of outbreaks of infectious diseases and protecting other members of the community has also been mentioned by Karabulut and Zengin (1).

The COVID-19 outbreaks had devastating effects on the people's well-being worldwide, not only health wise but also economically and socially. This would eventually raise the question of whether vaccination should be a topic of discussion, when the present data supports the lighter effects of the COVID-19 (and the variants) on vaccinated individuals, necessitating less intensive care unit hospitalizations and faster recovery possibilities having better overall effects on the people in general.

It is worth considering - while authors very kindly suggest "increasing the awareness of the anti-vaxxers behaviors damaging effect on public health"(1)- whether more direct and strict precautions to be taken for the good of community and public health.

In my opinion, the importance and necessity of education cannot be denied, and providing reliable information to anti-vaccine community is the best way to promote the overall health of the populations, but as this takes a long time, in urgent events such as the unexpected pandemic we faced recently, strict global measures may have to be taken for the good of the people.

Ethics

Peer-review: Internally peer-reviewed.

Financial Disclosure: The author declared that this study received no financial support.

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DOI: 10.4274/gulhane.galenos.2022.96729-reply
Gulhane Med J 2022;64:287-8

Reply to: Comment on "Parents' and healthcare professionals' views and attitudes toward anti-vaccination"

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Date submitted:
26.08.2022
Date accepted:
30.08.2022
Online publication date:
08.09.2022

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Keywords: Anti-vaccination, bioethics, individual autonomy, risk and benefits, slippery slope arguments

Dear Editor,

We would like to thank the author for his comments on our article "Parents' and healthcare professionals'' views and attitudes towards anti-vaccination" (1).

As the author has mentioned, we have emphasized the importance of childhood vaccination in order to protect the best interests of children based on scientific knowledge. The group we have focused on was children younger than 18 years of age and therefore considered vulnerable. The responsibility of this vulnerable group rests with the parents. The point that parents should consider is parents have the responsibility and authority to make medical decisions on behalf of their children. They are, however, not as free in making the decision about their child as they would be about themselves and are responsible for caring for their children from the scientific perspective when it comes to life and health. Giving parents' beliefs and cultural values a priority and acquiescing in their decision not to vaccinate their children results in too much focus on their wishes rather than their children's benefits (2).

It is, an important ethical value that we should respect the right of people to make choices of their own free will. However, what is generally overlooked is that limiting one's actions that harm other individuals is not the same as curtailing one's freedom (2). This issue should be approached from John Stuart Mill's Harm Principle, which holds that one should be free to act however one wishes unless one's actions cause harm to somebody else (3). Mill also argues that not only one's actions but also one's failure to act may cause harm, for which one is therefore held responsible. From that perspective, vaccine hesitancy is about failure to act. It should always be kept in mind that a person who causes harm, whether by acting or failing to act, is held responsible for that harm either for acting or for failing to act (3).

However, the sentence" in urgent events such as the unexpected pandemic we faced recently, strict global measures may have to be taken for the good of the people" that the author has suggested (4) needs to be thought through and carefully evaluated. This discourse emphasizes the concept of "compulsory vaccination".

It would not be the right action for us to use the concept of "compulsory vaccination" so clearly when we evaluate this case, especially for autonomous people over the age of 18, whose benefit-risk assessment results are unclear yet. Because in ethics, "slippery slope" arguments lead us to think. The following is what is intended to be explained with this argument; an innocent step to be taken in the direction of situations that are likely to lead to unacceptable consequences for ethical values may cause undesirable situations. Starting to slide on the slope is used in the sense that the values drift to an uncontrollable and unstoppable point. If we formulate it, if the first step, step A, is taken, step B, which

defines an undesirable situation for which we have no reason to refuse it, will follow, followed by step C and will result in an unstoppable course from C to N. Unfortunately, step N is an ethically unacceptable step. If an action of type A is accepted, many actions of type B, type C, and from C to N actions will become accepted. For this reason, it is necessary not to take the first step, that is, step A, or to draw justified boundaries before reaching that area by noticing in advance the gray area where the loss of control begins. However, it is important that this limit is meaningful and drawn in order to protect ethical values (5-7). Based on this argument, it would be appropriate for the expression “strict global measures” to be evaluated by a scientific commission, including ethical experts in particular, and even if it is, its boundaries should be very clearly defined.

We would like to thank the author again for his interest and valuable contributions to our study.

Ethics

Peer-review: Internally peer-reviewed.

Financial Disclosure: The author declared that this study received no financial support.

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