ISSN 2149-7893 • EISSN 2536-507X

Indexed in Web of Science

Official journal of
Cyprus Turkish Medical Association

CYPRUS JOURNAL OF MEDICAL SCIENCES

VOLUME 5 • ISSUE 4 • DECEMBER 2020

Official journal of
Cyprus Turkish Medical Association

CYPRUS JOURNAL OF MEDICAL SCIENCES

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Publication Type
Local periodical

Printed Date
December 2020

Printed at
Share Ajans, Şehit Fevait Ali Sok. Dük. No: 4 C, Sönmezler Apt, Göçmenköy, Nicosia, Cyprus

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Aims and Scope

Cyprus Journal of Medical Sciences (Cyprus J Med Sci) is the scientific, peer reviewed, open access international publication organ of Cyprus Turkish Medical Association. The journal is published three times a year, in April, August, and December. As of 2020, the journal has become a quarterly publication, publishing in March, June, September, and December. The journal's publication language is English.

The aim of the journal is to publish original research papers of the highest scientific and clinical value in all medical fields. Cyprus Journal of Medical Sciences also publishes reviews, rare case report and letters to the editors.

The target audience of the journal includes healthcare professionals physicians, and researchers who are interested or working in in all fields of medicine.

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publication Ethics (COPE), European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice).

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3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Title page: A separate title page should be submitted with all submissions and this page should include:
- The full title of the manuscript as well as a short title (running head) of no more than 50 characters,
- Name(s), affiliations, highest academic degree(s), and ORCID IDs of the author(s),
- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number), and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfill the authorship criteria.

Abstract: An abstract should be submitted with all submissions except for Letters to the Editor. The abstract of Original Articles should be structured with subheadings (Background/Aims, Material and Methods, Results and Conclusion). Please check Table I below for word count specifications.

Keywords: Each submission must be accompanied by a minimum of three to a maximum of five keywords for subject indexing at the end of the abstract. The keywords should be listed in full without abbreviations. The keywords should be selected from the National Library of Medicine, Medical Subject Headings database (https://www.nlm.nih.gov/mesh/MBrowser.html).

Main Points: All submissions except letters to the editor should be accompanied by 3 to 5 "main points" which should emphasize the most noteworthy results of the study and underline the principle message that is addressed to the reader. This section should be structured as itemized to give a general overview of the article. Since "Main Points" targeting the experts and specialists of the field, each item should be written as plain and straightforward as possible.

Manuscript Types
Original Articles: This is the most important type of article since it provides new information based on original research. Acceptance of original papers will be

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based upon the originality and importance of the investigation. The main text of original articles should be structured with Introduction, Material and Methods, Results, and Discussion subheadings. An original article can be signed by maximum 6 authors unless it is a multi-center study or that it required extensive labour. Please check Table I for the limitations for Original Articles.

Clinical Trials

Cyprus Journal of Medical Sciences adopts the ICMJE's clinical trial registration policy, which requires that clinical trials must be registered in a publicly accessible registry that is a primary register of the WHO International Trials Registry Platform (ICTRP) or in ClinicalTrials.gov.

Instructions for the clinical trials are listed below.

- Clinical trial registry is only required for the prospective research projects that study the relationship between a health-related intervention and an outcome by assigning people.
- To have their manuscript evaluated in the journal, author should register their research to a public registry at or before the time of first patient enrollment.
- Based on most up to date ICMJE recommendations, Cyprus Journal of Medical Sciences accepts public registries that include minimum acceptable 24-item trial registration dataset.
- Authors are required to state a data sharing plan for the clinical trial registration. Please see details under "Data Sharing" section.
- For further details, please check ICMJE Clinical Trial Policy at http://www.icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html

Data Sharing

As of I January 2019, a data sharing statement is required for the registration of clinical trials. Authors are required to provide a data sharing statement for the articles that reports the results of a clinical trial. The data sharing statement should indicate the items below according to the ICMJE data sharing policy:

- Whether individual deidentified participant data will be shared
- What data in particular will be shared
- Whether additional, related documents will be available
- When the data will be available and for how long
- By what access criteria will be shared

Authors are recommended to check the ICMJE data sharing examples at http://www.icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html

While submitting a clinical trial to Cyprus Journal of Medical Sciences;

- Authors are required to make registration to a publicly accessible registry according to ICMJE recommendations and the instructions above.
- The name of the registry and the registration number should be provided in the Title Page during the initial submission.
- Data sharing statement should also be stated in the Title Page even the authors do not plan to share it.

Clinical trial and data sharing policy of the journal will be valid for the articles submitted from I February 2020.

Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). Information on statistical analyses should be provided with a separate subheading under the Materials and Methods section and the statistical software that was used during the process must be specified.

Type of manuscript	Word limit	Abstract word limit	Reference limit	Table limit	Figure limit
Original Article	4000	250 (Structured)	35	6	5 or total of 10 images
Review Article	5000	250	50	6	10 or total of 15 images
Case Report	1200	200	15	No tables	4 or total of 8 images
Letter to the Editor	400	No abstract	5	No tables	No media

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Units should be prepared in accordance with the International System of Units (SI).

Editorial Comments: Invited brief editorial comments on selected articles are published in The Cyprus Journal of Medical Sciences. Editorials should not be longer than 1000 words excluding references. Editorial comments aim to provide a brief critical commentary by reviewers with expertise or with high reputation in the topic of the research article published in the journal. Authors are selected and invited by the journal to provide such comments. Abstract, Keywords, and Tables, Figures, Images, and other media are not included.

Review Articles: Reviews prepared by authors who have extensive knowledge on a particular field and whose scientific background has been translated into a high volume of publications with a high citation potential are welcomed. These authors may even be invited by the journal. Reviews should describe, discuss, and evaluate the current level of knowledge of a topic in clinical practice and should guide future studies. The subheadings of the review articles should be planned by the authors. However, each review article should include an "Introduction" and a "Conclusion" section. Please check Table 1 for the limitations for Review Articles.

Case Reports: There is limited space for case reports in the journal and reports on rare cases or conditions that constitute challenges in diagnosis and treatment, those offering new therapies or revealing knowledge not included in the literature, and interesting and educative case reports are accepted for publication. The text should include Introduction, Case Presentation, and Discussion with an unstructured abstract. Please check Table 1 for the limitations for Case Reports.

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

Figures and Figure Legends

Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References
Both in-text citations and the references must be prepared according to the Vancouver style.

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Journal Article: Yazıcı A. The efficacy of endoscopic ventilation tube insertion in pediatric populations. Cyprus J Med Sci 2019; 4(2): 73-6.

Book Section: Suh KN, Keystone JS. Malaria and babesiosis. Gorbach SL, Barlett JG, Blacklow NR, editors. Infectious Diseases. Philadelphia: Lippincott Williams; 2004.p.2290-308.

Books with a Single Author: Sweetman SC. Martindale the complete drug reference. 34th ed. London: Pharmaceutical Press; 2005.

Editor(s) as Author: Huizing EH, de Groot JAM, editors. Functional reconstructive nasal surgery. Stuttgart-New York: Thieme; 2003.

Conference Proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92.

Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki öğrencilerin beslenme durumları, fiziksel aktiviteleri ve beden kitle indeksleri kan lipidleri arasındaki ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

Manuscripts Accepted for Publication, Not Published Yet: Slots J. The microflora of black stain on human primary teeth. Scand J Dent Res. 1974.

Epub Ahead of Print Articles: Cai L, Yeh BM, Westphalen AC, Roberts JP, Wang ZJ. Adult living donor liver imaging. Diagn Interv Radiol. 2016 Feb 24. doi: 10.5152/dir.2016.15323. [Epub ahead of print].

Manuscripts Published in Electronic Format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodIEID/cid.htm.

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When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be canceled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

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Editorial

Dear Colleagues,

I hope you are doing well, and I wish so. I know that every one of us is tired of this disastrous infection disease. I hope, like everyone else, that vaccines produced in certain countries and used worldwide will eventually cure the disease. But COVID-19 infection points out that infections will be the most observed diseases, and we should be well prepared to overcome them.

I wish you all a very great Christmas and a happy new year with healthy, peaceful life.

As life goes on, we are trying to put new knowledge into the literature. We will be welcomed to publish new knowledge and good quality papers about COVID-19 as soon as possible.

With my editorial, we are very grateful and would like to thank to all reviewers and authors for their tremendous efforts.

My best kind regards.

Sonuç Büyük
Editor in Chief

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Original Article

DOI: 10.5152/cjms.2020.1075

Factors Affecting Strangulation and Necrosis in Incarcerated Abdominal Wall Hernias

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Cite this article as: Şimşek A, Kocaaslan H, Dirican A, Ateş M. Factors Affecting Strangulation and Necrosis in Incarcerated Abdominal Wall Hernias. Cyprus J Med Sci 2020; 5(4): 279-83.

BACKGROUND/AIMS
The goal of the present study was to determine the factors affecting strangulation and necrosis in incarcerated abdominal wall hernias (AWHs).

MATERIAL and METHODS
This was a retrospective study conducted by reviewing the medical records of 44 patients who underwent surgery for incarcerated abdominal wall hernia at a university hospital between April 2009 and January 2018.

RESULTS
Of the 44 study patients, 28 were men and 16 were women of mean age 68.95 years. Of all, 30 patients (68.2%) had groin hernias (femoral: 7, inguinal: 23) and 14 (31.8%) had ventral hernias (incisional: 8, umbilical: 5, epigastric: 1). The intraoperative findings were strangulation in 24 (54.5%) and necrosis in 20 (45.5%) cases. Accordingly, omental resection was performed in 4, small bowel resection in 14, and large bowel resection in two patients. The hernia was repaired using a polypropylene mesh in 33 patients (of them 20 were Lichtenstein, 8 were on-lay, and 5 were plug-mesh) and without using a mesh in ten patients. The hernia was not repaired in one patient. The mean duration of hospitalization was 7.43 (range: 1-5) days. The mortality rate was 13.6%. In univariate analysis, the mortality was positively related to necrosis (p=0.045). The duration of these symptoms was the only factor that affected strangulation and necrosis.

CONCLUSION
The mortality rate was high in incarcerated abdominal wall hernias, while necrosis was the most significant variable associated with mortality. The duration of the symptoms was the most significant factor that affected necrosis; therefore, it is essential to perform surgical intervention within the first 24 h of admission.

Keywords: Abdominal wall hernia, incarcerated hernia, irreducible hernia, necrosis, obstruction, strangulated hernia

INRODUCTION

Although majority of the abdominal wall hernias (AWHs) are asymptomatic, they can cause serious problems if they are complicated or left untreated. An incarcerated hernia may not require immediate surgery, but it may develop into strangulated hernia, which can be fatal. The studies on complicated hernias generally focus on strangulated groin hernias of rates 0.29–2.9% (1). It is the most severe complication, representing a higher risk for mortality (2). The World Society of Emergency Surgery (WSES) guideline recommends emergency hernia repair in case of strangulation suspicion (3). However, early diagnosis of strangulation may be difficult (4, 5).

The aim of this study was to determine the factors affecting strangulation and necrosis in incarcerated AWHs.

MATERIAL and METHODS

Case Selection Criteria

A retrospective study was conducted by reviewing the medical records of the patients who underwent surgery for incarcerated AWHs at a university hospital between April 2009 and January 2018. The electronic database of the hospital

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Received: 31.05.2019
Accepted: 11.07.2019

were searched with all codes for hernia repair. The exclusion criteria included patients aged<18 years, presence of hernia other than AWHs, presence of hernia unsuitable for the diagnosis of incarcerated hernia, and patients with insufficient data; based on this criteria, 44 patients who underwent surgery for incarcerated AWHs were recruited for the study. The diagnosis of incarcerated hernia (irreducible hernia) was based on the physical examination when a hernia could not be reduced or pushed back manually. The diagnosis of strangulation (referring to ischemia caused by a compromised blood supply leading to gangrene when not relieved) and necrosis (referring to irreversible injury to the contents of the hernia resulting from insufficient blood flow) were made during the surgery. The duration of symptom presentation was defined as ''the time elapsed from the start of the first symptom until the start of surgery.'' The term ''mortality'' refers to death associated either directly or indirectly with the surgery.

The factors of age, gender, presence of additional diseases, type and lateralization of hernia, the duration of symptoms, type of anesthesia, presence of strangulation, and necrosis were recorded and selected as variables to conduct statistical analysis associated with mortality. The factors of age, gender, presence of additional diseases, types and lateralization of hernia, and duration of symptoms were selected as variables to conduct statistical analysis associated with strangulation and necrosis.

Surgical Technique

The resection of the necrotic content (such as omentum and small and large bowels) was performed in all cases with necrosis. Open hernia repair with or without mesh was conducted in accordance with the surgeon's preferences. All patients received prophylactic antibiotics prior to the surgery, and antibiotics were continued in patients with clinically and/or microbiologically verified infections.

Statistical Analysis

Descriptive statistical analysis and Chi-square test of independence were conducted by using the Statistical Package of the Social Sciences software version 17.0 (SPSS Inc.; Chicago, IL, USA).

Ethics Committee Approval

Ethics committee approval was received for this study from the Ethics Committee of İnönü University (Approval Date: 19.02.2019, Approval Number: 2019/4-38). Informed consent was not necessary due to the retrospective nature of this study.

RESULTS

The mean age of the 28 men and 16 women patients was 68.95 years (age range: 41–102 years). A total of 30 patients (68.2%) had groin hernias (femoral: 7, inguinal: 23) and 14 patients (31.8%) had ventral hernias (incisional: 8, umblical: 5, epigastric: 1). Ventral hernias were more frequently noted in women (62.5% vs. 14.3%), and groin hernias in man (85.7% vs.

Main Points:

- Newly diagnosed AWHs should be repaired electively to avoid urgent surgery.
- Surgical repair have to be performed within the first 24 h for incarcerated AWHs.
- In spite of emergency repair, mortality remain high in incarcerated AWHs.

TABLE 1. The factors affecting mortality in incarcerated abdominal wall hernias

Characteristic	Mortality (-)	Mortality (+)	p
Age			
<70	16 (84.2%)	3 (15.8%)	0.717
≥70	22 (88%)	3 (12%)	
Gender			
Male	24 (85.7%)	4 (14.3%)	0.868
Female	14 (87.5%)	2 (12.5%)	
Additional disease (+)	26 (86.4%)	5 (16.1%)	0.457
Hernia type			
Groin hernia	26 (86.7%)	4 (13.3%)	0.932
Ventral hernia	12 (85.7%)	2 (14.3%)	
Lateralization			
Right	17 (85%)	3 (15%)	0.880
Left	9 (81.8%)	2 (18.2%)	
Bilateral	1 (100%)	-	
Median	11 (91.7%)	1(8.3%)	
Duration of symptoms			
24 hour	22 (95.7%)	1 (4.3%)	0.06
>24 hour	16 (76.2%)	5 (23.8%)	
Strangulation			
Negative	19 (95%)	1 (5%) 0.128	
Positive	19 (79.2%)	5 (20.8%)	
Necrosis			
Negative	23 (95.8%)	1 (4.2%)	0.045
Positive	15 (75%)	5 (25%)	
Mesh usage			
Negative	7 (63.6%)	4 (36.4%)	0.011
Positive	31 (93.9%)	2 (6.1%)	
Type of anesthesia			
Local	1 (50%)	1 (50%)	0.186
Spinal	7 (100%)	-	
General	30 (85.7%)	5 (14.3%)	

TABLE 2. The mesh usage in cases without or with necrosis and/or strangulation

Characteristic	Mortality (-)	Mortality (+)	p
Necrosis (Cases resection performed)			
Negative	3 (12.5%)	21 (87.5%)	0.036
Positive	8 (40%)	12 (60%)	
Strangulation			
Negative	3 (15%)	17 (85%)	0.162
Positive	8 (33.3%)	16 (66.7%)	

TABLE 3. The factors affecting strangulation in incarcerated abdominal wall hernias

Characteristic	Strangulation (-)	Strangulation (+)	p
Age			
<70	9 (47.4%)	10 (52.6%)	0.824
≥70	11 (44%)	14 (56%)	
Gender			
Male	15 (53.6%)	13 (46.4%)	0.153
Female	5 (31.3%)	11 (68.8%)	
Additional disease			
Negative	8 (61.5%)	5 (38.5%)	0.165
Positive	12 (38.7%)	19 (61.3%)	
Hernia type			
Groin hernia	16 (53.3%)	14 (46.7%)	0.124
Ventral hernia	4 (28.6%)	10 (71.4%)	
Lateralization			
Right	10 (50%)	10 (50%)	
Left	5 (45.5%)	6 (54.5%)	0.556
Bilateral	1 (100%)	-	
Median	4 (33.3%)	8 (66.7%)	
Duration of symptoms			
24 hour	15 (65.2%)	8 (34.8%)	**0.006**
>24 hour	5 (23.8%)	16 (76.2%)	

TABLE 4. The factors affecting necrosis in incarcerated abdominal wall hernias

Characteristic	Necrosis (-)	Necrosis (+)	p
Age			
<70	10 (52.6%)	9 (47.4%)	0.824
≥70	14 (56%)	11 (44%)	
Gender			
Male	16 (57.1%)	12 (42.9%)	0.647
Female	8 (50%)	8 (50%)	
Additional disease			
Negative	8 (61.5%)	5 (38.5%)	0.546
Positive	16 (51.6%)	15 (48.4%)	
Hernia type			
Groin hernia	19 (63.3%)	11 (36.7%)	0.087
Ventral hernia	5 (35.7%)	9 (64.3%)	
Lateralization			
Right	11 (55%)	9 (45%)	
Left	7 (63.6%)	4 (36.4%)	0.571
Bilateral	1 (100%)	-	
Median	5 (41.7%)	7 (58.3%)	
Duration of symptoms			
24 hour	17 (73.9%)	6 (26.1%)	**0.007**
>24 hour	7 (33.3%)	14 (66.7%)	

37.5%). Femoral hernias occurred more frequently in women (25% vs. 10.7%). Incisional hernias also predominated in the women (43.8%) and inguinal hernias in men (75%). The presenting symptoms were pain (93.1%), bulging (63.6%), nausea and vomiting (34%), and the absence of defecation and flatus (13.6%). Of all cases, 47.72% had been symptomatic for >24 h and only 40.9% underwent surgery within 6 h of admission. A total of 31 (70.5%) patients had one or more additional diseases (cardiovascular system (n=24), respiratory system (n=16), endocrine system (n=5), central nervous system (n=3), and hepatopancreatobiliary system (n=1).

The intraoperative findings were strangulation in 24 (54.5%) and necrosis in 20 (45.5%) cases. Omental resection was performed in 4 (9.09%) patients, small bowel resection in 14 (31.8%) patients, and large bowel resection in 2 (4.54%) patients. The hernia was repaired using a polypropylene mesh in 33 patients (of them, 20 were Lichtenstein, 8 were on-lay, and five were plug-mesh) and without using a mesh in ten patients. The hernia was not repaired in a 58-year-old woman who was operated for giant recurrent incisional hernia requiring bowel resection. She had chronic obstructive pulmonary disease and hypertension and died 5 days after the operation due to pulmonary complications.

The mean duration of hospitalization-stay was 7.43 (range: 1–35 days) days. The overall mortality rate was 13.6%. Univariate analysis revealed that age, gender, type and lateralization of hernia, strangulation, the presence of additional diseases, and the type of anesthesia were not associated with the mortality rate. The mortality rate was however positively related to necrosis (p=0.045), but inversely related to mesh usage (p=0.011). (Table 1). Reinforcement of the hernia with mesh was statistically lower in cases who underwent resection for necrosis due to the preference of the surgeon (Table 2). In univariate analysis, the factors of age, gender, type and lateralization of hernia, and the presence of additional diseases were associated with neither strangulation nor necrosis. The duration of the symptoms was the only factor affecting both strangulation and necrosis (Tables 3 and 4). The characteristics of six patients who died are presented in Table 5. One of them was aged 102 years and the remaining 5 had coexisting diseases that resulted in pneumonia and/or liver rupture.

DISCUSSION

The management of complicated AWHs remain one of the most challenging surgical emergencies (3). The WSES guideline recommend emergency hernia repair on suspicion of intestinal strangulation (3). However, early diagnosis of strangulation may be difficult (4, 5). While the WSES guidelines strongly recommend systemic inflammatory response syndrome, contrast-enhanced computerized tomography findings as well as the levels of lactate, serum creatinine phosphokinase, and D-dimer are predictive of bowel strangulation, albeit their quality of evidence is low (grade IC recommendation) (3). Thus, difficulty in diagnosis can lead to delayed treatment and result in the development of serious complications.

There exists a few reports on the relationship between the complications of incarcerated hernia and the time elapsed

TABLE 5. The characteristics of the patients who died in the study

Patient no	P 1	P 2	P 3	P 4	P 5	P 6
Age	58	66	68	73	78	102
Gender	Female	Male	Male	Female	Male	Male
Additional disease	COPD HT	CHF HT CVA	CRF HT	COPD HT CVA RCC (liver met)	CAD	-
Hernia type	Insicional	Inguinal	Femoral	Insicional	Inguinal	Inguinal
Duration of symptoms	>24 hour	>24 hour	>24 hour	>24 hour	>24 hour	>24 hour
Strangulation	(+)	(+)	(+)	(+)	(+)	(+)
Resected content	Small intestine	Large intestine	Small intestine	Large intestine	Small intestine	(-)
Necrosis	(+)	(+)	(+)	(+)	(+)	(-)
Mesh usage	(-)	(-)	(+)	(-)	(+)	(-)
Type of anesthesia	General	General	Local	General	General	General
Complication	Pneumonia	(-)	Pneumonia Wound infection	Liver laceration (perop)	(-)	Pneumonia
The day of death	5	1	29	3	3	35

from the start of the first symptom until the start of surgery (6-10). Some past authors have reported that symptoms lasting for >6 h were associated with necrosis resulting in more severe complications (6, 7). In a study conducted by Kulah et al., the rates of strangulation and bowel necrosis were found to be significantly higher in patients who presented after 24 h [for strangulation (58.3% vs. 24.1%); for bowel necrosis (23.9% vs. 7.4%)]. Although the rate of mortality was higher in patients who presented after 24 h (6.6% vs. 1.9%), it was not statistically significant. The authors found that bowel necrosis had a significant effect on mortality (2% for cases without necrosis, 19.4% for cases with necrosis). They also found that coexisting diseases had a significant effect on mortality (9). These results may be attributed to the elderly subjects in whom the coexisting diseases increased. Koizumi et al. (10) also stated a statistical difference in the rate of bowel necrosis among patients operated within 24 h and those after 24 h (29% vs. 49%). Derici et al. (8) operated 182 patients for incarcerated AWHs within 24 h and found that only bowel necrosis was significantly associated with mortality. Unfortunately, delay in prompt surgical intervention continues to be an issue that needs to be resolved for most surgeons. According to the Danish hernia registry, incarcerated hernias are not always treated as rapidly as can be expected, even in Europe. It has been revealed that 60% of the cases are symptomatic for >48 h and only 23% undergo surgery within 8 h of admission (11).

Although conflicting results on the effect of duration of symptoms on mortality have been reported, there is no doubt that delayed surgery increases the rate of strangulation and necrosis in incarcerated AWHs. In the current study, 47.72% of the cases had been symptomatic for >24 h and only 40.9% had undergone surgery within 6 h of admission. Therefore, surgeons had to face the bitter truth about the consequences of delayed surgical intervention with a mortality rate of 13.6%, which is similar to that reported in the literature (ranging from 1.4 to 13.4%) (8). In concordance with the study conducted by Kulah et al., the duration of the symptoms was not a risk factor for increased mortality, rather it was associated with an increased risk of strangulation and necrosis (cut-off value was 24 h). The rates of strangulation increased from 34.8% to 76.2% and necrosis from 26.1% to 66.7% in patients who presented after 24 h of the onset of the symptoms. The mortality rate, which was 4.2% in patients without necrosis, increased up to 25% in cases with necrosis. The resection of any organ, especially of the bowel, may exacerbate surgical stress leading to severe complications, especially in high-risk patients. One of the six patients who died was aged 102 years and the remaining 5 had coexisting diseases, which caused pneumonia and liver rupture eventually.

Owing to its retrospective nature, this study was limited by the records of patients. It was also limited by the small number of cases.

In conclusion, a delay in surgery of >24 h since the onset of the symptoms for incarcerated AWHs is more likely to develop necrosis, which is associated with a higher mortality rate, irrespective of statistical significance. Therefore, it is essential to perform surgical intervention within the first 24 h. In spite of emergency repair, mortality remain high in incarcerated AWHs. Thus, elective repair should be advised for patients with a newly diagnosed AWHs to avoid complications that may necessitate urgent surgery.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of İnönü University (2019/4-38).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept - A.Ş., H.K., M.A., A.D.; Design - A.Ş., M.A., H.K.; Supervision - A.Ş., H.K., M.A., A.D.; Resource - A.Ş., H.K., M.A., A.D.; Materials - A.Ş., H.K.; Data Collection and/or Processing - A.Ş., H.K.; Analysis and/or Interpretation - A.Ş.; Literature Search - A.Ş.; Writing - A.Ş.; Critical Reviews - A.Ş., H.K., M.A., A.D.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1156

Evaluation of Balance Test Outcomes in Children with Poor Vision

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Cite this article as: Baş B, Karabulut M, Müjdeci B. Evaluation of Balance Test Outcomes in Children with Poor Vision. Cyprus J Med Sci 2020; 5(4): 284-8.

BACKGROUND/AIMS
Balance is constituted through the integration of vestibular, proprioceptive, and visual and motor neurophysiology systems. Any discrepancies in any one of these systems may negatively influence the overall balance and postural stability. This study aimed to evaluate balance in children with poor vision.

MATERIAL and METHODS
The subjects of the present study were children with poor vision. The mean age of the children in the study group (n=20) was 7.35±1.92 years, while that of the children in the control group with healthy vision was 8.2±1.10 years. All subjects were assessed with the Functional Reach Test, Romberg Test, Single Leg Stance Test (eyes open and closed), Pediatric Berg Balance Scale (PBBS), Modified Clinical Test of Sensory Interaction on Balance (mCTSIB), and The Timed Up & Go (TUG) Test.

RESULTS
The results showed that children with poor vision performed relatively poorer than healthy children in the Functional Reach Test, Romberg Test, Single Leg Stance Test (eyes open and closed), PBBS, mCSTIB, and TUG tests (p=0.00). We noted that children with poor vision had to strain more to preserve their postural stability in the Single Leg Stance Tests and the mCSTIB Test with closed eyes relative to that with opened eyes (p=0.00).

CONCLUSION
Our findings indicate that children with poor vision have weaker balance relative to their healthy peers. Thus, inadequate level of visual input negatively influences the body balance in children.

Keywords: Vision, low, balance, children

INTRODUCTION

Balance is constituted through the integration of the vestibular, proprioceptive, and visual system. Since children's first interactions with their environment occur through a sense of touching and vision, it is crucial for children to develop and maintenance their balance skills. Any discrepancies in any one of these systems may negatively impact the overall balance and postural stability in an individual (1).

Past studies have reported that vision is pivotal in terms of the preservation of postural stability (2-4). It has been proposed that the decrease in the postural stability increases the risk of fall in individuals with poor vision (5, 6).

According to the World Health Organization (WHO) (6), poor vision is defined as visual acuity of <6/18 but ≥3/60 or a corresponding visual field loss of <20°, in the better eye with the best possible correction (ICD-10 visual impairment categories 1 and 2) (6, 7). Poor vision may be congenital or hereditary and reflect the situation in which vision cannot be repaired with the help of glasses, contact lenses, and medical or surgical treatments. Individuals with poor vision utilize from the vestibular and proprioceptive systems more in order to compensate for their visual dysfunctions (6).

It is already known that vision plays a dominant role in the process of coding and maintenance of other sensory information. It has been discovered that, when healthy and visually impaired subjects are evaluated in accordance with eyes-closed experiments, the stability is decreased and other sensory inputs cannot completely compensate for the visual

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Received: 19.07.2019
Accepted: 15.10.2019

inputs (3, 8). Whether poor vision is congenital or hereditary is also crucial for determining the balance (9).

Some past studies have compared the balance scores of visually impaired and healthy individuals (5, 9), and only a limited number of studies compared the balance scores between healthy individuals and individuals with low vision (3, 5, 10). These studies especially indicate that single-leg stance on a foam surface with eyes opened and closed increases the postural instability and body sway (5, 11).

The aim of the present study was to evaluate balance in children with poor vision.

MATERIAL and METHODS

The study was approved by Ankara Yıldırım Beyazıt University ethics committee with the date / decision no of 25.10.2017 / 02. The data collection process was initiated after ethical consent. Verbal and written information was given to the children and parents about the purpose and scope of the study, and their written consent was obtained.

Participants

We first ensured that the inclusion of 20 children each in the study and control groups was suitable for statistical evaluation. The study included 20 children with congenital poor vision of mean age 7.35±1.92 years (study group) and 20 children with healthy vision of mean age 8.20±1.10 years. The ethical committee approval was received for this study. Written permissions from the subjects' families were received. The inclusion criteria for the participants were determined as follows: having no hearing loss, being able to receive commands, being able to walk without help for 10 m, having no additional impairment, and having no neurological or vestibular system issues. In addition, for the study group, an intensive visual evaluation was conducted by ophthalmologists (e.g., vision loss screen tests, optimal visual acuity test, and ophthalmological examination), and the criteria for poor vision was established in accordance with the WHO specifications for participation. In the control group, children with no history of vision loss and with 20/20 rations in the Snellen eye chart (who could answer correctly for E's in 40 - 20 feet line).

Methods

All children were evaluated with the Functional Reach Test, Romberg Test, Single Leg Stance Test (eyes open and closed), Pediatric Berg Balance Scale (PBBS), Modified Clinical Test of Sensory Interaction on Balance (mCTSIB), and The Timed Up & Go (TUG) Test. Although the evaluation of each child varied with respect to their physical traits, the evaluations lasted for 1 and half hours, which included the resting breaks. There were resting sessions of 10–15 min between each tests considering the children's needs. All tests were applied on the same day with adequate breaks between them. The necessary safety precautions against falling hazard throughout the evaluation process were practiced. In order to maximize the performance of children with poor vision, the necessary adjustments (e.g., walking path with contrasting colors, lightings on the finish lines) were already done for the tests. All tests were performed by the same specialist audiologist at the special education and rehabilitation center.

Main Points:

- Visual inputs are important for balance.
- Children with low vision may fail to maintain postural control in difficult conditions.
- Early detection of imbalance with balance assessment in children with low vision is important in preventing possible falls.

Functional Reach Test

The Functional Reach Test is used to evaluate both balance and the dynamic reach value. Initially, the children were asked to point their arms in front of them in a straight line, and the reach value was recorded. Then, they were asked to lean forward without lifting their heels and the maximum distance, to which the children could lean forward and back without losing their balance, was recorded (12).

Romberg test

Romberg Test is a neurological function test that evaluates the integrity among different sensory organs and neuronal conduction pathways utilized in the maintenance of balance. The test offers information about the central and peripheral vestibular system function and peripheral proprioception. Children were asked to stand upwards for 30 s with their eyes closed in this test (13, 14).

Single Leg Stance Test

In this test, the vestibular function and proprioception sense were evaluated. The participants were asked to lift their one leg without touching the other and wait for 30 s in that posture, initially with their eyes opened and then with eyes closed. The test was terminated in cases in which the lifted leg touched the ground or the other leg or skipping, hopping, or grabbing of the surrounding objects in order to maintain balance was noted (15).

Modified Clinical Test of Sensory Interaction on Balance (mCTSIB)

This test was used to evaluate the sensory system dysfunction on a firm and foam surface, with both eyes opened and closed. In tests on the foam surface with eyes closed, the effects of visual and somatosensory inputs were eliminated and the effects of vestibular inputs on postural stability were evaluated more efficiently. In the test, the children were asked to preserve their balance in 4 different conditions: 1. Eyes opened, on a firm surface, 2. Eyes closed, on a firm surface, 3. Eyes opened, on the foam surface, and 4. Eyes closed, on the foam surface, and the maximum time values for which the children could manage to stand in balance were recorded (16).

The Timed Up and Go Test [TUG]

This test is a conveniently applicable and reliable test used to evaluate the functional mobility and balance. Different variables such as the walking speed, postural control, functional mobility, and balance were evaluated in this test (17). In this test, the participants were first seated on a chair and then asked to stand up without handling the grip, walk for 3 m, and then sit again. During this task, the observers recorded the time with a chronometer.

Pediatric Berg Balance Scale [PBBS]

PBBS is a highly valid and reliable test used to evaluate the functional balance in routine life activities. It is the pediatric version of the Berg Balance Scale developed by Franjoine et al. (18). The highest point was 56 on the scale, which consisted of 14 sections and it was evaluated between 0 and 4. These sections were designed to have increasing functional difficulty levels. In the PBBS, the overall scores were recorded. On the PBBS scale, the overall score of 0–20 was considered as a balance disorder, while the score of 21–40 was considered as an acceptable balance performance and 41–56 corresponded to a good performance (18).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences software version 18 (IBM SPSS Corp.; Armonk, NY, USA). The compatibility of the data with respect to the normal distribution was evaluated with histograms, probability plots, and Kolmogorov Smirnov/Shapiro-Wilk's test. For every balance test and age variant between the groups, the values of mean±standard deviation were used. In a comparison of all test results between the groups and independent *t*-test were used. The paired *t*-test was used to compare the test scores of single-leg stance test and mCTSIB between the eyes open and eyes closed test conditions. p<0.05 was considered to be statistically significant.

RESULTS

The results of the Single Leg Stance Test, Romberg Test, mCTSIB, and Functional Reach Test are depicted in Table I. In all these tests, children with poor vision could only maintain their balance for a relatively short duration (p=0.00).

In the Romberg test, the mean duration for which the children with poor vision managed to maintain their balance was determined to be 27.65±5.82 s. In the control group, all children with healthy vision managed to complete the task in 30 s. Between the two groups, no statistically significant difference in terms of the Romberg Test scores were noted (p=0.08; Table I).

In the TUG test, children with poor vision completed the walking task in a greater amount of time relative to that by the control children (p=0.00; Table 2).

The overall PBBS score was relatively lower for the children with poor vision than for the control children (p=0.00; Table 2).

In both the children with poor and healthy visions, the performance in the Single Leg Stance test was found to be better when the test was conducted in 'eyes opened' position (p=0.00). In the mCTSIB test on children with poor vision, it was determined that children performed better in 'eyes opened' position with respect to 'eyes closed position on firm and foam surfaces (p=0.00). The children in the control group managed to reach the maximum time interval of 30 s on both firm and foam surfaces in the mCTSIB test (Table 3).

DISCUSSION

In the present study, children with poor vision performed poorly in the vestibulospinal tests. External stimuli and motor development played a significant role in terms of the development of postural control among the children. The fact that there was not adequate visual input for the children with poor vision negatively affected the extent of external stimuli and motor development (6).

Balance was maintained with respect to the visual, proprioceptive, vestibular, and motor development systems. The fact that there was no or insufficient amount of visual stimuli for children with poor vision negatively affected the ability to control the postural stability.

Several past studies have emphasized that vision impairment decreases postural stability (3, 5, 7, 11). In our study, we determined that children with poor vision showed decreased postur-

TABLE I. The Results of Romberg Test, Single Leg Stance Test, Modified Clinical Test of Sensory Interaction on Balance and Functional Reach Test

Test	Study Group Mean±SD	Control Group Mean±SD	p
Romberg Test-EC (sec)	27.65±5.82	30.00±0.00	0.08
Single Leg Stance-EO (sec)	5.64±2.66	20.88±1.33	0.00*
Single Leg Stance-EC (sec)	2.16±0.77	9.72±1.13	0.00*
mCSTIB (firm surface-EO) (sec)	26.45±5.39	30.00±0.00	0.00*
mCSTIB (firm surface-EC) (sec)	20.45±6.15	30.00±0.00	0.00*
mCSTIB (foam surface-EO) (sec)	24.30±4.49	30.00±0.00	0.00*
mCSTIB (foam surface-EC) (sec)	15.50±6.61	30.00±0.00	0.00*
Functional Reach Test (cm)	24.65±7.30	33.15±1.98	0.00*

*p<0.05, independent t-test. mCTSIB: Modified Clinical Test of Sensory Interaction on Balance

TABLE 2. The Results of Time Up & Go Test and Pediatric Berg Balance Scale

Test	Study Group Mean±SD	Control Group Mean±SD	p
TUG (sec)	22.43±7.80	9.82±1.00	0.00*
PBBS (score)	42.90±9.90	56.00±0.00	0.00*

*p<0.05, independent t-test. TUG: Time Up & Go test, PBBS: Pediatric Berg Balance Scale

TABLE 3. Comparison of Eyes Opened and Eyes Closed Scores of Single Leg Stance Test and Modified Clinical Test of Sensory Interaction on Balance

	STUDY GROUP		
	EO Mean±SD	EC Mean±SD	p
Single Leg Stance (sec)	5.64±2.66	2.16±0.77	0.00*
mCSTIB (firm surface) (sec)	26.45±5.39	20.45±6.15	0.00*
mCSTIB (foam surface) (sec)	24.30±4.49	15.50±6.61	0.00*
	CONTROL GROUP		
	EO Mean±SD	EC Mean±SD	p
Single Leg Stance (sec)	20.88±1.33	9.72±1.13	0.00*
mCSTIB (firm surface) (sec)	30.00±0.00	30.00±0.00	-
mCSTIB (foam surface) (sec)	30.00±0.00	30.00±0.00	-

*p<0.05, paired t-test. mCTSIB: Modified Clinical Test of Sensory Interaction on Balance, EO: Eyes Opened, EC: Eyes Closed

al stability in the Single Leg Stance, mCTSIB, Functional Reach Test, PBBS, and TUG tests, which conforms to the findings of previous studies.

The fact that it is relatively harder to maintain balance without vision indicates that the balance control mechanisms are mostly dependent on vision (7, 11, 19). Kayıhan et al. (20) analyzed the balance and touch perception. In their study, 29 visually impaired children and 41 children with low vision of ages 11–20 years were compared to 40 children with healthy vision of ages 16,17,18. The cumulative results of this study indicated that children with healthy vision had longer Single Leg Stance time. In addition, researches determined that the inefficiency of a vision-balance mechanism negatively affected the coordination activities (20, 21). In children with poor vision in our study, the duration of postural stability was determined to be shorter relative to those of children with healthy vision; this finding coincides with that of previous studies. Moreover, our study revealed a significant difference between the results with "eyes opened" position and "eyes closed" position for children with poor vision. Furthermore, in mCTSIB tests, in the "eyes closed" position on both firm and foam surfaces, this time interval was relatively shorter in children with poor vision. The balance control on a single-leg stance was mostly dependent on the visual information. In challenging positions, the proprioceptive input decreased and, in this case, the visual and vestibular inputs became more significant. Considering that children with poor vision received lesser amount of visual input, only vestibular input was utilized to maintain the postural control, and the overall performance of the children in terms of postural stability was negatively affected (11). This finding illustrates whether the tests were conducted with opened or closed eyes and whether the surface texture had a meaningful impact on the balance scores of the study group. This conclusion can be explained by the fact that visual proprioceptive information was relatively more sensitive than mechanic proprioceptive information, which was received from the vestibular and somatosensory systems (5, 9). In children with hereditary poor vision, all stages of motor development were improved through exploration of the environment. Since significant motor skills were already established in cases with congenital poor vision, it is possible to plan and manipulate actions accordingly (6). We believe that disruptions in the balance scores in challenging positions can be explained in comparison to the fact that the participants in our study group had hereditary poor vision; thus, they were unable to adapt to these positions. In our study, the finding that children with hereditary poor vision performed relatively poorer in the balance action on foam surface and in "eyes closed" position may indicate the necessity of familiarity and adaptation in terms of postural control in the proprioceptive and vestibular systems (5, 9).

Kayıhan (22) emphasized that visually impaired children required more experience in their routine life due to the delay in the development of motor skills. Bauchard et al. (23) compared 30 visually impaired children with 30 healthy-vision children of ages 8–13 years and concluded that visually impaired children had relatively poorer motor skill development. Murphy and O'driscoll (24) evaluated the factors of speed, agility, balance, and coordination skills in 6 visually impaired children of ages 5–6 years for over 2 years. At the end of their study, they noted issues in the visually impaired children in terms of these skills. Piereira (21) indicated that when individuals with healthy vision and visually impaired individuals of the same age were compared with respect to their overall performance in balance control, the latter group performed relatively poorly. In our study, the overall completion time of the TUG test was determined to be longer for children with poor vision when compared with those in the control group, which conforms to the findings of previous studies (8, 21, 22). The poorer performance of the study group in the TUG test with respect to the control group can be explained based the delay in the development of their motor skills (18), weaknesses in their motor skills (6), and disruptions in the speed, agility, balance, and coordination skills (24).

In the past studies, PBBS was often used to evaluate the balance performance in sitting and standing stance and the functional balance. In our study, a meaningful difference was noted between the study and control group in terms of the PBBS scores. Despite this difference, the average overall scores of both the study and control groups correspond to a "good balance" score. This result indicated that the study children in our study could perform independently in their routine activities with respect to their current visual condition. Together with this idea, we determined that the balance control in children with poor vision was negatively affected by challenging conditions such as the foam surface or "eyes closed" position in the Single Leg Stance Test, mCSTIB, and Functional Reach Test owing to the inefficient visual input. This finding implies that children with poor vision were more susceptible to the falling hazard in the challenges presented in everyday situations (soft ground, inclined ground, and dark or dimmed environment). We believe that this conclusion can be applied as a policy-determining factor in terms of the fall-prevention strategies against falling hazard among children with poor vision and in rehabilitation programs that aim to increase the vestibular adaptation.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ankara Yıldırım Beyazıt University (25.10.2017/02).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - B.B.; Design - B.B.; Supervision - B.M.; Resource - B.B., M.K.; Materials - B.B., B.M.; Data Collection and/or Processing - B.B., M.K.; Analysis and/or Interpretation - B.M.; Literature Search - B.B., M.K.; Writing - B.B.; Critical Reviews - B.M.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1431

The Association of Ki-67 and the Stage change between AJCC 7th and 8th Edition in Breast Cancer

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Cite this article as: Edebal Z, Turan S, Kılıç H. The Association of Ki-67 and the Stage change between AJCC 7th and 8th Edition in Breast Cancer. Cyprus J Med Sci 2020; 5(4): 289-94.

BACKGROUND/AIMS
The new pathological prognostic staging in the 8th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual uses biomarkers, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) for breast cancer staging, but not Ki-67. This study was designed to evaluate the relationship of Ki-67 with pathological prognostic staging parameters and its possible correlation with this new staging system.

MATERIAL and METHODS
We performed a retrospective analysis on 59 invasive ductal breast carcinoma patients. We restaged all the patients using anatomic staging (AS) and pathological prognostic staging (PPS). The correlation of Ki-67 with ER, PR, HER2, histological grade, tumor size, and lymph node status were compared using the Chi-square test.

RESULTS
When patients classified according to AS were restaged using PPS, 21 (36%) retained their original stage, while 34 (58%) were downstaged and 4 (6%) were upstaged. There was no correlation between the stage change and Ki-67, HER2, tumor grade, or size. Both, ER and PR positivity were markedly higher in the downstaged group (p=0.014 and p<0.001). Ki-67 was not significantly different between AS patients; however, stage 3 PPS patients had a significantly more positive Ki-67 ratio than stage-1 and stage-2 patients (p=0.007). Moreover, Ki-67 had a significant negative correlation with ER and PR and positive correlation with the tumor grade, HER2, and lymph node involvement.

CONCLUSION
Ki-67 is not useful for predicting the staging change from AS to PPS. However, it is strongly correlated with markers related to the biological features and prognosis in breast cancer. In order to increase its usefulness, more comprehensive studies are required.

Keywords: Breast cancer, Ki-67 antigen, cancer staging, biomarkers, prognosis

INTRODUCTION

Breast cancer is one of the most common malignancies across the world. About 2 million new cases are detected every year, and one of every 4 newly diagnosed cancer cases is that of breast cancer (1).

Until recently, the staging system developed by the American Joint Committee on Cancer (AJCC) that relies on the tumor size, lymph node involvement, and distant metastasis (TNM) was used for breast cancer management and prognosis estimation. With a deeper understanding of the biological factors related to breast cancer, the determination of various biomarkers has become a necessity (2). Thus, The AJCC Breast Cancer Expert Panel described a new "prognostic staging" that considers factors, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in addition to the TNM classification. They published this new staging system in the AJCC Cancer Staging Manual in 2016 and revised it in 2017 (3, 4). The pathological prognostic staging (PPS) is applicable for every patient who has undergone surgical excision for initial treatment without neoadjuvant therapy. This new staging is applied by using primary tumor size, lymph node involvement, distant metastasis, histological grade, ER, PR,

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Received: 15.10.2019
Accepted: 30.12.2019

and HER2. The AJCC Breast Cancer Expert Panel also recommended that a proliferation marker, such as Ki-67 and a genetic prognostic panel be performed at the time of initial diagnosis, if available (4). Ki-67 is an antigen expressed in the GI, S, G2, and M phases of the cell cycle, but not G0. The most common method for determining the Ki-67 status is immunohistochemistry (5, 6). Although Ki-67 is a recommended biomarker for assessing the proliferation status, it is not implemented in the PPS because it does not possess sufficient reliability owing to reproducibility issues and lack of agreement for cut-off points (4). Moreover, the results of the studies performed to establish a valid relationship between Ki-67 and other PPS biomarkers have been inconsistent (5, 7-10).

Here, we aimed to investigate the relationship between Ki-67 and other pathological prognostic factors in breast cancer as well as examine the effects of the 7th and 8th AJCC classifications on the staging change in the same patient. We also aimed to determine whether Ki-67 or any other biomarkers used for classification affect the staging changes.

MATERIALS and METHODS

Study Group and Pathological Evaluation

Our study protocol was approved by the Health Sciences Ethical Committee of Near East University, with approval number YDU/2019/70-853. As our study was a retrospective trial and did not involve the use of personal data, the need for informed consent was waived off. We retrospectively collected the data of patients who were operated at the Konya Beyhekim State Hospital between January 2014 and May 2019 for invasive ductal carcinoma. We excluded patients who were in the carcinoma-in-situ stage or had distant metastasis, were missing pathologic prognostic staging biomarkers, had received neoadjuvant therapy, or had not undergone lymph node dissection. The histological grades, ER, PR, HER2, and Ki-67 statuses of the patients were reevaluated by using the existing slides in the same pathology laboratory by the three pathologists. Grading was performed following the Elston/Nottingham modification of the Bloom-Richardson system (Scarff- Bloom-Richardson Grading system, Nottingham Modification) by rating the following three morphological features: tubule formation, nuclear pleomorphism, and mitotic figure count of the tumor. Each parameter was assigned a score from 1 to 3, and the tumor was classified as grade I, 2, or 3, if the sum of these was 3–5, 6–7, and 8–9, respectively. Staining over 1% was accepted as positive for ER and PR (11). For HER2, staining of 3+ was accepted as positive (12). We accepted the cut-off value for Ki-67 positivity as 20%, following the 2013 International St. Gallen Expert Consensus (13). Thereafter, the patients were restaged as per both, the 7th edition (anatomic staging, AS) and 8th edition (pathological prognostic staging, PPS) of the AJCC Cancer Staging Manual.

Main Points:

- Estrogen receptor (ER), Progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are included in the pathological prognostic for staging (PPS) breast cancer, but not Ki-67.
- Ki-67 positivity is significantly increased in PPS stage 3 patients.
- Ki-67 is not useful for predicting the staging change from anatomic staging to PPS. However, Ki-67 has a significant positive correlation with tumor grade, HER2, and lymph node involvement and it is also negatively correlated with ER and PR.

Statistical Analysis

All the statistical analyses were performed using the Statistical Package for Social Sciences software version 15 (SPSS Inc., Chicago, IL, USA). Patient age is reported as mean and standard deviation; tumor size and the absolute value of Ki-67 are reported as median and interquartile range values. Categorical variables are reported as frequencies and percentages. Parametric factors were compared using the t-test, and non-parametric factors were compared using the Mann Whitney U test or Kruskal-Wallis analysis. Categorical factors were compared using the Chi-square test and Fisher's exact test, where appropriate. If the p-value was <0.05, it was regarded significant.

RESULTS

The study population comprised of 97 patients. In thirty-eight of these patients, either one or more of the main markers used for breast cancer staging (ER, PR, HER2, Ki-67) was not analyzed and these patients were excluded from the study. We evaluated the data of the remaining 59 patients. One of the subjects was a man. The mean patient age was 61.4 y, and only 4 were aged <40 y. Table I shows the demographic and clinicopathological features of the patients, and Figure I shows the frequency distribution of Ki-67 absolute values.

We restaged the patients who were previously categorized as per the 7th edition of the AJCC Cancer Staging Manual using the PPS. The consistency rate of the new staging was 36% because 21 of the 59 patients remained in the same stage, while 34 (58%) were downstaged by at least one step, and 4 (6%) were upstaged. Stage 3C and 2A patients exhibited a staging change

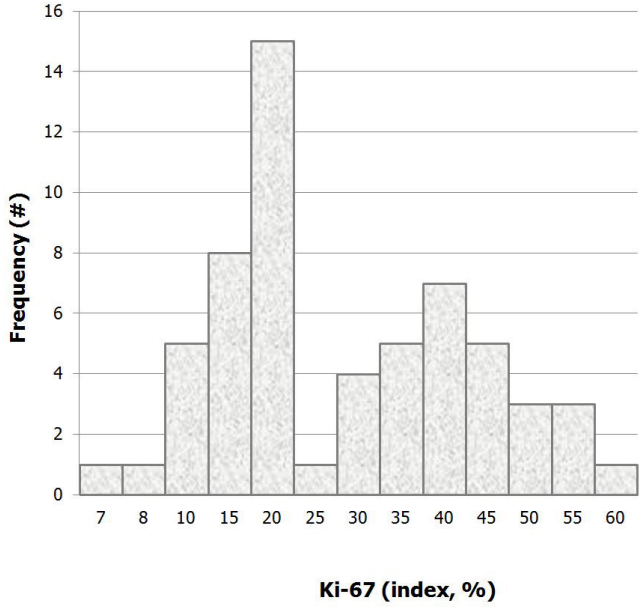

FIGURE I. Frequency distribution of the absolute Ki-67 values

TABLE I. Patient characteristics

	No. of Patients (n=59)	%
Localization		
Left	36	61
Right	23	39
ER		
Negative	16	27
Positive	43	73
PR		
Negative	24	41
Positive	35	59
HR (ER or PR)		
Negative	13	22
Positive	46	78
HER-2		
Negative	45	76
Positive	14	24
Histological Grade		
Grade I	11	19
Grade 2	35	59
Grade 3	13	22
Ki-67		
Negative	30	51
Positive	29	49
Lymph Node Metastasis		
0	18	31
I to 3	16	27
4 to 9	11	19
10 or more	14	23
Tumor size		
≤2 cm	19	32
>2 cm	40	68
	Mean	**SD**
Age (years)	61,4	13,9
	Median	**Interquartile Range**
Ki-67 (% Index)	20	25
Tumor Size (cm)	2,5	1,9
Node Count	3	9

ER: Estrogen receptor
PR: Progesterone receptor
HER2: Human epidermal growth factor receptor-2.

most frequently. Detailed staging distribution of the patients before and after restaging is presented in Table 2.

When the patient group whose staging remained unchanged after restaging was compared with the groups with a changed staging, a significant difference was found between stages I, 2, and 3 of Anatomical Staging (p=0.018, Table 3). Stage 2 and 3 patients had markedly more staging changes as compared to stage-I patients (p=0.014 and p=0.001, respectively). When the upstaged, downstaged, and unchanged cases were compared, the groups showed no difference in the tumor grade, tumor size, HER2, or Ki-67. However, the downstaged group had significantly more ER and PR positivity (p=0.014 and p<0.001). Furthermore, all 4 patients who were upstaged were PR negative; only I had ER positivity.

Ki-67 was not different among stage-I, stage-2, and stage-3 patients in AS; however, there was a significant difference between the stages in PPS. The Ki-67 positivity of the patients in PPS with stage 3 breast cancer was significantly higher than in those with stage I or 2 (p=0.007). When compared to the other clinicopathological features, Ki-67 had a significant negative correlation with ER and PR (p=0.015 and p=0.026), and positive correlation with HER2 (p<0.001), histological grade (p<0.001), and lymph node involvement (p=0.047, Table 4).

DISCUSSION

Ki-67 is an important biomarker for understanding the biology and behavior in breast cancer. It has an established prognostic property, and the determination of Ki-67 status is encouraged by the AJCC Breast Cancer Expert Panel even though it is not included in the PPS (4, 14). However, studies that have investigated the relationship between Ki-67 and breast cancer biomarkers, such as ER, PR, and HER2, have produced conflicting results.

In the present study, we found that Ki-67 was correlated with ER and PR negativity, HER2 positivity, increased histological grade, and lymph node involvement. Yip et al. reported similar correlations with ER, grade, and HER2, but not ER. Contrary to our results, their findings showed a relationship between Ki-67 and tumor size (10). There was a significant correlation between Ki-67 and tumor size, ER, PR, and grade, but not HER2 (Marwah et al.) (9). Another study reported that Ki-67 was related to the tumor grade, but not the tumor size (15). Ahmed et al. (8) reported findings similar to our findings in that Ki-67 was inversely correlated to ER and PR and had a positive correlation with grade and HER2, with no correlation to the tumor size. In contrast to some of these results and our findings, Kamranzadeh et al. (5) stated that Ki-67 was not correlated to ER, PR, tumor grade, or HER2. We believe that one possible explanation for these conflicting results might be the cut-off values chosen for Ki-67 in these studies. Yip et al. and Ahmed et al. chose 14%, Shetty et al. and Kamranzadeh et al. used 10%, and Marwah et al. determined 2 decision points as 5% and 20%, for grouping the Ki-67 values. We used 20% as the cut-off point as per the recommendations of the International Ki-67 in Breast Cancer Working Group and the 2013 St. Gallen consensus (13, 16). There is no universal agreement with respect to the cut-off point for Ki-67. There are different Ki-67 cut-off points in various studies, from 5% to 34% (17). Moreover, Ki-67 has different cut-off values that have the same clinical significance in certain clinical conditions. Denkert et al. (18) reported that many different Ki-67 cut-off values have similar significance for evaluating disease-free survival, response to neoadjuvant therapy, and overall survival, and it is impossible to state which cut-off value is the most appropriate. We presume that it may be beneficial to specify different Ki-67 cut-off values for different clinical purposes. Another reason for these conflicting results may be the differences in the study designs and variations in the patient attributes, such as mean age or race. Prospective and retrospective study designs might

TABLE 2. Distribution of upstaging and downstaging in patients with anatomic staging and pathological prognostic staging

	Pathological Prognostic Staging															
	IA		IB		2A		2B		3A		3B		3C		Total	
Anatomic Staging	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
IA	5	8.4	1	1.7											6	10.2
IB			1	1.7	1	1.7									2	3.4
2A	2	3.4	7	11.9	6	10.2	1	1.7							16	27.1
2B			1	1.7	3	5.1	3	5.1							7	11.9
3A					5	8.4	2	3.4	5	8.4	1	1.7			13	22.0
3B									1	1.7	---	0			1	1.7
3C											13	22.0	1	1.7	14	23.7
TOTAL	7	11.8	10	17	15	25.4	6	10.2	6	10.2	14	23.7	1	1.7	59	100

TABLE 3. Rates of stage changes in patients classified as per anatomic staging

Anatomic Stage	Unchanged	Down Staged	Up Staged	P*
Stage I	6 (75%)	0 (0%)	2 (25%)	0.003
Stage II	9 (39%)	13 (57%)	1 (4%)	
Stage III	6 (21%)	21 (75%)	1 (4%)	
Total	21 (36%)	24 (58%)	4 (7%)	

* Chi-square test

provide different outcomes; further, the inclusion of Caucasian, Asian, and/or Indian subjects in these studies may have caused the diverse results.

Ki-67 is also an established prognostic marker that is important for distinguishing among certain types of breast cancer. This marker helps in determining the proliferative capacity of the tumor, an important characteristic of tumor biology. Therefore, its relation with the other biomarkers related to the biological status of the tumor has been a matter of concern. We found significant relationships between Ki-67 status and ER negativity, PR negativity, HER2 positivity, increased histological grade, and lymph node involvement. First, mitotic index is one of the three parameters that are used for assigning the histological grade and is directly proportional to Ki-67. Thus, tumors with a higher mitotic index are expected to have a higher Ki-67 value that manifests with a higher grade. In fact, several studies have reported a significant correlation between Ki-67 and grade (8-10, 15, 19), while one research has shown a contradictory result (5). We found a strong positive correlation between Ki-67 and HER2 such that all the HER2 positive cases except one were also positive for Ki-67. Aziz et al., Yip et al., and Ahmed et al. state that HER2 positive tumors have a significantly higher Ki-67 value. In contrast, Kamranzadeh et al. and Marwah et al. reported that HER2 and Ki-67 were not significantly correlated. These studies used a cut-off value for Ki-67, while the previous 3 studies compared the median Ki-67 values of the groups. In addition, we found a significant correlation between Ki-67 and hormone receptor negativity. Following the implementation of PPS in daily clinical practice, patients with a positive hormone receptor have usually been assigned to lower stages. With the help of targeted therapy, these markers exert a favorable effect on prognosis. The negative prognostic features of Ki-67 have been reported in various studies (15, 19, 20). Although we were unable to evaluate its effect in this regard, we presume that the inverse relationship of Ki-67 with favorable biomarkers present in our study supports its undesirable effect on patient prognosis.

In our study, we inspected the agreement between AS and PPS as well as the factors that may affect the staging consistency. We found that 36% of our patients remained in the same stage, 58% were downstaged by at least one step, and 6% were upstaged. The consistency rate of restaging from the 7th edition to the 8th edition of the AJCC varied from 40.63% to 54.5% in other studies (7, 21-24). Most of these studies have reported upstaging rates of 5.3%–9.9% and downstaging rates were varying between 35.6% and 48.2% (21-24). But one study reported a much higher upstaging rate of 39.76% and a lower downstaging rate of 19.61% (7). Our upstaging rate was slightly higher than those reported by these studies. This could be because our patient population had a markedly higher mean age than the subjects in these studies. Moreover, most of our patients were in stage 3, making up 47% of all our patients; 78% of these patients were assigned to a lower stage after restaging. In the other studies, 13%–21% of the patients were in stage 3. We think that the high mean age of our study population and the high rate of advanced-stage patients compared to the aforementioned studies can be suggested as factors that influence our consistency and restaging rates.

AS expected, ER and PR were significantly different in the unchanged, upstaged, and downstaged groups. However, Ki-67, HER2 status, tumor grade, and tumor size were similar in these groups. Thus, we concluded that Ki-67 did not have any power in predicting the staging change. We performed the same analysis using 10%, 14%, 25%, and 34% values for Ki-67, as stated in other studies; however, we did not find a significant correlation between Ki-67 and the staging change (data not shown). This finding is in contradiction with the findings reported by Ding et al (7). In the mentioned study, Ki-67, tumor size, and lymph node involvement were independent individual factors for predicting staging change. To our knowledge, no other single-center study has investigated the predictive ability of Ki-67 on staging change from AS to PPS. We recommend that more comprehensive studies on larger patient populations be performed to evaluate this ability of Ki-67.

TABLE 4. Correlation of Ki-67 expression with clinicopathological features

	Ki-67		
	≤20	>20	p†
Age			
≤50	6 (38%)	10 (62%)	0.211
>50	24 (56%)	19 (44%)	
Localization			
Left	19 (53%)	17 (47%)	0.792
Right	11 (48%)	12 (52%)	
ER			
Negative	4 (25%)	12 (75%)	0.015*
Positive	26 (61%)	17 (39%)	
PR			
Negative	8 (33%)	16 (67%)	0.026*
Positive	22 (63%)	13 (37%)	
HER-2			
Negative	29 (64%)	16 (36%)	<0.001*
Positive	1 (7%)	13 (93%)	
Grade			
1	5 (46%)	6 (54%)	<0.001*
2	25 (71%)	10 (29%)	
3	0 (0%)	13 (100%)	
Anatomic Stage			
Stage I	5 (63%)	3 (37%)	0.086
Stage II	15 (65%)	8 (35%)	
Stage III	10 (36%)	18 (64%)	
Pathologic Prognostic Stage			
Stage I	12 (71%)	5 (29%)	0.007*
Stage II	13 (62%)	8 (38%)	
Stage III	5 (24%)	16 (76%)	
Tumor Stage			
T1	13 (68%)	6 (32%)	0.168
T2	14 (44%)	18 (56%)	
T3	3 (38%)	5 (62%)	
Node Status			
N0	11 (73%)	4 (27%)	0.047*
N1	9 (53%)	8 (47%)	
N2	7 (54%)	6 (46%)	
N3	3 (21%)	11 (79%)	

*Statistically significant.
†Chi-square test.

Lymph node involvement is one of the most important prognostic factors in breast cancer. However, some patients with similar tumor size and lymph node involvement have completely different prognosis (25). Thus, presenting the relationship of biomarkers with the nodal status might be beneficial for prognostic grouping. We demonstrated a significant positive correlation between Ki-67 and lymph node involvement. Most of our N0 cases were negative for Ki-67, and the rate of the patients in N1 and N2 stages did not have a meaningful difference from N0 cases in terms of Ki-67 positivity. However, most of the N3 patients were Ki-67 positive. This implies that tumors with a high proliferation rate are prone to lymphatic spread. This correlation between Ki-67 and lymph node involvement confirms that Ki-67 is an important prognostic biomarker. But, our finding contradicts certain recent reports (5, 9, 19). Nonetheless, a review of early breast cancer has shown that studies with a higher number of patients tend to demonstrate a significant positive correlation between Ki-67 and positive lymph node count (17). These contradictory results indicate the need for more comprehensive and larger population-based studies for investigating the correlation of Ki-67 with lymph node status in breast cancer.

In the present study, the Ki-67 positivity did not vary significantly in stages 1, 2, and 3 in AS; however, we found that stage 3 patients in PPS had markedly higher Ki-67 positivity than stage-1 and stage-2 patients. Ki-67 is a proliferation marker with widespread availability and ease of application; however, the lack of reproducibility and universal cut-off value do not allow its implementation in PPS. Nevertheless, Ki-67 is recommended by the expert panel to be determined at the time of initial diagnosis as a proliferation marker (4). Denkert et al. (14) recommended that the best strategy to demonstrate tumor biology in the adjuvant settings is to use Ki-67 as a continuous marker, rather than as a cut-off. Based on this information, we also compared the median values of Ki-67 among stage-1, stage-2, and stage-3 patients in AS. We did not find any difference using a cut-off value as stated before, and stage 3 patients had a significantly higher Ki-67 median value than stage-1 and stage-2 patients in AS (Kruskal-Wallis test, p=0.043, data not shown). We believe that this finding supports the suggestion of Denkert et al. and that using Ki-67 as a continuous marker may be a better approach. Denkert et al. (14) also suggested that especially intermediate Ki-67 levels that have low analytic validity have limited applicability in clinical practice and that we should not determine whether Ki-67 is positive based on marginal differences. Almost 50% of our patients had Ki-67 values of 15%–25%; this finding is important because it shows that this situation affects a large population of breast cancer patients.

This was a single-center study; this is a major strength of our study. Every sample was prepared in the same laboratory and evaluated by all three pathologists at the same time, minimizing the aforementioned variability of Ki-67 staining. In addition, to our knowledge, our study is one of the few single-center studies to evaluate the new PPS and its effects as well as the relationship of Ki-67 with the biomarkers used for PPS in a Turkic population.

This study has certain limitations; first, we studied a relatively small population because the study was performed at a single institution, and because our hospital does not specialize in breast care. In addition, the study population was relatively old and was diagnosed late for breast cancer; therefore, our sample was not representative of all breast cancer patients and was formed majorly of subjects who were in advanced stages. This may have influenced our consistency and restaging rates. Finally, we did not study the effects of the biomarkers on the prognosis, given our study design. Further, prognosis evaluation would have significantly contributed to our findings.

In conclusion, Ki-67 is not useful for predicting the staging change between AS and PPS. However, it is significantly correlated with most biomarkers used for PPS, emphasizing its importance in understanding the biological behavior of the tumor. Moreover, its correlation with stage and lymph node involvement strengthens its prognostic features. However, there remains a need for more comprehensive studies based on larger populations to increase the usefulness of Ki-67 in understanding breast cancer biology.

Ethics Committee Approval: Ethics committee approval was received for this study from the Health Sciences Ethical Committee of the Near East University (YDU/2019/70-853).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept - Z.E.; Design - Z.E.; Supervision - Z.E., H.K., S.T.; Resource - Z.E., H.K., S.T.; Materials - Z.E., H.K., S.T.; Data Collection and/or Processing - Z.E., H.K., S.T.; Analysis and/or Interpretation - Z.E.; Literature Search - Z.E.; Writing - Z.E.; Critical Reviews - Z.E., H.K., S.T.

Acknowledgements: The authors want to acknowledge the efforts of Mehmet Kul for retrieving archived patient data and organizing pathological reports.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1509

Calcium and Sodium Channel Blockers and Gastrointestinal Motility

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Cite this article as: Koç E. Calcium and Sodium Channel Blockers and Gastrointestinal Motility. Cyprus J Med Sci 2020; 5(4): 295-8.

BACKGROUND/AIMS
Calcium and sodium channels are necessary in all excitable cells to maintain their functions. Calciumchannel blockers are generally used clinically to treat various pathological conditions such as cardiovascular diseases. Moreover, sodium channel blockers are widely used clinically, such as in dentistry. Given their widespread use, these blockers may have effects on gastrointestinal motility.

MATERIAL and METHODS
This study used healthy adult Wistar rats for the experiments. The ileal segments were isolated and suspended in tissuebath. Contractile responses induced by acetylcholine (ACh) were recorded. To study the effects of calciumchannel blocker nifedipine (l,4-dihidropyridine calcium channel blocker) and sodiumchannel blocker prilocaine [2-(propylamino)-o-propionotoluidide], ileal segments were incubated with these agents, and ACh-induced contractions were then recorded.

RESULTS
All doses of calcium and sodium channel blockers significantly decreased the ACh-induced contractions in isolated ileal segments.

CONCLUSION
Calcium and sodium channel blockers have significantly decreasedgastrointestinal motility.

Keywords: Calcium channel blocker, sodium channel blocker, gastrointestinal motility

INTRODUCTION

Calcium channels are essentialfor many functions in excitablebody cells, such asin transmitter release, hormone secretion, excitation, and excitation–contraction coupling. L-type calcium channels play a key role in muscles that need extracellular calcium for contraction. Calcium channel blockers are generally used to treat cardiovascular diseasesas well as hypertension.As such, L-type calcium channel blockers are used in many clinical situations, such asin the treatment of hyperinsulinemichypoglycemia (l), respiratory system diseases (2), cardiac disorders, Parkinson's disease (3), and hypertension. Moreover, nifedipine may have a partially protective effect on noise-induced hearing loss (4). For many years, calciumchannel blockers have been used in the treatment of hypertension, and their side effectshave been well studied. Calcium blockers hypothetically exhibit relaxation effects not only on vascular smooth muscles but also on gastrointestinal smooth muscles. Although studies have focused on the effect of calcium channel blockers on vascular smooth muscle, only a few have investigated gastrointestinal motility.

Local anesthetics that have sodium channel blocking potentials are agents that reversibly block action potentials in excitable membranes. The exact mechanism of the effects of local anesthetics on various cellular physiological functions remains unknown. Thus, this study was carried out to investigate the effects of calcium channel blocker nifedipine andsodium channel blockerprilocaine on the amplitude of acetylcholine (Ach)-induced contractions on isolated rat ileum. As channel blockers are used commonly and systematically, specifically, this study aimed to investigate the possible effects of these blockers on gastrointestinal motility.

MATERIAL and METHODS

Healthy adult Wistar rats (n=l0) weighing an average 0f l50–250 g were used in theexperiments. This study was approved by the local ethics committee of Near East University (reference no. 2019/06). The animals were anesthetized lightly with

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Received: 06.11.2019
Accepted: 27.11.2019

pentobarbital (35 mg/kg i.p.) and were slain by decapitation and exsanguination. Ileal segments were suspended in an isolated tissue bath containing Tyrode's solution (mM: NaCl 137, KCl 2.68, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, and 5.5 glucose) and on bubbles with 95% O_2, 5% CO_2 mixture at 37°Cat pH 7.4. Tissue segments were brought into equilibrium for 60 minunder the optimal resting tension of 0.3 g. Contraction of ileal segments were then induced by adding ACh (6.7×10^{-5} M) which is accepted as maximal contractions in the control group. The amplitude of ACh-induced contraction was measured in millimeters from the recorded traces and was calibrated as 1g per 10 mm. When ileal segments were contracted with ACh (6.7×10^{-5} M),these parameters were accepted as ACh-induced control group. Local anesthetic prilocaine were used in two doses at 4.7×10^{-5}M and 9.4×10^{-5} M. After the addition of one of the prilocaine doses, Ach was added after 3 minutes a few minutes into the medium, and the results were recorded. Calcium channel blocker nifedipine was used in four different doses: 3.6×10^{-6}M, 7.2×10^{-6}M, 1.4×10^{-5}M, and 7.2×10^{-5}M. The ileal segments were washed several times and left for 60 minbefore each agent was added into the bath solution. Calcium channel blockerswere added first, followed by the addition of prilocaine and AChinto the medium, and the results were recorded.

RESULTS

The effects of calcium channel blocker nifedipine and sodium channel blocker prilocaine on ACh-induced contraction of isolated rat ileal segments were examined. The average peak amplitudes of ACh-induced contractions bytwo doses of prilocaine have decreased significantlycompared with those of the control groups (without prilocaine) (Figure 1).

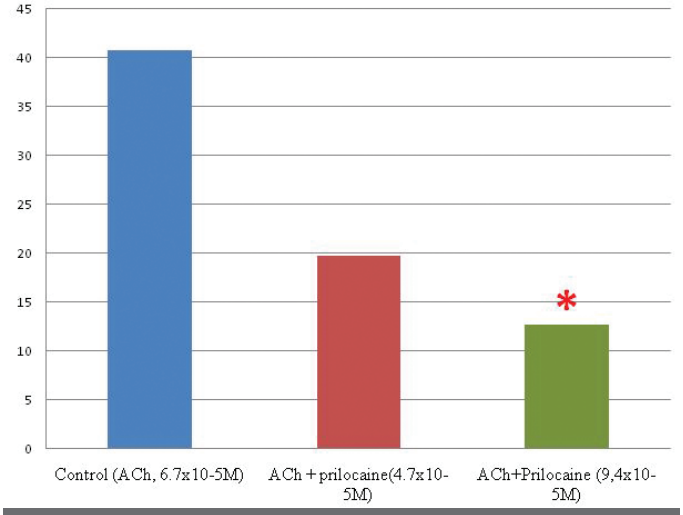

FIGURE 1. TheACh-inducedcontractionbydifferentdoses of Prilocaine

Main Points:

- Calcium and sodium Channel blockers have decreased the isolated ileal contractility.
- Local anaesthetic prilocaine significantly reduced ACh-induced contraction.
- Prilocaine has decreased calcium and sodium entry to the gastrointestinal smooth muscle.

In Figure 2, the average peak amplitude of ACh-induced contractions were significantly lower in all doses of nifedipine in the Tyrode group than in the control (without nifedipine) group.

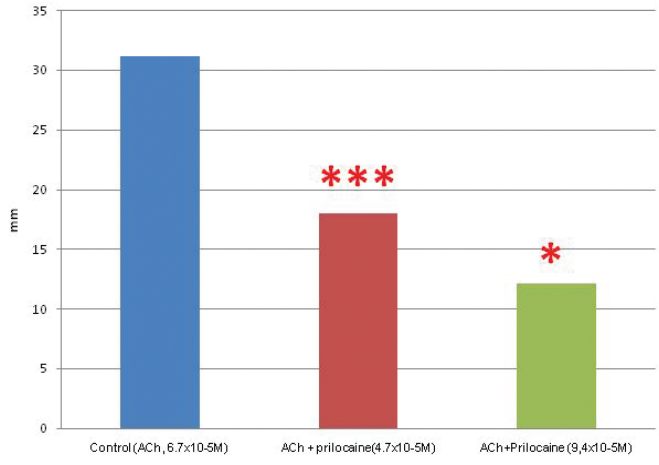

FIGURE 2. The ACh-induced contraction of different doses of prilocaine with 3.6×10^{-6}M nifedipine

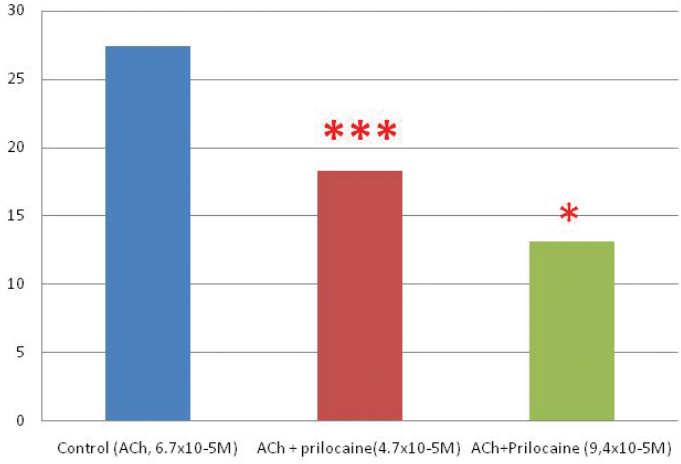

FIGURE 3. The ACh-induced contraction of different doses of prilocaine with 7.2×10^{-6}M nifedipine

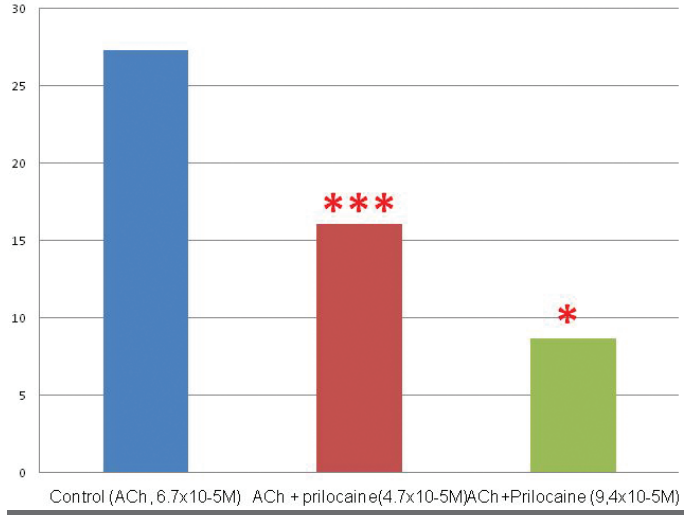

FIGURE 4. The ACh-induced contraction of different doses of prilocaine with 1.4×10^{-5}M nifedipine

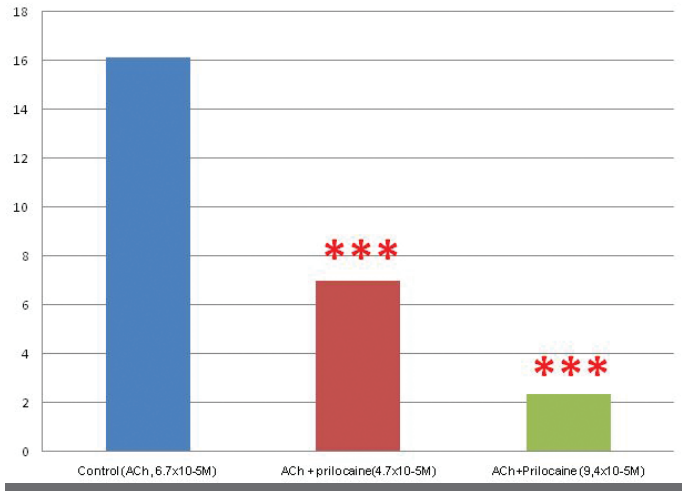

FIGURE 5. The ACh-induced contraction of different doses of prilocaine with 7.2xI0^{-5}M nifedipine

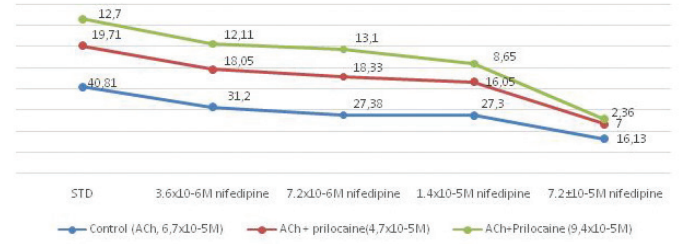

FIGURE 6. TheACh-inducedcontraction in differentdoses of prilocaineandnifedipine

The effect of nifedipine on the isolated ileal contractility was dose-dependent.

In Figures 2, 3, 4, 5 and 6, the effectof prilocaine on ACh-induced contractions in fourdoses of the nifedipine–Tyrode perfusion medium was significantly lowerthanthose of the control (without nifedipine) group.

Statistical Analysis

PASW I8 SPSS were used. Statistical analyses were performed with unpaired t-test. Significant difference from control were accepted as p<0.00I and p>0.05. Values were presented as mean±standard error of mean.

DISCUSSION

This study showed that the application of calcium and sodium channel blockers on perfusion medium has significantly decreased the contractility of isolated ileal segments.In this study, an increase in prilocaine and nifedipineconcentrations hascaused a decrease in the contractility of the ileum, and their effects were dose-dependent.

Several studies have reported the effects of local anesthetics on sodium channels (5, 6). The electrophysiological basis for the action of local anesthetics on nerves was established only within the past several decades. Although several studies have explored the effect of local anesthetics on other ion channels, such as calcium andpotassium channels (7), most of the investigations have focused on the sodium channel (8). However, the exact mechanism of the effect of local anestheticson excitation–contraction coupling and contractile proteins has not been thoroughly examined. Tsuda et al. explored the effects of local anesthetics on actomyosin and reported the actions of local anesthetics on purified proteins at a molecular level (9). They have suggested that the binding and ATPase of actomyosin were governed predominantly by weak and strong ionic binding, which was barely affected by local anesthetics.

In this study, calcium channel blockers significantly decreased the ACh-induced contraction of isolated ileal segments. The inhibition of contraction of rat ileal segments was dependent on nifedipine concentrations, and the effect of nifedipine wasdose-dependent.

Calcium antagonists or calcium channel blockers reduce the contractions of smooth muscles by inhibiting calcium ions (I0). In this study, nifedipine was used asa calcium channel blocker. Patai et al. (II) suggested that nifedipine has inhibited KCI-induced inward Ca^{+2}-induced contraction in a concentration-dependent fashion in the contraction of guinea pig trachea.

Dorkkan (I2) reported that active duodenal calcium transport was completely abolished by L-type nifedipine as well as inhibitors of the major basolateral calcium transporters.

Bladen et al. (I3) showed that a number of I,4-dihidropridine not only blocked L-type calcium channels but also blocked low-voltage-activated T-type calcium channels.

In conclusion, the calcium channel blocker nifedipine and sodium channel blocker prilocainehave significantlydecreased the contractility of isolated rat ileal segments. The effect of prilocaine and nifedipine on ACh-induced contraction was found to be dose-dependent. Prilocaine has decreased calcium and sodium entry to the gastrointestinal smooth musclessimilar to any other smooth muscles in the body. Nifedipine may have inhibited calcium influx through both potential and receptor-operated calcium ion channels. In this study, channel blockers decreased the gastrointestinal contractility. Nevertheless, cellular mechanisms underlying these effects still remain unclear; moreover therefore, further studies are required and results should be confirmed by human investigations.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Near East University (2019/6).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Conflict of Interest: Author has no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1770

Peripheral Block Education and Level of Competency: A Survey of Turkish Anesthesiologists

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Cite this article as: Selvi O, Tulgar S, Senturk Ö, Taş Z, Köse HC, Topçu Dİ, et al. Peripheral Block Education and Level of Competency: A Survey of Turkish Anesthesiologists. Cyprus J Med Sci 2020; 5(4): 299-304.

BACKGROUND/AIMS
Presently, in Turkey, there is no work being conducted on the period of regional anesthesia education, and there are no statistics available on the frequency with the administration of basic extremity blocks.The present survey was conducted on anesthesia doctors throughout Turkey to explorethe personal knowledge and competency of the participants. Simultaneously, this survey aimed to evaluate the information sources for regional anesthesia training in Turkey.

MATERIAL and METHODS
The present study surveyed 377 anesthesia doctors in March 2017 through a questionnaire formulated on the Delphi platform. The voluntary participants were recruited from professional organizations, hospital portals, and Turkey's anesthesia departments. Descriptive analyses were conducted for statistical evaluation.

RESULTS
Of the total participants, 3.2% were professors, 3.2% were associate professor doctors, 7.4% were assistant professors, 64.5% were specialist doctors, and 21.8% were residents. In the segment concerning skill and information level self-evaluation for blocks, the most commonly performedact was infraclavicular block (26.8%). With regard to their "information source," 40% of the participants chose "from my colleagues" for upper extremity blocks. The specialist training was the least popular information source for ultrasound-guided interscalene block, while the digital visual information sources were often consulted for the same.

CONCLUSION
Various sources have been identified as the source of information as much as the specialty training, more so in some blocks. A significant majority of the participants avoided block applications. In the future, it would be useful to collect data on a more comprehensive national scale to overcome the limitations of the present issue.

Keywords: Nerve block, anesthesiology, internet, education

INTRODUCTION

The techniques applied in regional anesthesia are increasingly becoming importance with each passing day, compounding the need for regional anesthetics training in Turkey and across the world (1). While it requires a certain level of manual dexterity, the safe and successful application of these techniques is correlated to the ability to access information on their correct application. However, proficiency in certain techniques in this field cannot be guaranteed by relaying on information alone. Such proficiency can only be obtained and consolidated by repeated performance, that is, by performing the procedures a certain number of times in clinic settings (2).

Anesthesia education combines anatomy, physiology, pharmacology, clinical evaluation, experience, knowledge, and manual dexterity into one unified field (3). As with every other type of contemporary education, this filed has also been

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Received: 05.02.2020
Accepted: 05.04.2020

implicated with a rise in the use of digital and virtual models for training in anesthesia (4). Beyond the standard source books, expert guidance, and experiences shared with contemporaries, modern anesthesia education employs digital models comprising video-based visual applications. Several universities and officially recognized institutes have invested in preparing videos and materials on regional anesthesia in order to meet this particular need (5).

National guidelines have been prepared for the delivery of regional anesthesia training to students during their professional medical training in Turkey. In this field, the instruction period continues beyond professional training, with regular courses, cadaver studies, conferences, and peer group discussions. To the best of our knowledge, no work has yet been conducted in relation to this period of anesthesia education in our country. Furthermore, there are no statistics on the frequency with which anesthesia physicians are involved in administering basic lower and upper extremity blocks in Turkey, nor are there any data available currently relating to the types of blocks and the skill levels involved.

This study conducted a survey of 377 anesthesia doctors spread across Turkey with the aim of exploring the personal knowledge and skill level of these physicians with regard to the basic regional anesthesia techniques conducted routinely in their clinic. Simultaneously, we intended to discover the rate at which these blocks were performed and the types of platforms used as information sources for regional anesthesia training in Turkey.

MATERIAL and METHODS

The target audience of the study was voluntary Turkish anesthesia and reanimation physicians who agreed to participate in the study. For this reason, the Local Ethics Committee did waived off approval by the Clinical Research Regulation, and emphasized that the identity information from the participants was reserved. In addition, on the survey entrance page, each participant had to agree on giving permission to obtain results of the survey published in order to participate in the survey.

The present study surveyed 377 anesthesia doctors working in Turkey between March 1 to 31, 2017 using a questionnaire formulated based on the Delphi platform. The participants were recruited on a voluntary basis from professional organizations, hospital portals, and the Department Heads of Turkey's anesthesia departments, who were contacted with a request for volunteering by filling out an online questionnaire form.

Main Points:

- This study exhibits the first survey outcomes regarding regional anesthesia education and statistics on the frequency with the administration of basic extremity blocks in Turkey.
- According to the results of this survey almost one-third of all participants who had some information on a block nevertheless stated that they lacked the requisite skill level to conduct it in a clinical environment.
- There is no clear framework for delivery training of regional anesthesia in Turkey.
- Internet-based information sources regarding regional blocks have gained popularity among Turkish anesthesiologists.

The first three questions on the questionnaire requested the institution in which the clinician works (such as the university hospital, training and research hospital, state hospital, private hospital), the clinician's academic status (whether professor, associate professor, assistant professor, specialist, resident physician), and their professional experience in years (<5 years, 5–10, 11–20, ≥21 years).

The second and third sections of the questionnaire investigated the information sources and the levels and the skill level of each participant in relation to both ultrasound-guided and conventional peripheral nerve stimulator for the upper extremity brachial plexus blocks. The following statements were presented as options for information and skill level: "I often carry out this procedure–I have adequate information and skill level for this kind of block," "I rarely carry out this procedure–I have adequate information and skill level for this kind of block," "I do NOT carry out this procedure–although I have adequate information and skill for this kind of block," "I have information about this kind of block, but I am not sufficiently skilled to carry it out on my own," and "I do not have sufficient information or skill for this kind of block." The options regarding information sources were as follows: "specialist training", "regional anesthesia courses", "source books, guides and journals", "from my colleagues," "internet videos" (e.g., New York School of Regional Anesthesia-NYSORA, YouTube®). The blocks mentioned included interscalene block, supraclavicular block, infraclavicular block, and axillary block.

The fourth section of the questionnaire dealt with femoral, sciatic, popliteal, and obturator nerve blocks, both ultrasound-guided and through the use of conventional peripheral nerve stimulator, and adopted similar questioning pattern to establish the information level, sources, and skill level of the participants.

This exclusive online survey could be accessed via a link sent to the participants who could only fill the form single time so as to produce the required results for statistical analysis.

Statistical Analysis

Data cleaning and analysis *were performed* using R 3.6.1. Descriptive statistics was applied to present quantitative descriptions of the data. For statistical analysis, the frequency distributions were calculated for all items of the questionnaire. The data regarding frequency distributions was considered as percentages in the plots.

RESULTS

The survey was completed by 377 people. Of the participants, 3.2% were professors, 3.2% were associate professor doctors, 7.4% were assistant professors, 64.5% were specialist doctors, and 21.8% were assistant/resident physicians. On the question about professional experience, 8.2% stated ≥21 years, 18.8% stated 11-20 years, 44% stated 5–10 years, and 28% stated <5 years of professional working experience. Of these, 26.3% currently worked at university hospitals, 23.9% worked at state hospitals, 35% worked at training and research hospitals, and 14.9% worked at private hospitals (Table 1).

In the segment concerning skill and information level self-evaluation for the upper extremity blocks, the most commonly performed block was the infraclavicular block accompanied by ultrasound (26.8%). The least performed block was the supraclavicular block with nerve stimulator (9%). Of the lower extremity blocks, the most common one was ultrasound-guided femoral block, while the least performed was the obturator block (Figure 1).

TABLE I. The demographics of the participants

Information:	n
Academic degree:	
Professor:	12
Associate professor doctor:	12
Assistant professors	28
Specialist:	243
Assistant/resident physicians:	82
Professional experience:	
21 years or more	31
11 to 20 years	71
5 to 10 years	169
Less than five years	106
Hospital choice:	
University hospital	99
State hospital	90
Training and research hospitals	132
Private hospitals	56

With regard to the training period, 40% of the participants opted for "from my colleagues" as their "information source" for the upper extremity blocks; this rate reduced to 30% for the lower extremity blocks. For specialist training, the most commonly given information source was the execution of axillary blocks with a nerve stimulator (58.6%). The least popular choice for specialist training information source was ultrasound-guided interscalene block (30%) and obturator block (24%). Information obtained through the designated courses was most commonly associated with ultrasound-guided interscalene blocks and most rarely with nerve stimulator obturator blocks. In terms of the digital visual information sources (such as Nysora and YouTube), these were often consulted for ultrasound-guided interscalene blocks and least consulted as an information source for nerve stimulator axillary blocks and femoral blocks (Figure 2).

For all blocks, the source books and guides were stated as information sources at a rate of 33-40%.

DISCUSSION

Based on the results of the present study, the most prevalent peripheral blocks administered in Turkey were ultrasound-guided upper extremity blocks. Furthermore, this study revealed an increase in the use of internet-based digital sources as the information source for this type of block. Other than a study by Gürkan et al., no information has been obtained on the use of peripheral blocks previously in Turkey. Moreover, there are no

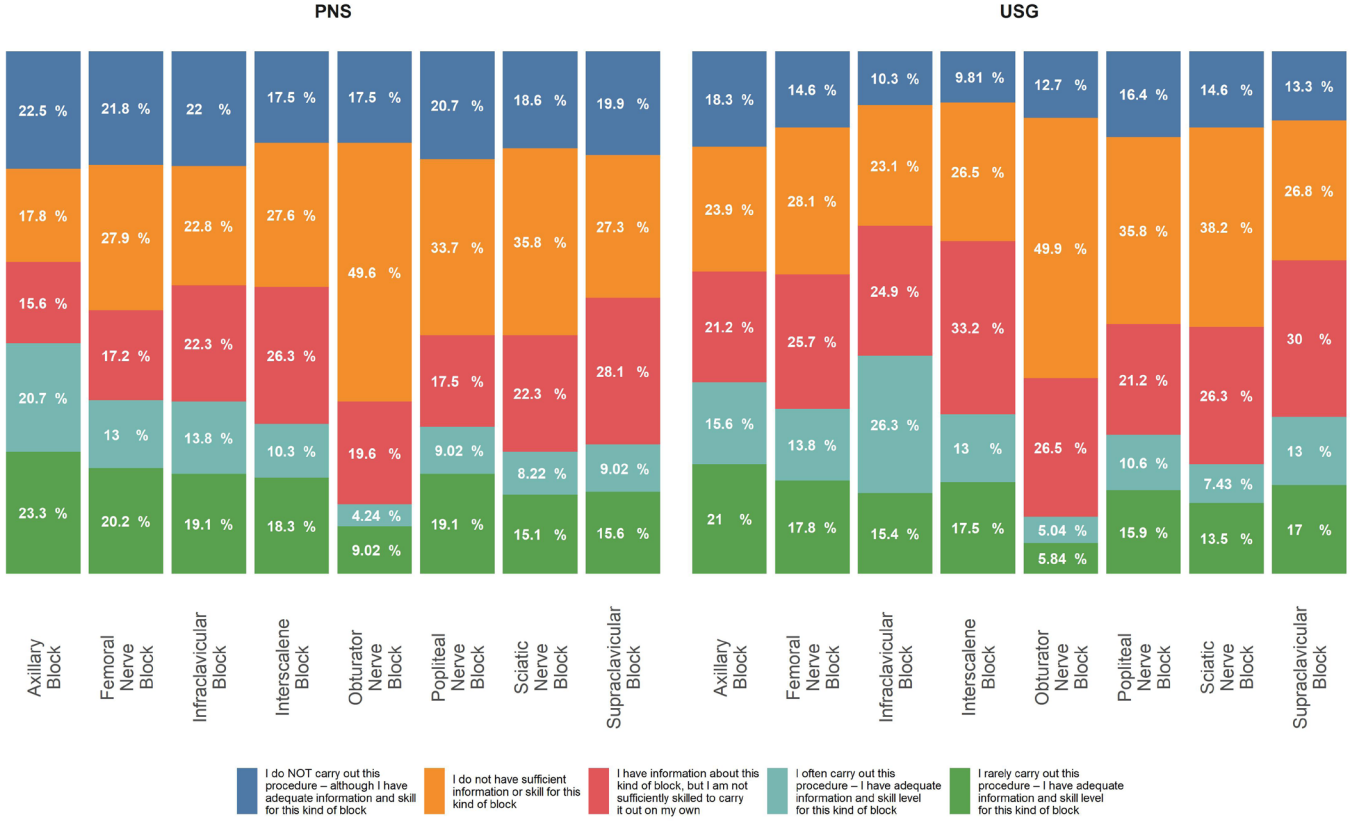

FIGURE I. Self-evaluation for level of skill / information and application frequency for upper and lower extremity blocks
PNS: Peripheric nerve stimulator USG: Ultrasound-guided

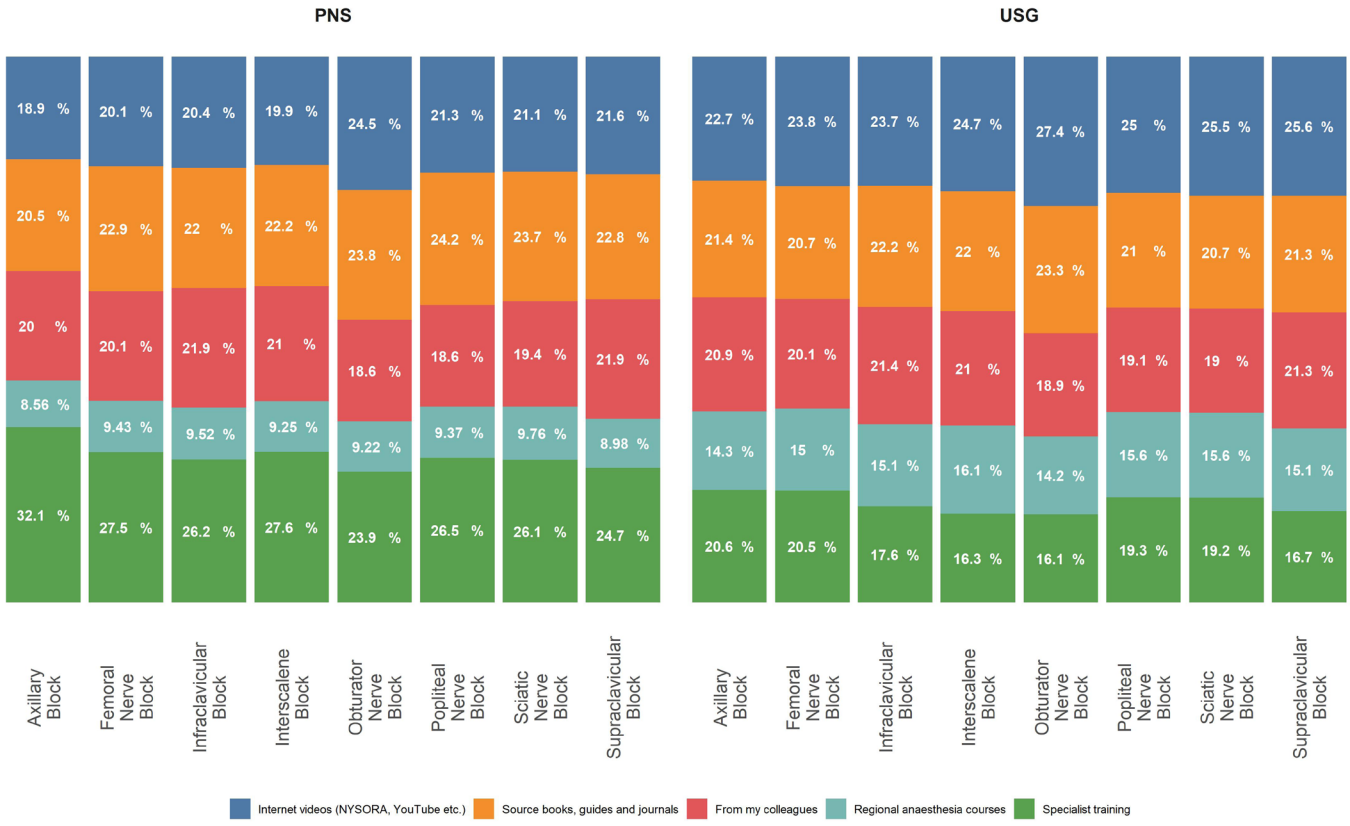

FIGURE 2. Information sources for learning regional regional block techniques
PNS: Peripheric nerve stimulator USG: Ultrasound-guided

data available on the information sources used in regional anesthesia training, which makes the present study one of its first kind in Turkey.

The study conducted by Gürkan et al. (6) identified that peripheral blocks accounted for only 12% of all regional anesthesia procedures. In the present study, while 30% of all anesthetists performed USG upper extremity blocks, this rate dropped to approximately 25% for the lower extremity blocks. Thus, there was a preference in all types of blocks for the ultrasound-guided form over the nerve stimulator form of the procedure. In Turkey, more specifically, the fact that ultrasound has become the norm for blocks leads one to believe that they may not be carried out without ultrasound. This tendency toward applying ultrasound is particularly pronounced for interscalene and infraclavicular blocks, a result that ties in with the global data on these preferences (7). The popularity of the upper extremity blocks has been correlated with the enhanced effectiveness and safety that is associated with the use of ultrasound (8). Among anesthesia training centers, it was found that the success rate of ultrasound-guided blocks was higher than that of nerve stimulator, and this success rate has been reported to be almost as high as 97% (9). In short, it can be fairly stated that the rising popularity of conducting peripheral blocks with ultrasound is closely related to the increased efficacy and safety accompanied with the use of ultrasound use.

With an average of 25% of all participants stating the lack of information on the upper extremity blocks, this rate increased to 38% for the lower extremity blocks and even approached 50% for obturator nerve blocks. According to the regional anesthesia "fellowship" training and acute pain treatment guides, candidates should learn 24 types of block to gain expertise; however, this number is not sufficient for real expertise (10). Not only does expert training in Turkey insufficient to meet the minimum recommended requirement for the number of types of block taught, there is no clear framework for delivery this type of training. For example, a past study noted that expert training required 45 attempts at epidural anesthetic and 60 at spinal anesthetic, with a success rate of 90% (11). Moreover, individual differences could also stretch the standards one way or the other. For instance, another study found that, while some candidates could reach the required proficiency with a 90% success rate over 57 intubation attempts, other candidates reported failing to achieve even 80% success rate after 100 attempts (2). Thus, all candidates should be subjected to personal evaluation and be provided requisite exposure to both conceptual and practical information during the regional anesthetic training program. Medical procedures should be taught via simulations recorded with advanced video-recording devices, and giving feedback on the recorded attempts of students has been shown to increase the success of the training outcome (12). Developing procedure-specific metrics and using proficiency-based progression training may also bring improvement in the patient outcomes for complex medical interventions (13).

Due to the practice of various curricula within the field of anesthesia and the varying programs for different types of specialism, physicians emerge from specialist training with different knowledge sets with regards to the use of techniques of region-

al anesthetics. When questioned on the survey in regard to the information sources for block procedures, anesthetists stated that they received information from other colleagues. Reaching a rate of 40% for certain blocks, this rate of receiving help and information from colleagues continued after professional training had been completed. It can be inferred from these results that this type of information source is particularly valuable for anesthetists who come from an unusual educational background or for whom an information gap could be quickly bridged with the help of input from colleagues.

Alongside clinical knowledge, ambidexterity, hand-eye coordination, the ability to interpret sonographic visualizations, and the ability to think in three dimensions are all necessary for regional anesthesia procedures. For this purpose, before operating on actual patients, regional anesthesia training utilizes multimedia technologies, simulation systems, and cadaver studies for practical training purpose (14). Once proficiency had been obtained through training, the measurement of an anesthetist's capability could also present a problem. Unfortunately, despite the invasive nature of anesthesia, the measurement and evaluation of medical candidates' technical suitability for the successful performance of these procedures continues to lag (15). No data is available on the contents or evaluation criteria for the regional anesthesia components of advanced anesthesia training in Turkey. According to the present survey, advanced training was considered as the information source at a level of approximately 50% for some blocks, while this rate was lower for more general courses. However, the same survey revealed that, about one-third of all participants who had some information on a block nevertheless stated that they lacked the requisite skill level to perform it. This outcome indicates that the regional anesthetic training offered within advanced training courses is insufficient for clinical proficiency. When teaching procedures that require motor skills within an adult education environment, the behavioral steps of the learning process should be monitored. In this way, didactic instruction can be considered through observation and motor planning performed by the posterior parietal cortex at an early "informational" level of the candidate's training. At the second "associative" level, memory is laid down in relation to the procedure being learned, and, at the third "autonomous" level, the student is finally able to conduct the required set of motor skills in an accurate and automatic manner (9). According to educational experts, despite the fact that several doctors are aware of what needs to be taught, very few know how it should be taught (16).For this reason, even if the training does provide advanced candidates encouragement in performing block procedures, the lack of suitable training staff, the absence of appropriate supervision, and the fact that some instructors are not sufficiently in command of their own proficiency level suggests that individuals undergoing regional anesthesia training may not reach the level of autonomous proficiency.

Internet technologies, interactive training applications, online courses, and digital visual sources have taken anesthesia training to a new level. More than one-third of all participants in the survey stated that they had benefited from these types of sources for all types of blocks, and, for certain blocks, nearly half of the respondents used such technologies as information and training sources. The ability to rely upon these digital sources and the question about who is preparing the content is important from the point of view of patient safety (17). For example, the majority of publicly available videos on YouTube concerning regional anesthetics have been found to be inadequate from the perspective of content and safety. The videos produced by institutes and universities, on the other hand, tend to be more trustworthy (5). In this case, it seems sensible to consult video guides published by designated foundations and associations both during an advanced training program and beyond it. Similarly, professionally produced training videos in the Turkish language is expected to fill the gap that currently exists for advanced anesthesia training courses and beyond in Turkey. On this point, the content of videos produced by associations and university foundations that take on this role is improving (18).

The present study is based on a survey conducted on a voluntary basis. As the participant selection was not a randomized process, the results obtained may not be applicable to the more general situation of Turkish regional anesthesia procedures across the board. Doctors working in the same clinic or hospital can sometimes display a common approach that may be an obstacle for the homogeneity of the study in its entirety. Thus, as there have been no other studies in this area in the literature, the present study outcomes may still constitute a valuable contribution to the research in this field.

In conclusion, the results of this study indicate that various information sources, including internet-based visual data, have been identified as the source of information as much as the specialty training. In some of the blocks, their importance overweighs the traditional specialty training. This finding proves that the trend in the education of the regional anesthesia has been gradually changing in Turkey as in the rest of the world. According to the outcome of this study, a significant majority of the participants avoided block applications. In order to overcome such problems and having a national roadmap to improve the regional anesthesia education in Turkey, it would be useful to collect the data on a more comprehensive national scale.

Ethics Committee Approval: N/A

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept - O.S., S.T.; Design - O.S., S.T., D.İ.T.; Supervision - Z.Ö.; Resource - Ö.Ş., H.C.K.; Materials - Z.T., Ö.Ş.; Data Collection and/or Processing - D.İ.T., S.T.; Analysis and/or Interpretation - S.T.; Literature Search - O.S.; Writing - O.S.; Critical Reviews - Z.Ö.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1848

Anticancer Activities of *Allium sativum* L. Against MCF-7 and MDA-MB-231 Breast Cancer Cell Lines Mediated by Caspase-3 and Caspase-9

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Cite this article as: Isbilen O, Volkan E. Anticancer Activities of Allium sativum L. Against MCF-7 and MDA-MB-231 Breast Cancer Cell Lines Mediated by Caspase-3 and Caspase-9. Cyprus J Med Sci 2020; 5(4): 305-12.

BACKGROUND/AIMS
Allium sativum (garlic) has been used as a medicinal herb for centuries, and commonly among cancer patients as herbal supplements. The aim of this study is to explore the in vitro antitumoral effects of *Allium sativum* L. (ASB) on human breast cancer (BCa) cell lines, MCF-7 and MDA-MB-231.

MATERIAL and METHODS
Trypan blue assay and LDH assays were used to quantitatively determine the cytotoxicity effects of ASB extract on BCa cell lines. Cytotoxicity experiments were followed up by caspase-3 and -9 activity assays to get a mechanistic insight into the associated molecular pathways. The MTT assay was used to demonstrate the antiproliferative activity, whereas the lateral motility assay was used to gain insight into the migration potential of BCa cells upon incubation with the ASB extract. The bioactive molecules in the ASB extract were delineated through GC-MS analysis.

RESULTS
Exposure to ASB extract caused a significant cytotoxicity effect on MCF-7 and MDA-MB-231 cell lines. A significant activation of caspase-9 was observed in both tested cell lines, indicating that the cytotoxic activity is mediated in an apoptotic manner. The results of the MTT assay revealed a significant antiproliferative effect on both tested cell lines at all tested time points. The lateral motility experiments showed a significant reduction in BCa cell motility demonstrating the antimotility potency of the ASB extract. An abundance of bioactive molecules in ASB were revealed via GC-MS analysis, many of which have been previously associated with anticancer activities.

CONCLUSION
Overall, *Allium sativum* has significant antitumoral and antimotility effects on MDA-MB-231 and MCF-7 human breast cancer cells which are attributed to its bioactive molecules.

Keywords: *Allium sativum* L., anticancer, apoptosis, proliferation, cytotoxicity

INTRODUCTION

Globally, breast cancer is the most frequently diagnosed type of cancer and the main cause of cancer related mortality in women (1). Non-gender discriminatory data also suggests that breast cancer is the second main cause of cancer-related mortalities in both sexes, representing 11.6% of all cancer deaths, led only by lung cancer at 18.4% (1). The statistics representing cancer cases and cancer-related deaths are expected to rise by 70%, causing the annual cancer-related deaths to reach 17 million by 2030 (1). Although it has been established that current therapies used against cancer have reached some success at preventing or delaying cancer-related deaths, current statistics are indicative of the need for further research to improve the patients' quality of life and reduce the death rates. It is imperative to research novel molecules as well as commonly used resources to learn basic biological aspects and treatment/prevention of the disease and to reveal novel therapeutics.

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Received: 03.03.2020
Accepted: 23.08.2020

Several drugs that are currently used have originated from therapeutic plants (2). Furthermore, various bioactive molecules obtained from plants are used for the development of new medicines. Around 28.187 different species of plants are used for medicinal purposes or the development of new therapeutics (3).

In vitro and *in vivo* anticancer studies of garlic, *Allium sativum*, have revealed that molecules within the bulbs utilize several mechanisms to prevent cancer formation, such as the induction of drug metabolizing enzymes (4), by serving as an antioxidant agent (5) and by inhibiting tumor formation (6). Altering cell signaling mechanisms and inducing apoptosis (7) as well as boosting immune system cells against cancer (8) and inhibiting angiogenesis (9) were demonstrated as anticancer mechanisms associated with *Allium sativum. In vivo* research also revealed that the consumption of *Allium sativum* plays a role in significantly reducing the risk of some gastrointestinal cancers (10, 11).

Although various studies on *Allium sativum* and its effects on cancer have been conducted, the mechanistic details of its anticancer effects on breast cancer cells are yet to be investigated. To this end, the aim of this study is to mechanistically investigate the antiproliferative, cytotoxic, antimetastatic, and apoptotic effects of the ethanolic extracts of *Allium sativum* L. bulb on weakly metastatic MCF-7 and strongly metastatic MDA-MB-231 BCa cell lines.

MATERIALS and METHODS

Collection of garlic cloves

The fresh forms of *Allium sativum* L. were obtained from a local market in North Cyprus and the identification of garlic cloves was performed by Prof. Dr. Mehmet Koyuncu (CIU, Pharmaceutical Botany Dept).

Extraction of garlic cloves

Allium sativum L. bulb (ASB) samples were separated, sliced, and kept at the room temperature for drying. The dried plant material was then powdered and the extraction was done by mixing powdered material with 95% ethanol with a 1:10 w/v ratio. The sample was then macerated at room temperature for 24 h and filtered with Whatman No°1. Rotary-evaporator (Heidolph, Germany) was used for the concentration of samples at 40 °C. ASB yield was 0.344%. ASB extracts were labeled accordingly and stored at 4 °C for further analysis.

Main Points:

- Allium sativum L. induces cytotoxicity on MCF-7 and MDA-MB-231 breast cancer cell lines in a caspase-3, and caspase-9 mediated manner.
- Allium sativum L. inhibits the proliferation of MCF-7 and MDA-MB-231 breast cancer cell lines with a higher antiproliferative effect on the strongly metastatic MDA-MB-231 line in a concentration-dependent manner.
- Allium sativum L. exerts antimotility activity on both strongly and weakly metastatic breast cancer cell lines, MCF-7 and MDA-MB-231.
- The GC-MS analysis of Allium sativum L. bulb revealed several bioactive molecules with anticancer activities, including diallyl sulfide, diallyl trisulfide, dimethyl sulfide, 1,2-benzenedicarboxylic acid, and hexadecanoic acid.

Culture conditions for BCa Cell Lines

Strongly and weakly metastatic BCa cell lines, MDA-MB-231 and MCF-7, were grown at 37 °C, 5% CO_2, and 100% relative humidity in Dulbecco's Modified Eagle Medium (DMEM) (Gibco by Life Technologies TM, USA) supplemented with 4 mmol/l L-glutamine and 10% fetal bovine serum until 90-100% confluence as previously described by Fraser et al. (12). This study was conducted in accordance with the World Medical Association Declaration of Helsinki.

Trypan blue exclusion assay

The cytotoxicity effects of *Allium sativum* L. extracts on BCa cells were evaluated using trypan blue exclusion assay. Cells were plated with 3×10^4 /ml density and incubated overnight. MCF-7 and MDA-MB-231 breast cancer cells were incubated for 24, 48, and 72 h with *Allium sativum* extract. The percentage of alive cells was determined from 20 randomly selected areas under an inverted microscope (Leica, Germany) (13).

Lactate Dehydrogenase (LDH) Cytotoxicity assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme released as a result of damaged plasma membrane which is directly proportional to cellular cytotoxicity. The LDH activity of ASB incubated cells (0.5×10^6 /ml cell density) was measured quantitatively using the LDH Cytotoxicity Assay Kit (Thermo Scientific, USA) in accordance with the manufacturers' instructions.

Caspase-3 and Caspase-9 activity

The caspase-3 induction by *Allium sativum* L. extract in breast cancer cell lines (0.5×10^6 /ml) was evaluated quantitatively using the Caspase-3 Colorimetric Activity Assay Kit (Merck Millipore, USA) in accordance with the manufacturer's directions. Similarly, caspase-9 activity measurements were determined quantitatively using the Caspase-9 Colorimetric Activity Assay Kit (Merck Millipore, USA) on MDA-MB-231 and MCF-7 cells (0.5×10^6) in accordance with the manufacturer's directions.

Methyl-thiazolyltetrazolium (MTT) assay

The change in the cell numbers was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Invitrogen, ThermoScientific) assay as previously explained by Isbilen et al. (13). Both BCa cell lines were plated (3×10^4 /ml cells) and kept overnight in an incubator followed by the *Allium sativum* L. bulb extract treatment (at 24, 48, and 72 h). All cells received 1 mg/ml MTT containing DMEM and were incubated at 37 °C for 3 h. Moreover, MTT was removed and replaced with 0.89 ml DMSO (Sigma-Aldrich) and 0.11 ml glycine buffer. Measurements to determine cell numbers were performed at 490 nm on a multi-well plate reader (ELx800, Biotek Instruments) (13). EXCEL software was used to calculate the IC_{50} value of the extract concentration that killed 50% of the cells.

Wound healing (lateral motility) assay

To determine the inhibition of motility on BCa cells, lateral motility assay experiments were performed. Breast cancer cells were plated at a density of 4×10^5 /ml and incubated overnight at 37 °C and 5% CO_2. Three wounds were produced with a width of 0.5–0.8 mm using 200 µl micropipette tips. Moreover, DMEM me-

dia was replaced with fresh media containing *Allium sativum* L. extracts, and wounds were visualized using a digital inverse microscope camera (Leica, Germany). At the end of the 24 h incubation with extracts, the wound widths were re-measured and re-evaluated via the ImageJ software (14).

Gas Chromatography–Mass spectroscopy (GC-MS) analysis

Gas chromatography–mass spectroscopy analysis was performed as previously described by Leikshmi et al. (15). *Allium sativum* L. extracts were filtered and analyzed using the GC-MS-QP2010 PlusSystem (Shimadzu). By using the MS data library WILEY7.LIB, the spectrum was analyzed, and the bioactive compounds were identified.

Statistical analysis

The experiments of this study were performed at least three times and in triplicates. The data obtained were presented as means±S.E.M. Experimental data were analyzed using Student's unpaired *t-test* and one-way ANOVA followed by Newman-Keuls post hoc analysis (INSTAT Software). Statistical significance was set at p<0.05(*) or p<0.01 (**).

RESULTS

Allium sativum L. induces cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cell lines

Viability assay (trypan blue assay) was used to assess the toxicity of the ASB extract on MCF-7 and MDA-MB-231 breast cancer cells at different time points (24, 48, and 72 h). The ASB extract demonstrated a significant cytotoxicity on MCF-7 (at 24 h, 86.27% decrease in viability; at 48 h, 91.65%; at 72 h, 89.63%) (control *vs* 10000 µg/ml; p<0.01; n=3; Figure 1) and MDA-MB-231 breast cancer cells (at 24 h, 94.87% decrease in viability; at 48 h, 93.69%; at 72 h, 95%) (control *vs* 10000 µg/ml; p<0.01; n=3; Figure 1). Statistical analysis on the viability assay results revealed that starting from the 1250 µg/ml incubations of the ASB extract, a high cytotoxicity level on both MCF-7 and MDA-MB-231 cells was observed. In conclusion, the ASB extract caused a sharp

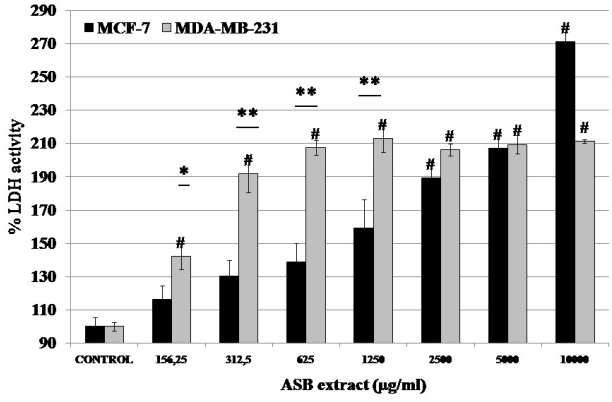

FIGURE 2. The Allium sativum bulb (ASB) extract induces LDH-related cytotoxicity in MCF-7 and MDA-MB-231 BCa cells. Data represents mean±S.E.M. #, p<0.05 vs control*, p<0.05 and **, p<0.01.

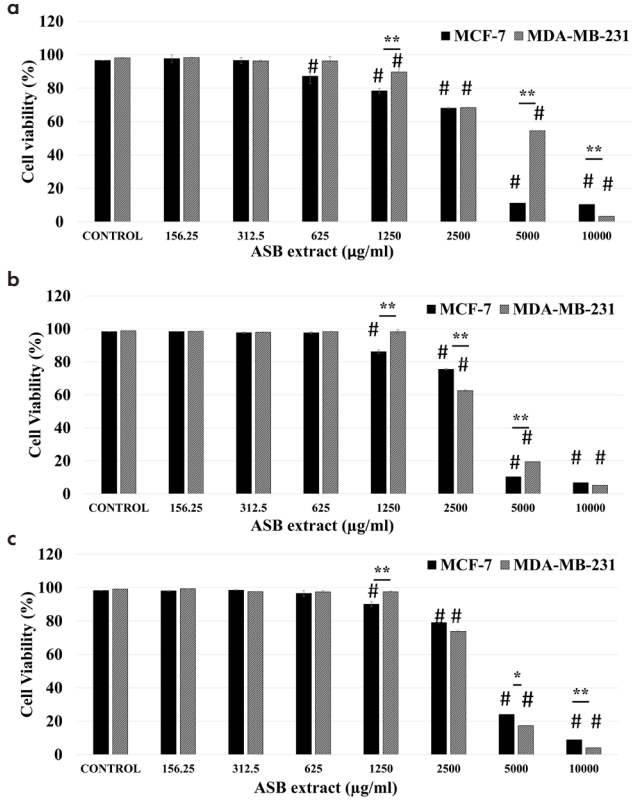

FIGURE 1. a-c. The Allium sativum bulb (ASB) extract decreases the viability of MCF-7 and MDA-MB-231 cells.% viability of BCa cell lines for 24 h (a), 48 h (b), and 72 h (c) periods. Data represents mean±S.E.M. #, p<0.05 vs control*, p<0.05 and **, p<0.01.

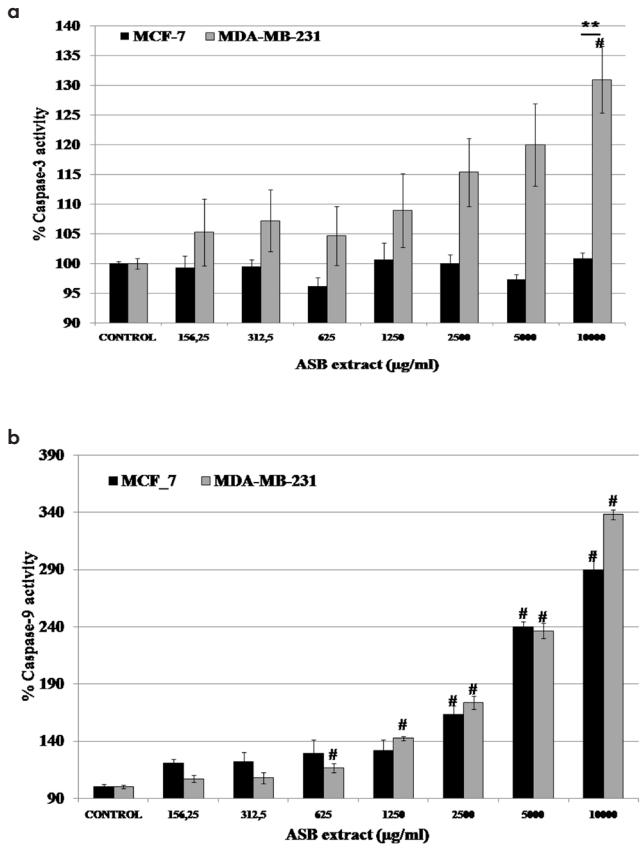

FIGURE 3. a, b. The Allium sativum bulb (ASB) extract induces caspase-3 (a) and caspase-9 (b) activities in MCF-7 and MDA-MB-231 BCa cells. Data represents mean±S.E.M. #, p<0.05 vs control*, p<0.05 and **, p<0.01.

decrease of cell viability specifically at high concentrations at all tested time points in both breast cancer cell lines.

Allium sativum L. induces LDH activity in both MCF-7 and MDA-MB-231 breast cancer cell lines

The LDH-related cytotoxic effects of ASB on both MCF-7 and MDA-MB-231 cells were evaluated. Control experiments were taken as 100%. The experimental results revealed that the incubations of ASB caused a significant LDH release in MDA-MB-231 cells (24 h: 211.57±1.05%; control *vs* 10000 mg/ml; p<0.05; Figure 2). Similarly, MCF-7 cells were incubated with ASB extracts for 24 h where ASB extract caused a significant induction of LDH release in MCF-7 cells as well (24 h: 271.45±5.63%; control *vs* 10000 mg/ml; p<0.05; Figure 2).

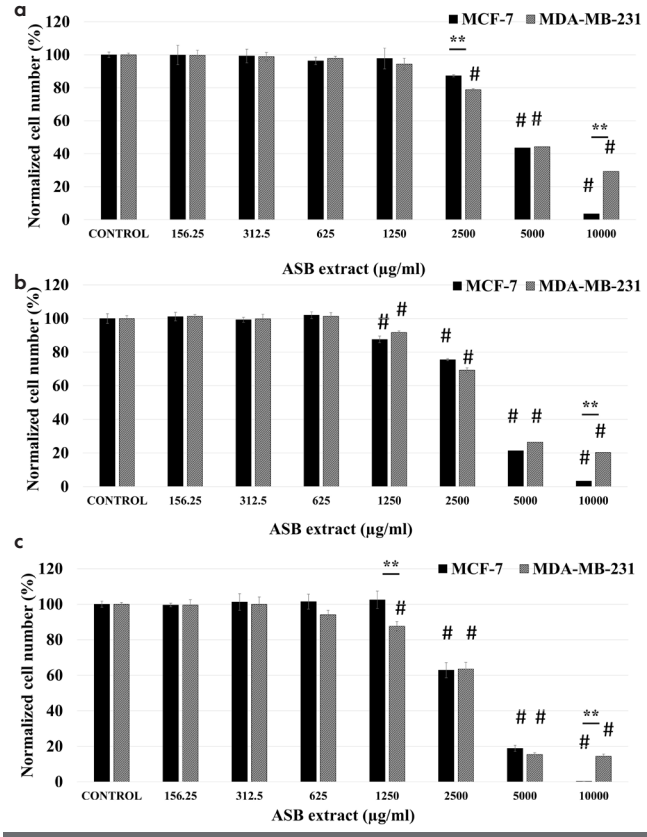

FIGURE 4. a-c. The Allium sativum bulb (ASB) extract exerts significant antiproliferative effect on MCF-7 and MDA-MB-231 cells. Data represents mean±S.E.M. #, p<0.05 vs control*, p<0.05 and **, p<0.01.

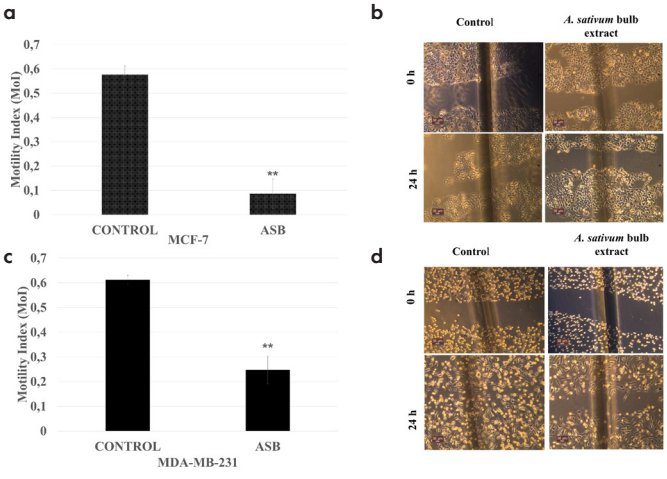

FIGURE 5. a-d. The Allium sativum bulb (ASB) extract inhibits the lateral motility of MCF-7(a) and MDA-MB-231(c) cells. *, p<0.05 and **, p<0.01 relative to control. Images of ASB incubation of MCF-7(b) cells and MDA-MB-231(d). Scale bars: 50 µm.

Allium sativum L. induces caspase-9 in both MCF-7 and MDA-MB-231 breast cancer cell lines

Caspase-3 activity experiments were performed on MCF-7 and MDA-MB-231 cells upon incubations with ASB extract for 24 h. Control experiments were taken as 100%. Experimental results revealed that incubations with ASB increased the caspase-3 activity significantly only at a concentration of 10000 mg/ml in MDA-MB-231 cells (24 h: 130.36±5.64%; control *vs* 10000 mg/ml; p<0.05; Figure 3a). On the other hand, treatments with ASB on MCF-7 cells did not show any significant increase in caspase-3 activity (24 h: 100.81±1.01%; control *vs* 10000 mg/ml; p>0.05; Figure 3a).

The caspase-9 activities of MCF-7 and MDA-MB-231 BCa cells incubated with ASB extracts were evaluated. Control experiments were taken as 100%. Experimental results showed that the incubation of MDA-MB-231 cells with ASB caused a significant increase in caspase-9 activity compared to the control experiments (24 h: 337.9 2±4.47%; control *vs* 10000 mg/ml; p<0.05; Figure 3b). Similarly incubations of MCF-7 cells with ASB caused a significant increase in caspase-9 activity at 24 h (24 h: 289.67±16.46%; control *vs* 10000 mg/ml; p<0.05; Figure 3b).

Allium sativum L. inhibits the proliferation of MCF-7 and MDA-MB-231 breast cancer cell lines

MTT assay was used to determine the antiproliferative effects of the ethanolic *Allium sativum* L. extract on MCF-7 and MDA-MB-231 BCa cells at different tested time points (24, 48, and 72 h). *Allium sativum* was previously shown to have effects on MCF-7 cell lines (16). To investigate the antiproliferative effects of the ASB extract, different concentrations of the ASB extract were tested on both BCa cell lines. A significant antiproliferative effect was determined in MDA-MB-231 cells at a concentration of 2500 µg/ml at 48 and 72 h (Figure 4), suggesting a stronger effect on highly metastatic BCa cell line (MDA-MB-231 cell line) based on the IC_{50} calculations (Table 1).

Allium sativum L. inhibits the lateral motility of MCF-7 and MDA-MB-231 breast cancer cell lines

The antimotility potency of *Allium sativum* L. bulb in both MCF-7 and MDA-MB-231 cells was evaluated using the lateral motility assay. An extract concentration of 156.25 µg/ml was selected wherein no significant cytotoxic or antiproliferative activity was observed. During the control experiment, the MoI of MCF-7 cells was 0.57±0.03, whereas during the ASB extract treatment (24 h), the MoI of MCF-7 cells decreased to 0.086±0.06 (p<0.01) (Figure 5a). Moreover, during the control experiment, the MoI of MDA-MB-231 cells was 0.40±0.01, whereas during the incubation with ASB extract at 24 h, the MoI of MDA-MB-231 cells decreased to 0.24±0.05 (p<0.01) (Figure 5c). The changes in MoI during the incubations with ASB extract strongly indicate an inhibitory effect of ASB on the lateral motility of MCF-7 and MDA-MB-231 breast cancer cells.

GC-MS analysis of *Allium sativum* L. bulb extract

The bioactive compounds of ethanolic *Allium sativum* bulb extract were analyzed using gas chromatography–mass spectroscopy. The results obtained from the analysis showed the presence of acetic acid (2.53%), dimethyl trisulfide (0.30%), diallyl disulfide (1.05%), methyl allyl trisulfide (0.76%), diallyl trisulfide (1.6%), diallyl sulfide (0.13%), guanosine (5.78%), 1,2-benzenedicarboxylic acid (3.58%), hexadecanoic acid (1.58%), heptadecanoic acid (0.48%), 1H-purin-6-amine (2.16%), octadecamethylcyclononasiloxane (6.03%), tetramethylcyclotetrasiloxane (6.95%), octadecamethylcyclononasiloxane (6.03%), 4-hydroxy-1H-purine (2.2%), hexacontan (2.22%), 2, 4, 6, 8-tetramethyl-cyclotetrasiloxane (1.25%), and flavone 4'-OH,5-OH,7-di-O-glucoside (1.47%) (Figure 6; Table 2). These findings also support the data from Leikshmi et al. (15). and Park et al. (17) who previously performed a GC-MS analysis of the *Allium sativum* L. extract (15, 17).

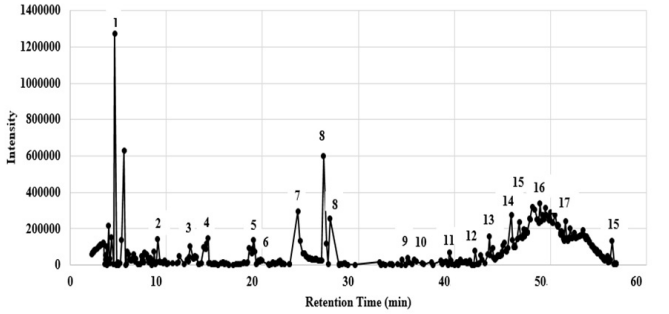

FIGURE 6. Chromatogram of gas chromatography–mass spectroscopy analysis: bioactive compounds obtained from ethanolic extracts of Allium sativum bulb (ASB). Numbers on peaks correspond to the molecules in Table 2.

TABLE 1. IC50 values of MCF-7 and MDA-MB-231 cells incubated with Allium sativum L. bulb (ASB) extract. Data represents mean ±S.E.M.

	IC_{50} concentration µg/ml	
	ASB	
	MCF-7	MDA-MB-231
24h	4822.2±325	4664.3±457
48h	4646.5±338	2874.2±201
72h	3924.5±298	2771.9±178

DISCUSSION

Breast cancer affects 2.1 million women every year (18). The mortality rates among breast cancer patients depend on the type of breast cancer and nature of the cancer tissue. Moreover, highly metastatic breast cancers, specifically triple negatives, cause an increase in the percentage of mortality (19) implicating a need for development of novel therapies and preventatives with antitumoral and antimetastatic activities.

Allium sativum, also known as common garlic, is believed to have anticancer effects and therefore is consumed frequently by cancer patients as well as the general public as a means to avoid cancer. In recent decades, antitumoral activities of *Allium sativum* were widely studied in different *in vitro* and *in vivo* models. Experimental studies suggest that direct exposure of different cancer cells to garlic extract is more effective than the oral administration of the extract suggesting that bioactive molecules might be losing their anticancer potency through absorption via the epithelial lining of the gastrointestinal tract

TABLE 2. Compounds determined through GC-MS analysis of Allium sativum L. bulb (ASB) extract and their bioactivities

	Compound name	*Allium sativum* L. bulb% compound*	Biological activity
1	Acetic acid	2.53	Antibacterial and antifungal (26)
2	Trisulfide, dimethyl	0.30	Antiproliferative (26) and antimicrobial (27)
3	Diallyl disulfide	1.05	Inhibiting tumor cell proliferation, tumor cell apoptosis promoter, antimetastatic, anti-inflammatory (17), antibacterial (28)
4	Methyl allyl trisulfide	0.76	-
5	Diallyl trisulfide	1.60	Anticancer, antioxidant, blood pressure lowering, platelet aggregation (29,30,31)
6	Diallyl sulfide	0.13	Anticancer, antimicrobial, anti-angiogenic, and immunomodulatory (32)
7	Guanosine	5.78	Neuroprotective and neuromodulator (33)
8	1,2-Benzenedicarboxylic acid	3.58	Anticancer (34), antimicrobial, antifungal, anti-malarial (35,36)
9	Hexadecanoic acid	1.58	Antitumoral (37), antimicrobial, antioxidant, decreases blood cholesterol, anti-inflammatory (35)
10	Heptadecanoic acid	0.48	Antibacterial and antioxidant (38)
11	1H-Purin-6-amine	2.16	Anti-inflammatory and cytoprotective (39)
12	Tetramethylcyclotetrasiloxane	6.95	-
13	Octadecamethyl cyclononasiloxane	6.03	Antifungal (40)
14	4-Hydroxy-1H-purine	2.20	-
15	Hexacontan	2.22	-
16	Cyclotetrasiloxane, 2,4,6,8-tetramethyl-	1.25	-
17	Flavone 4'-OH,5-OH,7-DI-O-glucoside	1.47	Larvacidal (41)

*% peak area

(20). Previously, it was shown that the intraperitoneal treatment of sarcoma 180 and EL4-induced lethal ascites with raw garlic extract diminished the cancer formation in C57BL/6 mice (20). However, the oral administrations of the extract did not cause any significant anticancer effects in the in vivo model. Furthermore, this study revealed that the direct injection of *Allium sativum* in tumors is a promising candidate for the development of new applications of *Allium* species (20).

In line with the above *in vivo* findings, our study showed that the direct application of *Allium sativum* extract on tumor cells exerts significant anti-tumoral effects on both highly metastatic and weakly metastatic human breast cancer cell lines. The cytotoxicity effects exerted by *Allium sativum* on both MCF-7 and MDA-MB-231 breast cancer cell lines in a time- and concentration-dependent manner (Figure 1) are potentially due to the presence of diallyl trisulfide (1.6%) and diallyl disulfide (1.05) (Figure 6, Table 2). Diallyl trisulfide was previously shown to cause the induction of cytotoxicity in U937 leukemia cells (17). Diallyl trisulfide and diallyl disulfide which are organosulfur compounds found in *Allium sativum* were previously shown to inhibit PI3K/Akt/mTOR and induce apoptosis in colon, gastric, prostate, and breast cancers (21). The LDH cytotoxicity experiments of the ASB extract on MCF-7 and MDA-MB-231 BCa cells were consistent with the trypan blue exclusion assay experiments wherein a similar LDH cytotoxicity level was observed after incubations with the ASB extract at 24 h (Figure 2). Incubations with the ASB extract caused a significantly increased LDH release in both MCF-7 and MDA-MB-231 cell lines which presents supportive data on the cytotoxicity effects of the ASB extract on breast cancer cell lines.

Caspase-3 and caspase-9 activity experiments were conducted to investigate the apoptotic effects of the ASB extract on both breast cancer cell lines. Incubations with the ASB extract at a time period of 24 h showed a significant increase in caspase-9 activity in MCF-7 and MDA-MB-231 BCa cells, whereas this activity was observed to be higher in the highly metastatic breast cancer cell line MDA-MB-231 (Figure 3). This finding suggests a slow initiation process for apoptosis in MCF-7 cells as the initiator caspase (caspase-9) was significantly elevated at the 24 h period in both cell lines in which the only significant increase in the executioner caspase levels (caspase-3) was observed in MDA-MB-231 cells at this time period (Figure 3). The increase in caspase-3 activation in MDA-MB-231 cells suggests a faster apoptotic response in this strongly metastatic cell line.

Our cells have an infinite capacity to divide until they enter into a dormant state or cell death mechanisms are activated, whereas cancer cells have an unlimited capacity to proliferate (22). Currently, an approach to inhibit or reduce cell division in the process of tumorigenesis is being developed. To this end, the antiproliferative effects of the ASB extract were evaluated using MTT assays. Incubations with the ASB extract for both MCF-7 and MDA-MB-231 BCa cell lines revealed that the extract has a significant antiproliferative effect in a time- and concentration-dependent manner at designated time periods. Specifically, at a concentration of 2500 µg/ml, no cytotoxic effect was observed in MDA-MB-231 cells with the trypan blue assay, but significantly lower numbers of BCa cells were observed with the MTT assay, suggesting that at this concentration, instead of inducing cell death, the extract inhibits cancer cell proliferation. A similar effect was also observed at 48 h incubations with a concentration of 2500 µg/ml. For the 72 h incubations, the antiproliferative impact on MDA-MB-231 cells was assessed at a concentration of 1250 µg/ml wherein no significant decrease in viability was observed using the trypan blue viability assay, indicating that a longer exposure at lower concentrations also causes the inhibition of cancer cell proliferation.

Breast cancer is the leading cause of cancer-associated mortalities in women as well as one of the most diagnosed cancer types. Primary tumor treatment approaches have significantly improved over time, although current treatments for the inhibition of metastasis, which is the underlying reason for most of the mortalities attributed to cancer, are highly limited (23). Lateral motility assays that were performed to determine the antimotility effect of the ASB extract revealed that the incubation of MCF-7 and MDA-MB-231 cells with the ASB extract demonstrated a significant antimotility potency ($p<0.05$) on both tested cell lines. Experimental data revealed that the ASB extract significantly inhibited the motility for both MCF-7 and MDA-MB-231 cells, in which a more robust effect was observed in MCF-7 cells. This result is expected as MCF-7 is a model for lowly metastatic breast cancer cells. The antimotility effect of the ASB extract is potentially due to the presence of both hexadecanoic acid (1.58%) and diallyl disulfide (1.05%) in which both bioactive compounds were previously shown to exhibit antimetastatic properties (24, 17). Diallyl disulfide which is one of the major components of *Allium sativum* can block the NF-kB signaling pathways to suppress metastasis and the invasion of breast cancer cells (25). Furthermore, it has been reported that *Allium sativum* extracts induce the suppression of cyclooxygenase (COX)-2 (25). Since COX-2 plays an important role in several cellular processes such as cancer cell migration, metastasis, and tumor-associated angiogenesis, the suppression of COX-2 by Allium extracts is a significant modulator for the inhibition of tumor development (25).

Our GC-MS experiments were performed comparatively with those on well-known bioactive molecules and showed the presence of important bioactive molecules with eminent anticancer activities such as diallyl sulfide, diallyl trisulfide, dimethyl sulfide, 1,2-benzenedicarboxylic acid, and hexadecanoic acid (26, 33, 38).

Overall, the results of this study indicate the cytotoxic, antiproliferative, apoptotic, and antimotility effects of the ethanolic extract of *Allium sativum* bulb on weakly and strongly metastatic breast cancer cell lines MCF-7 and MDA-MB-231, revealing the mechanistic nature of the observed cell death and highlighting the anticancer potency of bioactive molecules found in *Allium sativum* L.

Ethics Committee Approval: N/A.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - O.I., E.V.; Supervision - E.V.; Resource - O.I.; Materials - O.I., E.V.; Data Collection and/or Processing - O.I.; Analysis and/or Interpretation - O.I., E.V.; Literature Search - O.I., E.V.; Writing - O.I., E.V.; Critical Reviews - O.I., E.V.

Acknowledgements: The authors would like to extend their acknowledgements to Prof. Dr. Mehmet Koyuncu for identification of *Allium sativum* L. Bulbs and Biotechnology Research Center for supporting this research.

Conflict of Interest: Authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has been supported by Cyprus International University Biotechnology Research Center.

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1911

Neonates Hearing Screening Results: A Comparison of Chirp and Click Stimuli with an Automated Auditory Brainstem Response Device

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Cite this article as: Hüseyin D, Yazıcı A, Coşkun ME. Neonates Hearing Screening Results: A Comparison of Chirp and Click Stimuli with an Automated Auditory Brainstem Response Device. Cyprus J Med Sci 2020; 5(4): 313-6.

BACKGROUND/AIMS
Automated Auditory Brain Response (AABR) devices are unique tools for neonate hearing screening programs. Contemporary utilization of AABR devices is based on two stimuli called chirp and click. The first study objective was to identify any possible differences in chirp and click stimulus results of the AABR devices in neonates. The second aim was to investigate any possible risk factor that could affect the results of each stimulus.

MATERIAL and METHODS
Chirp and click stimuli were applied to each neonate using the AABR devices. Results were recorded automatically as pass or failed. Those with failed results were called after month for a retest. Hearing loss risk factors were obtained from the parents and caregivers.

RESULTS
Twenty-one of the fifty-eight chirp stimuli gave negative results on the AABR in the second AABR test. Twenty-nine of the seventy-six click stimuli on the AABR gave a failed result on retest. The most common risk factors were consanguineous marriage of parents (n=184), history of being admitted to the neonatal intensive care unit (n=119), and jaundice (n=102). In addition, hearing loss was not detected in any neonate and was therefore not considered to significantly affect the results of the chirp or click stimulus on the AABR.

CONCLUSION
We were unable to prove that the chirp stimulus could be replaced by the click stimuli in neonatal hearing screening using AABR device.

Keywords: Sensorineural, hearing, loss, auditory brain response

INTRODUCTION

Early detection of hearing loss is essential for the development of social, linguistic and cognitive functions; thus, neonatal hearing screening programs are performed worldwide. An ideal neonatal hearing screening test could detect hearing loss of ≥ 35 decibels (dB) in the better ear in infants aged ≤ 3 mon (1). Auditory brainstem response (ABR) and otoacoustic emissions (OAE) are the most commonly accepted and performed universal tests (2). Despite being a time-consuming procedure compared to OAEs, some reports have shown that ABRs are more sensitive than otoacoustic emissions (3, 4).

Automated auditory brainstem response (AABR) is a useful type of ABR for rapid screening of the hearing ability of newborns. AABR is performed using click or chirp stimuli. A stimulus is a wave that results in a neural activity response. Theoretically, click stimulus starts an earlier neural activity in the broad areas of the basillar membrane than the apical part of the cochlear nerve. This tonotopic activation of the cochlear nerve by click stimulus could cause temporal delays. For preventing these temporal delays, the chirp stimuli were created. Chirp stimuli include both higher and lower frequencies. Theoretically, chirp stimuli stimulates different parts of the cochlear nerve simultaneously (5). The chirp stimulus is superior for the identification of waves or latencies resulting from auditory neural response (6, 7).

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Received: 09.04.2020
Accepted: 29.09.2020

It has been hypothesized that chirp stimulus in newborn scans provides faster and safer results than click stimulus in the evaluation of auditory brain responses (8). However, few studies have assessed the clinical application of screening for neonate hearing ability using the chirp versus the click stimuli with an automated ABR device. The main purpose of this article was to identify if there was any significant difference in the neonate hearing screening between the chirp and click stimuli using an AABR device.

MATERIAL and METHODS

This prospective study includes the results of hearing screening tests performed from November 2018 to February 2019. Hearing screening was performed using Maico Diagnostic MB II Beraphone (Maico Diagnostics, Berlin, Germany®) (chirp stimuli) and GSI AUDIOscreener (Grason-Stadler, Minnesota, United States®) (click stimuli) AABR devices with a different kind of stimulus in soundproof rooms. Maico Diagnostic MB II Beraphone (®) device (CE-Chirp TM) includes a chirp wave with a repetition rate of 93/s delivered at 35 dB HL. GSI AUDIOscreener (®) has a click wave 100 µs width, and a stimulus rate of 32–62 per second. The input frequency range of the GSI AUDIOscreener (®) ranges from 30–3000 Hz.

AABR was performed in natural sleep and sedation. Only one kind of stimuli (chirp or click) was applied to each baby. Two groups were created as per the types of stimuli.

The results of each newborn hearing screening were interpreted as "pass" or "fail" automatically by the Maico Diagnostic MB II Beraphone (®) and GSI AUDIOscreener (®) AABR devices. The passing criteria for neonatal hearing screening were defined as the detection of hearing level at 35 dB HL at a single device. Those for whom "fail" result was obtained were scheduled for a reevaluation after 1 mon. As per our hearing policy, if one of the ears did not pass AABR, we accepted the result as a failure and planned further investigation.

Information regarding demographic variables and risk factors was obtained from the parents or caregivers. The risk factors were categorized as follows. Based on the age, the subjects were classified as newborns (≤28 d old) and infants (>28 d old). A cut off of 1500 g was used to classify the subjects as per birth weight. Duration of pregnancy was classified as >38 wk or <38 wk. Intensive care history was defined by the implementation of mechanical ventilation for at least 5 d.

The following risk factors were estimated: consanguineous marriage of parents, hearing loss event in the family, speech disorder event in the family, history of phototherapy treatment, and drug use by the mother during pregnancy. The effect of these risk factors was investigated in those two stimuli groups separately.

Main Points:

- Neonatal hearing screening is crucial for the early detection of hearing loss individuals.
- AABR is a universal gold standard for detecting hearing loss in neonates.
- Despite the broad usage of chirp stimulus, click stimulus also demonstrates accurate results.

Written informed consent was obtained from parents or caregivers of all the patients. Ethical approval was obtained from the University of Gaziantep Ethical Committee (approval number of 2019/116).

Statistical Analysis

The categorical variables of two independent groups were compared using Chi-Square test. For predicting multiple risk factors, multiple logistic regression models were used. Odds ratios were calculated for each risk factor with a 95% confidence interval. All Statistical analyses were performed using the Statistical Package for Social Sciences software version 22.0 (IBM SPSS Corp.; Armonk, NY, USA).

RESULTS

Both the ears of 609 subjects (294 girls and 315 boys) were screened with an AABR device at the University of Gaziantep Audiology department. Three hundred and thirteen of these patients were provided the chirp stimulus and the others were provided the click stimulus. The mean age (day) of the subjects was 23,4±8,9 (range 1–45); the average age of those in the chirp group was 23,9±9,2 (range 1–45) and that of those in the click group was 22,9±7,6 (range 1–44). The chirp group had 144 men and 152 women; the click group had 151 men and 162 women. The chirp and click stimulus groups did not show any significant difference based on age (p>0,005) or sex (p>0,005).

Total 134 patients failed in the initial hearing assessment. Fifty-eight of those who failed were screened using the click stimulus, and 76 were screened using the chirp stimulus. Further, 44 of those in the click group failed bilaterally, that is, in both ears. In contrast, 49 subjects in the chirp group failed bilaterally. One month thereafter, the chirp and click stimuli were applied again for the 134 subjects who failed the test. The same stimuli were given to the same subject, and the same stimuli were applied in the first test and the retest. Failed chirp results were found in 21 of the 58 subjects who failed the first test. Twenty-nine of 76 babies failed the AABR control tests with the click stimuli. However, the parents of only 1 patient have applied to our clinic for clinical ABR after the failure of two AABR tests.

Based on the results of the second hearing screening, the effect of stimulus type (click and chirp) used in AABR on hearing screening was not significant (p=0,817). The risk factors that were evaluated have been shown in Table 1 and 2 for different stimuli.

The most common risk factors for hearing loss were consanguineous marriage of parents (n=184), followed by history of admission to neonatal intensive care unit (NICU) (n=119), and jaundice (n=102). One multivariate analysis was performed for the click and chirp groups after multiple binary regression models were created. The risk factors were displayed separately for chirp stimulus in Table 1 and for click stimulus in Table 2. Multivariate analysis identified that the evaluated risk factors did not significantly influence the results of the ABR to chirp or click stimulus.

TABLE I. Results of the multivariate analysis for chirp stimuli

Variable	p	OR	95%CI for OR Lower	95%CI for OR Upper
Sex	0.437	1.539	0.517	4.585
Age (days)	0.258	1.333	1.314	1.99
Birth weight	0.18	1.618	0.07	0.922
Consanguineous marriage	0.612	0.741	0.232	2.367
Family history of hearing loss	0.278	0.946	0.876	1.022
Family history of speech disorder	0.18	0.919	0.835	1.011
Pregnancy duration	0.181	1.050	0.954	1.155
Intensive care history	0.696	0.727	0.146	3.614
Hyperbilirubinemia with exchange transfusion	0.278	0.946	0.876	1.022
Ototoxic Drug Use	0.447	0.973	0.922	1.027

TABLE 2. Results of the multivariate analysis for click stimuli

Variable	p	OR	95%CI for OR Lower	95%CI for OR Upper
Sex	0.082	0.39	0.149	1.018
Age (days)	0.993	0.998	0.596	1.67
Birth weight	0.26	1.644	1.37	1.97
Consanguineous marriage	0.936	1.042	0.384	2.825
Family history of hearing loss	0.3	0.293	0.025	3.391
Family history of speech disorder	0.727	0.609	0.37	10.12
Pregnancy duration	0.068	1.074	0.973	1.186
Intensive care history	0.072	0.42	0.163	0.635
Hyperbilirubinemia with exchange transfusion	0.134	0.327	0.072	1.489
Ototoxic Drug Use	0.727	0.609	0.037	10.124

DISCUSSION

As per our findings, AABR neonatal hearing screening results using the chirp stimuli were not superior to those obtained using the click stimuli.

There was no statistical evidence regarding the type of stimuli as per the results of the hearing screening in neonates. However, there was a marked trend for the use of the chirp stimuli for the screening of neonate hearing.

Both the stimuli were equally effective. Previous research suggests that narrow band chirp stimuli were expressed as a fast and reliable assessment of auditory thresholds as compared to click stimuli (9, 10). Moreover, the chirp stimuli provide more evident V wave configuration than the click stimuli in the evaluation of the auditory neural pathway (5, 6, 11). This discrepancy in the findings and previous reports may be related to the automated ABR devices rather than the stimulus type. When compared to diagnostic ABR, nearly 40% of the infants with hearing loss over 45 dB hearing level passed the test using the AABR device (12). This finding was believed to be related to non-auditory neural activity or electromagnetic background noise that could be misinterpreted using the AABR device (12).

The most common risk factor for neonate hearing loss in this study was consanguineous marriage of parents; this result was contradictory to that reported by previous studies (13, 14) and may be related to social factors in our region. The second common risk factor was a history of NICU admission. MBII BERAphone with chirp stimuli was compared with a standard conventional ABR with click stimuli; the sensitivity was 100% and specificity was 96.8% by Melagrana et al. at NICU(ok) (15). Based on studies with a different design, Gustini et al. and Meier et al. displayed similar results in the comparison of chirp and click stimuli with AABR (16, 17). Consistent with our findings, the aforementioned articles did not absolutely recommend the replacement of one stimulus by another.

The following was a study limitation: low application rates of patients who did not pass the second AABR disallowed further investigation that would enable a comparison of our results with those of diagnostic ABR. The second limitation of this study was that we did not apply two different stimuli in the same neonates. A further study of applying both click and chirp stimulus to the same neonate may enhance the findings of this study.

In conclusion, we believe that the chirp stimulus cannot be replaced by the click stimuli in neonatal hearing screening using an AABR device. Moreover, we could not conclude that a certain type of stimulus had a noticeable effect on hearing screening in newborns with risk factors.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gaziantep University (2019/116).

Informed Consent: Written informed consents were taken from the parents and caregivers of the patients.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - H.D.; Design - H.D., A.Y.; Supervision - A.Y., M.E.C.; Resource - H.D.; Materials - H.D.; Data Collection and/or Processing - H.D.; Analysis and/or Interpretation - A.Y., M.E.C.; Literature Search - A.Y., M.E.C.; Writing - A.Y., M.E.C.; Critical Reviews - A.Y., M.E.C.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1937

Conservative Treatment for Locally Advanced Carcinoma of the Larynx Using Alpha-Crystalline B as a Prognostic Molecular Marker

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Cite this article as: Alzahrani R, Alghamdi AN, Shami A, Elsawy WH, Gharib AF, Amin HS, et al. Conservative Treatment for Locally Advanced Carcinoma of the Larynx Using Alpha-Crystalline B as a Prognostic Molecular Marker. Cyprus J Med Sci 2020; 5(4): 317-22.

BACKGROUND/AIMS
This study was performed to understand the prognostic role of alpha-crystalline B (αBC) in locally advanced laryngeal cancer.

MATERIAL and METHODS
Forty-five patients were enrolled; they were treated with induction chemotherapy followed by definitive radiotherapy. Alpha-crystalline B (αBC) was evaluated using quantitative real time-polymerase chain reaction.

RESULTS
Complete response to induction chemotherapy was observed in 16 patients, and partial response was achieved in 27 patients. After radiation therapy, 32 patients showed complete response. The αBC level was significantly higher in the tumor tissues than in the normal adjacent tissues. This level was significantly correlated with the patient's age, clinical stage, and treatment response. High αBC levels were significantly associated with short overall and disease-free survival.

CONCLUSION
Induction chemotherapy can efficiently preserve the larynx in a high proportion of patients. αBC can be used as a molecular marker for predicting the treatment response and survival in patients undergoing induction chemotherapy.

Keywords: Locally advanced cancer larynx, induction chemotherapy, αB-crystalline, qRT-PCR, prognostic factors

INTRODUCTION

Total laryngectomy with cervical lymph nodes dissection accompanied by radiotherapy has been recognized as a suitable treatment for locally advanced laryngeal cancer (1). Patients treated with total laryngectomy experience the following postoperative complications: loss of speaking ability and impaired swallowing, with their impact on the quality of living leading to many social issues (2). The problems arising from total laryngectomy raise the need for the establishment of a novel treatment strategy that has similar treatment efficacy and survival rate, with acceptable complications (3). The concept of preserving the larynx has been approached by various modalities, including chemotherapy to downstage the tumor so that the patient can be treated with less radical surgery or by using radiotherapy. Induction chemotherapy in locally advanced head and neck cancer has been studied during the previous three decades. However, there is no consensus for the optimal use of induction chemotherapy in head and neck malignancies except in locally advanced laryngeal cancer (4).

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Received: 05.04.2020
Accepted: 29.08.2020

The chain of alpha-crystalline B (αBC) is a protein encoded by the CRYAB gene in humans (5). It is part of the family of heat shock proteins and acts as a molecular chaperone that mainly binds misfolded proteins to prevent protein aggregation, inhibiting apoptosis and contributing to the intracellular architecture (5). Defects in this gene/protein have been associated with cancer and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Alpha-B chain crystalline (αBC) can be triggered by heat shock, ischemia, and oxidation and belongs to the family of heat shock proteins. They behave as molecular chaperones although they do not re-naturalize proteins and release them as a real chaperone; instead, they bind improperly folded proteins to prevent their aggregation (6). In addition, by inhibiting the processing of the pro-apoptotic protein caspase-3, αBC can confer stress resistance to cells. Two unique features of alpha crystallines are autokinase function and intracellular architecture integration (7). Alpha-A and alpha-B gene products are expressed independently; alpha-A is preferentially limited to the lens and retina, while alpha-B is commonly expressed in several tissues and organs (8).

Although significantly expressed in eye lenses and muscle tissues, αBC may also be observed in several cancers, including squamous cell carcinoma in the head and neck (HNSCC) and breast carcinomas (9, 10). αBC expression is associated with the metastases in HNSCC and breast carcinomas and other types of cancer; further, its expression is commonly associated with bad prognosis (9-11).

This study aimed to study the expression level of αB-crystalline in locally advanced squamous cell carcinoma of the larynx with normal nearby tissues adjacent to the tumor. We also studied the relationship between its expression and the clinicopathological features.

MATERIAL and METHODS

From August 2013 to January 2020, 54 patients with locally advanced cancer of the larynx were referred to the Clinical Oncology Department and Head and neck surgical Oncology Unit, Zagazig University Hospitals, Zagazig, Egypt and East Jeddah Hospital, Jeddah, SA were evaluated for this study. The study was performed with the understanding and written consent of each patient and was approved by the Ethics Committee of the Faculty of Medicine, Zagazig University (257/2013).

Eligibility criteria:

1. No previous treatment
2. Biopsy-proved squamous cell carcinoma of the larynx
3. Locally advanced disease (T3 or T4 with No, N1 or N2) as per the American Joint Committee for cancer staging (12)
4. Performance status of ≥2 on the ECOG scale (13)
5. Adequate hepato-renal, cardiac, and bone marrow functions
6. Adequate nutritional and auditory status
7. Written informed consent for study participation
8. Age<70 y

Main Points:

- Induction chemotherapy can be used effectively in locally advanced laryngeal cancer followed by radiotherapy and can be efficient in conserving the larynx in a certain proportion of patients without compromising survival.
- High level of αBC may be correlated with aggressive behavior of SCC of the larynx and unfavorable outcome.

The initial evaluation included a history and physical examination, complete blood cell count, routine serum chemistries, creatinine clearance test, chest radiography, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the head and neck, and bone scan. Local tumor extent and regional metastases were further assessed using triple endoscopy, and a biopsy was obtained from the tumor tissues and adjacent normal tissues.

Induction Chemotherapy:

The induction chemotherapy comprised the following three cycles of docetaxel 75 mg/m^2 (day 1), cisplatin 75 mg/m^2 (day 1), and a continuous fluorouracil infusion at 500 mg/m^2 per day (days 1–5) every 4 wk.

Response to induction chemotherapy was evaluated clinically via endoscopic examination before each cycle and radiologically using CT scan or MRI that was performed after the second cycle. Assessment of palpable lymph node(s) was done also by clinical examination and palpation. Responding patients (Complete response (CR) or PR) received a maximum of three courses of chemotherapy before to definitive radiation. After the third cycle, patients with CR or PR were treated with irradiation while patients with any evidence of disease progression underwent surgical resection and postoperative radiation therapy. Tumor responses were defined as per the Response Evaluation Criteria in Solid Tumors criteria (RECIST) (14).

Radiation Therapy:

All patients received radiotherapy, either immediately after chemotherapy in CR or PR patients or postoperatively in patients who failed to respond to chemotherapy. After chemotherapy, comprehensive radiation therapy was delivered with 5000 cGy supplemented by a boost of 2000 cGy to the primary tumor site and persistent lymph nodes, when present. A dose of 5000cGy was administered after the surgery and a booster dose of 14 Gy was administered to sites with positive margins and/or extracapsular spread and/or three or more involved lymph nodes, if any. The response was re-evaluated 12 wk after radiation therapy. Patients with persistent laryngeal disease underwent salvage laryngectomy, while those with persistent neck disease and those whose primary tumor was controlled underwent dissection of the neck alone.

Surgery:

The extent of surgical resection was determined as per the original assessment of the extent of the tumor before chemotherapy. Classic wide-field total laryngectomy was performed for all primary tumors. Regional neck dissections were performed in all surgical patients except those with T3N0 or those with midline supraglottic T4N0 tumors for whom it could not be determined which side of the neck was chiefly at risk of occult metastases. Salvage surgery was performed after the confirmation of residual tumor on biopsy. All the patients were followed up and examined on a 3-month basis for the first year after treatment and every 6 mon thereafter.

Quantitative real time-polymerase chain reaction:
Total RNA was obtained using a commercial reagent (Trizol, Gibco Inc., Grand Island, NY, USA) from tumor tissues and nearby normal tissues. Using oligo-dT primers and M-MLV reverse transcriptase, total RNA (2 µg) was reverse transcribed. RT-PCR products comprising αB-crystalline and β-actin were amplified using gene a specific primer (αB-crystalline: F- 5′-GGAATTGATCGCCATCCACCAC-3′, R- 5′-CCGCTCGAGCTATTTCTTGGGGGCTGCGG-3′, β-actin: F- 5′-GCA CCA CAC CTT CTA CAA TG-3′, R- 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′). A mixture (40 µL) of 3 µL cDNA, 10 µM primers, 2.5 µg/mL Go Tag Flexi DNA polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH, 9.0), 3.0 mM MgCl2, and 0.2 mM dNTPs, was prepared to perform PCR. In a 9700 Thermocycler (Perkin-Elmer), PCR was performed, and the general thermocycling conditions were as follows: 1 cycle of initial denaturation at 95°C for 2 min, supplemented by 30 cycles at 95°C for 30 s, annealing at 55°C for 40 s and reaction at 72°C for 100 s, followed by supplementation by a final extension for about 5 min at 72°C. The relative value of αB-Crystalline mRNA was evaluated using the relative cycle threshold (Ct) means (15).

Statistical Analysis
Statistical analyses were performed using the Statistical Package for Social Sciences software version 12.0 (SPSS Inc.; Chicago, IL, USA). Student's t-test and one-way analysis of variance (ANOVA) test were used to compare the mean±standard deviation (SD) values of the αB-crystalline expression levels of the different groups. Kaplan Meier survival curves were used to compare the overall and disease-free survival of the groups.

RESULTS

The study involved 54 patients, including 45 men and 9 women. The median patient age was 51 y (range 40–62 y). After 2 cycles of induction chemotherapy, complete clinical response was noted in 16 patients. Partial response was achieved by 27 patients. After 3 cycles of chemotherapy, 43 patients received radiation therapy. In 32 patients (32/43), CR was noted (Table 1). Salvage surgery was performed after chemotherapy in 11 patients and 11 patients with residual disease after chemotherapy and radiotherapy (9 PR and 2 SD) (Table 2).

TABLE 1. Clinical response of patients after induction chemotherapy and 12 wk after radiotherapy

	Response after chemotherapy	Response after Radiotherapy
Number of patients	54	43
Complete response (%)	16 (29.6)	32
Partial response (%)	27 (50)	9
Stable disease (%)	11 (20.4)	2

TABLE 2. Surgical salvage after induction chemotherapy and radiation therapy

		Surgical salvage after neoadjuvant chemotherapy (n=11)	
		Unilateral regional neck dissection	Bilateral regional neck dissection
Total laryngectomy with partial pharengectomy	2		
Total laryngectomy	5	3	2
Hemi laryngectomy	4	4	-
Surgical salvage after chemotherapy and radiation therapy (n=11)			
Total laryngectomy	-	-	-
Hemi laryngectomy	4	1	3
Neck dissection only	7	5	2

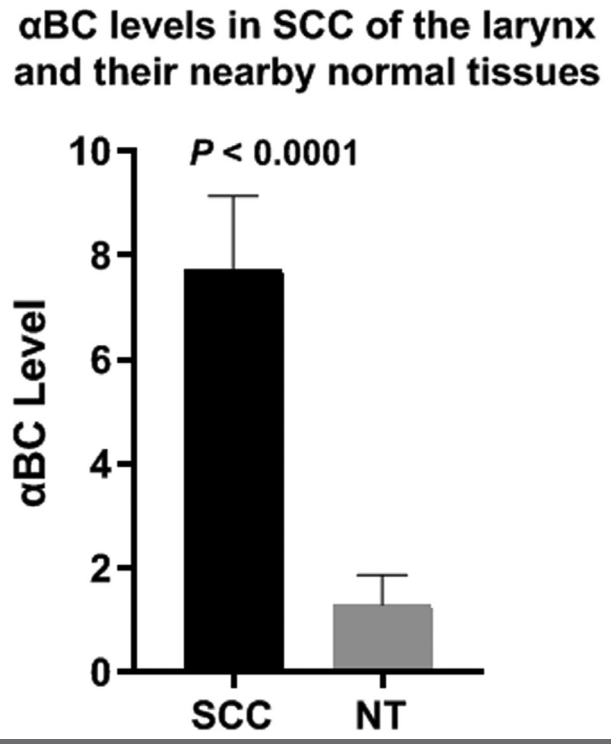

FIGURE 1. αBC levels in laryngeal cancer tissues (SCC) and their nearby normal tissues (NAT)

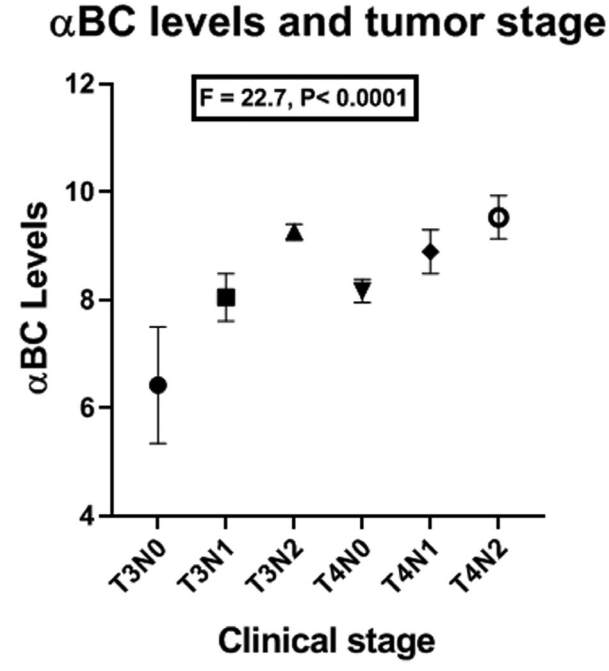

FIGURE 2. Levels of αBC as per the tumor stage

After chemotherapy and radiotherapy, all the patients with PR had CR in the primary site without CR in cervical lymph nodes; five patients underwent regional neck dissection, and two patients underwent modified radical neck dissection.

The αB-crystalline expression level was assessed in squamous cell carcinoma (SCC) of the larynx relative to the nearby normal tissues adjacent to the tumor. In SCC, the αBC level ranged from 4.01 to 9.92. The mean±SD level was 7.73±1.41 SD; meanwhile, its level ranged from 0.14 to 2.41 with a mean±SD level was 1.29±0.57 in the nearby normal tissues. The difference was statistically significant p<0.001. (Figure 1)

TABLE 3. Relationship between age and αB-crystalline level in SCC

Patients characteristics	αB-crystalline				
Age	**No**	**Mean**	**SD**	**t-test**	**P**
<50 years	19	5.2	1		
≥50 years	35	6.6	1.7	3.16	0.003
Sex					
Male	45	6.89	0.99		
Female	9	6.61	1.24	0.73	0.47
T					
T2	8	5.64	1.37		
T3	33	7.80	1.08		
T4	13	8.82	0.61	23.47**	<0.0001
N					
N0	12	7.01	1.22		
N1	27	8.24	0.56		
N2	15	7.39	2.16	4.24**	0.0198
Stage					
III	33	6.26	0.59		
IVA	21	7.76	0.89	7.44	<0.0001
Response to treatment as per the pretreatment αBC levels					
Complete response (CR)	32	6.12	0.45		
Partial response or stable disease (PR/SD)	11	7.35	0.32	8.42	<0.0001

**Analysis of variance (ANOVA) test

The level of αBC in SCC was correlated with the characteristics of all the patients; data are presented in Table 3. There was a significant correlation with the age of patients (p=0.003). The sex of the patients was not related to the αBC level. Significant correlations were observed with the size and extent of the primary tumor (T), (p<0.0001), regional lymph node involvement (N), p=0.0198), and tumor stage (p<0.0001). We observed that the αBC level increases with the tumor stage; detailed analyses of stage and the αBC levels are shown in Figure 2. Patients with T3N0 tumors had a mean level of αBC of 6.43, while those with T4N2 had a mean level of 9.53, the difference was significant (p<0.0001).

The level of αBC was significantly related to the tumor stage; low stage tumors had a low level of αBC level and vice versa, one-way analysis of variance, F=22.7, p<0.0001.

The treatment response was assessed with the level of αBC; the mean level of αBC in patients with CR was 6.12, while that in those with partial or no response was 7.35 with a significant difference (p<0.0001), Table 3.

At 36 mon, the patterns of failure (locoregional, or distant) in 22 patients with no response to treatment who underwent surgical resection with or without postoperative radiotherapy were as follows: 10 patients died; 7 had locoregional recurrence; 3 had distant metastases; and 2 had both, locoregional and distant relapse. In contrast, of the 32 patients who achieved CR after chemotherapy-radiotherapy, 2 died without evidence of disease because of other causes; 10 had locoregional recurrence of disease; 2 had distant metastasis; and 1 had both, local and distant relapse, leaving 17 patients living with laryngeal preservation.

We further explored the effect of αBC level on a patient's survival in laryngeal cancer. Our evaluation revealed that both, over-

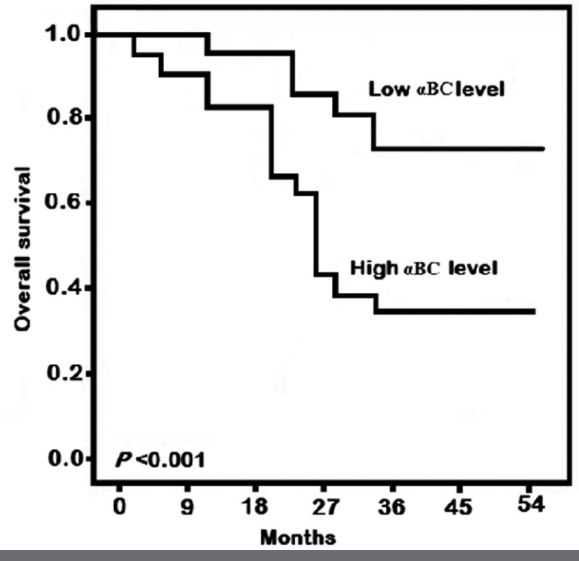

FIGURE 3. Kaplan Meier overall survival curve for patients with locally advanced laryngeal cancer as per the αBC levels

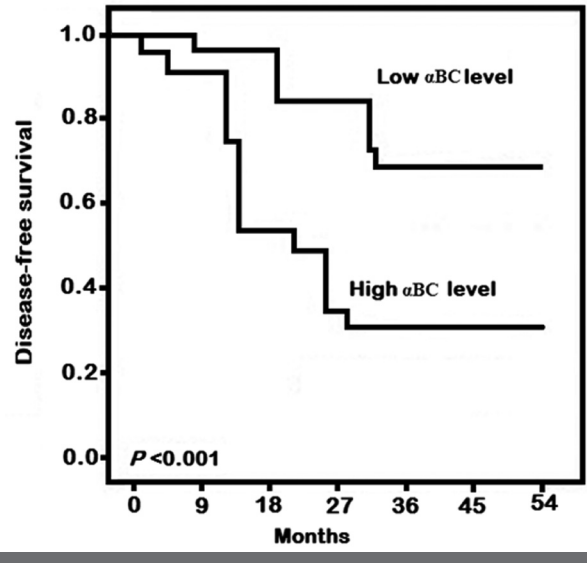

FIGURE 4. Kaplan Meier disease-free survival curve for patients with locally advanced laryngeal cancer as per the αBC levels

all survival and disease-free survival in patients with high αBC levels were all shorter than those in patients with low αBC level. (p<0.001, Figure 3, 4).

The overall survival in patients with low levels of αBC was 74% versus 35% in patients with high levels of αBC (p<0.001).

The disease-free survival in patients with low levels of αBC was 72% versus 32% in patients with high levels of αBC (p<0.001).

DISCUSSION

In the treatment of head and neck cancer, two issues are most importance. The first is survival and the second is the preservation of organ function (quality of life) (3, 16, 17). Although survival differs by the cancer site within the head and neck, it is generally poor in stages III and IV. Since the end of the 1970s, several studies have been published on induction or neoadjuvant chemotherapy in patients with head and neck cancer. Most investigators have shown that induction chemotherapy provided poor results because there was no improvement in survival or disease control. However, several studies suggest that induction chemotherapy may play an important role in preserving laryngeal function (phonatory speech) (18, 19). Recent data from a large number of published studies indicate that induction platinum and fluorouracil chemotherapy followed by radiotherapy can achieve laryngeal preservation in 30%–50% of patients, even with long-term follow-up (20-22). Furthermore, no compromise in survival was associated with the delay in surgery and radiotherapy in cases where chemotherapy failed.

Our results of a 31.5% laryngeal-preservation rate in laryngeal cancer are consistent with other studies and support further investigation of laryngeal-preservation strategies in patients with locally advanced laryngeal cancer (23-25). In order to maintain the larynx, comprehensive radiation therapy was used in selected cases, with laryngectomy intended for patients with tumor recurrence following radiation; however, the overall cure rates have been decreased (26-28). The largest studies found 3-year disease-free survival rates of 20%–50% for patients with advanced-stage III or IV cancers, with larynx preservation in less than half of the cured patients (29, 30). Geretschläger reported 3-year locoregional control (LRC), distant metastasis-free survival, and overall survival (OS) rates of 77%, 96%, and 63%, respectively (29). Our patients had more advanced disease than those reported by Geretschläger. Those patients were not eligible for radical radiation therapy with salvage surgery as this approach was associated with a low cure rates. The encouraging results achieved in these patients with advanced cancers suggest that initial chemotherapy improved the effectiveness of definitive radiation therapy (26). We believe that a shift in the clinical strategy is necessary to reduce the laryngeal cancer-related mortality. In an attempt to support this view, we studied Alpha-B-crystalline (αBC) as a possible molecular marker for predicting response to induction chemotherapy and survival in locally advanced SCC of the larynx. We examined the levels of αBC in SCC of the larynx and their nearby normal tissues. A significant difference was observed in its levels in SCC of the larynx and their nearby normal laryngeal tissues. We also analyzed the relation between its levels and different clinical factors. We observed that an advanced tumor stage was associated with a high αBC level. Moreover, a significant increase in its level was observed in older patients. The patient's sex was not related to the αBC level. Another significant observation was its relation to the response to induction chemotherapy and radiation therapy. Patients with CR had lower αBC levels than those with partial or no response.

We further evaluated the effect of αBC level on patient survival; the OS and disease-free survival were shorter in patients with high αBC levels than in those with low αBC levels (p<0.001).

Our results support the observations of Mao et al. (31) who found that the αBC level was higher in laryngeal SCC than in their adjacent normal tissues and tumor stage. Contrary to our findings, their results indicated no significant relationship to patient age. Our observations also support those of Yilmaz et al. (32) who studied the αBC expression in metastatic, non-metastatic laryngeal SCC, and normal tissue samples and found a significant correlation between the level of αBC and laryngeal SCC, but an insignificant correlation with the tumor stage and lymph node metastases.

To the best of our knowledge, this is the first to evaluate the αBC levels in patients with SCC of the larynx and the response to induction chemotherapy. αBC is expressed in several types of malignant tumors. In ovarian cancer, Tan et al. (11). found a high αBC expression in the ovarian cancer tissues and its level was significantly correlated with the tumor size (p=0.028), lymph node metastasis (p=0.000), distant metastasis (p=0.005), tumor node metastasis stage (p=0.002), and survival (p=0.000). Shi et al. (33) revealed that the αBC levels were considerably elevated in colorectal cancer tissues as compared to that in the corresponding non-cancerous tissues (p<0.05 and p=0.014, respectively). They also stated that this elevation was significantly related to distant metastasis (p=0.040) and OS (p=0.003). Chen et al. (34) found that in gastric cancer tissues, the αBC expression is up-regulated relative to matched ordinary tissues and is closely correlated with cancer metastasis and shorter survival time.

We conclude that while more comprehensive trials with a larger sample and longer follow-up are required to confirm these findings, induction chemotherapy can be used effectively in locally advanced laryngeal cancer followed by radiotherapy or efficient conservation of the larynx in a certain proportion of patients without compromising survival. A high αBC level may be correlated with the aggressive behavior of SCC of the larynx and unfavorable outcomes.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Zagazig University (257/2013).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept - R.A., S.A.; Design - U.G., W.E., R.A.; Supervision - R.A., M.A., M.E.Y.; Resource - W.E., A.F.G., H.A., M.E.Y., U.G.; Materials - A.F.G., M.E.Y., U.G.; Data Collection and/or Processing - H.A., A.F.G., M.E.Y., U.G.; Analysis and/or Interpretation - A.F.G., M.A., U.G.; Literature Search - A.N.A., H.A., M.E.Y.; Writing - R.A., A.F.G., A.N.A., M.E.Y.; Critical Reviews - R.A., S.A., A.F.G., U.G.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.1979

Digital and Extradigital Glomus Tumors: A Clinicopathological Analysis

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Cite this article as: Kilitci A, Elmas ÖF, Tuncer K. Digital and Extradigital Glomus Tumors: A Clinicopathological Analysis. Cyprus J Med Sci 2020; 5(4): 323-6.

BACKGROUND/AIMS
Glomus tumors (GTs) refer to a rare group of perivascular tumors that usually affect the digital region. The lesions are histologically composed of vessels and glomus cells in varying proportions. The present study was designed to reveal the clinicopathological findings of the entity.

MATERIAL and METHODS
We reviewed the demographic, clinical, and histopathological features of the patients with GTs who were admitted to our tertiary center in the previous decade.

RESULTS
Total 16 patients were enrolled, including 9 men and 7 women. The mean patient age was 57.8 y. The most common location was the finger followed by other locations that included the chest wall (2), elbow (1), forearm (1), nose (1), anterior abdominal wall (1), hip (1), back (1), thigh (1), knee (1), and foot (1). Seven patients presented with pain and localized tenderness. Subungual involvement was more common among women (4). All the lesions were solitary, soft in consistency, yellowish/brown in color, and regular in shape with smooth contours. The size of the lesions ranged from 0.5 to 2.5 cm (average size: 1.25 cm). Histopathologically, the patients were classified into 3 main types, including solid type, glomangioma type, and glomangiomyoma type. Edematous and extensive myxoid stromal changes were found in 4 patients. Immunhistochemical study was performed to support the diagnosis in 8 patients. Ki-67 expressions were "low" for all 8 specimens. All the patients were treated with total excision, and there was no recurrence in any case during at least 1 y of follow-up.

CONCLUSION
Extradigital GTs are more common than digital tumors. It should be kept in mind that a glomus tumor has the ability to involve different sites in the body. The differential diagnosis of painful and painless subcutaneous nodules in any location should include GTs.

Keywords: Glomus tumor, histopathology, digital, extradigital

INTRODUCTION

Glomus tumor (GT) describes a benign neoplastic proliferation that originates from the glomus body. Glomus bodies, also called neuromyo-arterial bodies, are involved in body temperature regulation and are most numerous in the fingers and toes (1). About 75% of GTs are located in the fingers and 65% of them are located in the subungual space in particular. Solitary GTs are typically painful, while multiple GT can be painless (2). Extradigital GTs can be small in size, asymptomatic, non-palpable, and painless (1-4). Owing to these subtle clinical features, the diagnosis of extradigital GT may be challenging. However, in cases of painful lesions, the pain can be considered as "idiopathic pain", and the patients may be referred to irrelevant departments, including rheumatology, neurology, and psychiatry; leading to the administration of inappropriate treatment regimens, such as cortisone injection, physiotherapy, and nerve decompression (5).

Histologically, a GT is composed of glomus cells, blood vessels, and smooth muscle cells in varying proportions. GTs are histologically classified into solid type, glomangioma, or glomangiomyoma as per the predominant histological component (3, 6). GTs rarely show malignant nature (7).

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Received: 10.04.2020
Accepted: 24.05.2020

Few studies have investigated the clinical and histological characteristics of GTs, and most of these studies are single case reports and small case series. The present study aimed to contribute toward an understanding of the clinicopathological nature of the entity.

MATERIAL and METHODS

This retrospective study included a study of the clinical, demographic, and histopathological features of patients with a histopathological diagnosis of GT over a period of 10 y (January 2010 to January 2020). Hematoxylin Eosin (H&E) stained slides and the slides subjected to immunohistochemical staining were reviewed to confirm the diagnosis. Patients for whom insufficient clinical information was available were excluded. Descriptive statistical analyses were performed using the Statistical Package for Social Sciences Version 22.0 (IBM SPSS Corp.; Armonk, NY, USA) for Windows program. Informed consent was obtained from all participants. All the procedures were performed as per the principles in the Helsinki Declaration, and the study was approved by the local ethics committee (Decision date and number: 2020-02/20).

RESULTS

Total 16 patients, including 9 men and 7 women were enrolled. The mean patient age was 57.8 y. The most common localization was in the subungual area (n=5) followed by that in the trunk (n=3), elbow (n=1), forearm (n=1), nose (n=1), gluteal region (n=1), and back (n=1). Most lesions (n=11) had an extradigital localization. The subungual involvement was more common in women (n=4). The only symptom was pain that was present in 7 patients. The remaining 9 patients had cutaneous nodular growth (Figure 1). All the patients presented with a single lesion. The mean tumor diameter was 1.25 cm (diameter range: 0.5–2.5 cm). Only digital specimens (n=5) were submitted with a preliminary diagnosis of GT. None of the extradigital lesions had a preliminary diagnosis of GT. Total excision was the only treatment method used for all the included lesions.

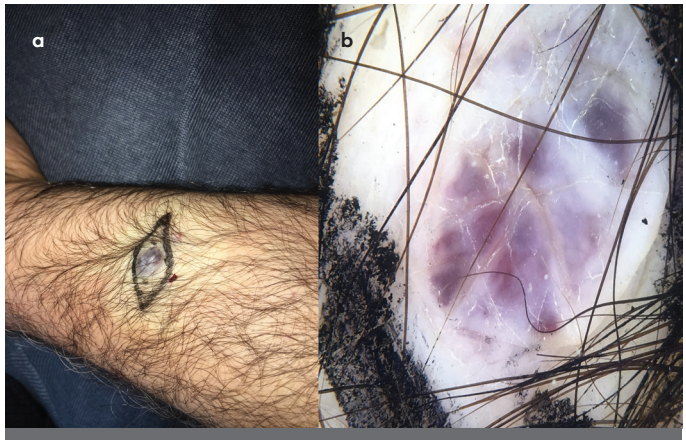

Figure 1. a, b. (a) A painful purple nodule located on the extensor surface of the left arm (b) Dermoscopic examination of a glomus tumor. Large purple clods arranged in a jigsaw-like fashion on a white structureless background

Main Points:

- Glomus tumor has the ability to involve different sites in the body.
- The differential diagnosis of painful and painless subcutaneous nodules in any location should include glomus tumor.
- In addition to classical histological features, GTs may show secondary histological changes, including large myxoid areas, hyalinization, edema, and rarely, calcification.

Macroscopically, all the lesions were soft in consistency, yellowish/brown in color, and regular in shape with smooth contours. With respect to the histopathological features, 50% of the lesions (n=8) were located in the lower dermis, while the other 50% were located in the subcutaneous tissue. The most common histopathological subtype was solid type (n=10, 62.2%) followed by glomangioma (n=5, 31.3%), and glomangiomyoma (n=1, 6.2%). All digital GTs were classified as solid type.

Seven lesions showed nerve fibers within or around the tumor, while the other lesions were free of the nerve fibers (Figure 2). Four lesions showed large myxoid edematous areas, while 2 showed hyaline

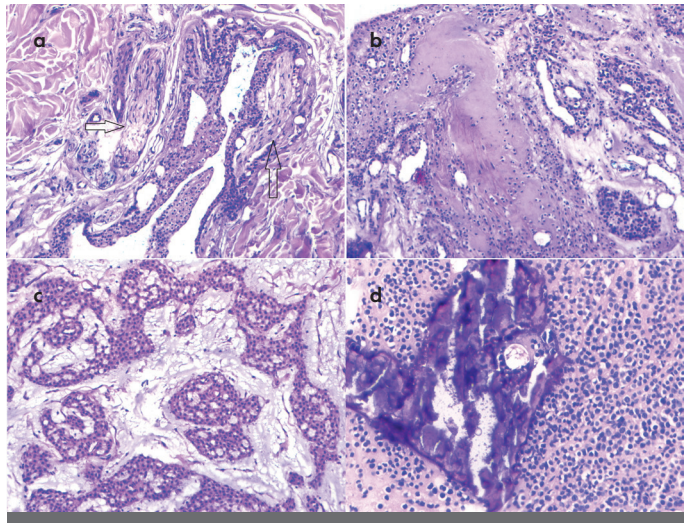

Figure 2. a-d. (a) Glomus tumor containing nerve bundles; arrows (H&E, x100) (b) The tumor shows extensive hyalinization (H&E, x100) (c) Tumoral stroma is myxoid and edematous (H&E, x100) (d) Calcification is visible in the tumor (H&E, x200)

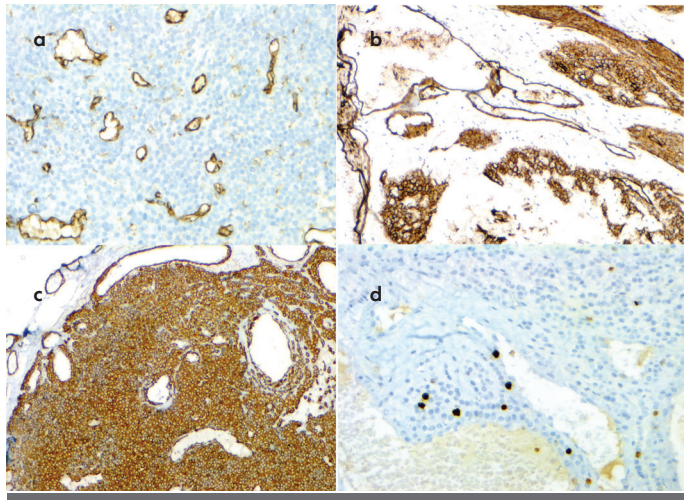

Figure 3. a-d. (a) Vessels staining with CD31, negative glomus cells (H&E, x200) (b, c) Glomus tumor reacts with CD34 and SMA (H&E, x100; H&E, x100) (d) Ki-67 proliferative index is very low in the glomus tumor (H&E, x200)

TABLE I. Clinicopathological data of the cases (D: digit, SMA: smooth mucle actin)

Case	Age	Gender	Anatomical localization	Diameter of tumor	Pain	Peripheral nerve intensity	Histological localization	Pathological subtype	Immunohistochemistry	
									glomus cells	vessels
1	78	M	anterior abdominal wall	2 cm	no	no	dermis	glomangioma	not available	
2	17	F	foot	1 cm	no	moderate	dermis	solid	CD31- CD34+ Desmin- SMA+	CD31+ CD34+ Desmin- SMA+
3	47	F	subungual, left hand D2	0,5 cm	yes	moderate	subcutaneous tissue	solid	not available	
4	75	M	hip	2 cm	no	moderate	dermis	glomangioma	CD31- CD34- Desmin- SMA+ S100-	CD31+ CD34+ Desmin- SMA+ S100-
5	40	F	thigh	1,5 cm	no	no	subcutaneous tissue	solid	not available	
6	41	M	back	1,5 cm	no	no	dermis	glomangiomyoma	not available	
7	62	M	elbow	1,2cm	no	mild	dermis	glomangioma	CD31 focally+ CD34 focally+ Desmin- SMA+	CD31+ CD34 + Desmin- SMA+
8	81	F	nose	1,5 cm	no	no	dermis	solid	not available	
9	68	M	right knee	2,5 cm	no	no	dermis	glomangioma	not available	
10	74	M	chest wall	0,8 cm	no	mild	dermis	glomangioma	CD31- CD34 focally+ Desmin- SMA+	CD31+ CD34+ Desmin- SMA+
11	36	M	chest wall	2 cm	yes	no	subcutaneous tissue	solid	CD31- CD34- Desmin- SMA+ panck-	CD31+ CD34+ Desmin- SMA+ panck-
12	82	M	subungual, left hand D5	0,6 cm	yes	mild	subcutaneous tissue	solid	CD31- CD34+ Desmin- SMA+	CD31+ CD34+ Desmin- SMA+
13	35	F	subungual, right hand DI	0,7 cm	yes	no	subcutaneous tissue	solid	not available	
14	56	F	subungual, right hand D4	0,5 cm	yes	mild	subcutaneous tissue	solid	CD31- CD34+ Desmin- SMA+ Vimentin+ S100-	CD31+ CD34+ Desmin- SMA+ Vimentin+ S100-
15	56	F	subungual, left hand D4	0,8 cm	yes	no	subcutaneous tissue	solid	CD31- CD34+ Desmin- SMA+ panck-	CD31+ CD34+ Desmin- SMA+ panck-
16	77	M	forearm	0,9 cm	yes	no	subcutaneous tissue	solid	not available	

degeneration. Dystrophic calcification was evident in another lesion (Figure 2). None of the lesions showed nuclear atypia, pleomorphism, or mitosis. Total 8 (50%) lesions were also evaluated using immunohistochemical stains to confirm the diagnosis (Figure 3). The demographic, clinical, histopathological, and immunohistochemical data of the patients are demonstrated in Table I.

DISCUSSION

The exact etiopathogenesis of GT is unknown; however, trauma and hereditary factors have been implicated. Some authors suggested that glomus bodies proliferate in response to trauma (8, 9). In a study, a history of trauma was present in 20%–30% of the patients diagnosed with GTs (1). In our study, none of the patients had a history of trauma.

Pain is a common symptom of GTs. The pathogenetic mechanism of pain in GT is clearly unknown; however, various hypotheses involving the sensitivity of the capsule to pressure; the release of chemical substances, such as histamine and heparin from the mast cells; substance P and TRPV1 expression in glomus cells; and an increase in nerve fibers penetrating into the tumor have been proposed (3, 8). In the present study, 3 of the 7 patients who had a painful lesion histologically showed a mild to moderate number of peripheral nerve fibers in the tumor. It is noteworthy that 4 of the 9 patients without pain also demonstrated a low number of nerve fibers. The pain status does not appear to have a significant correlation with the presence or absence of peripheral nerve fibers.

Extradigital cases are usually painless, and typically, a cutaneous mass or discoloration is the main complaints (1, 4). In keeping with the relevant literature, in our study, only 2 of the 11 patients with extradigital GTs had pain and localized tenderness, while all digital subungual lesions (n=5) were tender and painful. The asymptomatic nature of the extradigital GTs poses a clinical diagnostic challenge. They are usually clinically misdiagnosed as nevus, melanoma, hemangioma, pyogenic granuloma, neuroma, leiomyoma, and spiradenoma (1, 10). In a study, only 20% of the extradigital tumors received a correct preliminary diagnosis by a clinician (3). In this study, none of the extradigital GTs was submitted with a preliminary diagnosis of GT.

In GTs, the size of the lesion is typically small and does not exceed 1 cm in most patients (1, 11). The lesions located in the lower extremi-

ties can exceed 2 cm in diameter (12). In this study, 8 lesions (50%) were < 1 cm, while 7 lesions (44%) had sizes ranging from 1 cm to 2 cm. Only one lesion was > 2 cm. In our study, the mean tumor size was slightly larger than those reported in other studies (1, 11, 12).

Solid GTs are predominantly composed of glomus cells, while glomangiomas mainly include vascular structures. Glomangiomyomas are composed of fusiform smooth muscle cells (3, 10). In this study, the most common histopathological subtype was the solid type (n=10, 62.2%) followed by glomangioma (n=5, 31.3%), and glomangiomyoma (n=1, 6.2%). These rates were respectively reported as 73%, 19%, and 8% by Einzinger and Weiss; as 76.5%, 23.5%, and 0% by Kim SH et al.; and as 77.8%, 22%, and 0% by Kim MG et al (6, 10, 13).

The main differential diagnosis of solid GTs includes eccrine spiradenoma and hidradenoma that are characterized by focal ductal differentiation and positivity for epithelial markers unlike GT. Glomangioma and glomangiomyoma can be confused with hemangioma and leiomyoma wherein glomus cells distributed in the vessel wall are absent (14).

In suspected cases, immunohistochemical studies should be performed to avoid misdiagnosis. In GTs, the tumor cells are positive for smooth muscle actin (SMA) and rarely, focally positive for desmin. CD34 may also be positive. In this study, 8 (50%) lesions were evaluated using immunohistochemical stains to confirm the diagnosis. In keeping with the relevant literature, SMA was positive for all the lesions, while desmin was negative. CD34 was positive in 6 lesions in varying degrees. Only one lesion showed positive CD31 staining.

GTs may exhibit secondary histological changes, including large myxoid areas, hyalinization, edema, and in some cases, calcification. In this study, 4 lesions showed large myxoid edematous areas, while two demonstrated hyaline degeneration. Calcification was evident in another lesion. Edema and myxoid changes were reported in 11.8% of the patients in a 17-patient series performed by Kim SH et al. and in 22.2% of the 27 tumors evaluated by Kim MG et al (6, 10). To prevent misdiagnosis, possible secondary changes in GTs should be considered carefully.

The overwhelming majority of GTs is benign; however, malignancy may be present rarely. The histological criteria for malignancy include a high mitotic count (5/50 HPF), a diameter > 2 cm, and the presence of necrosis and pleomorphism that were not observed in any of the included lesions (15).

The single effective treatment method for GT is total excision (1). Inadequate excision is associated with early recurrence. Late recurrence may indicate multiple tumors that are initially overlooked. The reported recurrence rates range from 12% to 33% (1, 2, 10). In the present study, all the excision specimens that were submitted had tumor-free margins, and none of the patients experienced recurrence during the 1-year period of follow up.

Our study has certain limitations in terms of the retrospective nature and relatively small sample size. The reason for the small number of patients with a histopathological diagnosis of GT despite a period of 10 y may be the high number of asymptomatic patients who did not consult any department. The high number of patients who were clinically misdiagnosed or treated conservatively may be other causes.

In conclusion, considering these subtle clinical features, the diagnosis of extradigital GT may pose a diagnostic challenge. Thus, the differential diagnosis of painless and painful nodular growths should include GT in addition to other relevant preliminary diagnoses for the enhancement of a clinicopathological correlation that is key for the diagnosis of many cutaneous tumors.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ahi Evran University Faculty of Medicine Clinical Research (2020-02/20).

Informed Consent: Informed consent was obtained from all participants.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - A.K., Ö.F.E.; Design - A.K.; Supervision - A.K., Ö.F.E.; Resource - Ö.F.E., K.T., A.K.; Materials - A.K.; Data Collection and/or Processing – Ö.F.E., A.K.; Analysis and/or Interpretation - K.T.; Literature Search - K.T.; Writing - A.K., Ö.F.E., K.T.; Critical Reviews - Ö.F.E.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.2133

Prevalence of Pathologic Plica Following Anterior Cruciate Ligament Injury

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Cite this article as: Aydın D, Polat B. Prevalence of Pathologic Plica Following Anterior Cruciate Ligament Injury. Cyprus J Med Sci 2020; 5(4): 327-32.

BACKGROUND/AIMS
The present study was designed to determine the relationship between the time from anterior cruciate ligament injury to operation as well as the presence of pathologic plica. We also assessed the relationship between the presence of pathologic plica and plica-associated cartilage injury as well as preoperative anterior knee pain.

MATERIAL and METHODS
All the data for this retrospective observational study was obtained from hospital records and arthroscopy videos. Preoperative complaints, such as giving-way episodes, anterior knee pain, and knee locking, and intraoperative findings, such as the presence and absence of the pathologic plica, cartilage injury, and meniscus lesion were evaluated. Patients operated within 3 mon of the injury and those operated ≥ 3 mon after the injury were classified into Group I and Group 2, respectively.

RESULTS
We enrolled 76 patients; group I included 46 patients, and group 2 comprised 30 patients; all the patients underwent anterior cruciate ligament reconstruction. The rate of preoperative anterior knee pain and pathologic plica formation was significantly higher in group 2 ($p<0.05$) as compared to that in group I. The reported preoperative anterior knee pain and cartilage injury in the pathologic plica positive group was significantly higher than that in the plica negative group ($p<0.05$).

CONCLUSION
Pathologic plica formation rate increases as the time period from anterior cruciate ligament injury to reconstruction increased. Preoperative anterior knee pain and intraoperative knee cartilage damage were more common in patients with pathologic plica. We recommend that pathologic plica investigation and resection be considered during anterior cruciate ligament reconstruction surgeries.

Keywords: Anterior cruciate ligament, reconstruction, anterior knee pain, suprapatellar plica, medial plica

INTRODUCTION

Synovial plica is a term that is used to define intraarticular folds that are remnants of the embryonic membranous septation of the joint and are accepted as normal structures of the knee joint that are observed incidentally during routine arthroscopy (1). Plica is classified into the following four distinct types. Infrapatellar plica is the most common plica; medial patellar plica has the highest potential to cause knee symptoms followed by suprapatellar plica; lateral patellar plica is very rare (2). As the plica is a remnant, plica of some size may exist in all knees. In most arthroscopic studies, the incidence of some kind of synovial plica was reported to be 70%–91% (2). Synovial plica rarely becomes symptomatic, usually following a knee trauma (1). In normal knees with no trauma, plica is thin, elastic, and harmless (3, 4). They could accommodate knee motions with no injury to the surrounding structures. Repetitive knee movements, trauma, and surgical knee interventions can cause irritation of the synovial plica. In this case, inflammation and hemarthrosis cause fibrosis and thickening of the plica (3). Impingement of the thickened plica may cause plica-related symptoms, such as anterior knee pain, recurrent knee effusion, giving way, clicking, and locking (5).

Diagnosis of plica syndrome can be challenging. The symptoms and clinical findings of plica syndrome have poor specificity and are similar to more common intraarticular pathologies, such as meniscal lesions and articular cartilage injuries (2).

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Received: 10.05.2020
Accepted: 20.06.2020

Imaging modalities are insufficient for diagnosis in most cases. Magnetic resonance imaging is able to demonstrate plica; however, this modality is ineffective for determining whether the plica is pathologic (6). The gold standard for the diagnosis of plica syndrome is knee arthroscopy because surgical treatment and further evaluation of the associated cartilage pathologies are possible during the same surgery.

Anterior cruciate ligament (ACL) injuries are common, and surgical reconstruction is the accepted treatment in young, active patients. During diagnostic arthroscopy that is performed to make a decision regarding reconstruction, plica structures are generally not evaluated in detail. We hypothesized that in some patients with ACL injury, the normal plical structures that existed before the trauma may become thicker and inelastic after a latent period, and plica-related mechanisms may be responsible for anterior knee pain. This study aimed to determine the relationship between the time from ACL injury to operation and the presence of pathologic plica. Moreover, the relationship between the presence of pathologic plica and plica-associated cartilage injury and preoperative anterior knee pain was assessed.

MATERIAL and METHODS

We obtained ethical approval for this study from the Institutional Review Board of the Near East University Medical Faculty (Reference No. 58-598). All the patients provided written informed consent for the publication of their individual data.

This study was planned as a retrospective observational study. We enrolled 87 patients who had undergone arthroscopic ACL reconstruction surgery with quadrupled hamstring tendon grafts in the authors' clinic from January 2015 to October 2017. Patients who had undergone arthroscopic ACL reconstruction surgery and had agreed to participate in the study were included in our analyses. Arthroscopy videos are routinely recorded for all ACL reconstruction surgeries. In this context, eight of the patients whose arthroscopic video qualities were insufficient for investigation were excluded. Furthermore, three of the patients who did not consent to participate within the study were also excluded. Thus, finally, 76 patients who met the inclusion criteria were enrolled.

The hospital records and interviews with patients were used to collect information about their demographic characteristics and initial complaints. Trauma mechanism, time from trauma to operation, and preoperative complaints, such as giving-way episodes, anterior knee pain, and knee locking were recorded. Anterior knee pain was positively accepted when the patient experienced pain while performing one or more of the following daily activities: walking, running, jumping, climbing stairs, squatting, and sitting with bent knees for a prolonged period. Arthroscopy videos and operation reports were evaluated by two researchers independently for the determination of intraarticular pathologies. The patients were evaluated as per the presence or absence of pathologic plica formation, cartilage, and meniscus related pathologies. Descriptions of the investigated pathologies are presented below.

Main Points:

- Pathologic plica formation rate increases with increase in the duration from anterior cruciate ligament injury to reconstruction.
- Pathologic plica is related to anterior knee pain and knee cartilage damage.
- In delayed reconstruction cases, pathologic plica existence should be evaluated in detail to prevent further cartilage damage and postoperative anterior knee pain.

Pathologic Plica: Hypertrophic, non-transparent, tight, and inelastic plica was recorded as pathologic (Figure I-3). They were classified as medial and/or suprapatellar as per the location of the pathologic plica.

Cartilage injury: Cartilage injuries were graded as follows: Grade I, focal areas of softening with normal contour of the cartilage surface. Grade II, damage compromising <50% of the thickness of the cartilage. Grade III, damage compromising>50% of the thickness of the cartilage, but not extending to the subchondral bone tissue. Grade IV, total cartilage wearing with exposed subchondral bone. Cartilage injury was considered to be present when there was cartilage damage in any location of the knee of Grade 2 or more.

Meniscal Lesion: A meniscal lesion was considered when any of the following types of tears were present and treatment was required: parrot beak tear, radial tear, longitudinal tear, horizontal tear, bucket-handle, and complex tear.

Our patients were divided into two groups as per the duration between the injury and operation. Patients operated within 3 mon of the injury and those operated ≥ 3 mon after the injury were classified into group I and group 2, respectively. We compared the demographic characteristics, preoperative complaints, and intraoperative findings of these two patients groups. Accordingly, the relationship between the presence of pathologic plica and plica-associated cartilage damage and preoperative anterior knee pain was analyzed.

Statistical Analysis

In the descriptive statistics of the data, mean, standard deviation, median lowest, highest, frequency, and ratio values were used. The distribution of the variables was measured using the Kolmogorov-Smirnov test. Independent samples t-test and Mann–Whitney U test were used for the analysis of quantitative independent data. The chi-square test was used for analyzing qualitative independent data, and Fischer test was used when the chi-square test was not suitable. All the statistical analyses were performed using the Statistical Package for Social Sciences software version 22.0 (IBM SPSS Corp.; Armonk, NY, USA). Two authors analyzed the operation video of each patient independently. The examiners were blinded to the group information of the subject. In case of disagreement between the examiners, the data were re-evaluated until a consensus was reached. Interobserver reliabilities of the operation findings were assessed using the 'κ' statistical test. A kappa value of 0.8–I was considered to indicate perfect agreement.

RESULTS

Primary ACL reconstruction was performed for 76 patients, including 65 men and II women. The average age of the study

subjects was 29.8 y (range, 18–46 y). Surgery was performed on the right knee of 48 patients and the left knee of 28 patients. Reported trauma mechanisms were related to football in 43 patients, sprain in 14, basketball in 11, skiing in 4, traffic accident in 3, and kickboxing in 1 patient. The rate of investigated preoperative complaints were giving-way episodes in 67 (88.2%) patients, clicking or locking in 39 (51.3%), and anterior knee pain in 15 (19.7%) patients. Pathologic plica formation was noted in 30 (39.5%) of the patients. Of these pathologic plica, 15 were suprapatellar, 8 were medial, and 7 were both. Meniscal lesions were observed in 35 (46.1%) of the patients, where 21 of the lesions were medial, nine were lateral, and five were both-sided meniscal tears. Cartilage injury was observed in 22 (28.9%) of the patients, and their distribution was as follows: 11 patients had medial condyle, 11 patients had patella, 5 patients had lateral condyle, and 2 patients had trochlea cartilage injuries.

There were 46 patients in the first group and 30 in the second group. The average duration from the time of injury to the time of operation was 7.2 wk (range, 4–11 wk) in group 1 and 16.5 mon (range, 3–48) mon in group 2. A comparison of the demographic features, preoperative complaints, and arthroscopic findings of these two groups has been presented in Table 1. The rates of recurrent giving-way episodes, preoperative clicking, and locking were not significantly different ($p>0.05$) between group 1 and group 2. The rate of preoperative anterior knee pain was significantly higher in group 2 as compared to that in group 1 ($p<0.05$). In addition, the pathologic plica formation rate was significantly higher in group 2 than in group 1 ($p<0.05$). The medial pathologic plica existence ratio did not differ significantly ($p>0.05$) between group 1 and group 2s. However, suprapatellar pathologic plica formation was significantly higher in group 2 as compared to that in group 1 ($p<0.05$). The rate of meniscus lesions was not

TABLE 1. Comparison of the demographic features, preoperative complaints, and arthroscopic findings of the two groups

		Group 1		Group II			
		Mean ± sd/n-%	Median	Mean ± sd/n-%	Median	p	
Age		29.0±7.3	28.5	30.9±6.8	31.0	0.250	t
Sex	Female	10 21.7%		1 3.3%		0.026	X^2
	Male	36 78.3%		29 96.7%			
Side	Right	32 69.6%		16 53.3%		0.152	X^2
	Left	14 30.4%		14 46.7%			
Plica	(-)	37 80.4%		9 30.0%		0.000	X^2
	(+)	9 19.6%		21 70.0%			
Medial Plica		6 13.0%		9 30.0%		0.069	X^2
Suprapatellar Plica		5 10.9%		17 56.7%		0.000	X^2
Meniscus Lesion	(-)	26 56.5%		15 50.0%		0.577	X^2
	(+)	20 43.5%		15 50.0%			
Medial		12 26.1%		14 46.7%		0.065	X^2
Lateral		12 26.1%		2 6.7%		0.033	X^2
Cartilage İnjury	(-)	40 87.0%		14 46.7%		0.000	X^2
	(+)	6 13.0%		16 53.3%			
Patellar		0 0.0%		11 36.7%		0.000	X^2
Medial Condyle		2 4.3%		9 30.0%		0.002	X^2
Lateral Condyle		3 6.5%		2 6.7%		0.980	X^2
Trochlea		2 4.3%		0 0.0%		0.516	X^2
Preop Ant Knee Pain	(-)	43 93.5%		18 60.0%		0.000	X^2
	(+)	3 6.5%		12 40.0%			
Injury Mechanism	Basketball	8 17.4%		3 10.0%		0.574	X^2
	Football	26 56.5%		17 56.7%		0.822	X^2
	Kick Box	0 0.0%		1 3.3%		0.394	X^2
	Ski Sport	1 2.2%		3 10.0%		0.333	X^2
	Sprain	9 19.6%		5 16.7%		0.987	X^2
	Traffic Accident	2 4.3%		1 3.3%		0.703	X^2
Recurrent Giving-Way Episodes	(-)	7 15.2%		2 6.7%		0.259	X^2
	(+)	39 84.8%		28 93.3%			
Preoperative Clicking And Locking	(-)	24 52.2%		13 43.3%		0.451	X^2

[t] t test / m Mann-Whitney u test / X^2 Chi-square test (Fischer exact)

TABLE 2. Relationship between the presence of pathologic plica and plica-associated cartilage injury and preoperative anterior knee pain

		Plica (-)		Plica (+)		
		n	%	n	%	p
Cartilage Injury	(-)	44	95.7%	10	33.3%	0.000 X^2
	(+)	2	4.3%	20	66.7%	
Preoperative Anterior Knee Pain	(-)	45	97.8%	16	53.3%	0.000 X^2
	(+)	1	2.2%	14	46.7%	

X^2 Chi-square test

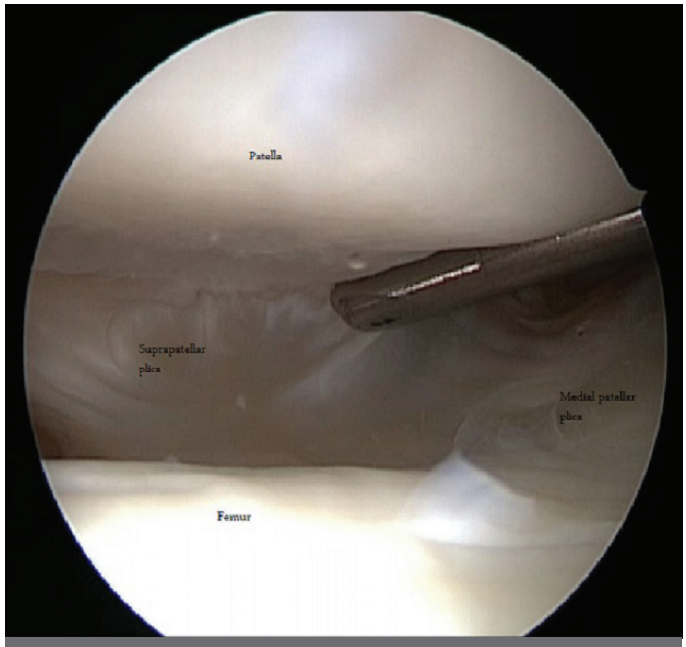

FIGURE I. Hypertrophic and non-transparent appearance of pathologic suprapatellar and medial patellar plica

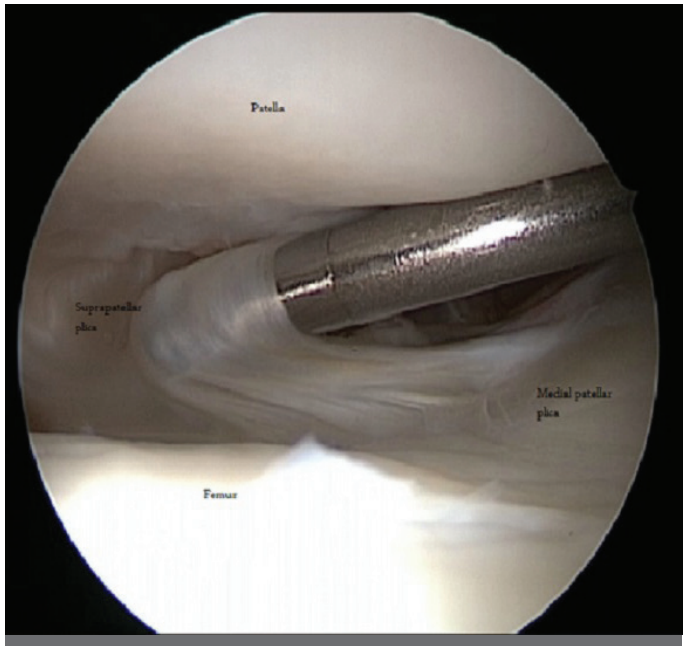

FIGURE 2. Inelastic and non-fragile structure of pathologic plica

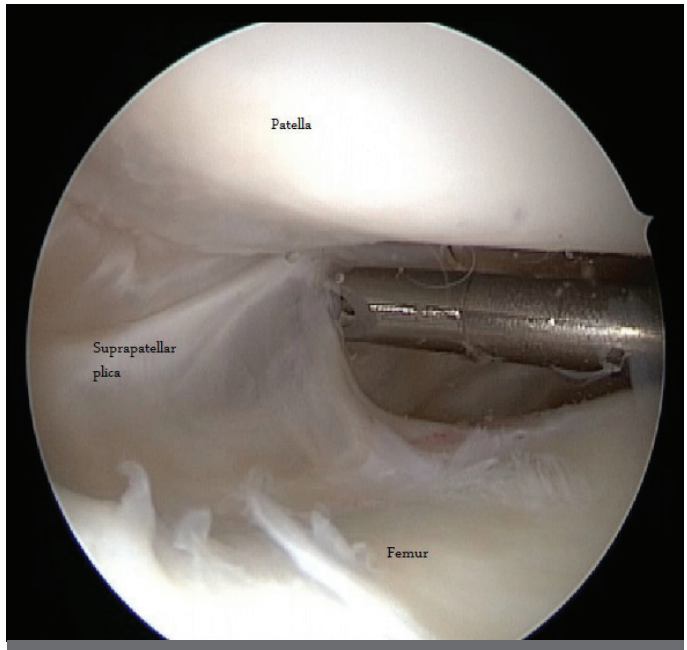

FIGURE 3. Resection of pathologic plica with an arthroscopic shaver

significantly different between the groups (p>0.05). The medial meniscus lesion ratio was similar between the two groups (p>0.05); however, the lateral meniscus lesion ratio was significantly higher in group I as compared to that in group 2 (p<0.05). The cartilage injury rate was significantly higher in group 2 than in group I (p<0.05). Patella and medial condyle cartilage injury was significantly higher in group I than in group 2 (p<0.05); however, there was no significant difference between the groups in terms of lateral condyle and trochlea cartilage injuries (p>0.05) (Table I). The reported anterior knee pain and cartilage injury in the pathologic plica positive group was significantly higher than that in the plica negative group (p<0.05) (Table 2). Interobserver agreement (k) values in the assessment of pathologic plica were 0.83 and 0.87, respectively, indicating perfect agreement.

DISCUSSION

The most important finding of this study was that the rate of pathologic plica increased as the duration between ACL injury and operation increased. We assumed that ACL injuries may cause pathologic transformation of the plica over time, and this could be an overlooked cause of anterior knee pain after this injury.

Some possible anterior knee pain pathologies following ACL injury are Hoffa syndrome, patellofemoral chondral injury, patellar tendinitis, quadriceps tendinitis, patellar bone bruise, anterior horn injuries of the meniscus, and synovial plica syndrome. Symptoms and clinical findings of these pathologies are generally indistinguishable for a certain disease (I).

In our study, the cause of statistically higher anterior knee pain rate in the second patient group was thought to be due to high patellar cartilage damage prevalence. However, in the subgroup analysis of patients with and without plica, the relationship between the presence of plica and anterior knee pain was significant. Therefore, this study showed that anterior knee pain after ACL injury may be related to pathologic plica. Imaging

modalities, such as radiography, ultrasonography, and magnetic resonance imaging have limited efficiency in diagnosing plica syndrome. Exclusion of other anterior knee pain pathologies with the suspicion of plica syndrome is necessary for diagnosis. Definitive diagnosis is performed using arthroscopy. However, it is easy to miss the existence of suprapatellar plica during arthroscopy. Medial plica is like a shelf on the medial synovial wall (7) that sometimes travels in front of the medial femoral condyle; however, suprapatellar plica resembles the roof of the knee joint, particularly if it is a complete type (8).

Plica is normal structures commonly observed during routine knee arthroscopy. Plica syndrome is considered to occur because of structural changes of the plica following an inflammatory process (9). Inflammation and swelling of the plica is responsible for acute complaints, and long-term thickened plica may cause chondral damage. Christoforakis et al. reported increased incidence of articular cartilage wearing in knees with synovial plica, particularly in the patella and medial femoral condyle (5). Any factor that causes intraarticular hematoma and synovitis could cause pathologic transformation of the plica. Elastic tissues of the plica became fibrotic with prolonged inflammation. Occult trauma, mechanical irritation with repetitive knee movements, intraarticular pathologies (meniscal injury, loose body, osteochondritis dissecans, patella subluxation etc.), and surgical interventions are reported in the etiology (1). In our study, we investigated the presence of pathologic plica after ACL injury, the most common cause of traumatic hemarthrosis in the knee (10).

The criteria used to define plica as pathologic have not been well established. Plica causing anterior knee pain and recurrent knee effusion without associated intraarticular lesion should be classified as pathologic (3). Pathologic plica is diagnosed during arthroscopy if it is hypertrophic, has lost its transparency and fragile structure, and causes chondral damage with impingement. However, plica evaluation is subjective; some surgeons overlook it as the etiology of anterior knee pain, while others identify it as the cause in most cases. Further, there is no threshold for classifying plica as pathologic or normal. Pathologic plica is treated with arthroscopic resection. The investigation and resection of normal plica structures during routine knee arthroscopy may be associated with a longer surgical time and extra damage during the procedure. The necessity of normal plica resection is a controversial subject in the literature (2). To our knowledge, there are no data on the time required for pathologic plica transformation after intraarticular inflammation, such as ACL injury, in the literature. We determined a 3-month period as a threshold value in this retrospective study because in our clinical practice, we encountered more pathologic plica intraoperatively in patients who had been operated after 3 mon of ACL injury. In ACL injury cases, patients experience a period of hematoma and immediately after regression of the hematoma, they start daily activities; this may cause further inflammation in the synovial membranes. Chronic synovitis causes fibrotic transformation of the plica and may be the cause of anterior knee pain in patients who have experienced ACL injury.

The underlying mechanism by which suprapatellar plica becomes symptomatic remains controversial. It is assumed that complete type suprapatellar plica is more likely to manifest symptoms (11, 12). Different causal mechanisms have been reported for the symptoms. Inflammation of the plica caused by any reason leads to structural changes in the plica. Hypertrophic plica exerts increased forces on the articular surfaces and causes degenerative changes (13). Impingement of the hypertrophic plica between the extensor mechanism and the medial femoral condyle during knee flexion beyond 70° is another reported mechanism for symptoms (14, 15). Fibrotic suprapatellar plica acts on the superior patella and changes its dynamics during flexion and extension. Malalignment of the patella in this manner is also a reported cause of anterior knee pain (16). Intraarticular volume and joint fluid distribution may also be affected by a large suprapatellar plica; thus, increased pressure applies forces on the articular cartilage (13).

In this study, pathologic plica was detected in 9 (19.6%) of the 46 patients who underwent reconstruction within 3 mon of ACL injury, and pathologic plica was detected in 21 (70%) of the 30 patients who underwent reconstruction ≥ 3 mon after ACL injury. This significant difference showed that plica hypertrophy occurs after a latent period from the time of injury. It could be said that plica works as the gatekeeper of the knee joint because it is hypertrophied after intraarticular inflammation. In some cases, the plica structures will become hypertrophic and cause plica-related symptoms. In the present study, anterior knee pain and cartilage damage of the knee, especially in the patella and medial condyle, was significantly more common in patients with pathologic plica.

This study has certain limitations, including the relatively small sample size, retrospective study design, and the absence of a comparison group without plica resection. We believe that further research that compares patient satisfaction in ACL reconstructions with and without plica resection is warranted. Although this study proved that pathologic plica is associated with anterior knee pain and cartilage damage, long-term follow-up cohort studies are needed to improve the understanding regarding the significance of pathologic plica.

In conclusion, Pathologic plica formation rate increases with an increase in the duration between ACL injury and reconstruction. Preoperative anterior knee pain and intraoperative knee cartilage damage were more common in patients with pathologic plica. We recommend that pathologic plica investigation and resection be considered during ACL reconstruction surgeries.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Near East University Scientific Research (Reference No. 58-598).

Informed Consent: Written informed consent was obtained from the patients.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - D.A.; Design - D.A., B.P.; Supervision - B.P.; Resource - D.A., B.P.; Materials - D.A., B.P.; Data Collection and/or Processing - D.A., B.P.; Analysis and/or Interpretation - D.A., B.P.; Literature Search - B.P.; Writing - D.A., B.P.; Critical Reviews - D.A., B.P.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.2163

The Impact of Preoperative Nutritional Status of Elderly Patients on the Postoperative Outcome: Comparison of Two Nutritional Assessment Tests and Biochemical Tools

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Cite this article as: Sezen Ö, Çevik B. The Impact of Preoperative Nutritional Status of Elderly Patients on the Postoperative Outcome: Comparison of Two Nutritional Assessment Tests and Biochemical Tools. Cyprus J Med Sci 2020; 5(4): 333-8.

BACKGROUND/AIMS
Our main goal was to compare the two validated nutritional screening tools and serum proteins that are used as routine biochemical markers for the prediction of the postoperative outcome.

MATERIAL and METHODS
The current study was design to evaluate the prevalence of malnutrition among elderly patients undergoing elective curative gastrointestinal cancer and trochanteric hip fracture surgery. On admission to the preoperative holding area, all patients underwent the two nutritional screening tests: Mini Nutritional Assessment (MNA) and Nutritional Risk Screening 2002 (NRS - 2002). After assessment, all participants were followed-up throughout their hospital stay. We calculated the lymphocyte-monocyte ratio (LMR) and albumin-globulin ratio (AGR) according to the laboratory results. The surgical outcome (discharge to ward/intensive care) and postoperative infection (systemic or surgical site) were recorded.

RESULTS
The group of patients with MNA < 17 (malnutrition) had a significantly high ICU admission rate and incidence of systemic or surgical infection, which led to the prolonged length of hospital stay. According to the NRS-2002 screening tool, the ICU admission rate was higher in patients with a score ≥ 3. The surgical site infection rate in this group was also significantly higher than that of the patients with an NRS-2002 score < 3. When comparing the two screening tools, the incidence of ICU admission was significantly higher in patients with MNA>17. The mean serum albumin and globulin levels and the AGR was comparable between before and after the surgery. However, the mean LMR was significantly lower than the preoperative value.

CONCLUSION
Measurements with easy-to-perform tests will provide guidance in terms of identifying potential perioperative risks.

Keywords: Malnutrition, older adults, nutritional biomarkers, MNA, NRS-2002

INTRODUCTION

People are living longer worldwide, and the proportion of the population that is over 60 years is expected to increase from 12% to 22% between 2015 and 2050 (1). With increasing age, people have various age-related co-morbidities, and many of them require hospitalization. A larger proportion of the elderly population undergoes surgical procedures due to advances in surgical, anesthetic, and intensive care interventions.

Malnutrition leads to a progressive decline in the health condition, reduced physical and cognitive functional status, increased utilization of health care services, premature institutionalization, and increased mortality in the elderly population (2).

Determining the malnutrition risk is an important area of concern, and precautions need to be taken in order to initiate appropriate nutritional support. However, physicians do not always have enough time to treat malnutrition. Due to functional alterations caused by malnutrition, falls are frequent in the geriatric population, and hip fracture is

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Received: 19.05.2020
Accepted: 21.06.2020

one of the main reasons of hospitalization. Early hip surgery within 48 hours was recommended because of the decrease in mortality risk and perioperative complications (3). A recent meta-analysis indicated that the prevalence of malnutrition in patients with hip fracture was approximately 18.7% using the Mini-Nutritional Assessment (MNA) test. Using the Body Mass Index (BMI) as a diagnostic tool, the prevalence increased up to 45.7% (4). Malignancies are the other main cause of malnutrition, and weight loss can sometimes be the first symptom of cancer. A systematic review revealed that malnutrition was significantly positively associated with increased risk of all-cause mortality (5). In a retrospective study that included 709 adult patients in 25 Brazilian hospitals, the incidence of complications among patients with malnutrition was 27% (relative risk [RR] = 1.60) compared with 17% among the well-nourished counterparts. The mortality of patients with malnutrition and well nutrition was 12.4% *vs* 4.7%, respectively (RR = 2.63) (6).

Nutritional assessment is a systemic process and can be done by using the ABCD methods, that is, the anthropometry, biochemical/biophysical, clinical, and dietary methods (7). Although many screening and diagnostic tools have been introduced in clinical practice, no single test is sufficient for the assessment of the nutritional status (8). The Nutritional Risk Screening 2002 (NRS 2002) test and Mini Nutritional Assessment (MNA) are the most common screening tools for hospitalized patients (9, 10). The MNA is a more useful tool for the identification of frail patients (11).

Many nutritional assessments and screening tools use biochemical markers such as albumin, prealbumin, transferrin, C-reactive proteins, and total lymphocyte count. Albumin is mostly used as a laboratory marker to assess the nutritional status in daily clinical practice. However, its sensitivity and specificity is a controversial issue. A recent meta-analysis indicated that serum albumin level was strictly affected by the nutritional status and that hypovolemia is a negative prognostic index in the elderly population (12). Globulin is another major component of serum proteins that plays a role in the immune and chronic inflammatory process. Both serum levels of albumin and globulin alone could be easily affected by many factors; therefore, albumin-to-globulin ratio (AGR) was recommended as a valuable prognostic factor, especially in the cancer patients (13).

The current study was design to evaluate the prevalence of malnutrition among elderly patients undergoing elective curative gastrointestinal cancer and trochanteric hip fracture surgery. The main goal was to compare the two validated nutritional screening tools and serum proteins that are used as routine biochemical markers for the prediction of the postoperative outcome.

MATERIAL and METHODS

This prospective, observational, cross-sectional study was conducted after receiving approval from the Institutional Ethics Committee (decision no:2018/514/144/3) according to the ethical principles outlined in the Helsinki Declaration and Good Clinical Practice guideline. Written informed consents were obtained from all the participants.

Study Population

We consecutively enrolled patients aged ≥ 65 years who were scheduled for elective curative gastrointestinal cancer and trochanteric hip fracture surgery between January and April 2019.

Exclusion Criteria

Patients undergoing palliative or emergency surgery, aged < 65 years, those who refused to participate in the study, having cognitive impairment, communication problems, multiple trauma patients,¡ and right-sided hip fractures were excluded.

Assessment of the Nutritional Risk

On admission to the preoperative holding area, all patients underwent the two nutritional screening tests; MNA and NRS-2002. After assessment, all participants were followed-up throughout their hospital stay.

MNA has two forms, including the short (MNA-SF) and long form (MNA-LF). MNA-SF consists of six sections including appetite, recent weight loss, mobility impairment, acute illness, dementia or depression, and body mass index. We used the MNA-LF, which consisted of twelve more sections: Living arrangements, medications, presence of pressure ulcers, quality and number of meals, fluid intake, autonomy of feeding, self-perception about health and nutrition, and mid-upper arm and calf circumferences. Scores below 17 indicated malnourished, 17–23.5 at risk of malnutrition, and 24–30 normal nutritional status (14).

NRS-2002 consists of body mass index, weight loss, recent decrease in food intake and severity of illness. This tool has three components: a severity of disease score, a nutritional score, and an age score. The score ranges between 0 and 6 according to the assessment. We categorized the patients as well-medium risk (<3) and nutritionally at risk (≥3) (15).

Anthropometric Measurements

Personal characteristics such as age, gender, weight, height, and body mass index were recorded. Right middle arm circumference was measured at the mid-point between the acromial process of the scapula and the olecranon process of the ulna with the arm hanging loosely by the side. Calf circumference was measured from the widest part of the right leg while the patient was in the supine position and the knee was in a right angle between the thigh and calf.

Laboratory Tests

All laboratory data were acquired from patients within seven days prior to any surgery in our institution. In hip fractures, the laboratory tests depend on the admission date of the patients, and they are scheduled for surgery within three days. Total lymphocyte count, monocyte count, and serum albumin and globulin levels are part of the standard preoperative assessment laboratory tests for surgical patients.

Data Collection

For this study, clinical data were collected from the patient's electronic health records, including age, date of admission, clinical diagnosis, and American Society of Anesthesiologist' (ASA) physical status. All anthropometric measurements were performed by the same investigator. If the patient was unaware of his/her height, the investigator estimated the approximate

height of the patient. In case of unknown body weight, we used the Buckley's bed side method for estimation of the body weight. This method uses the abdominal circumference (AC) and thigh circumference (TC) to estimate the actual body weight (ABW) of the patient. In male and female patients, ABW is formulated as -47.8 + 0.78 x AC + 1.06 x TC and -40.2 + 0.47x AC + 1.30 x TC, respectively (16). The scores of MNA-LF and NRS-2002 were recorded. We retrieved the laboratory findings from the hospital electronic database and calculated the lymphocyte-monocyte ratio (LMR) and albumin-globulin ratio (AGR) according to the laboratory results. Surgical outcome (discharge to ward/intensive care) and the postoperative infection (systemic or surgical site) were recorded.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8. While analyzing the study data, Student's t-test was used for comparisons between descriptive statistical parameters (mean, standard deviation, minimum and maximum value) and one-way ANOVA followed by the Tukey posttest for multiple comparisons.

The differences were considered statistically significant when p values were < 0.05 (*, p < 0.05; **, p < 0.01; ***, p < 0.001). The data has been reported as mean ± standard deviation (SD).

RESULTS

Totally, 100 patients were recruited into the study. We excluded 18 patients due to reoperation and missing data; therefore, statistical analysis was conducted using data from 82 patients (Figure 1). Patients' characteristics and anthropometric measurements are shown in Table 1. The mean serum albumin and globulin levels and the AGR were comparable between before and after operation. However, the mean LMR after surgery was significantly lower than the preoperative value (Table 2). The group of patients with MNA < 17 (malnutrition) had a significantly higher ICU admission rate and incidence of systemic or surgical infection, which led to the prolonged length of hospital stay (Table 3).

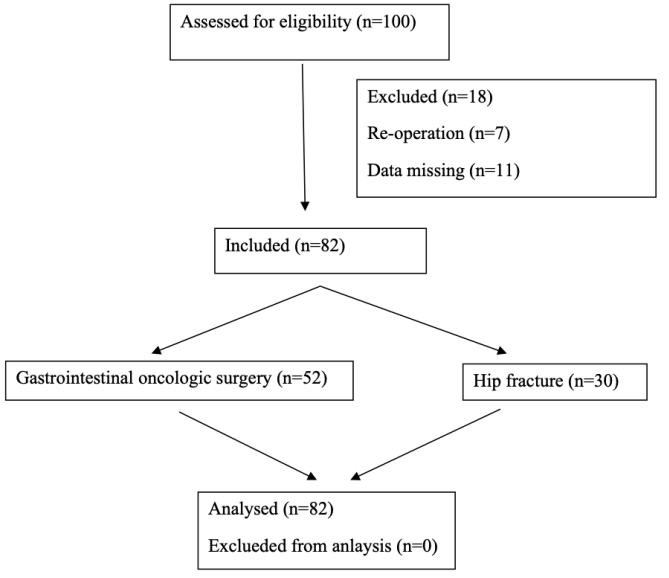

FIGURE 1. The consort flowchart of the study

According to the NRS-2002 screening tool, the ICU admission rate was higher in patients with a score ≥ 3. The incidence of surgical site infection in this group was also significantly higher than that of the patients with an NRS-2002 score < 3 (Table 4).

TABLE 1. Patients' characteristics and anthropometric measurements

Variables	Results
Age (years)*	73.57±8.25
Gender **	
Female	41 (50)
Male	41 (50)
Weight (kg)*	67.70±11.24
Height (cm)*	163.7±8.97
BMI*	22.99±3.46
ASA physical status**	
II/III/IV	50(60.98)/29 (35.37)/3(3.65)
Middle arm circumference (cm)*	25.40±4.32
Calf circumference (cm)*	41.12±6.05

Data was expressed as *Mean ±Standard Deviation (SD);**the number of patients (n) and the percentage (%). ASA: American Society of Anesthesiologist'; BMI: Body Mass Index; kg: kilograms; cm: centimeters

TABLE 2. Patients' clinical data

Variables	Results
Reason of admission[1]	
GIS oncologic surgery	52 (63.41)
Hip fracture	30 (36.59)
Outcome[1]	
Ward	48(58.54)
Intensive care unit	34(41.46)
Length of hospital stay (days)[2]	9.73±6.78
Serum albumin level (g/dL)[2]	
Preoperative	3.22±0.56
Postoperative	2.76±0.52
Serum globulin level (g/dL)[2]	
Preoperative	2.66±0.54
Postoperative	2.17±0.49
AGR[2]	
Preoperative	1.22±0.21
Postoperative	1.31±0.03
Total lymphocyte count (%)[2]	
Preoperative	15.44±8.57
Postoperative	8.85±5.16
Monocyte count (%)[2]	
Preoperative	6.76±3.25
Postoperative	6.24±3.04
LMR[2]	
Preoperative	2.52±1.53
Postoperative	1.56±1.01*

GIS: Gastrointestinal system; AGR: Albumin-globulin ratio; LMR: Lymphocyte-monocyte ratio
Data was expressed as [1]the number of patients (n) and the percentage (%) and [2]Mean ±Standard Deviation (SD);* highly significant.

TABLE 3. The correlation of MNA groups with the study parametersl (Mean ±SEM)

Variables	17<MNA (n=36)	17≤MNA<24 (n=32)	24≤MNA (n=14)	p
ICU admission (%)	77.78±7.03	15.63±6.52	7.14±7.14	*<0.0001
The presence of systemic infection (%)	52.78±8.44	6.25±4.35	0	*<0.0001
The presence of surgical site infection (%)	63.89±8.12	25.00±7.78	7.14±7.14	*<0.0001
Length of hospital stay (days)	12.89±1.48	7.47±0.54	6.79±0.37	*<0.0001
Postoperative AGR	1.26±0.05	1.33±0.05	1.42±0.09	0.225
Postoperative LMR	1.75±0.19	1.35±0.16	1.55±0.22	0.259

[1]Mann Whitney-U test; SEM: Standard Error of Mean; MNA: Mini Nutritional Assessment; ICU: Intensive care unit; AGR: Albumin-globulin ratio; LMR: Lymphocyte-monocyte ratio; * highly significant

TABLE 4. The correlation of NRS-2002 groups with the study parameters[1]

Variables	NRS-2002<3 (n=15)	NRS-2002 ≥3 (n=67)	p
ICU admission (%)	6.67±6.67	49.25±6.15	*0.002
The presence of systemic infection (%)	13.33±9.09	28.36±5.55	0.233
The presence of surgical site infection (%)	13.33±9.09	44.78±6.12	**0.024
Length of hospital stay (days)	8.33±1.44	10.04±0.86	0.380
Postoperative AGR	1.36±0.09	1.31±0.04	0.554
Postoperative LMR	1.56±0.19	1.56±0.13	0.999

[1]Student t test; SEM: Standard Error of Mean; NRS: Nutritional Risc Score; AGR: Albumin-globulin ratio; LMR: Lymphocyte-monocyte ratio; * very significant

TABLE 5. Data related to the comparison of two assessment tests in patients with malnutrition risk

Variables	17<MNA (n=36)	NRS-2002 ≥3 (n=67)	p
ICU admission (%)[1]	77.78±7.03	49.25±6.15	0.005*
The presence of systemic infection (%)[1]	52.78±8.44	28.36±5.55	0.014
The presence of surgical site infection (%)[1]	63.89±8.12	44.78±6.12	0.065
Length of hospital stay (days)[2]	12.89±1.48	10.04±0.86	0.078
Postoperative AGR[2]	1.26±0.05	1.31±0.04	0.715
Postoperative LMR[2]	1.75±0.19	1.56±0.13	0.398

[1]Student t test; [2]Mann–Whitney U test; SEM: Standard Error of Mean; NRS: Nutritional Risc Score; AGR: Albumin-globulin ratio; LMR: Lymphocyte-monocyte ratio; * very significant

Comparing the two screening tools, the incidence of ICU admission was significantly higher in patients with MNA>17 (Table 5), and MNA was a good predictor for ICU admission and the development of systemic infection (Table 6).

DISCUSSION

Elderly patients often suffer from malnutrition, which is a condition that is usually underestimated in surgical patients. However, the nutritional status is a main factor for the postoperative process. Our study indicated that patients with MNA < 17 and NRS-2002 ≥ 3 have significantly higher ICU admission rates and incidence of infection. When comparing the two screening tools, MNA has a better predictive value than NRS-2002. Regarding biochemical markers, LMR showed a significant decline after surgery, but this parameter has no correlation with the screening tools and postoperative outcome.

It is essential to assess the uniqueness of an elderly patient in order to achieve a successful perioperative management. The nutritional status has a paramount importance in the surgical risk stratification and the risk-modifying interventions that help to predict the surgical outcome. Malignancy is often associated with poor food intake. A prospective cohort study concerning elderly patients suffering from periampullary neoplasm indicated that 87% of patients were classified into the at-risk-of malnutrition or malnourished group by using the MNA screening tool. These patients showed a higher overall surgical morbidity (17).

The prevalence of malnutrition in older patients with hip fracture is higher than in older adults. This is due to the fact that there is an increased need for calories secondary to the systemic inflammatory response, poor nutrition due to pain and decrease in mobility. In a review evaluating the effect of malnutrition and nutritional treatment on outcomes and mortality in elderly patients with a hip fracture, it was stated that malnutrition increases mortality (30% within I year and up to 40% within 3 years.), affects functional recovery after the fracture, and increases health expenditures (18, 19).

Helminen et al. (20) stated in their study that they evaluated the prognosis of 594 elderly patients with hip fracture according to MNA-SF, MNA-LF, and serum albumin levels, and found that all of these tests were a strong indicator in determining short- and long-term mortality.

In our study, MNA-LF and NRS-2002 tests were used. MNA-LF was a good predictor for ICU admission and the development of systemic infection. In our study, no difference was found between preoperative and postoperative AGR. However, the LMR decreased in the postoperative period.

Norman et al. (21) found that pressure sores and infections were associated with malnutrition. Our results are consistent with the work by Norman et al. Surgical site infection rate was higher in patients with NRS-2002 ≥ 3 than in the group with NRS-2002 < 3. In patients with MNA < 17, both systemic and surgical site infection rates were high. Gurnieiro et al. (22) established that MNA-LF is suitable for predicting mortality. In our study, the incidence of ICU admission was significantly higher in patients with malnutrition according to the two nutritional assessment tests used (MNA-LF and NRS-2002).

TABLE 6. Correlation of two screening tests to the study parameters

	AUC	95% CI	Sensitivity (%)	Specivity	Cut-off	p
MNA-LF						
ICU admission	0.652	0.539-0.754	70.5	62.5	19.5	0.01*
The presence of systemic infection	0.722	0.612-0.815	90.4	49.1	20.5	0.0004*
The presence of surgical site infection	0.600	0.486-0.707	37.5	84.0	13.5	0.139
NRS-2002						
ICU admission	0.561	0.447-0.670	47.0	64.5	3	0.329
The presence of systemic infection	0.602	0.488-0.709	33.3	83.6	4	0.157
The presence of surgical site infection	0.532	0.418-0.643	25.0	82.0	4	0.622

AUC: Area Under Curve from ROC; CI: Confidence Interval, *statistically significant, MNA-LF: Mini Nutritional Assessment test-Long Form, NRS-2002: Nutritional Risk

JunDe et al. (23) compared MNA-SF, NRS-2002, biochemical markers among elderly patients and reported that MNA-SF may be a favorable test for nutritional analysis. Myoung-Ha et al. (24) compared five nutrition tests (MNA-LF, MNA-SF, GNRI, MUST, and NRS-2002) and reported that MUST is the best test for nutritional analysis. Koren-Hakim et al. (25) in their studies comparing the MNA-SF, NRS-2002, and MUST tests, found that all tests were performed well, although they reported that MNA-SF predicted readmissions and mortality better. In our study, when the two screening tests (MNA-LF and NRS-2002) were compared, the incidence of ICU admission was significantly higher in MNA-LF.

In a study where the nutritional status of 246 patients undergoing pancreotoduodenectomy was evaluated with the MNA test and biochemical markers (albumin, prealbumin, and transferrin), it was reported that malnutrition was related to poor postoperative outcomes (26).

In a retrospective, single-center study performed by Inoue et al. (27) in elderly patients with a hip fracture, who underwent MNA-SF, NRS-2002, MUST, and the Geriatric nutritional risk index (GNRI) tests prior to surgery, MNA-SF was found to be an optimal test for nutritional screening.

Limitations

We acknowledge various limitations in our study. If a patient did not know his/ her height, the investigator estimated the height value.

Our study was conducted in a single center. A multicenter study will be needed to disseminate our results to a larger patient population.

In conclusion, consequently, the nutritional status is a topic that is often overlooked in preoperative evaluations. However, measurements with easy-to-perform tests will provide guidance in terms of identifying potential perioperative risks.

Nutritional risk screening tools can indicate the negative consequences of hospitalized patients.

MNA is a good predictor for ICU admission and the development of systemic infection.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Kartal Dr. Lütfi Kırdar Training and Research Hospital Clinical Research ethics committee (2018/514/144/3).

Informed Consent: Written informed consent was obtained from patients who participated in this study

Peer-review: Externally peer-reviewed.

Author contributions: Concept - Ö.S.; Design - Ö.S.; Supervision - B.Ç.; Resource - Ö.S., B.Ç.; Materials - Ö.S., B.Ç.; Data Collection and/or Processing - Ö.S.; Analysis and/or Interpretation - Ö.S., B.Ç.; Literature Search - Ö.S., B.Ç.; Writing - Ö.S.; Critical Reviews - B.Ç.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.2291

Potential Predictive Factors for Breast Cancer Subtypes from a North Cyprus Cohort Analysis

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Cite this article as: Ulgen A, Gürkut Ö, Li W. Potential Predictive Factors for Breast Cancer Subtypes from a North Cyprus Cohort Analysis. Cyprus J Med Sci 2020; 5(4): 339-49.

BACKGROUND/AIMS
We present the first epidemiological survey from North Cyprus to determine the predictive factors for breast cancer subtypes.

MATERIAL and METHODS
More than 300 patients with breast cancer, with 90% of them having the cancer subtype information, were examined at the State Hospital in Nicosia between 2006 and 2015 for their demographic, reproductive, genetic, and epidemiological factors. The breast cancer subtypes and the estrogen receptor (ER) +/- progesterone receptor (PR) +/- and human epidermal growth factor 2 (HER2) +/- status were determined. Single and multiple variable regularized regressions, with predictive factors as independent variables and breast cancer subtypes as dependent variables, were conducted.

RESULTS
Our cohort differed significantly from larger cohorts (e.g., the Breast Cancer Family Registry) in terms of age, menopause status, age at menarche, parity, education, oral contraceptive use, and breastfeeding, but the distribution of breast cancer subtypes was not significantly different. The subtype distribution in our cohort was also not different from that of another Turkish cohort. We found that the ER+ subtype was positively related to age/postmenopause, ER+/PR+ subtype was associated positively with age but negatively with cancer stage, and HER2+ subtype that negatively correlated with ER+ and ER+/PR+ was associated positively with cancer stage but negatively with age/postmenopause.

CONCLUSION
Assuming ER+ and ER+/PR+ to have better prognostic, HER+ to have worse prognostic, then older age and postmenopause seem to be beneficial, smoking and family history of cancer seem to be detrimental. Further steps include exploring potential biomarkers and using cure models to determine long-term breast cancer survivors.

Keywords: Breast cancer subtypes, predictive factors, estrogen, progesterone, human epidermal receptors, regularized regression, LASSO, ridge, elastic nets

INTRODUCTION

Breast cancer is the most common type of cancer diagnosed in the western part of the world. In Europe, there were more than 523,000 breast cancer diagnoses and more than 138,000 deaths among women in 2018 (1). Worldwide, almost 2 million women are diagnosed with breast cancer each year, and approximately 30% of them die from this disease. Breast cancer is largely considered as a disease predominantly influenced by lifestyle-related risk factors (2), although twin studies of heritability of breast cancer have shown that genetic contribution could be significant (3). Recent research on the combined contribution from several genetic variants to breast cancer reports a >60% area-under-receiver-operator-curve prediction rate (4, 5), explaining 20% variance (6).

This study was presented at the International Genetic Epidemiology Society Meeting, 2-4 July 2020 (online meeting)
This study was presented at the European Mathematical Genetics Meeting, 16-17 April 2020 (online meeting).
This study was presented at the National and International Biostatistics Congress, 26-29 October 2019 Antalya, Turkey.

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Received: 12.06.2020
Accepted: 28.11.2020

Female hormones may affect breast cancer, and their status has been used to classify breast cancer types. In particular, estrogen receptor-positive (ER+) or -negative (ER-), progesterone receptor-positive (PR+) or -negative (PR-), and human epidermal growth factor 2-positive (HER2+) or -negative (HER-) are the major classification schemes of breast cancer subtypes. It has been demonstrated that ER+/-, PR+/- and HER+/- breast cancer subtypes have different clinical characteristics (7); the cancer etiology of these subtypes can be heterogeneous, and treatment strategies are also divergent. Especially, hormone receptor-positive (ER+ or PR+) subtype might have good prognosis using drugs such as tamoxifen/Nolvadex. Similarly, the more aggressive HER2+ subtype can be treated successfully using drugs such as trastuzumab/Herceptin. In contrast, the triple-negative subtype (ER-/PR-/HER2-) poses challenges in treatment strategies (8).

Although international and national studies of breast cancer have been conducted with large sample sizes, such as the Breast Cancer Family Registry (BCFR) (*www.bcfamilyregistry.org*), the Global Initiative for Cancer Registry Development (GICR) (*gicr.iarc.fr*), and the Breast Cancer Surveillance Consortium (BCSC) (*www.bcsc-research.org*), there has never been a breast cancer survey investigating the subtype distributions, potentially explanatory variables, and the correlation between these variables and breast cancer subtypes in North Cyprus (except some studies in Turkey) (9). To fill this research gap, we conducted the first epidemiological survey of approximately 300 patients with breast cancer from North Cyprus, among whom approximately 230 patients were Turkish Cypriots.

We collected and analyzed data regarding reproductive (age at menarche, number of children (0 for nulliparity), menopause status, hormone therapy, oral contraceptive use, breastfeeding, and left/right breast with cancer), demographic (age at diagnosis, education level, and housewife/employed), genetic (first-degree relative having cancer), and epidemiological (smoking and other cancer types) characteristics. The majority of these characteristics are known to be risk factors for breast cancer, e.g., early menarche, late menopause, nulliparity, long hormone replacement therapy, older age, and family history of breast cancer; however, it is unclear which factor is predictive for breast cancer subtypes.

Our analysis strategy is as follows: We considered ER, PR, and HER2 as dependent variables and others as independent variables. As we did not have control (non-cancer) samples, this survey was a case-only analysis or subtypes-with-case analysis (I0, II). The first analysis was conducted to compare the distribution of our independent and dependent variables with that reported in major public breast cancer databases. Second, we determined the correlation between the cancer subtypes. Third, univariate, multiple, and regularized logistic regressions were performed to detect any factor–subtype association, i.e., to identify potential predictive factors for breast cancer subtypes. Although we observed some minor surprising results, our study cohort conforms to some other studies concerning predictive factors for breast cancer subtypes.

Main Points:

- A Turkish Cypriot cohort of breast cancer patients show a similar distribution of subtypes based on hormone receptor status as other international cohorts.
- Older age and postmemopause status tend to have the ER+ or PR+ subtype with a better prognosis.
- Although having not achieved statistical significance, breastfeeding seems to be positively associated with ER+PR+ subtype, hormone therapy negatively being associated with ER+ subtype, and family history of cancer being positively associated with HER2+ subtype.

MATERIAL and METHODS

Sample collection: We included the data of 324 subjects (321 women; 3 men) collected retrospectively from Dr. Burhan Nalbantoğlu State Hospital (BNSH) in Nicosia, North Cyprus, during 2006–2015, largely from the years 2011–2015 (93%). This sample represented approximately 40% of total breast cancer cases that existed in the archives during this period. The data consisted of reproductive factors, histology, and biomarker information such as the status of ER, PR, and HER2. Permission was obtained from the Ministry of Health from the Turkish Republic of Cyprus for the scientific use of the data. In addition, ethical approval to conduct the study was obtained from the Eastern Mediterranean University Ethics Committee in Famagusta, with the IRB approval number AAAP8950. Patient consent forms were not required. Telephone interviews were conducted when necessary to collect information from patients to fill in the missing factor values.

For the study sample, pathologists from the BNSH ascertained the ER and PR status based on the immunohistochemistry (IHC) and/or pathology reports of the patients' tumor tissues using a standardized protocol and pathology reporting forms. For all cases, the available HER2 status (approximately 290 cases) was provided from patient medical reports. In cases where tumor tissue was available, pathologists used IHC testing for ER and PR and categorized tumors as ER and PR positive if ≥10% of tumor cells stained positive. When the ER or PR +/- status is not labeled, but with a specific percentage, we considered it as unknown. Menopause status and other information were extracted either from the medical records (with guidance/approval from an oncologist) or by telephone interviews.

Preprocessing of data: We excluded the three male samples, reducing the sample size from 324 to 321. Regarding the hormone receptor status, if the left/right breast had a different value, it was labeled as NA (unknown). Moreover, if the hormone receptor status was not binarized but represented by a percentage, it was labeled as NA.

Other recoding of data included (smoking) seldom=0, quit=1, x-number-pocket=1; (family history) first-degree relatives are parents, children, and siblings; (other cancer) anything not "no" was considered as yes (including metastasis); (education) 0, 1, 2, and 3 for no school, primary/middle school, high school, and college or more, respectively; (housewife/employed) retired was considered as the same as employed; (tumor stage) "high stage" was considered as 3, inoperable was considered as 4, A/B/C were ignored; (invasive cancer) invasive ductal carcinoma/invasive cribriform cancer/invasive secretory cancer were considered as invasive, and all others were considered as not invasive.

Of the 321 samples, 300 had ER or PR status information, 291 had HER2 status information, all samples had age information, but only 214, 215, 217, and 222 patients had details regarding breastfeeding, age at menarche, use of oral contraceptives, and use of hormone therapy. The amount of missing data for other factors is given in Tables 1 and 2. An independent variable was removed when the missing rate was too high (e.g., >0.2). The remaining missing values were imputed from the values of known variables (e.g., if x is the independent variable, two values are missing, they are replaced by (R code): *sample(x[!is.na(x)][1:2]).*

Turkish Cypriot and nonTurkish Cypriot patients: Of the 321 patients, 314 reported their birth country, which indicated that the majority of them were born in Cyprus (n=233), 53 were born in Turkey, and the remaining 28 were born in other countries, including UK, Turkmenistan, and Bulgaria. Although our analysis did not focus on the genetic or ethnicity contribution to the breast cancer subtypes, and foreign-born does not automatically imply non-Turkish Cypriots, we performed all analyses twice, one using 321 samples and the other using 233 Turkish Cypriot-only samples.

Statistical Analysis

All statistical analyses were conducted using either R 3.5.1 (*www.r-project.org*, released July 2018) or the Statistical Package for Social Sciences software version 17.0 (SPSS Inc.; Chicago, IL, USA). The *Rtsne* R package was used for the t-SNE analysis (*github.com/jkrijthe/Rtsne*), with default parameter settings (e.g., perplexity=30, dims=2). The *glmnet* R package (*web.stanford.edu/~hastie/glmnet*) (12) was used for the following regularized regressions: least absolute shrinkage and selection operator (LASSO; alpha=1, family="binomial"), elastic net (alpha=0.5), and ridge (alpha=1). The logistic regression was conducted using the standard R function *glm(... family=binomial(link="logit"),* and Fisher's test was conducted using the R function *fisher test*. The independent two-sample t-test between age distribution, one from raw data and the other from summary statistics, was conducted using our customized R script.

RESULTS

Visual inspection of data by t-SNE: The t-distributed stochastic neighbor embedding (t-SNE) (13) is a commonly used method

TABLE 1. Factors that are distributed differently between North Cyprus cohort and BCFR

	North Cyprus				BC Fam Registry		NC vs BCFR pv		
factor	**Whole n=321**	**ER+PR+ n=204**	**ER-PR- n=64**	**++vs- pv**	**ER+PR+ n=2486**	**ER-PR- n=920**	**all**	**++**	**--**
age	57.4±12.8	58.7±12.5	55.3± 13	0.06	47.1±9.3	44.5±9.8		**6E-29**	**1E-8**
menopause	201 (64.8%)	136 (68.3%)	36 (57.1%)	0.13	951 (40%)	310 (35%)	**5E-18**	**1E-14**	**6E-4**
Not	109 (35.2%)	63 (31.7%)	27 (42.9%)		1431 (60%)	574 (65%)			
NA	11	5	1						
menarche	13.14±1.31	13.11±1.30	13.32±1.25	0.3					
age ≤ 11	19 (8.8%)	12 (8.5%)	3 (5.7%)	0.43	528 (22%)	183 (21%)	**1E-5**	**3E-4**	6E-3
12	54 (25.1%)	40 (28.4%)	11 (20.8%)		590 (24%)	215 (24%)			
≥ 13	142 (66%)	89 (63.1%)	39 (73.6%)		1317 (54%)	482 (55%)			
NA	106	63	11						
parity	2.34±1.42	2.47±1.5	2.17±1.18	0.1					
no.kid=0	31 (10%)	21 (10.5%)	6 (9.4%)	0.44	565 (23%)	191 (21%)	**9E-8**	**8E-5**	0.04
1-2	159 (51.1%)	91 (45.5%)	35 (54.7%)		1015 (41%)	391 (42%)			
≥ 3	121 (38.9%)	88 (44%)	23 (35.9%)		906 (36%)	338 (37%)			
NA	10	4	0						
edu: < HS	174 (56.5%)	113 (57.1%)	33 (51.6%)	0.47	710 (29%)	289 (32%)	**1E-20**	**4E-15**	**0.002**
≥ HS	134 (43.5%)	85 (42.9%)	31 (48.4%)		1740 (71%)	602 (68%)			
NA	13	6	0						
OC use	76 (35%)	46 (32.4%)	19 (35.8%)	0.73	1795(73%)	680 (77%)	**3E-31**	**6E-23**	**5E-10**
no	141 (65%)	96 (67.6%)	34 (64.2%)		648(27%)	198 (23%)			
NA	104	62	11						
breast feed	168 (78.5%)	112 (80.6%)	38 (71.7%)	0.24	1359 (55%)	454 (50%)	**2E-13**	**1E-9**	0.003
No	46 (21.5%)	27 (19.4%)	15 (28.3%)		1105 (45%)	448 (50%)			
NA	107	65	11						

Factors that are significantly different between the North Cyprus cohort and the BCFR cohort: age at diagnosis, postmenopause or premenopause status, age at menarche (first occurrence of menstruation), parity (number of births), education level (HS: high school), oral contraceptive use, breastfeeding. Pv (++ vs - -) is the Fisher's test p value comparing the North Cyprus ER+PR+ vs ER-PR- group. pv (NC vs BCFR) is the Fisher's test p value comparing the North Cyprus and BCFR groups. Missing data (NA) are not counted in calculating the percentage and not used in Fisher's test. All p values smaller than 0.001 (this threshold is recommended in (37)) are marked by boldface.

Abbr.: ER; Estrogen Receptor, PR; Progesterone Receptor, HER2; Human Epidermal Growth Factor 2, BCFR; Breast Cancer Family Registry, NC; North Cyprus, edu; Education, HS; High School, OC; Oral Contraceptives

TABLE 2. Other Factors

	North Cyprus				BC Fam Registry		NC vs BCFR pv		
factor	**Whole n=321**	**ER+PR+ n=204**	**ER-PR- n=64**	**++vs- pv**	**ER+PR+ n=2486**	**ER-PR- n=920**	**all**	**++**	**--**
HT	24 (19.5%)	14 (9.7%)	7 (13%)	0.6	424 (18%)	111 (13%)	0.03	**0.0093**	1
no	198 (80.5%)	131 (90.3%)	47 (87%)		19551 (82%)	7581 (87%)			
NA	99	59	10						
fam-hist	85 (26.5%)	55 (27%)	19 (29.7%)	0.75	714 (9%)	244 (27%)	0.65	0.63	0.56
no	236 (73.5%)	149 (73%)	45 (70.3%)		1761(29%)	673 (73%)			
stage : I	60 (23.4%)	41 (24.4%)	10 (18.2%)	0.55					
2	135 (52.7%)	89 (53%)	30 (54.5%)						
3	51 (19.9%)	31 (18.5%)	14 (25.5%)						
4	10 (3.9%)	7 (4.2%)	1 (1.8%)						
NA	65	36	9						
otherC	17 (5.3%)	11 (5.4%)	4 (6.3%)	0.76					
no	304 (94.7%)	193 (94.6%)	60 (93.8%)						
smoke	84 (26.2%)	53 (26%)	20 (31.2%)	0.42					
no	237 (73.8%)	151 (74%)	44 (68.8%)						
breast : L	162 (50.6%)	112 (80.6%)	38 (71.7%)	0.27					
R	149 (46.6%)	27 (19.4%)	15 (28.3%)						
both	9 (2.8%)	7 (3.4%)	0 (0%)						
NA	1	0	0						
housewife	118 (42.8%)	83 (45.9%)	21 (36.8%)	0.28					
employed	158 (57.2%)	98 (54.1%)	36 (63.2%)						
NA	45	33	7						
invasive	237 (75.5%)	155(76.4%)	48 (75%)	0.87					
no	77 (24.5%)	48 (23.6%)	16 (25%)						
NA	7	1	0						

Similar to Table 1, a list of factors that are not significantly different between the North Cyprus cohort and the BCFR cohort: hormone therapy (HT) and family breast cancer history. Other factors do not have available data in BCFR: tumor stage, whether the patient has other cancers, smoking, left (L) or right (R) breast or both with cancer, housewife or employed, invasive cancer. (note 1) never or former menopausal HT use.
Abbr.: ER; Estrogen Receptor, PR; Progesterone Receptor, HER2; Human Epidermal Growth Factor 2, BCFR; Breast Cancer Family Registry, NC; North Cyprus, BC; Breast Cancer, HT; Hormone Replacement, otherC; other Cancers

to represent high-dimensional data in two or three dimensions. We had previously used this technique in other applications in biology/genomics (14, 15).

In this study, we used three dependent variables (ER, PR, and HER2), five quantitative independent variables (age at diagnosis, age at menarche, number of children, education level, and cancer stage), and ten binary independent variables (left/right breast, menopause, first-degree relative with cancer, other cancer, smoking, hormone therapy, oral contraceptive use, breastfeeding, housewife/employed, and cancer invasiveness). The quantitative variables were standardized to have zero-mean and unit-variance (*z*-transformation).

Due to high missing rates for age at menarche (33%), hormone therapy (31%), oral contraceptive use (32%), and breastfeeding (33%), we retained only those samples that had information on these factors. This reduced the sample size from 321 to 211 for the t-SNE plot. Other missing data (of much lower missing rate) were imputed for these 211 patients.

Figure 1 shows one run of t-SNE. Because ER, PR, and HER2 are a component of the input, it is not surprising that their values are well partitioned in the plot (e.g., ER+ and ER- samples). It was observed that ER+ samples tended to be PR+, and HER2- and ER- samples tended to be PR- and HER2+. The seven samples with other cancers (including metastasis) formed a distinct cluster from the remaining samples. Although ER, PR, and HER2 values separated in an up-down direction in Figure 1, other factors such as menopause status, breastfeeding, and age appeared to be separated in (not completely) an orthogonal direction.

The nonTurkish Cypriots are marked with different symbols (Turkey-born patients in circles, other foreign-country-born patients in crosses) in the top two rows of Figure 1. There was no evidence indicating that the location of these points in the plot, or their collective features, are highly different from the remaining Turkish-Cypriot samples.

Distribution of patient factors: Table 1 shows that our study cohort (n=321) is distinct from the BCFR sample, which consisted of

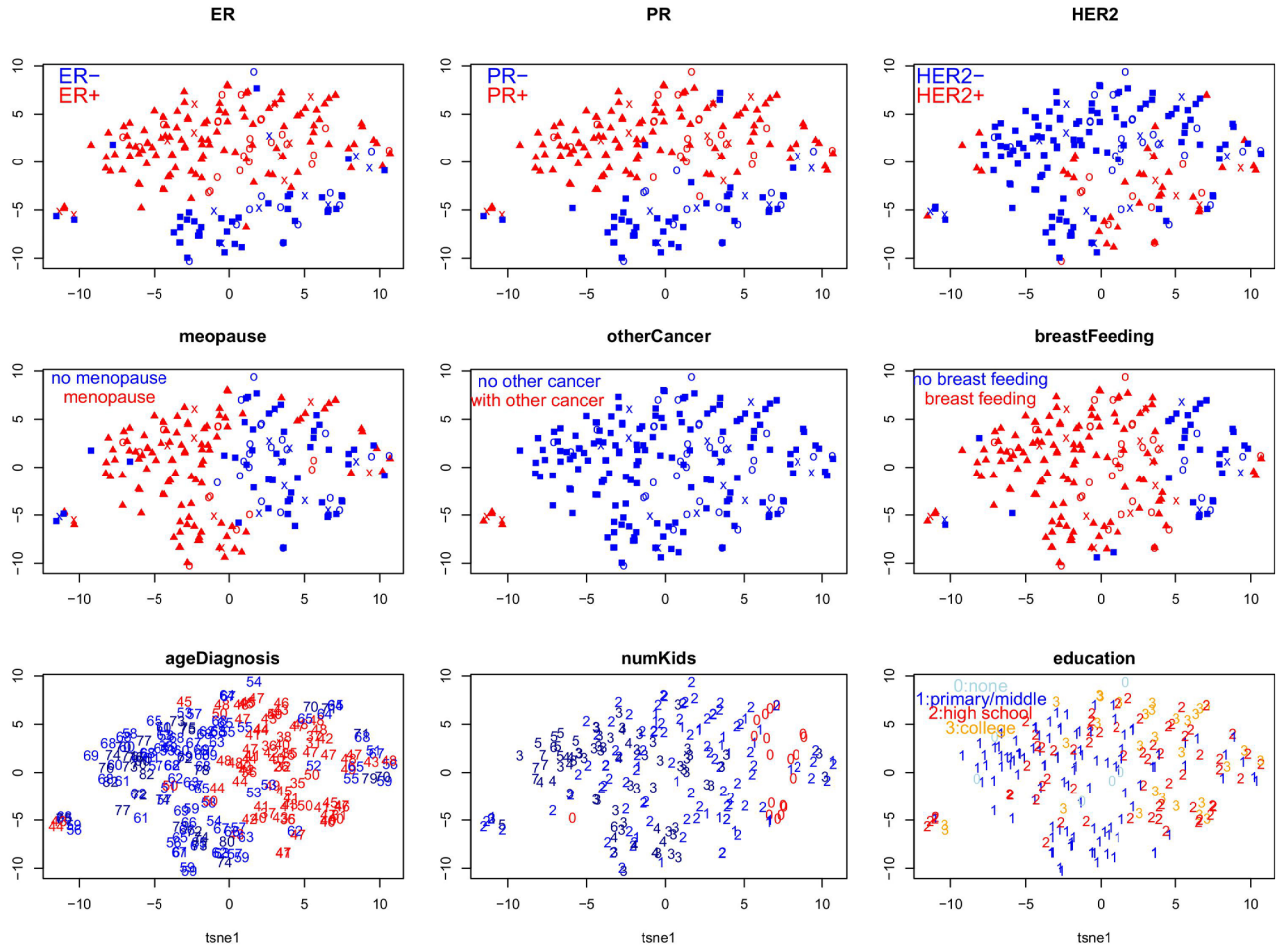

FIGURE I. t-SNE plot of North Cyprus samples. t-SNE plot of 2II breast cancer patients (out of 32I total) with enough non-missing factor values. The nine subplots are the same plot labeled with different information: ER subtype (red for ER+, blue for ER-), PR subtype, HER2 subtype, menopause status (post-menopause in red, pre-menopause in blue), if the patient has other cancer (red for yes, blue for no), breast feeding (red for yes, blue for no), age of diagnosis (red if younger or equal to 50 years old), parity/number of children, education level (0 for none, I for primary or middle school, 2 for high school, 3 for college or higher). The Turkey-born samples are marked with circle, and other foreign born samples are in crosses

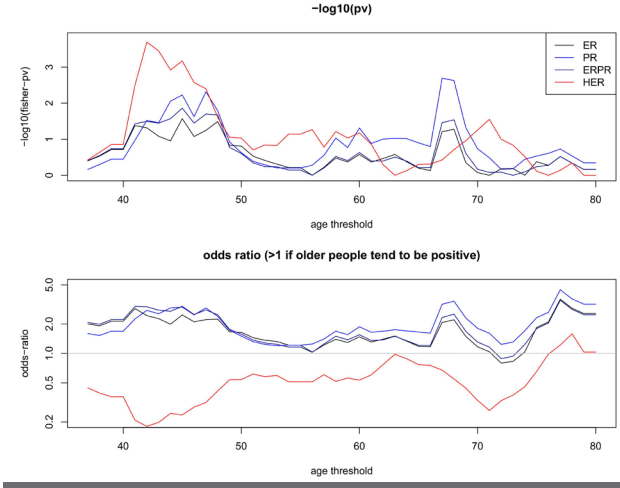

FIGURE 2. Tuning age threshold to convert age to age-group. (top) Fisher's p-value (in minus log with base I0) of age-group vs breast cancer subtype (ER, or PR, or ER/PR, or HER2) as a function of the age threshold. (bottom) odds-ratio as a function of the age threshold

subjects of primarily USA/Canada/Australia origin, in terms of several demographic or reproductive factors. The North Cyprus cohort was older in age, had lower education level and less use of oral contraceptives, and had greater number of postmenopausal subjects, lesser number of subjects with young (≤II years) age at menarche, fewer nulliparous subjects, and greater number of breastfeeding subjects.

The same analysis was conducted on the 233 Turkish-Cypriot-only patients, and the same results were obtained (data not shown).

There are two explanations for these significant differences. The first is due to the cultural and customary differences between countries (e.g.,, use of oral contraceptives). The second explanation is that our study sample was collected from the state hospital, and a higher percentage of affluent patients may opt for treatment at private hospitals or hospitals overseas. The differences remained even for the ER+/PR+ subgroup and for the ER-/PR- subgroup (although less significant due to smaller sample sizes).

Within our North Cyprus cohort, when the ER+/PR+ and ER-/PR- groups were compared in terms of these factors, only the ER-/PR- group was slightly younger (t-test p value=0.06) (Table I). The differences in other factors were not yet significant, probably because either they were actually not different or there was no sufficient sample size to confirm the difference.

TABLE 3. Count and testing of ER, PR, HER2 distributions

(A) Distribution of breast cancer hormone receptor subtypes in North Cyprus cohort

total sample/ Turkish Cypriot N=290/212	HER2- (n=220/166, 75.9%/78.3%)		HER2+ (n=70/46, 24.1%/21.7%)	
	PR-	PR+	PR-	PR+
ER- (n=74, 25.5% /n=52, 24.5%)	40 (triple negative)/32	8/5	23 (HER2+ only)/14	3/1
ER+ (n=216, 74.5% /n=160, 75.5%)	9/6	163/123	9/6	35/25
	PR- (n=81, 27.9%/n=58, 24.5%)			
	PR+ (n=209, 72.1%/n=154, 72.6%)			

(B) Distribution of ER and PR subtypes in BCFR

total sample (N=4011)	PR- (n=1317, 32.8%)	PR+ (n=2694, 67.2%)
ER- (n=1128, 28.1%)	920	208
ER+ (n=2883, 71.9%)	397	2486

(C) Distribution of HER2 subtypes in BCFR

total sample (N=792)	HER2- (n=607, 76.6%)	HER2+ (n=185, 23.4%)
ER- and PR- (n=206, 26%)	139 (triple negative)	67
ER+ and/or PR+ (n=586, 74%)	468	118

(D) Fisher test p-value between Cyprus cohort and BCFR/BCAC

subtype	BCFR vs n=321 set	BCFR vs Turkish Cypriot n=233
ER+ vs ER-	0.38	0.27
PR+ vs PR-	0.09	0.1
ER+/PR+ vs ER-/PR-	0.81	0.61
4 ER/PR group	0.08	0.04
HER2+ vs HER2	0.81	0.65
triple-negative vs not	0.17	0.47
HER/(ER-PR- or not)	0.49	0.65
triple-negative vs not (BCAC)	0.79	0.48
HER/(ER-PR- or not) (BCAC)	0.44	0.69

(A) Breast cancer subtype counts in the North Cyprus cohort (using either all samples or Turkish-Cypriot-only samples). Hormone receptor-positive (including luminal A and luminal B) consists of 8+9+163=180 counts (62.1%) if all samples are used; and (5 + 6 + 123) / 212=63.2% if Turkish Cypriot samples are used. HER2+ and hormone receptor-positive consists of 3 + 9 + 35=47 counts (16.2%) or (1 + 6 + 25) / 212=15.1% if only Turkish Cypriot samples are used. (B) ER and PR subtype distribution in BCFR (data taken from (17). (C) HER2 subtype distribution in BCFR (data taken from (17). (D) Fisher's test p value of subtype distribution difference between BCFR (or BCAC for the last two rows) and Cyprus cohorts (all and Turkish-Cypriot-only sample).
Abbr.: ER; Estrogen Receptor, PR; Progesterone Receptor, HER2; Human Epidermal Growth Factor 2, BCFR; Breast Cancer Family Registry, BCAC; Breast Cancer Association Consortium

There were also some other factors distributed not very differently between our cohort and the BCFR cohort, as summarized in Table 2. These factors included hormone therapy usage and having a first-degree relative with cancer. The remaining factors shown in Table 2 did not have the information corresponding to the BCFR, including other cancers, smoking status, left/right breast with cancer, housewife/employed, and cancer invasiveness. Only for the ER+/PR+ subtype, the North Cyprus cohort was significantly less likely to have hormone therapy than the BCFR samples.

We also examined the correlation between factors. Using the data of all patients with breast cancer without considering the subtypes, the following correlations were observed: (1) patients who breastfeed are less likely to undergo hormone therapy (OR=7.1, Fisher's p value 9×10^{-5}), (2) patients who are employed are more likely to smoke than housewives (OR=3.1, p value 1.3×10^{-4}), and (3) patients who are employed are more likely to be in premenopause than housewives (OR=2.8, p value 1.3×10^{-4}).

Conversion of age into age group: Age is a special factor different from others because it is a continuous variable spanning a wide range of values. Discretizing or categorizing a continuous variable is an involved topic by itself. Age is a well-known target for such categorization (16). We categorized the study sample into younger and older age groups based on an age threshold. When an age threshold is chosen, a 2-by-2 count table can be constructed according to the binary age group and binary breast cancer subtype. Age group versus breast cancer subtype association can be judged using Fisher's test.

Figure 2 shows (-log) Fisher's p value (top) and odds ratio (bottom) as a function of age threshold for converting age into age group. For HER2, the best p value was 0.0002 when the age threshold was 42, and there was a broad range of age threshold (41–47) where the Fisher's test was significant at 0.01 level. For ER, PR, and ER/PR, this age threshold range also led to some significant test results, indicating that patients younger than mid-40s may form a distinct group, which tended to be ER, PR, and ER/PR negative and HER2 positive. At the age threshold of 67–68, there was a second peak, indicating that patients older than that age tended to be ER, PR, and ER/PR positive.

Distribution of breast cancer subtypes: We compared the hormone receptor-defined subtype distribution between the North Cyprus cohort and the BCFR cohort as shown in Table 3. The count in each of the eight ER/PR/HER2 subtypes in the n=321 set and the n=233 Turkish-Cypriot-only set is listed in Table 3(A). These counts were not available for the BCFR cohort (17), but the distribution according to ER/PR subtypes and that according to HER2/(ER-PR- and not) subtypes were available, which are reproduced in Table 3(B) and (C).

The Fisher's test for the following subtype groupings was conducted: ER, PR, ER+/PR+ vs ER-/PR-, four ER/PR groups, HER2, triple-negative vs remaining, and the p values are listed in Table 3(D). The lack of a significant difference in subtype distribution between the North Cyprus cohort and the BCFR cohort, indicating certain similarity, is in strong contrast to the dissimilarity of several demographic and reproductive factors as shown in Table 1.

The largest difference was probably in the proportion of PR subtypes (higher PR+ proportion in the North Cyprus cohort than in the BCFR cohort), which may also cause a relatively larger difference for the four ER/PR groups. The highly significant correlation between ER and PR may make PR measurement redundant. In fact, it has been argued that the added value of PR is questionable (18). More specifically, the ER-/PR+ subtype is rare and may not be reproducible (i.e., it can be reclassified into another subtype by another method) (18).

TABLE 4. Significant factors in univariate or multiple logistic regression (and the corresponding p-values)

factor	ER	PR	HER2	ER	PR	HER2
		all-samples		Turkish Cypriot		
age	0.024	0.025	0.0018(-)		0.034	0.019
menopause	0.0076		0.0016 (-)	0.065		0.0023
	0.08 (multiple)		0.08 (multiple) (-)			0.017 (multiple)
numkids		0.037				
famhist			0.0078			
			0.018 (multiple)			0.017 (multiple)
smoking						0.017

Factors that are significantly (at 0.05 level) related to breast cancer subtypes according to either single variable or multiple logistic regressions using either all samples or Turkish-Cypriot-only samples. The values are single variable logistic regression p values (or multiple variate if marked). "famhist" refers to the presence of any cancer (not necessarily breast cancer) in any first-degree relative.
Abbr. ER;
Abbr.: ER; Estrogen Receptor, PR; Progesterone Receptor, HER2; Human Epidermal Growth Factor 2, famhist; Presence of any cancer (not necessarily breast cancer) in any first-degree relative

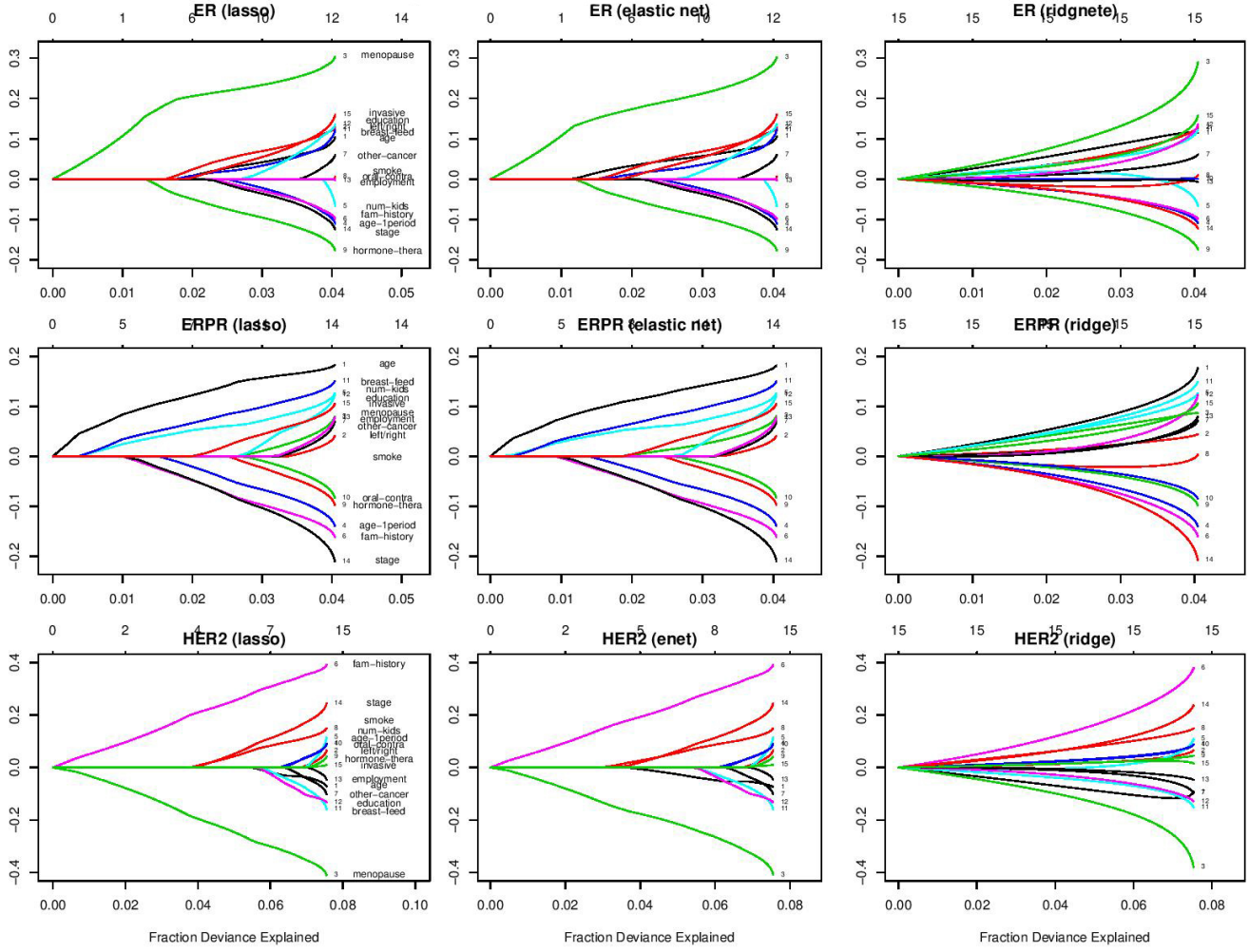

FIGURE 3. Regularized regression on ER, ER/PR and HER2. Variable tracing/selecting plot of LASSO, elastic net, ridge logistic regressions (columns I-3) for ER, ER/PR, and HER2 (rows I-3). Each line is a factor, and positive x direction represents a more relaxed constraint, allowing more variables. The y axis is the coefficient of a factor/variable: positive (negative) coefficient means a positive (negative) correlation between the factor and the subtype status (ER+, ER+PR+, HER2+ are I's, ER-, PR-, HER2- are 0's). The x axis is deviance explained by the (regularized) logistic regression

The distribution of breast cancer subtype, defined as the combination of HER2 and ER-PR- (see, e.g.,, Table 3 (C)), was also strikingly similar between our cohort and the cohort of the Breast Cancer Association Consortium (BCAC) conducted in the UK (19), which primarily consisted of Europeans/Caucasians. The subtype information was obtained from (20), and the Fisher's p value for testing triple-negative-only proportion in the two cohorts was not significant (Table 3 (D)). The testing result for the

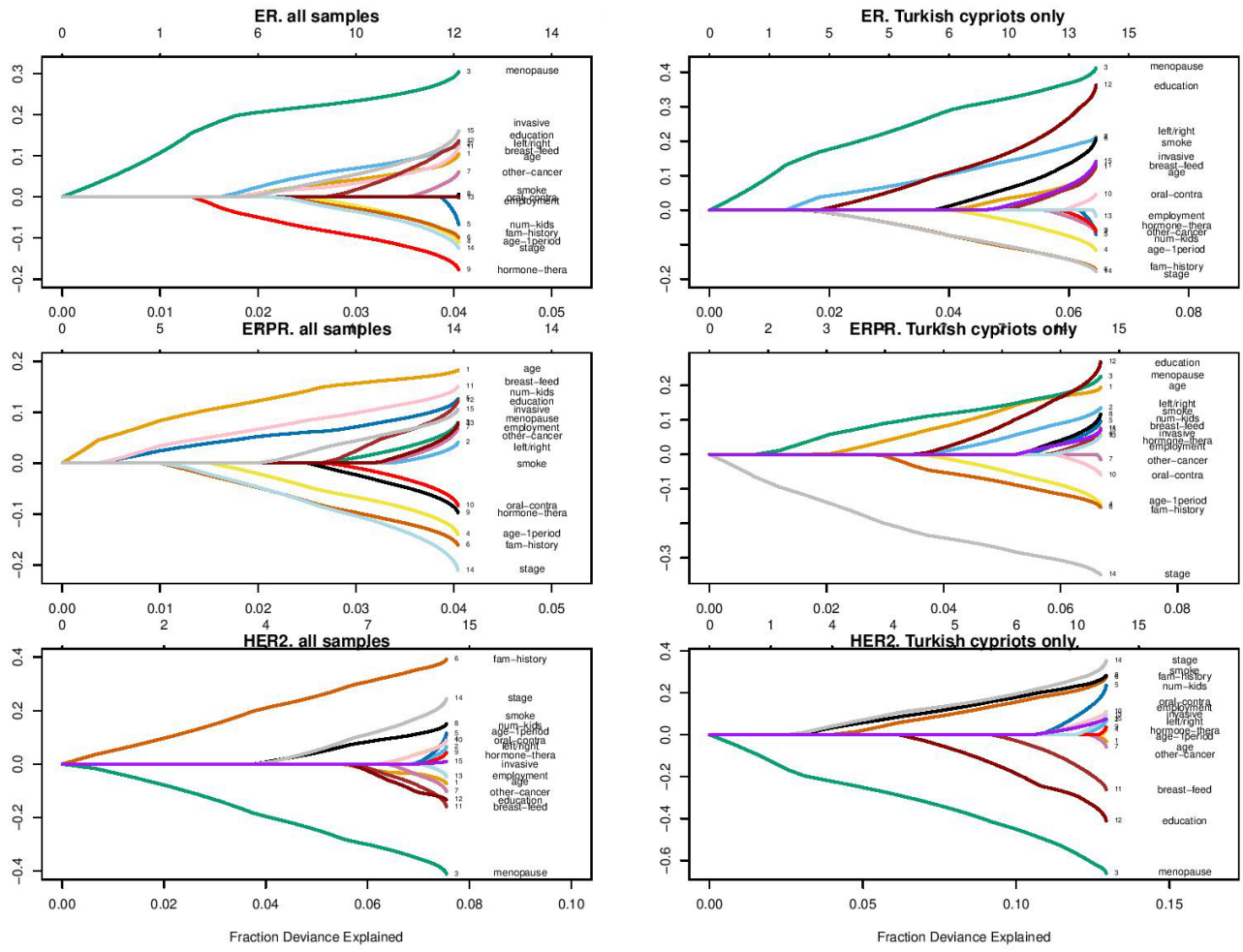

FIGURE 4. Comparison of LASSO regression between n=321 and n=233 sets. LASSO regression results for all n=321 patients (left) and for n=233 Turkish Cypriot patients (right), for ER, ER/PR, HER2 (rows 1-3). See Fig. 3 for more explanation of the y and x axes

proportions of four subtypes (triple-negative, HER2+ only, ER+ and/or PR+ and HER2+, and ER+ and/or PR+ and HER2-) was also not significant.

Predictive factors for breast cancer subtypes: The comparison of factor values between ER+ or PR+ and ER- or PR- samples can also be cast into a regression of ER/PR (dependent variable) over individual factors (independent variables). Table 4 shows all the results that are significant at the 0.1 level from regressing ER or PR or HER2 by either single factor using univariate logistic regression or all factors using multiple logistic regression, and for the n=321 sample and for the n=233 Turkish-Cypriot-only sample.

As shown in Table 4, age correlated positively with ER+ and PR+ but negatively with HER2+. These results are similar to those shown in Table 1 as well as Figure 2, where ER+PR+ patients are older in age. Due to the positive correlation between ER and PR, ER+ patients and HER2- patients are older in age.

Table 4 also shows that postmenopause status correlated positively with ER+ but negatively with HER2+. It can be stated that menopause plays a protective role as postmenopause patients are more likely to be in the more curable ER+ subtype and less likely to have the worse prognosis ER- type (21). The positive correlation between menopause status and age is self-explanatory, and the association between menopause status and ER or HER2 can also be easily explained by age. The last three minor conclusions are that HER2+ patients are more likely to have a first-degree relative with cancer, PR+ patients tend to have more children, and HER2+ patients tend to be smokers.

Between univariate and multiple regressions, we also applied three closely related regularized regressions, LASSO, elastic net, and ridge (22), to analyze the situation with a few explanatory variables. The regularized regressions accomplished the task of variable selection [e.g., (23, 24)] by imposing constraint on the sum of the absolute value of all fitting coefficients, effectively setting several coefficients to be zero, thus removing the contribution from these variables. Figure 3 shows how the coefficient of each explanatory variable increases, from left to right, when the number of nonzero-coefficient variables increases, for (rows) the dependent variables of ER, ER/PR, and HER2, and for (columns) LASSO, elastic net, and ridge.

First, we observed that LASSO, elastic net, and ridge regression showed a similar trend as that of the breast cancer subtype. Therefore, we might focus only on the LASSO plot, which is shown in the first column in Figure 3. Second, to observe how the n=321 dataset may differ from the n=233 Turkish-Cypriot-only dataset, we plotted the LASSO results side by side in Figure 4.

For the ER subtype, the dominant contribution from menopause status was not only consistently observed for both the n=321 and n=233 datasets, but it was also consistent with Table 4. Hormone therapy was a promising signal for the n=321 dataset but not for the n=233 dataset. For ER/PR, the cancer stage was a consistent signal that negatively correlated with the ER+/PR+ status. This signal was also emphasized in the BCFR article (17). Breastfeeding was a promising signal for the n=321 dataset, but it appeared to be less important for the n=233 dataset. Further discussion on the benefits of breastfeeding in reducing the probability of acquiring poor prognostic breast cancers, such as triple-negative subtypes, has been described previously (25, 26). For the HER2 subtype, similar to univariate and multiple regressions (Table 4), menopause status was a dominant factor with negative correlation. Interestingly, there was a signal for the n=321 dataset from first-degree-relative cancer history, but that signal became weaker for the n=233 dataset.

DISCUSSION

Without using control samples, we conducted a case-only analysis of potential predictive factors for different subtypes of breast cancer. The case-only design has been implemented in previous breast cancer studies, and it is "an important initial step in understanding the extent of etiologic heterogeneity between tumor subtypes" (10, 11). Because different subtypes of breast cancer have different prognoses, it is important to evaluate their distribution.

One of the striking results we obtained in this study was that our North Cyprus cohort had highly similar ER+, ER+/PR+, and HER2+ status as that of the BCFR cohort, even though the our cohort was much older in age and had greater number of postmenopausal subjects, lower education status, less use of hormone therapy, and greater number of breastfeeding subjects. If older age/postmenopausal patients tend to have ER+, our older cohort should have a higher proportion of the ER+ subtype than the BCFR cohort. Although it was in fact the case (74.5% ER+ in the n=321 North Cyprus dataset and 71.9% ER+ in the BCFR dataset), the difference in the underlying factors (age or menopause status) was highly significant between the two cohorts, but it was not significant in the ER distributions.

This aspect can be discussed in general terms, i.e., can the correlation at one level be translated into correlation at another level? In our study, we examined the potential similarity/dissimilarity of the distribution of a factor in two datasets (low-level), and contemplated whether it can be translated into the similarity/dissimilarity of the distribution of a subtype affected by these predictive factors (high-level) in those two datasets. In our previous investigation of a very different issue, i.e., the linkage/association analysis of multiple correlated phenotypes in a lipid panel, we had observed that the correlation at the high-level (phenotype) does not necessarily translate into a correlation at the low-level (linkage disequilibrium or colocalization between genetic variants) (27).

The causal link between the two levels could also be not sufficiently strong to translate correlation from one level to another. In our LASSO analysis (Figure 3, 4), it can be observed that the fraction of the explained deviation (range of *x*-axis) of ER, ER/PR, and HER2 is at the most a few percentage, even using all factors. Random forest run on the same data also showed that the classification rate in terms of the ER or ER/PR or HER2 status was not high, i.e., on average, it was scarcely >50% (results not shown). This highlights the fact that several true predictive factors for breast cancer subtypes are not yet included in our data (e.g.,., body mass index (BMI)), and moreover, the known genetic causes of breast cancer (e.g.,., BRCA1 and BRCA2) are not part of the analysis.

In a recent systematic meta-analysis of African breast cancer subtypes (28), it was found that the proportion of ER+ and PR+ samples fluctuated significantly from study to study. There are also data indicating that the triple-negative subtype rate is much higher in African women than in European/Caucasian women (29, 30). To double-check whether the breast cancer subtype distribution in our North Cyprus cohort was still the same as in another study, we selected a published summary statistics from a southeastern Turkish cohort (31). The ER+, ER+/PR+, and HER2+ proportions in the Turkish cohort were 73.5%, 81.8%, and 30.4% compared to the proportions of 74.5%, 75.9%, and 24.1% in our North Cyprus cohort, respectively, leading to Fisher's test p values of 0.8, 0.086, and 0.076, respectively (number of samples in the Turkish cohort with the subtype information: 438, 437, and 434, respectively). These differences are within the ranges and are not significant.

It could be of great interest to compare our breast cancer subtypes statistics with those of a Greek Cypriot cohort. We found two surveys on breast cancer in Greek Cypriots, one with 1100 patients conducted from 1999 to 2005 (32) and another with more than 4000 patients conducted from 2005 to 2013 (33). Unfortunately, there was no hormone receptor subtype information for the Greek Cypriot cohorts in both time periods. However, we could compare the distribution of other factors when the comparable data are available. We found that for the 1995–2005 period, the distribution of children was almost identical between the Greek and Turkish Cypriot cohorts; age and smoking status were not significantly different; and Greek Cypriot patients had higher education level (p value=1×10^{-8}), less incidence of family breast cancer history (p value=5×10^{-7}), more early (age ≤11 years) menarche (p=0.04), less breastfeeding (p=0.02), and less use of oral contraceptives (p=0.004). The Greek Cypriots 2005-2013 cohort had more patients with invasive type of breast cancer compared to the Turkish Cypriots (p-value=1E-14), but level of smoking status was comparable (not significant).

Our regularized regressions (LASSO, elastic net, and ridge) (Figure 3, 4) revealed potential predictive factors for breast cancer subtypes. However, these weak signals can only be considered as a "trend" that has not yet been confirmed by statistical tests, as those shown in Table 4. In a previous study (34), the risk of benign disease proliferation was found to be higher in patients with the ER+ subtype than in patients with the ER- subtype. This finding can be compared to the positive contribution observed in our study from other cancers (including metastasis) and the ER+ or ER+/PR+ subtype (Figure 4). In another previous research (17), breastfeeding was not associated with the ER-/PR- subtype, which can be compared with our result that breastfeeding positively correlated with the ER+/PR+ subtype. In the study conducted by (21), it was observed that the ER- cancer rate stopped increasing at a certain age, whereas the ER+

rate continued to increase. This observation can be compared to our result that postmenopause positively correlated with the ER+ subtype (Figure 4). Colditz et al. (35) reported significant differences in age, menopause status, and past use of hormone therapy in four ER/PR groups. Yang et al. (36) found that early age at menarche (≤12 years) was less common in the PR- group than in the PR+ group, and this was also true in our data comparing the ER-/PR- and ER+/PR+ groups. To summarize, many of the predictive factors for breast cancer subtypes observed in our study are consistent with those reported in the literature. The positive correlation observed between cancer family history and HER2+ subtype (Figure 4) remains intriguing.

In conclusion, we used a unique cohort of breast cancer in an understudied population to examine the breast cancer subtypes and related factors. A simplified analysis framework was used, keeping the breast cancer subtypes at one level and all factors at another level. The distribution of several factors was extremely different from that of another large breast cancer registry, whereas the subtype distribution was similar. This indirectly shows that we have not exhaustively measured all the predictive factors for breast cancer subtypes. The relationship between the two levels was investigated by regression using one variable, all variables, or a subset of variables. These regression analyses indicated that postmenopause and/or older age patients with breast cancer are more likely to have the ER+ subtype and the HER2- subtype. We also observed several other trends that need to be validated in a larger cohort.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Columbia University, NY, USA and Eastern Mediterranean University (IRB approval number AAAP8950).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept - A.U.; Design - A.U.; Supervision - W.L.; Resource - O.G.; Materials - A.U., W.L.; Data Collection and/or Processing - O.G., A.U.; Analysis and/or Interpretation - A.U., W.L.; Literature Search - A.U., W.L.; Writing - A.U., W.L.; Critical Reviews - O.G.

Acknowledgments: The authors would like to thank the Ministry of Health at the Turkish Republic of Northern Cyprus for granting access to the breast cancer archives at the Burhan Nalbantoğlu State Hospital in Nicosia and Dr. Nilay Acar, Dr. Mehmet Ali Alpdoğan, Dr. Fuat Ağlarcan, and Dr. Whitney A. Onuorah from the School of Medicine, Eastern Mediterranean University, for their help and guidance in data collection. The authors would like to thank Prof. Mary Terry Beth and her colleagues at the BCFR at the Department of Epidemiology at Columbia University in New York and the Robert S Boas Center for Genomics and Human Genetics for their help and support.

Conflict of Interest: Authors have no conflicts of interest to declare.

Financial Disclosure: This study was supported through the Fulbright Visiting Research Scholarship Grant by the US Department of State.

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2020.2355

Complete Removal of the Infrapatellar Fat Pad in Total Knee Arthroplasty Does Not Affect the Patellar Height

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Cite this article as: Güler S, Capkin S, Özmanevra R. Complete Removal of the Infrapatellar Fat Pad in Total Knee Arthroplasty Does Not Affect the Patellar Height. Cyprus J Med Sci 2020; 5(4): 350-4.

BACKGROUND/AIMS
Most orthopedic surgeons tend to remove the infrapatellar fat pad (IPFP) in total knee arthroplasty (TKA). The present study was designed with the aim of determining whether complete removal of the IPFP during TKA affects the patella height.

MATERIAL and METHODS
Knee radiographs of 85 patients who underwent primary TKA that involved complete IPFP removal were reviewed retrospectively. We measured the patella height using the Insall–Salvati (IS) ratio, modified IS (mIS) ratio, Cathon–Deschamp (CD) index, and Blackburne–Peel (BP) index on preoperative radiographs and at the I-year follow-up. Two orthopedic surgeons independently analyzed all 85 radiographs and were blinded to the measurement taken by the other surgeon. The average values of the data recorded by the two observers was used in the final analyses.

RESULTS
The average preoperative patella height was higher than the mean patella height at I year after TKA using all the measurement methods. Although this may reflect patellar tendon shortening or elevation of the joint line, this decrease was not significant using all methods ($p>0.05$).

CONCLUSION
Complete removal of the IPFP during TKA for patients with primary osteoarthritis did not affect the patellar height at the I-year follow-up.

Keywords: Total knee arthroplasty, fat pad, height, patella, complete, removal

INTRODUCTION

Hoffa's fat pad, commonly called the infrapatellar fat pad (IPFP), is an extra-synovial structure that consists of fibrous adipose tissue and is located between the synovium and joint capsule (I). Generally, it is partially or completely removed during total knee arthroplasty (TKA) to allow better visualization of the lateral compartment of the joint (2, 3). However, the results of IPFP removal are unclear, and the issue of preserving or removing the IPFP remains controversial (4). IPFP removal may cause shortening of the patellar tendon, increase anterior knee pain, and reduce flexion (2-5). Complete removal of IPFP during TKA may cause injury to the lateral genicular artery and damage the vascular supply of the patellar tendon, causing tendon scarring (5). Patellar tendon shortening and scarring of the IPFP can result in a patella baja after TKA (3, 5). Patella baja (PB) is identified by abnormally low placement of the patella on lateral radiography compared to the femoral trochlea. Reduced patella height is a common finding after TKA and is mainly caused by shortening of the patellar tendon or elevation of the joint line (6). In the literature, various methods have been suggested to measure the height of the patella using pre-TKA and post-TKA radiography (7).

Patellar tendon shortening is a known complication after TKA; however, there is no consensus about the role perioperative IPFP removal in patellar tendon shortening in the literature (8). Some previous studies have reported that the patellar tendon shortened after IPFP removal, while others have shown no effect of IPFP removal on the patellar tendon length

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Received: 26.06.2020
Accepted: 06.09.2020

(2, 5, 9-11). Considering the increase in the number of patients undergoing TKA, there is no consensus on the effect of complete removal of the IPFP on patellar tendon shortening that reflects as a decrease in the patellar height. Therefore, we designed this study to determine whether complete removal of the IPFP during TKA affects the patella height before and after TKA. We hypothesized that complete removal of the IPFP may affect the patella height after TKA.

MATERIAL and METHODS

We retrospectively reviewed the electronic medical records of 112 patients who underwent primary TKA from January 2017 to April 2019 for potential inclusion in the study. We enrolled patients of any age and sex who had undergone primary TKA for primary knee osteoarthritis and for whom non-weight-bearing lateral knee radiographs taken in at at least 30° flexion preoperatively and first-year postoperatively were available. Patients with lateral release, patellar resurfacing, any other prior surgery in the same knee, ; any systemic inflammatory disease, ; a history of revision surgery for periprosthetic infection, fracture, or other causes, ; bilateral TKA, ; and patellar tendon injury during TKA who had undergone TKA for causes other than primary osteoarthritis were excluded. We also excluded patients for whom knee radiographs performed at at least 30° flexion before and after the operation were available and those whose knees were over-rotated, whose radiographs were not visible, and those who disrupted the correct measurement of patellar height. Furthermore, we did not include the data of patients who died during the postoperative follow-up period or were lost to follow-up. Of the 112 patients who were identified as potential study subjects, finally, 27 were excluded as per these criteria. Thus, the data of 85 patients were included in the final analyses Ethical approval was obtained from the local ethics committee Aksaray University Human Research Ethical Committee (2020/06-12), and the need for informed consent of the patients was waived owing to the retrospective nature of the study.

Surgical Technique

All the surgical procedures were performed by the first author under spinal anesthesia (S.G.). In all the procedures, a medial parapatellar approach was preferred, a tourniquet was used, and the IPFP was removed completely for all patients. The prosthesis used in all the patients was the Vanguard® Complete Knee System manufactured by Zimmer Biomet in Warsaw, Indiana. A cemented TKA that has fixed-bearing insert and sacrificing posterior cruciate ligament were used in all patients. The patellar surface was not replaced in any patient.

Main Points:

- Hoffa's fat pad, commonly known as the infrapatellar fat pad (IPFP) is an extra-synovial structure that consists of fibrous adipose tissue and is partially or completely removed during total knee arthroplasty (TKA) to allow better visualization of the lateral compartment of the joint.
- The results of IPFP removal are unclear, and the issue of preserving or removing the IPFP remains controversial.
- Patellar tendon shortening is a known complication after TKA; however, there is no consensus about the contribution of IPFP removal during surgery in the literature.
- We conclude that complete removal of the IPFP during TKA for primary osteoarthritis did not affect the patellar height at the 1-year follow-up.

Assessment of Radiologic Parameters

Radiological measurements were performed using the lateral view of the knee at 30° flexion preoperatively and at 1 y postoperatively. All the measurements were obtained electronically using lateral knee radiographs with the picture archiving and communication system (Infinity Healthcare Co., Seoul, South Korea). Two orthopedic surgeons independently analyzed all 85 radiographs and were blinded to each other's measurements. The average values of the data recorded by the two surgeons were used for the final analyses. The patella height was measured on radiographs using the Insall-Salvati (IS) ratio, modified IS (mIS) ratio, Blackburne–Peel (BP) index, and Caton–Deschamps (CD) index. In order to calibrate the patellar height measurements and minimize enlargement errors, the diameter of the femoral shaft was measured as a constant value at 8 cm proximal to the combination of the condyles and femoral shaft on each lateral radiograph, as previously described (2).

For each radiograph, the preoperative patellar height was measured using the following four distinct methods: IS, mIS, BP, and CD, as described in their respective original publications (12-15). The IS includes the ratio of the length of the patellar tendon (the distance between the distal pole of the patella and the tibial tuberosity) to the maximum length of the patella (between the distal pole and the proximal pole of the patella) (Figure 1a). Normal values range from 0.8 to 1.2 (12). The mIS ratio consists of the ratio of the distance between the tibial tuberosity and the articular surface of the patellar distal pole to the length of the articular surface of the patella (Figure 1b). A ratio>2 indicates a patella alta (13). The BP index is the ratio of the perpendicular height between the anterior superior corner of the tibia and the inferior aspect of the patellar articular surface to the length of the articular surface of the patella (Figure 1c). Normal values range from 0.54 to 1.06 (14). The CD index comprises the ratio of the distance between the antero-superior point of the tibial plateau and the distal pole of the patellar articular surface to the length of the articular surface of the patella (Figure 1d). Normal values range from 0.6 to 1.2 (15).

IS and mIS ratios were measured at 1 year postoperatively using the same method that was used preoperatively (Figure 1e, f). Postoperative CD and BP indices were measured using the modified versions because they were more applicable for measuring the patella height after TKA (7, 16). The modified BP (mBP) index was defined as the vertical height divided by the antero-superior corner of the polyethylene insert to the lower direction of the patellar joint surface and the length of the joint surface of the patella (Figure 1g). Normal values range from 0.54 to 1.06 (16). The modified CD (mCD) index is calculated by dividing the patella by the length of the joint surface between the lower end of the joint surface and the antero-superior corner of the tibial polyethylene insert (Figure 1h). Normal values range from 0.6 to 1.2 (7). These modifications enable the measurement of the height of the patella from the joint line; therefore, they are less affected by the thickness of the proximal tibial resection and tibial polyethylene insert (7, 16).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences software version 20.0 (IBM SPSS Corp.; Armonk, NY, USA). Descriptive statistics are presented as mean, standard deviation, and range values, as well as percentages. The inter-observer reliability for the two observers was determined with intra-class correlation coefficients (ICC). The scores were interpreted as follows: a score of 0–0.50 indicated poor reliability, 0.50–0.75 indicated moderate reliability, 0.75–0.90 indicated good reliability, and a score>0.90 showed excellent reliability (17). The data were tested for normality using the Shapiro-Wilks test. Paired t test was used to assess the statistical significance of the differences between the preoperative and postoperative measurements. A p- value of<0.05 was considered significant. The most important results are presented in the tables.

RESULTS

Among the 85 patients included in this study, 17 were men and 68 were women. At the time of the surgery, the average age of the study subjects was 66.96±8.42 y (range, 50–83 y), and their average body mass index was 27.47±1.59 kg/m^2 (range, 24.68–32.12 kg/m^2).

With regard to the preoperative measurements, moderate inter-observer reliability had an ICC of 0.74 (95% confidence interval [CI], 0.58–0.82) for BP index, while other measurements (IS, mIS, and CD) had good inter-observer reliability (Table 1). With regard to the postoperative measurements, moderate inter-observer reliability was shown with an ICC of 0.70 (95% CI, 0.54–0.80) for the mBP index; the other measurements (IS, mIS, and mCD) had good inter-observer reliability (Table 2).

Table 3 presents a comparison of the mean patellar heights before and after TKA. Shapiro-Wilks test was used to determine whether the data were normally distributed. Therefore, paired t test was used to assess the statistical significance of the differences between the preoperative and postoperative measurements.

The mean IS ratio preoperatively was 1±0.12, and this ratio decreased to 0.98±0.09 at the 1-year follow-up. The reduction in the average IS ratio was not significant (p=0.203). The IS ratio remained unchanged in 4 (4.70%) patients, decreased in 54 (63.53%), and increased in 27 (31.76%). The average preoperative mIS ratio was 1.57±0.17, and this ratio decreased to 1.55±0.17 at the 1-year follow-up. The reduction in the mean mIS ratio was not significant (p=0.45). The mIS ratio remained unchanged in 4 (4.71%) patients, decreased in 52 (61.18%), and increased in 29 (34.11%). The average preoperative CD index was 0.82±0.13, and this value decreased to 0.81±0.12 (mCD) at the 1-year follow-up. The reduction in the mean CD index was not significant (p=0.809). The CD index remained unchanged in 11 (12.94%) patients, decreased in 42 (49.41%), and increased in 32 (37.6%).

The average preoperative BP index was 0.77±0.12, and this value decreased to 0.74±0.1 (mBP) at the 1-year follow-up. The reduction in the mean BP index was not significant (p=0.081). The BP

TABLE 1. Interobserver reliability for the measurements of patellar height during the preoperative period

Methods	ICC	95% CI	p value
Insall–Salvati	0.88	0.84–0.93	<0.001
Modified Insall–Salvati	0.77	0.63–0.85	<0.001
Caton–Deschamps	0.81	0.72–0.87	<0.001
Blackburne–Peel	0.74	0.58–0.82	<0.001

ICC: intra-class correlation coefficients, CI: confidence interval

TABLE 2. Interobserver reliability for the measurements of patellar height at postoperative

Methods	ICC	95% CI	p value
Insall–Salvati	0.89	0.83–0.93	<0.001
Modified Insall–Salvati	0.76	0.63–0.84	<0.001
Modified Caton–Deschamps	0.84	0.76–0.89	<0.001
Modified Blackburne–Peel	0.70	0.54–0.80	<0.001

ICC: intra-class correlation coefficients, CI: confidence interval

TABLE 3. Comparison of mean patellar heights before and after TKA

Methods	Preoperative	Postoperative	p value
IS	1±0.12 (0.75–1.33)	0,98±0.09 (0.79–1.2)	0.203
mIS	1.57±0.17 (1.26–1.99)	1.55±0.17 (1.17–2)	0.45
CD/mCD	0.82±0.13 (0.49–1.14)	0.81±0.12 (0.51–1.06)	0.809
BP/mBP	0.77±0.12 (0.47–1.03)	0.74±0.1 (0.48–0.95)	0.081

IS: Insall–Salvati; mIS: Modified Insall–Salvati; CD/mCD: Caton–Deschamps/Modified Caton–Deschamps; BP/mBP: Blackburne–Peel/Modified Blackburne–Peel

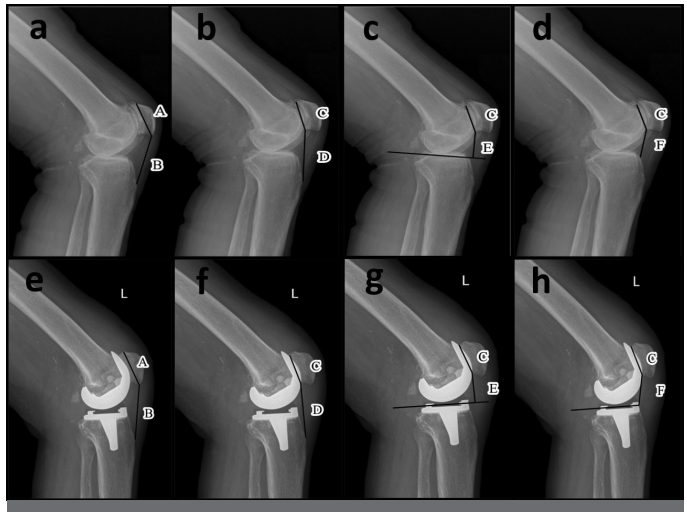

Figure 1. a-h. Representation of the four methods used for measuring patellar height. (a) Preoperative Insall–Salvati ratio: B/A, (b) Preoperative Modified Insall–Salvati ratio: D/C, (c) Preoperative Blackburne–Peel index: E/C, (d) Preoperative Caton–Deschamps index: F/C, (e) Postoperative Insall–Salvati ratio: B/A, f. Postoperative Modified Insall–Salvati ratio: D/C, (g) Postoperative Modified Blackburne–Peel index: E/C, (h) Postoperative Modified Caton–Deschamps index: F/C
A: The diagonal length of the patella, B: The length of the patellar tendon C: The length of the articular surface of the patella, D: The distance between the lower end of the articular surface of the patella and patellar tendon insertion to the tibial tubercle, E: The perpendicular height from the anterior superior corner of the tibial polyethylene insert to the inferior aspect of the patellar articular surface, F: The distance between the lower end of the articular surface of the patella and the anterior superior corner of the tibial polyethylene insert.

index remained unchanged in 5 (5.88%) patients, decreased in 46 (54.12%), and increased in 34 (40%).

According to the preoperative IS ratio, 82 (96.4%) subjects had a normal patellar height, 1 (1.2%) had a PB, and 2 (2.4%) had a patella alta. As per the preoperative mIS ratio, PB, or alta was not detected in 85 patients. As per the preoperative CD index, 83 (97.6%) patients had a normal patellar height, while 2 (2.4%) had a PB. Based on the preoperative BP index, 81 (95.2%) patients had a normal patellar height, 2 (2.4%) had a PB, and 2 (2.4%) had a patella alta.

According to the postoperative IS ratio, 84 (98.8%) patients had normal patellar height, while 1 (1.2%) subjects had a PB. According to the postoperative mIS ratio, 84 (98.8%) had a normal patellar height, while 1 (1.2%) had a PB. As per the postoperative mCD index, 83 (97.6%) subjects had normal patellar height, while 2 (2.4%) had a PB. Based on the postoperative mBP index, all (100%) the patients had normal patellar height. None of the patients was found to have a patella alta postoperatively using any index.

DISCUSSION

We analyzed the effect of complete removal of IPFP during TKA on the patellar height, preoperatively, and at 1 y postoperatively. The preoperative average patellar height was higher than the value at 1 y postoperatively, using all measurement methods. Although this may reflect patellar tendon shortening or elevation of the joint line, this decrease was not significant using any method ($p>0.05$). Our study indicates that complete removal of the IPFP during TKA had no significant effect on the height of the patella.

The removal or preservation of IPFP during TKA remains controversial (3-5, 8). Various studies have shown that complete removal of IPFP can cause patellar tendon shortening (2, 5, 11). Tanaka et al. (11) observed a significant decrease in the length of the patellar tendon in 54 patients with rheumatoid arthritis who underwent IPFP removal than in 54 patients whose IPFP was preserved. A significant shortening of the patellar tendon in the IPFP removal group was shown by Lemon et al. (2). Chougule et al. (5) showed the shortening effects of IPFP removal during TKA. Compared to preoperative measurements, the length of the patellar tendon at 1 year was significantly shortened in 133 knees on which TKA with IPFP resection was performed (5).

In contrast, Maculé et al. (9) stated that IPFP removal did not appear to change the patellar tendon length during the first 6 mon after TKA. Imren et al. (10) found similar results; the IPFP was completely removed in 228 patients who underwent TKA. They showed that IPFP removal during TKA did not alter the length of the patellar tendon at the 5-year follow-up (10). In a recent study, patellar tendon length and functional outcome scores after TKA for osteoarthritis were compared for patients in whom IPFP was completely removed and those for whom it was preserved. No significant differences were observed in the IS, mIS, CD, and BP indices preoperatively, at 1 y, and at 5 y after the procedure between the resection group and preservation group. In our study, the length of the patellar tendon (evaluated using IS and mIS ratios) and height of the patellar (evaluated using CD and BP indices) were evaluated radiographically in patients who underwent complete IPFP removal during TKA. Complete removal of IPFP during TKA did not affect the length of the patellar tendon or the height of the patella at the 1-year follow-up.

PB is described as a distally positioned patella relative to its normal position, measured using lateral radiography. It results from shortening of the patellar tendon or elevation of the joint line after TKA (6). PB is classified as true PB or false PB (6). True PB is caused by shortening of the patellar tendon, and it results in the pathological values of the indices (IS index<0.8 or mIS index<1.2 and BP index<0.54 or CD index<0.6) (5-6,18). The pseudo-PB causes elevation of the joint line without shortening of the patellar tendon and causes changes in the CD (<0.6) or BP (<0.54) values. Moreover, the IS and mIS ratios remain normal (no patellar tendon shortening). IS and mIS ratios reflect the length of the patellar tendon, while the BP and CD indices show the distance between the distal pole of the patella and the tibial plateau (5, 6, 18).

In our study, 1 (1.2%) patient had true PB, while 4 (4.8%) had pseudo-PB during the preoperative period. Moreover, 2 (2.4%) of the patients had true PB, while another 2 (2.4%) had pseudo-PB at the 1-year follow-up. Pseudo-PB is a common complication after TKA and occurs when the patellar tendon is not shortened, but the level of the joint line increases. A large polyethylene insert used after tibial or femoral excessive resection to restore knee stability or excessive soft tissue release may also result in pseudo-PB (6). In a recent study, the incidence of pseudo-PB after TKA was 14.4%; however, this was contradictory to our findings (6). However, Gatha et al. did not report a significant difference between the preoperative and postoperative patella height (19).

Chougule et al. (5) reported lengthening of the patellar tendon (according to IS ratio) in 133 knees of 16 (12.1%) patients who underwent TKA with IPFP. They stated that this was owing to preoperative pain and disability caused by the degenerative process; in other words, the corrected patellar tendon shortened, as evidenced by improved postoperative functioning. In our study, patellar height increased in 27 patients (31.76%) as per the IS ratio and in 29 (34.11%) patients as per the mIS ratio at the 1-year follow-up. These increases reflect the lengthening of the patellar tendon. The patellar height increased in 32 (37.6%) patients as per the CD index and in 34 (40%) patients as per the BP index at the 1-year follow-up. These increases reflect a decline in the joint line and can be explained by shortening of the patellar tendon and a reduction in the patella height owing to the degenerative process in the preoperative knee joint. Thus, the patella height that had already decreased preoperatively increased after TKA. In fact, this increase indicated that the patella height returned to the normal value.

Our study has some strengths. The surgeries were performed by a single surgeon, while the same implant and technique were used for all the patients. Our study also has certain limitations. The study was designed retrospectively, the sample size was small, and the results were presented at the 1-year follow-up. Further, we did not perform a comparison between early and late postoperative radiographs. In addition, no other group was included for the comparison, such as patients who underwent partial removal or preservation of the IPFP.

In conclusion, we conclude that complete removal of the IPFP during TKA for primary osteoarthritis did not affect the patellar height at the 1-year follow-up. Larger case series and/or randomized controlled studies are needed to confirm our results.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Aksaray University (2020/06-12).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Author contributions: Concept - S.G., S.C., R.Ö.; Design - S.G., S.C.; Supervision - S.G., S.C., R.Ö.; Resource - S.G., S.C.; Materials - S.G., S.C.; Data Collection and/or Processing - S.G., S.C.; Analysis and/or Interpretation - S.G., S.C.; Literature Search - S.G., S.C., R.Ö.; Writing - S.G., S.C., R.Ö.; Critical Reviews - S.C., R.Ö.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Review

DOI: 10.5152/cjms.2020.1029

Whitening Dentifrices: A Review

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Cite this article as: Değer C, Müjdeci A. Whitening Dentifrices: A Review. Cyprus J Med Sci 2020; 5(4): 355-60.

Whitening dentifrices are used in dental procedures for achieving aesthetic effects, such as tooth cleaning and stain removal. Today, owing to the effect of media, there is an increasing interest among individuals to have a healthy and beautiful smile, resulting in higher use of easily accessible whitening dentifrices. The whitening effect of whitening dentifrices is mostly attributed to their constituent abrasives or chemical, optical, or enzymatic ingredients. Thus, it is important to have updated knowledge regarding the ingredients, whitening efficacy, and mechanisms regarding the action of whitening dentifrices. Several dentifrices are available in the global market, and dentists should be aware that the selection of the most appropriate dentifrice is possible with correct knowledge about the content of each dentifrice. Based on their ingredients, certain whitening dentifrices may negatively affect dental hard tissues. Thus, dentists should warn patients about the potential adverse effects of whitening dentifrices on teeth and the negative consequences of dentifrices with unproven whitening claims.

Keywords: Whitening dentifrices, abrasive, blue covarine, charcoal, activated carbon

INTRODUCTION

The color of a tooth is a combination of the internal color and external pigmentations that may present on the tooth surface. The internal color of the tooth is affected by the absorption and reflection properties of the dentine and enamel. However, the most important determinant of the overall tooth color is the dentin structure (1). Tooth discoloration can be classified as extrinsic or intrinsic as per the location of the stain. Extrinsic stains are present on the tooth surface or in the acquired pellicle. They can be consequences of smoking habit; poor brushing technique; the use of certain cationic agents, such as chlorhexidine; and/or a diet rich in colored foods or drinks, such as coffee, tea, and red wine. Intrinsic discolorations occur with changes in the structural composition or thickness of dental hard tissues. Systemic factors, such as excessive fluoride intake, erythroblastosis fetalis, and systemic medication, can cause internal discolorations (2).

Owing to the influence of media, patients are showing an increasing interest in getting whiter and brighter teeth. Various methods have been developed to resolve discoloration and make teeth look whiter to fulfill patient requirements. These methods include the use of whitening dentifrices, enamel microabrasion, vital bleaching as home/office, non-vital bleaching, and prosthetic treatments (3).

Dentist–supervised home bleaching technique is the most commonly used whitening procedure (4). In this treatment, 10% carbamide peroxide gel is placed in a tray, and patients apply the tray themselves at certain hours during the day for a period of 2 wk. The benefits of the home bleaching technique and patient satisfaction have encouraged the marketing of over-the-counter products (5). Gels, rinses, whitening strips, and dentifrices are some examples of the over-the-counter whitening products (3).

Whitening dentifrices with their ease of use and low cost have been getting increasing attention from consumers (1) and are perhaps the most accessible product type for many patients and consumers who want to whiten their teeth (6). Different whitening dentifrices that have different mechanisms of action are available in the market. Whitening dentifrices are generally formulated to remove external stains using physical methods and to prevent the reformation of these discolorations (7). Today, it is widely accepted that the primary mechanism effective in whitening dentifrices is the abrasive mechanism although other ingredients, including chemical agents, enzymes, and optic agents are added into these dentifrices to remove stains and prevent them from re-appearing (1).

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Received: 14.05.2019
Accepted: 27.06.2019

Mechanism of Action and Ingredients of Whitening Dentifrices

Abrasives

Abrasives added as insoluble particles in toothpastes have been used for tooth cleaning in combination with toothbrush bristles for about 2000 y (7). Abrasives are physically harder than the stain (1), and they remove stained dental plaque on tooth surface mechanically. Although abrasives constitute the main mechanism in the elimination and prevention of external stains, they are only effective on external stains and reach only those areas that can be accessed using a toothbrush. Moreover, they have limited effects in malocclusion regions, interdental areas, and gingival areas (1), and they do not change the color of the teeth.

Whitening dentifrices containing abrasives have been formulated with specifically designed higher content of abrasives than that in standard dentifrices (2). It is important for whitening dentifrices to have maximal cleaning and stain-removing effect with minimal hard tissue wear (8). The stain removal effect of whitening dentifrices that contain abrasives depends on the abrasive particle size, shape, distribution, concentration (9), as well as pH of the dentifrices (10). When the abrasive particles are too large, the toothbrush bristles are unable to catch these particles and the particles are swept away from the tooth surface and become ineffective (1). The hard tissue wear effect of the dentifrices and the relative safety of abrasivity have been standardized for enamel and dentine as radioactive dentin abrasion (RDA) and radioactive enamel abrasion methods. These methods enable a comparison of the abrasiveness of dentifrices and evaluate their appropriate lifetime use by patients (11). The International Organization for Standardization (ISO) has determined the abrasivity limit for dentifrices as 250 RDA (12). Whitening dentifrices having RDA 60–100 are considered to have medium abrasiveness and those with RDA>100 are considered highly abrasive (11).

Whitening dentifrices can contain the abrasives as monoabrasives or a mixture of different abrasives. Abrasives having chemically different types may exert varying cleaning effectiveness; chemically similar abrasives may have different cleaning ratios. Moreover, the mixture of chemically different abrasives can have a different effect than individual abrasive components (13). The following abrasives are used in whitening dentrifices: hydrated silica, calcium carbonate, dicalcium phosphate dihydrate, calcium pyrophosphate, alumina, perlite, and sodium bicarbonate (1, 2).

Main Points:

- Chemical and mechanical substances have been added to whitening dentifrices to increase the effect of abrasive cleaning in areas that cannot be reached by a toothbrush and to ensure the effectiveness of dentifrice.
- Dentifrices containing proteolytic enzymes are good alternatives for patients with dentin hypersensitivity to dentifrices containing abrasives that cause enamel and dentin wear.
- Today, many dentifrices are sold on the market for whitening. Dentists should be aware of the ingredients in these dentifrices, recommend which is right for their patients, and warn about possible side effects.

Silica abrasive technology has generally been used in dentifrices. The most common abrasives are hydrated silica and calcium carbonate. Hydrated silica is a gentle abrasive that has the ability to clean teeth stains very effectively (13). It is compatible with most active ingredients in dentifrices, and the Mohs' scale hardness of hydrated silica is 5 (14).

A type of silica abrasive, "soft silica" abrasive, has been recently introduced and has reduced Mohs' scale hardness (conventional silica=5.9, soft silica=4.2) and dentine abrasion (conventional silica RDA=110, soft silica RDA=87). When soft silica is added into a toothpaste formulation, in vitro studies have showed a significant improvement in the pellicle-cleaning ratio than that with conventional silica formulations. This result has been further supported in a clinical study on chlorhexidine/tea-induced stain removal and a natural stain prevention study (15).

Another type of abrasive, high cleaning silica that is not abrasive on teeth and provides maximum stain removal effect has also been incorporated into whitening dentifrices (16). Significant natural stain removal with a high cleaning silica dentifrice was found comparable to that with silica-containing dentifrice over a period of 6 wk (17).

Perlite is an amorphous glassy silicate used as a polishing agent in prophylaxis products that are associated with excellent stain removal and low abrasivity. Perlite added into a silica-based dentifrice or a calcium carbonate dentifrice has shown significant improvement in stain removal as compared to a control silica dentifrice in both, in vitro and in vivo studies (18, 19).

Sodium bicarbonate has low abrasivity and Mohs' scale hardness of 2.5; moreover, it is compatible with active ingredients in dentifrices (14). The whitening effect of toothpastes containing sodium bicarbonate in a high concentration is greater than that of toothpastes containing silica and calcium phosphate. This result has been explained by the low abrasiveness, low internal hardness, and high solubility of calcium phosphate. Dentifrices containing sodium bicarbonate in different concentrations have been found in vitro to be more effective in removing intrinsic yellow stains than water or classical dentifrices (20). Koertge et al. (20) have examined the whitening effect of a classic dentifrice and a dentifrice containing 65% sodium bicarbonate in 115 volunteers over a period of for 12 wk. The dentifrice containing sodium bicarbonate was significantly more effective in reducing the discoloration and increasing the whiteness compared to a classic dentifrice. Yankell et al. (21) have found that sodium bicarbonate/calcium peroxide containing dentifrices are more effective than silica-based tartar dentifrices for controlling extrinsic stains. Bollmer et al. (22) have investigated the effect of 3.3% pyrophosphate containing tartar control dentifrice and classic dentifrice containing hydrated silica and disodium pyrophosphate on chlorhexidine induced stain formation in vivo. They have observed that both the dentifrices significantly reduced the coloration on the buccal surfaces of the anterior teeth.

Chemical Agents

It is difficult to remove intrinsic stains with dentifrices that remove stains mechanically with abrasive ingredients. Therefore, various chemical agents that have non-abrading whitening effect have been added into whitening dentifrices (1).

The following chemical agents are used in whitening dentifrices: hydrogen peroxide, calcium peroxide, sodium citrate, sodium pyrophosphate, sodium tripolyphosphate, sodium hexametaphosphate, and sodium chlorite (1).

Hydrogen peroxide is used as a tooth whitening agent for about 100 y and was first used in 1989 when Haywood and Heymann introduced home-type bleaching. Today, peroxides are added into dentifrices to oxidize intrinsic stain molecules and change their absorption spectrum to lower visibility and bleach the enamel. The American Dental Association has stated that hydrogen peroxide concentrations of ≥ 3% should not be considered for frequent or extended use owing to the risk of damage to oral tissues (23). Therefore, in dentifrices, the concentration of peroxides is very low (usually hydrogen peroxide 1%, calcium peroxide 0.5%–0.7%), and the percentage of hydrogen peroxide allowed in Europe is only 0.1%. A low concentration of peroxide is permitted; thus, the difficulty in incorporation of peroxide into dentifrice and short application time can limit the effectiveness of peroxide containing dentifrices in removing intrinsic stains (1, 14). Therefore, both, chemical and mechanical agents have been added into whitening dentifrices to contribute to the effect of abrasive cleaning and ensure the effectiveness of the dentifrice in areas that cannot be reached with a toothbrush (1).

A dentifrice containing 1% hydrogen peroxide as a chemical agent has shown a significant reduction in the extrinsic stains as compared to toothpastes containing only silica and silica/hexametaphosphate in vitro (23). Hydrogen peroxide in whitening dentifrices has interfered with chromatic alterations of the teeth within 2–6 weeks of brushing (24). Kleber et al. (25) have compared a 1% hydrogen peroxide/sodium bicarbonate containing toothpaste to a toothpaste with silica/sodium bicarbonate. They reported that 1% hydrogen peroxide/sodium bicarbonate containing toothpaste was more effective in reducing yellowness and increasing brightness. In an in vivo study with whitening dentifrices containing hydrogen peroxide in the range of 1% concentration, an improvement was noted in the tooth color (24). No superiority of whitening dentifrices with chemical whitening agents has been reported. A whitening dentifrice containing hydrogen peroxide, calcium pyrophosphate, and tetrapotassium phosphate as chemical agents has shown similar extrinsic stain removal performance as compared to an ordinary dentifrice in a study by Soares et al. (26).

Chemical agents, such as pyrophosphate, tripolyphosphate, and hexametaphosphate have binding affinity to enamel, dentin, and tartar. They remove stain components during adsorption and prevent the formation of new pigments. Pyrophosphates dislocate anions and negatively charged macromolecules that are present in the acquired enamel pellicle to remove stains (27). Sodium pyrophosphate is frequently used in whitening dentifrices in addition to its use in tartar removal. Dentifrices containing sodium tripolyphosphate and pyrophosphate combination are more effective than non-bleaching silica dentifrices containing only sodium tripolyphosphate (27).

Sodium hexametaphosphate, another chemical agent, is a long-chain pyrophosphate variant. It attaches to the surface of the teeth with multiple bonding zones, and when compared to pyrophosphate, it prevents the formation of tea/coffee-induced stains (28).

Enzymes

Extrinsic stains are primarily incorporated into the pellicle. Enzymes, such as proteases, that break down proteins can be effective on the protein portion of the pellicle and can be used to remove extrinsic stains without abrasive actions (1). Therefore, whitening toothpastes that contain natural enzyme extracts derived from plants have been manufactured as non-abrading whitening agents (29). Nowadays, enzymes incorporated into the dentifrices for whitening purposes are usually natural proteolytic enzymes, such as papain and bromelain, that belong to a group of proteases (30). Papain is derived from the plant Carica Papaya, and Bromelain is derived from Ananas Camosus (31). Both the enzymes hydrolyze and break the protein pellicles down on the tooth surface, thus inhibiting the attachment of oral microorganisms and stain on the tooth surface (32). An enzymatic dentifrice with papain and bromelain has been found to be more effective for stain removal than control dentifrice in vitro (33). Moreover, this enzyme-containing dentifrice has shown considerable residual bleaching effect even after at the end of the 2 months owing to its proteolytic and antibacterial actions (29). In clinical studies, the efficacy of enzymatic dentifrices has been reported, and a dentifrice containing papain, alumina, and citrate has shown to reduce discolorations (34).

Dentifrices containing proteolytic enzymes that do not contain abrasive ingredients are reported to be a good alternative for patients with dentin hypersensitivity instead of dentifrices containing abrasives causing enamel and dentin wear (29). However, the shelf life of enzymatic dentifrices is shorter than that of regular dentifrices owing to their proteolytic structure (31).

Optical Agents

Perception of tooth color is a complex phenomenon that is influenced by the experience of the person evaluating the tooth color, the illumination of the environment, the presence of internal or external colorations, and the color of the lips. Commission International De L'Eclairage (CIE) introduced a three-dimensional mathematical color system (CIELAB) for identifying color perception in 1976. This system consists of the following 3 axis: L*, a* and b*; L* represents lightness of the object, where a* represents red-green scale and b* represents the yellow-blue scale (35).

The use of an optical principle is an alternative approach to tooth whitening. Decrease in b* value causes color change from yellow to blue tone and makes the patient perceive his/her own teeth as whiter (36). Considering that the deposition of blue colored agents onto the tooth surface causes a shift in the color from yellow to blue, a blue pigment like blue covarine has been added into dentifrices. Blue covarine pigment simulates the scattering wavelengths of the enamel in the blue extension and changes the appearance from yellowish to bluish. Blue covarine adds a thin blue layer to the enamel surface and alters the perception of the tooth color; thus, a silica-based whitening dentifrice containing blue covarine has been developed. Dentifrice**s** containing blue covarine cause a sudden and significant reduction in tooth yellowness and enhance the whiteness (37).

A study that compared dentifrices containing blue covarine in different concentrations and a dentifrice without blue covarine has showed that brushing once with the dentifrice containing blue covarine could increase the tooth whiteness compared to

the control dentifrice. In addition, as the concentration of blue covarine increased, tooth yellowness decreased with the decrease in b* values, and the whiteness increased (37).

Some whitening dentifrices contain another optical agent, titanium dioxide, a white pigment. Titanium dioxide attaches into surface irregularities, resulting in a whiter tooth illusion as only a surface phenomenon instead of internal color change (38).

Other Ingredients

Some agents, such as detergents and antimicrobials, that are included in regular toothpastes, can enhance the whitening effect.

Dentifrices containing sodium lauryl sulfate reduce the surface tension of the stain molecules and prevent the binding of these molecules to the enamel (7). Some plaque bacteria are reported to have chromogenic properties that cause green, brown, and black discoloration on the surface of the tooth. Antimicrobial agents in whitening dentifrices might pass through the lipophilic bacterial membrane and cause disruption of the bacterial structure in the pellicle, thereby reducing extrinsic stains. Triclosan and methyl paraben are the most commonly used antimicrobial agents in whitening dentifrices (7).

Nathoo et al. (39) have compared the extrinsic stain removal efficacy of a dentifrice containing triclosan, PVM/MA copolymer, NaF, and specially designed silica for sensitivity relief to a negative control dentifrice containing NaF. They reported that the whitening effect of the dentifrice containing triclosan, PVM/MA copolymer, and NaF on extrinsic stains was significantly more than that in the negative control group.

Recent Developments in Whitening Dentifrices

Charcoal is a good absorbent, and charcoal-containing preparations have been used primarily for acute poisoning and drug overdose (40).

The use of charcoal for oral hygiene started with Hippocrates in the Ancient Greek period (41). Charcoal dust for tooth cleaning has been used in different ways, such as applying with finger on teeth, chewing sticks, or toothpaste (42).

Nowadays, many dentifrices containing active carbon or activated charcoal are manufactured and sold on internet sites and in the market for whitening purposes. Micro charcoal in dentifrices is claimed to absorb dirt and clean the teeth and gaps between the teeth that are difficult to reach (43). However, charcoal, has been explained as an abrasive mineral for the teeth or gingival tissues, and products containing charcoal have raised concerns about damage to these oral structures (44). A dentifrice containing charcoal, silica, and hydrated silica caused significant changes in the enamel surface roughness that exceeded the threshold of bacterial retention of 0.2 μm after brushing for an equivalent of 3 months. Star-shaped or so-called fractal shaped charcoal particles and larger average size of particles of the charcoal dentifrice are believed to be responsible for the increased surface roughness (43).

In a study on oral hygiene habits that was performed in Malaysia, Yaacob and Park (45) found that 8.9% of the population using charcoal or table salt has varying degrees of severity of concave cavities into dentin formed on the labial surfaces of the teeth, resulting in the teeth becoming less white, even yellowish, with enamel loss.

Despite the reported theory that older types of charcoal-containing products are harmful, a manufacturer claims that activated carbon must not be confused with raw charcoal powder dentifrices because activated carbon is created by mixing charcoal powder into the dentifrice at the stage of manufacturing, creating an homogenous non-abrasive product that whitens teeth by absorbing stains; in contrast, the charcoal powder whitens teeth by rubbing particles against the teeth (46). It is also claimed that dentifrices that contain activated carbon have an ingredient that coats the tooth surface with a blue tint to influence light reflection and provide additional whitening (44).

Brooks et al. (42) examined 50 activated charcoal-containing dentifrices and found that these dentifrices have been introduced to the market with consumer-appealing terms, such as ecological, herbal, and organic labels. However, only 4 of the 50 examined dentifrices are reported to contained fluoride. Although 96% of these dentifrices claim to exert a whitening effect, to our knowledge, no controlled clinical studies have proven these claims. Thus, dentists should inform their patients about the unproven effects of these dentifrices and the consequences that may result from the lack of fluoride (42).

Safety of Whitening Dentifrices

It is important to ensure that any oral care product does not have any adverse effect on the tooth, dental materials, and soft tissues. There are some concerns about the adverse effects of ingredients of whitening dentifrices on dental hard and soft tissues.

In high concentrations, protease enzymes, such as papain and bromelain can theoretically cause irritation in the soft tissues. However, papain and bromelain in dentifrices have not been associated with adverse effects (7).

DeSalva et al. (47) have showed that triclosan in the dental products can be tolerated by the human body. However, continuous exposure to antimicrobial agents may raise microbial resistance and result in an inadequate immune response when virulent microorganisms are encountered (7). There are insufficient clinical studies regarding the long-time use of whitening dentifrices; therefore, dentists should consider the long-time adverse effects of triclosan.

Pyrophosphates in tartar control dentifrices and whitening dentifrices have been reported in only one study wherein they caused cheilit and circulum dermatitis in patients (48).

A sodium lauryl sulfate containing dentifrice has been compared with a non-sodium lauryl sulfate containing-dentifrice by Veys et al. (49). They observed hyperceratinized epithelial changes in the mucosae exposed to sodium lauryl sulfate for a long-time.

As per one study, a whitening dentifrice containing titanium dioxide caused Yellow Nail Syndrome. It is known that this syndrome manifests symptoms, such as yellow nails, lymphedema,

and respiratory disorder, that are associated with titanium in adults (titanium dioxide-containing drugs, dental implants). Hsu et al. (50) reported the case of a 9-year-old patient with Yellow Nail syndrome who swallowed the dentifrice containing titanium dioxide during brushing. Thus, this syndrome was first noted in children and titanium dioxide-containing toothpastes are not recommended for use in children.

In conclusion, nowadays, increasing aesthetic concerns owing to the influence of the media have encouraged the use of easily accessible whitening dentifrices by patients and have made whitening technologies important for dentists. Despite a number of in vitro and in vivo studies on whitening dentifrices, there is no consensus on the best whitening technology with dentifrices. Thus, dentists should be aware of the contents of these dentifrices, recommend the appropriate onefor their patients, and warn about the possible abrasive effects.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - C.D., A.M.; Design - A.M.; Supervision - A.M.; Resource - C.D.; Materials - C.D.; Data Collection and/or Processing - C.D.; Analysis and/or Interpretation - C.D., A.M.; Literature Search - C.D.; Writing - C.D., A.M.; Critical Reviews - A.M.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Review

DOI: 10.5152/cjms.2020.1342

Exosome Mimetic Nanovesicles; Are They Next Best Alternative Therapeutic Approach Combating Cancer?

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Cite this article as: Özverel CS. Exosome Mimetic Nanovesicles; Are They Next Best Alternative Therapeutic Approach Combating Cancer? Cyprus J Med Sci 2020; 5(4): 361-4.

Cancer is regarded as one of the most dangerous diseases despite the advances in technology and therapeutic strategies against it. The current treatment strategies are ineffective as well as present with various disadvantages, such as drug resistances, ineffective uptake of the therapeutic agents at the tumor site, incompatible delivery of drugs, and immune-rejection, among others. Extracellular vesicles, especially exosome mimetic nanovesicles, have become one of the latest focuses of research in anticancer therapies. The invention of these nano-sized vesicles, which function in cell-to-cell communication, have promoted the development of innovative drug delivery systems due to their cargo-carrying abilities and targeted deliveries. Exosome mimetic nanovesicles have similar surface protein structures to exosomes and offer various important advantages over the exosomes, such as the production yield and isolation protocol. This review aims to summarize the current research studies on exosome mimetic nanovesicles together with their potential in combating cancer in the future.

Keywords: Cancer, cancer therapy, exosomes, nanovesicles

INTRODUCTION

Cancer has been regarded as one of the most dangerous disease affecting people across the world (1). Despite the advancements made in the fields of biotechnology and bioengineering, no effective treatment has yet been established against cancer. Conventional therapeutics, especially chemotherapeutics, are highly known for their abilities of tumoral deoxyribonucleic acid damage, thereby inducing a cell cycle arrest, eventually leading to cell death (2, 3). Although, they are widely being used in the clinical settings, when used aggressively, they offer major drawbacks of chemotherapeutic resistances and damage induction to healthy cells (4). Therefore, these drawbacks direct researchers to search for the next best alternative strategy in terms of effectiveness and reduced cytotoxicity.

Extracellular vesicles secreted between cells are known for their functions in cell-to-cell communication. They are also known for their regulatory activities of the immune system, creating niche for tumor growth, conditioning metastatic sites during tumorigenesis, and facilitating the spread of misfolded proteins in neurodegenerative diseases (5, 6). In addition, these extracellular vesicles are known for their activities of indicating pathogenesis and disease progression owing to their individual protein, peptide, and lipid expression profiles. Furthermore, their ability in cell-to-cell communication enables them to transport signaling molecules and localize at distant tissues, suggesting their potential usage in the development of efficient drug delivery systems (5).

Exosomes and exosome-derived nanovesicles are now considered as the hot topic in drug delivery systems against cancer. Past studies have demonstrated that they possess lower cytotoxicity and higher accuracy when compared to other drug delivery systems and mono-chemotherapies (7, 8).

MATERIAL and METHODS

We conducted aliterature review of studies on exosome mimetic nanovesicles, nanovesicle drug delivery systems, and nanovesicles used in cancer treatment. Data was searched via search engines analyzed widely in terms of the current status and future prospective of nanovesicle-based treatments.

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Received: 18.09.2019
Accepted: 20.11.2019

Disadvantages of Conventional Drug Delivery Systems

The development of antitumor strategies has been a huge field of research in the recent years owing to the ineffectiveness of the present conventional methods, including the drug delivery systems. Drug delivery systems have been developed as an efficient approach to induce cell-specific death of the tumor. Some of these conventional drug delivery systems involve the use of liposomes, carbon nanotubes, dendrimers, and gold nanoparticles (9-12). These conventional drug delivery systems present with major drawbacks, for instance, unnecessary release of therapeutic drugs to the neighboring tissues. Another disadvantage of these conventional delivery systems is the adverse immunogenic response as well as their accumulation in the organs such as liver and kidneys (13). Furthermore, the conventional systems fail to accumulate at the tumor site due to their unwanted recognition via the hosts' immune system (14).

Exosomes

Exosomes were first discovered as small vesicles inducing calcification of the long bones in a research study back in the 1960s (15). Later, they were detected in fluids such as blood and semen. In 1987, the term exosome was used for the first time ever in the literature to describe tiny membranous vesicles that are released into the extracellular space via exocytosis (16).

Specific targeting in tumor studies has been a major field with the goal of efficient cell death at lower toxicity in terms of collateral damage (17). During the past decades, several studies have been performed on drug delivery systems against cancer; however, their aforementioned disadvantages make them inefficient. Despite the vast number of studies performed on this subject, only a few of them, especially those on liposomal and polymeric nanoparticle formulations, have achieved the Food and Drug Administration, USA approval and are being used in clinical cancer therapies (11). No clinical trials have reported other drug delivery systems because of the challenges of unwanted distribution and higher toxicity levels (18).

Recently, exosomes have gained a lot of interest as a novel drug delivery system (18). Exosomes are <150 nm in dimensions and hence act as intermediatesin cell-to-cell communication. They can also be produced by almost all mammalian cells, including tumor cells (19). One of the major advantages of exosomes is their capability in transportation of endogenous biological cargos, such as proteins, small ribonucleic acids (sRNAs), and messenger ribonucleic acids (mRNAs) across the cells (20, 21). These capabilities provide advantages such as biocompatibility and decreased immune clearance rates in comparison to those with the conventional drug delivery systems (21). Some of the other advantages of exosomes are their better and longer accumulation at the organs or tumor sites and reduced toxicity levels (20). In addition to these advantages over the conventional systems, they also facilitate their uptake to the target cells due to the presence of surface proteins (22, 23).

Main Points:

- Exosome mimetic nanovesicles are promising candidates against cancer.
- Nanovesicles have exosome-mimicking properties of biocompatibility, easy cargo transportation, low immune clearance rate, and low resultant toxicities.
- Nanovesicles have major advantages over exosomes such as production yield and easy isolation protocol.
- Nanovesicles opened a new era in the drug delivery systems in combination with anticancer therapies.

Exosomes are presently being used to deliver various biological substances as well as chemotherapeutics, including doxorubicin, paclitaxel, curcumin, and some other peptide-based therapeutics, such as *signal transducer and activator of transcription 3* inhibitors, as well as genetic materials, such as small-interfering ribonucleic acids (siRNAs) (23-29). Moreover, they are extremely promising candidates for immunotherapy against cancer. Past studies have demonstrated the potential of dendritic cell-derived exosomes in stimulating patients' immune system against cancer (30). However, these new-era drug delivery systems also present disadvantages, especially in the protocol of isolation and yield obtained. These disadvantages unfortunately limit the usage of exosomes in the drug delivery systems. Exosomes require a huge yield of starting materials, such as cells and culture media, and the protocol takes enormous amount of time, which makes the overall process difficult and expensive to isolate (31).

Several strategies have been investigated in order to increase the production yield of exosomes. Some of these involve lowering of the pH of the culture media, increasing the initial tissue concentration, or prolonging the incubation time during a protocol. However, none of these strategies have been found efficient in increasing either the production yield of exosomes or in decreasing the time consumed in performing isolations (32).

Cell-Derived Exosome Mimetic Nanovesicles

Exosome mimetic nanovesicles are currently being investigated as alternatives to exosomes due to their superior properties over exosomes. They offer advantages over the production and time consumption during isolation. Exosome mimetic nanovesicles are isolated via the application of a physical force across the membranes of nano-scale dimensions. Several methods, including passing the cells through mini-extruders' micro channels or several rounds of centrifugation using custom devices, have been proposed (33-35). Nanovesicles provide advantages in terms of preserving their surface proteins from the parent cells and mimicking exosomal features. These also provide the advantages of decreased clearance rates from the body, targeted delivery of cargo, and efficient uptake mechanisms (36).

Starting from the same initial cell count, nanovesicle production generates a larger number of vesicles when compared to the conventional exosome-isolation protocols. They not only provide larger cell count but also require a shorter period of time for isolation, as short as 72 h (22).

Several studies have been performed on exosome mimetic nanovesicles to further characterize their therapeutic potentials. In a study by Goh et al. (37), nanovesicles derived from monocytes were used in the drug delivery system against cancer cells. They isolated nanovesicles and loaded chemotherapeutic drug doxorubicin to investigate their discriminatory approach between healthy and tumor cells. They found that monocyte-derived nanovesicles target cancerous cells and demonstrate a

clear discrimination toward cancer cells showing their potential in anticancer therapy (37). In another study, nanovesicle-isolated macrophages were loaded with chemotherapeutics, and their anticancer activity was investigated both *in vivo* and *in vitro*. Both *in vivo* and *in vitro* studies demonstrated a promising effect of nanovesicles against tumor cells. Exosomes and nanovesicles were further compared in the same study, and no significant differences were noted in their antitumor activities (32). Chemotherapeutic drug-loaded nanovesicles were investigated in a comparative *in vivo* study, and the data obtained suggested that drug-loaded nanovesicles have better cellular uptake and permeability, resulting in reduced tumor dimensions when compared to liposomal formulations and drug-only treatment (38).

Mesenchymal stem cells (MSCs)-derived exosomes are known for their anticancer activities. Chemotherapeutic agent-loaded MSCs have been determined as great vehicles for delivery. In a study by Kalimuthu et al. (39), the anticancer activities of paclitaxel-loaded exosome mimetic nanovesicles were investigated both *in vivo* and *in vitro*. Our results demonstrated lower cell viability of the breast cancer cells *in vitro* and reduced tumor dimensions *in vivo* (39).

Exosomes are also known for their capabilities in delivering ribonucleic acids (RNAs)such as mRNAs and micro RNAs that alter the phenotype of the target cells. Hence, exosome mimetic nanovesicles can not only be used asnovel drug delivery systems but also for use in various gene therapies due to their potential in carrying genetic materials. RNA interferences possess the ability to selectively attenuate specific genes, making it highly valuable in treating diseases, including cancers that are caused by overexpression of genes (40-42). Lunavat et al. (43) demonstrated the successful uptake of siRNA into nanovesicles and subsequent attenuation of target gene expression. The data suggested that nanovesicles can form a new platform for RNA delivery to successfully target the cell cytoplasm (43).

De-regulations in the cell cycle are the major hallmarks of cancer formation. Disruption of the cell-cycle control kinases (mainly cyclin-dependent kinases [CDKs]) cause the formation of various cancers, including lung, breast, liver, and blood (44-46). Targeting the CDK pathway is one of the major areas focused in cancer-treatment studies. These genetic materials have limited stability and the drug delivery systems are inefficient in relation to the cellular uptakes. Tumor-derived exosome mimetic nanovesicles have been suggested as an alternative method for delivery of these RNAs, which are biocompatible, non-immune reactive, and easily taken up. Yang et al. (47) demonstrated the successful delivery of RNAs in a breast cancer model both *in vivo* and *in vitro* and proved the specific downregulation of CDK4 target genes and induction of cell cycle arrest at the siRNA-delivered study groups (47).

In conclusion, drug delivery systems are important parts of most of the clinical therapeutics practiced in the present time. However, they present with certain drawbacks, such as inefficient immune clearance and uptake mechanisms. Exosomes, which are cell-to-cell communicators, are expressed nearly by all mammalian cells and reportedly possess the capability of better delivery due to their biocompatibility, capability of transportation of endogenous biological cargos, lower immune clearance rates, and lower resultant toxicities. They not only demonstrate their activities in drug delivery systems but also in immunotherapies. Exosome studies have however not moved forward for clinical application owing to their time-consuming isolation techniques and low production yield. Nanovesicles, smaller-diameter exosome mimetics, have therefore opened up a new era in the drug delivery systems in combination with anticancer therapies. These nanovesicles possess exosome-mimicking properties of biocompatibility, easy cargo transportation, low immune clearance rate, and low resultant toxicities and can be produced in a relatively higher quantity. The research field on nanovesicles is progressing quickly since their discovery. Recent studies have demonstrated their enormous potential in cancer therapy. The potential abilities of exosome mimetic nanovesicles in cancer therapy is worth exploring in the future.

Peer-review: Externally peer-reviewed.

Conflict of Interest: Author has no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Review

DOI: 10.5152/cjms.2020.2658

COVID-19 and Ozone

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Cite this article as: Yılmaz N, Eren E, Öz C. COVID-19 and Ozone. Cyprus J Med Sci 2020; 5(4): 365-72.

COVID-19 is characterized by an abnormal inflammatory response associated with endothelial dysfunction and microvascular complications. Unfortunately, no specific treatment for the disease and its life-threatening complications is available as of now. Ozone (O3) gas is a molecule consisting of three oxygen atoms in a dynamically unstable structure due to the presence of mesomeric states. Although O3 can have dangerous effects, it can have many therapeutic effects due to hormesis. The direct effect of ozone may be the direct inactivation of COVID-19, stimulation of oxygen metabolism, and activation of the immune system. Fortunately, COVID-19 contains sulfur-bound proteins that can be easily damaged through ozone oxidation. This structural content can be crucial to the antiviral effect because ozone can easily break down the double bonds in sulfur protein structures through a reaction called ozonolysis. Therefore, medical ozone can help reduce pneumonia, slow viral replication, regulate lung circulation and oxygenation, and prevent microvascular thrombosis. Ozone therapy can be considered as a cost-effective and easy-to-administer adjunct therapy while awaiting the development of a specific drug or vaccine for COVID-19. Furthermore, a growing number of studies have shown that ozone can be used as an adjuvant therapy for COVID-19.

Keywords: Ozone, COVID-19, SARS-CoV-2, ferritin, hormesis

INTRODUCTION

As of today, coronaviruses are still important human and animal pathogens. SARS-CoV-2 has been identified by the World Health Organization (WHO) as the cause of the COVID-19 pandemic that is still ongoing. In July 2020, when this article was written, about 11 million confirmed cases worldwide and unfortunately >700,000 deaths from COVID-19 were reported. Taxonomically, COVID-19 has the genus *Riboviria*, which is a coronavirus related with severe acute respiratory syndrome, origin of Nidovirales, sub-origin Cornidovirineae, family Coronaviridae, and subfamily Orthocoronavirinae. Like many other coronaviruses, COVID-19 spreads through airborne droplets or through direct contact, from person to person or from surface to person (1, 2).

Because COVID-19 is a small +ssRNA virus, its average diameter varies but is between 60 and 140 nm. The virus is very mild and can hang in the air because its density is about 1 g/mL. However, the spike (S) proteins on the surface of the virus are around 90. This provides a technical advantage for the virus to bind to the receptor. Moreover, these spikes can be considered quite long, each spike being approximately 9 to 12 nm in length, and the appearance is similar to a "sunbeam crown." The latent period of patients with COVID-19 is 3.69 days, and the infection period is 3.48 days. Moreover, the ordinary incubation period of COVID-19 is 5.1 days and the symptomatic formation time is 11.5 days. Depending on these values, a maximum duration of 12.5 days in 95 percentiles and a quarantine period of 14 days is sufficient. The basic number of reproduction (Ro) of COVID-19 is estimated to be 2.24–3.58 days (2). Although the primary route of transmission is through the respiratory tract, patients affected by COVID-19 also have viruses in the intestinal system, kidney, and sweat glands. Contrary to common knowledge, COVID-19 can also be thrown and smeared through feces, urine, and sweat (1, 2).

Based on the results of the current studies, it has been suggested that spike (S) protein, envelope protein, membrane protein, nucleocapsid protein, 3CL protease, papain-like protease, RNA polymerase, and helicase protein are potential targets for antiviral drugs. As of now, no licensed antiviral drug confirmed by the Food and Drug Administration (FDA) or the European Medicines Agency is available to treat COVID-19 patients. Moreover, the absence of approved vaccines

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Received: 17.08.2020
Accepted: 23.10.2020

and the absence of FDA-approved therapeutic agents against COVID-19 prevent the control of the outbreak and lead to the use of other treatment methods. Furthermore, the WHO reports confirmed that a "vaccine or specific pharmaceutical therapeutic for COVID-19" is not available as of now (2-4).

Ozone and Hormesis

Now let us take a closer look at the atomic structure of the ozone, which stimulates the body's antioxidant defense system, at least by triggering the oxidative stress (OS). Ozone is a naturally occurring gas produced from O_2 atoms in the atmosphere. The O_2 molecule consists of 2 O_2 atoms. These O_2 molecules are separated into the atoms by energy discharge or ultraviolet (UV) light from the sun during lightning storms. Single oxygen atoms cannot endure alone without being regrouped into di-atomic oxygen molecules. In this recombination phase, some atoms will transform into loosely bound triatomic O_3 (Figure 1). This novel molecule is called ozone or O_3 (3). In experiments conducted about a hundred years ago, water was shown to emit an electrical odor after electrolysis. The smell of this water is called "ozone," which means "odorant." In the gas phase, three oxygen atoms located in the ozone form an isosceles triangle with an angle of 1.26 A°. Ozone gas, discovered about a hundred years ago in the middle of the 19th century, is a gas made up of three oxygen atoms in a dynamically unstable structure due to the presence of mesomeric states. In fact, ozone has an even number of electrons in its outer orbit, meaning it has no unpaired molecules. As a result, although ozone is not a radical molecule, it is much more reactive than O_2 and easily produces some ROS produced by O_2 (2, 4).

O_3, which consists of O_2 in the ozone layer in the upper layers of the atmosphere, performs an extremely important function for life on earth. The main function of ozone is to keep humans from the detrimental effects of the UV radiation of the sun. Ozone is contained in the atmosphere in concentrations perfectly compatible with life and is formed on the earth's surface in less than 20 µg/m^3. In fact, ozone is a bidirectional gas (5).

The first effect of the ozone layer performs a vital task in blocking most of the carcinogenic UV radiation having the wavelengths of 100–280 nm from the electromagnetic waves spectrum emitted by the sun in the stratosphere, which is the upper part of the atmosphere. However, the second effect may not be very useful. Unfortunately, increasing carbon dioxide with global warming can damage the ozone layer. As a result, the increased ozone concentration in the troposphere, which is the lowest layer of the atmosphere, that touches the ground and where gases are the most intense, can cause serious pulmonary injury and raised mortality in humans. Although this effect of the ozone appears to be harmful, empirical and clinical evidence for the medical use of ozone has been growing for nearly a century (4, 5).

Paracelsus, laying the foundations of toxicology, made a well-known definition in the 16th century. He worked on the development of modern toxicology, saying that "nothing is actually poison and everything is nontoxic and only the accurate dose distinguishes a poison from a drug." Low-dose exposure of the cell/organism to a toxic, chemical, or environmental factor accelerates the species' adaptation process. This mechanism, which exists in order for organisms to survive and proliferate against various stimuli/stresses around them and to respond adaptively, can be observed in the process of hormesis, an indispensable phenomenon in the evolutionary process (5, 6). During the biological evolution of the earth, the use of oxygen by metazoans allowed a fantastic biological diversity and growth in living things but also created a slow-acting "poison," which was oxygen. Moreover, oxygen can slowly poison biological organisms through oxidation by disrupting their molecular structure (6, 7).

Hormesis explains that the response to internal or external physiological and pathogenic stimuli/injuries that threaten the life of the cell or organism is not single-phase (linear) but biphasic or even multiple (multiphasic). The adaptive process and stability (homeostasis) in the organism are preserved or impaired depending on the balance (dose, severity, and frequency) of these physiological-pathological stimuli. Hormetic stimuli elicit adaptive epigenetic phenomena. Unfortunately, contemporary life requires us to live in millions of chemicals. Therefore, the dose-poison relationship has become more important today. The biphasic dose-response curve of the ozone is a common finding of experiments in the field of toxicology, but this low-

Main Points:

- SARS-CoV-2 (COVID-19) is an RNA virus containing (S) spike proteins that contain abundant cysteine amino acids in its structure.
- No cure or vaccine for COVID-19 is available yet. Therefore, simple inexpensive adjuvant therapies are very important in COVID-19.
- Ozone, which has been safely involved in treatments for a long time with its triatomic O3 structure, can be used as an adjuvant treatment for COVID-19. Our first presentations in this field have attracted attention in the literature.
- Possible effectiveness of ozone on sulfhydryl groups and hormetic activity has been discussed in detail for the first time in the literature.

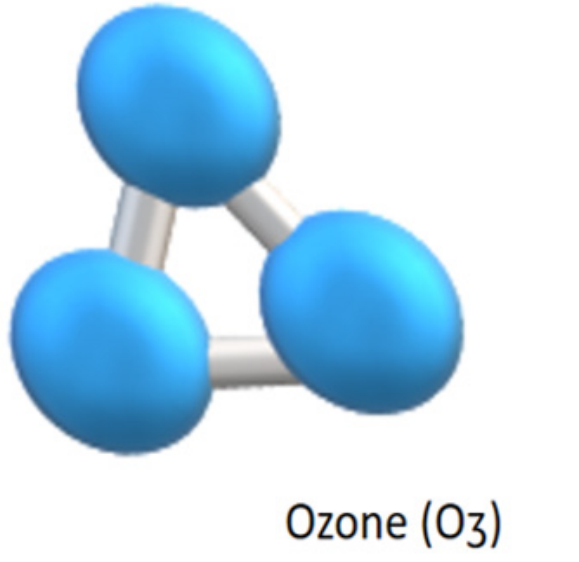

FIGURE 1. Molecular structure of ozone

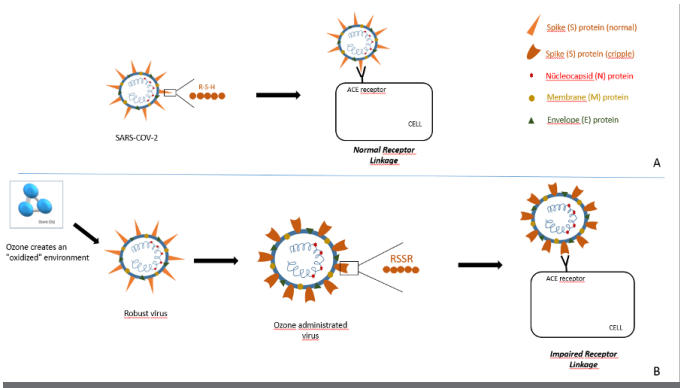

Figure 2. a, b. (a) Covid-19 and cell receptor linkage (b) The effect of ozone on the Covid-19's (S) protein and its results on the virüs receptor linkage

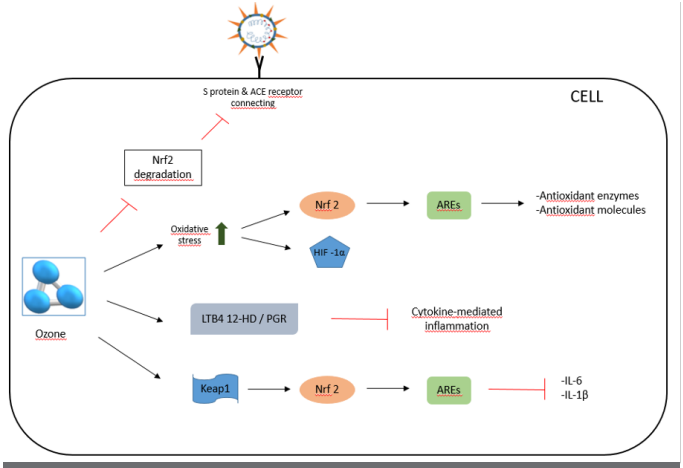

Figure 3. The effects of ozone in the cell

dose data is often overlooked, and the current appearance is that it is necessary to reduce toxin levels as much as possible. But in fact, in many cases, it has been found that these 'toxins' have important and beneficial effects, for example, at low doses of ozone. Moreover, many, but not all, drugs show hormone-dose behaviors (3, 6-9).

Despite this oxidation process, how is oxygen-dependent life possible? The answer to this is quite simple. We can say that the antioxidant system developed by living aerobic organisms, despite the presence of oxygen, has evolved over the past 2 billion years to counteract the destruction of biological life by oxygen. Indeed, the human antioxidant system has a difficult function in mitochondria to counteract the formation of a substantial amount of superoxide anions, such as approximately 3–5 g per day, and the sustained leaving of hydrogen peroxide (H_2O_2) released by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases found everywhere in the cells (7-9).

But during this task, the auxiliary of the antioxidant system is actually the oxidant agents created. Paradoxically, the appropriate dose definition and hormetic laws mentioned by Paracelsus come into play, and the signal message that is obligatory for life can be provided thanks to the physiological production of H_2O_2 in the cell. Indeed, the term "hormesis," which is very common today, can be defined as "the beneficial effect of display to a high dose of a harmful agent at a low dose," which is a double-sided effect. When we evaluate the behavioral activity of ozone, we can see a complete hormetic application. First of all, with the delivery of ozone in suitable conditions and doses to human blood, an OS occurs first. This is the first wave of the ozone effect. With the production of highly reactive messengers, a homeostatic change occurs in the person. An adaptive phenomenon, developed in the evolutionary process against this sudden OS occurring in the person, comes into play and the biochemical antioxidative reactions are induced at the cellular level. Stresses constitute an exceedingly helpful adaptation response represented by the revitalization of crucial defense mechanisms (9-11). For example, until 2002, pharmacologically low ozone doses were thought to be stimulating and high doses were preventive. Let us make a simple analogy, for example, although a low antigen dose like a vaccine has been suggested to be preventive from diseases. The fact that ozone is almost immune-stimulating is not very suitable. Although ozone acts in a complex manner and, even at high doses, can be differently effective, this high dose may be accompanied by some side effects (10, 11).

Ozone and Oxidation Potential

As it turns out that, structurally, although ozone is not a radical species in itself, it can be thought that the possible toxic effects of ozone are caused by reactions from free radicals. In conditions of oxidation potential (E °), O_3 (2.07 V) is a strong oxidant next to fluorine (3.06 V) and hydroxyl radical (2.80 V). Other common oxidants have low power compared to O_3: hydrogen peroxide (1.77 V), hypochlorous acid (1.49 V), and chlorine (1.36 V). The electrical potential of ozone can be evident in the presence of water (10, 11).

$$O_3 + 2e^- + 2H^+ \rightarrow H_2O + O_2 + 2.075V$$

Two types of different mechanisms can be proposed with ozone-induced radical-producing reactions: first is the classical strong radical type (hydroxyl radicals) which is formed and provides the oxidation of biomolecules, and the second is the mechanism that creates compounds, such as nonradical aldehydes (10-13).

Ozone is very unstable and has a half-life of about forty minutes at 20°C and is decomposed by an exothermic reaction. As is known, exothermic reactions are reaction types that can give energy out. In this case, ozone can release the released chemical energy to the organism. This complexity of the mechanism of action of ozone should always be considered in treatment. As a result, ozone can be considered as a pro-drug that produces biochemical messengers that can speed up electron transfer and general metabolism in the blood (9-13).

COVID-19 and Ozone

Ozone can be very useful in the treatment or prevention of viral infections. Usually, viruses are simple, free particles of macromolecules. Viruses, unlike bacteria, can only replicate in the infected host cell. In the years since the last two centuries, ozone has been applied in the treatment of many viral diseases. Ozone therapy was applied in addition to the standard treatment regimens in the first emerging SARS-CoV-I outbreak (2, 3, 9, 13). The fact that ozone is an energy-rich molecule in addition to its unique physico-chemical and biological properties enabled beneficial results to be obtained in SARS-CoV-I patients (9, 10, 13).

Unfortunately, fewer scientific studies have shown the effect of ozone application on SARS-CoV-2. Studies showing that ozone application can be effective in SARS coronavirus disinfection are included in the literature. Since the structure of COVID-19 is very similar to that of SARS-CoV-1, it is safe to say that the same effects will work on COVID-19 as well (3, 8). As mentioned previously, the main mechanism of ozone gas on human biochemical pathways is oxidative pre-conditioning. This notion has been shown in both proteomics and genomics trials (3, 9).

With the simplest definition, ozone destroys the nucleic acids in the nucleus by damaging the viral RNA, starting with the lipid/protein coating on their outer layers. At upper concentrations, ozone can demolish the capsid or outer protein shell of the viruses through oxidation. Numerous virus families (e.g., poliovirus 1 and 2; human rotavirus; Norwalk virus; parvoviruses; hepatitis A, B, and non-A, non-B) are viruses sensitive to the virucidal effects of ozone (2, 3, 11-14). Most studies on the virucidal effects of ozone have focused on the tendency of ozone to break down lipid and protein molecules in multiple bond configuration areas. Indeed, viral DNA or RNA cannot survive when the virus' lipid envelope breaks down. So, enveloped viruses are usually more sensitive to physicochemical distress than naked virions (13-15). However, the effects of ozone on unsaturated lipids are one of the well-documented biochemical effects. Moreover, ozone is known to interact with proteins, carbohydrates, and nucleic acids. SARS-CoV-2 is an enveloped virus, like all other coronaviruses and many other respiratory viruses. When ozone comes into contact with the protein coat or shell of a virus particle, capsid, protein hydroxides, and protein hydroperoxides are formed. Fortunately, the capsid proteins of viruses have no protection against increased OS. The oxidation of phospholipids and lipoproteins, possibly caused by ozone therapy, may also damage the viral spike (S) protein and may disturb the COVID-19 viral replication cycle by disrupting the virus-host cell contact with peroxidation (8, 15).

Sulfhydryl Groups of COVID-19 and Ozone

Regarding protein-formed viral structures, ozone may be mainly interfering with sulfhydryl groups due to its remarkable affinity. Structural proteins of COVID-19 contain a large number of sulfhydryl (R-S-H) residues from the cysteine amino acid. The virus S-H functional group is vital for COVID-19, meaning that thiol groups can be targeted in the fight against the virus. Therefore, many viral capsid glycoproteins must be in the form of reduced cysteine (SH) amino acids instead of oxidized cysteine amino acids (RSSR) to successfully penetrate host cells. For example, HIV and Ebola viruses also need reduced sulfhydryl (SH) groups, since the protection of the SH group is imperative for host receptor linkage (2, 3, 8, 15). Such viruses with such high virulence have cysteine-rich regions, including (S) protein and envelope proteins, similar to the structure of COVID-19. With these thiol groups, COVID-19 can often bind to the ACE enzyme receptor in the host cell, thereby contributing to viral fusion (Figure 2). Moreover, depending on the redox state, they can determine the ability to bind to this type of receptor as reduced thiol (SH) (open) or oxidized thiol (closed) (RSSR) (2, 3, 15).

In fact, the three-dimensional protein structure in viruses, which is rich in cysteine amino acids, is highly vulnerable to oxidation, because oxidant agents, such as ozone, cripple and disrupt the protein structure in (S) proteins and can degenerate the three-dimensional structure of the virus. Similarly, ozone, which creates an "oxidized" environment in the blood, can help the defense system to directly neutralize the amino acid thiols involved in the structure of viruses in the blood and tissues. Moreover, the spike protein (S) of COVID-19 is very prone to oxidation because the structure of the spike protein has a high number of tryptophan amino acids after cysteine, and these amino acids can easily be oxidized (15).

In fact, ozone is made up of compounds called "ozonides." Ozonides are oxidant agents that can produce the fine-tuning effect of ozone other than its direct effect. Because ozonides are oxidant molecules on their own, they produce molecules of peroxides, peroxyls, alkenes, and alkanes. These molecules can oxidize cysteine and tryptophan amino acids and keep them in a "closed" position. Similarly, ozone also reacts easily with other amino acids containing methionine, tryptophan, and sulfur. Moreover, while cysteine-dependent proteins are inactivated by ozone administration, many enzymes in viral structures are inevitably affected, for example, ozone administration may inhibit the papain enzyme. Papain enzyme inhibition can reduce viral fusion/replication. In short, we can oxidize the three-dimensional structure of cysteine/methionine, tryptophan amino acid, and lipid in the critical membrane or protein structure by using the direct oxidant property of ozone and benefit from this critical redox fragility potential in fusion regions. The free thiol group of captopril blocks the catalytic Zn^{+2} center of ACE-1. It is therefore desirable to use drugs with groups that can react covalently with COVID-19 essential proteins (13-15).

Similarly, even oxygen in the atmosphere can slowly destroy these sulfhydryl (thiol) groups in viruses through oxidation. The increase in heat can also strengthen the disrupting effect of oxygen on viruses at high atmospheric temperatures. Many viruses may gradually oxidize with atmospheric oxygen on external surfaces, such as COVID-19, and lose their ability to infect. Ozone can speed up the oxidation process made by atmospheric oxygen. For example, alcohol-based disinfectant solutions have a similar effect in preventing infections. Thiol (SH) bonds are much weaker than hydroxyl (OH) bonds in alcohols, so alcohol-containing hand disinfectants can easily oxidize viruses through oxidation. But we should not forget that oxygen-dependent life has a strong antioxidant defense system against oxygen attack in the evolutionary process. With the help of these antioxidant mechanisms, ozone cannot harm the sulfur-containing amino acids in our cells (15, 16).

The main cellular mechanisms that protect amino acids against ozone oxidation in cells and blood are the oxidation of glutathione, oxidation of NADH/NADPH coenzymes, and other antioxidative systems. Simple RNA viruses like COVID-19, which have been living since the early ages of the world, need iron atoms for their catalytic activity in the absence of oxygen in the atmosphere, and since the atmosphere has no oxygen at that time, the antioxidant protection mechanisms do not occur. Therefore, sulfur-containing protein structures, such as (S) protein can easily be damaged through oxidation. This reaction can be very important for the antiviral effect to occur, because it causes the double bonds in the ozone protein structures to easily split through a reaction called ozonolysis (15-17). Likewise, acting

ozone is a triatomic oxygen molecule, which reacts with bioorganic compounds holding double bonds, and the supplementation of three oxygen atoms to the unsaturated bond with the generation of ozonides can contribute to the fight against viral infections. Moreover, ozone can be widely used as an inexpensive, safe, and possibly effective agent in developing countries. Unfortunately, ozone therapy has not been widely applied as a treatment during the current COVID-19 outbreak. Italy and China are two examples of countries that have used ozone therapy in the treatment of COVID-19 patients. Much more work is needed for the development of the application of ozone therapy in future outbreaks. Ozone can be inexpensive, safe for use in combination with other treatments, and synergistically effective as an immunotherapy. Based on the data obtained, ozone therapy can be a useful method to control COVID-19 (16-18).

Immunomodulation and COVID-19

However, ozonides molecules are less reactive than the ozone' molecules, but their effects are resistant because they may activate several biochemical pathways and serve as biochemical signaling molecules that modulate the immune system. This immune modulatory effect can be helpful as a means of safely suppressing the "cytokine storm," including coronavirus (Figure 3). Our immune system, which is already developing in the evolutionary process, uses oxidant agents (e.g., hydrogen peroxide, superoxide, nitric oxide, hypochlorous acid) naturally for direct disinfectant purposes against microbes (18-20). It should be noted that the ozone gas exact has a double carbon bond and therefore reacts with unsaturated fatty acids present for the oxidative reaction, leading to the generation of peroxides following the hydrolytic breakdown of the poly-unsaturated lipid chains. Fortunately, COVID-19 is a very rich virus in terms of lipid presence (1, 8). The lipid chains are thus broken down by the loss of their hydrophobic properties and converted into hydrophilic (i.e., water-collapsible components). Also, lipoperoxides, derived from the breaking of an ozonide chain, become water-soluble because they are short-chain lipid compounds. Due to this feature, when we wash our hands with soap, we can neutralize the virus (1-3). Ozone can likewise disrupt the viral structure by dissolving lipids by acting at the molecular level. In particular, COVID-19, like many other viral infections, can be more lethal in the elderly population. Ozone therapy can also be used to regulate the biochemical metabolic activities among the elderly (4). Generally, low-dose prophylactic applications of ozone therapy can stimulate slowed metabolic pathways in older individuals and can make older individuals more prepared for outbreaks through immune modulation using adjuvant therapy. Of course, we can think that ozone can be applied preferably in low-dose intervals in elderly individuals, whereas, especially if older individuals encounter COVID-19, keeping their metabolism awake may be possible with intermittent ozone sessions. Unfortunately, the cellular mechanisms that could explain the positive effects of intermittent blood ozone sessions need to be investigated further (18-21).

A small-scale ozone study against the COVID-19 outbreak can be an encouraging example. In this small study conducted in China (16), the clinical and laboratory results of patients receiving ozone using the major autohemotherapy (MAH) technique were evaluated. In short, MAH was reported to be very effective and serious COVID-19 patients were discharged faster (16). The symptoms of these older patients, who were diagnosed with severe COVID-19, disappeared with MAH treatment, and their laboratory results and radiological findings were normalized (4, 16). Moreover, no side effects related to MAH have been reported in these elderly individuals with COVID-19 (16, 17). Patients who received MAH were found to be discharged in a shorter time compared to those who did not with the same age and findings. According to these preliminary study results and findings, the probability of ozone application as a treatment for COVID-19 should be considered.

Currently, no therapeutic agent has yet been confirmed as an effective treatment for COVID-19. For this reason, publications supporting alternative treatment applications are very important for the containment of the COVID-19 pandemic. However, additional laboratory and clinical research are needed to established the effectiveness of ozone in treating COVID-19. Determining the optimal ozone dose and suitable treatment time may elicit the molecular effect. Moreover, many previous studies in the literature have reported that ozone administration is effective and safe in the treatment of some strong viral infections, such as HIV, hepatitis C, and Ebola. (1, 14, 18, 19).

COVID-19 and Molecular Pathways of Ozone

Perhaps the widespread clinical application of ozone in the COVID-19 outbreak may yield successful results. In monkey cells, the ability of the virus to be inactive with ozone in the laboratory is a sign of the existence of a basic molecular mechanism. Data on such molecules from many studies in the literature are promising. Ozone application may also reduce Nrf2 degradation and is a multi-effective molecule. This effect can reduce the activity of (S) protein and ACE2 and the effectiveness of SARS-CoV-1 and COVID-19 receptors (1, 14-16).

Moreover, ozone can activate cellular antioxidant enzymes by affecting the transcription of antioxidant response elements (AREs) with the OS it temporarily creates. These enzymes include SOD, GPx, GST, CAT, HO-1, NADPH quinone (NQO-1), heat shock proteins (HSPs), and phase 2 enzymes involved in xenobiotic metabolism. This mechanism allows cells to stay awake by receiving a warning signal and fight viral infections (22, 23).

The stimulation of the Nrf2/AREs system with OS can stimulate the two intracellular antioxidant mechanisms, the glutathione system and thioredoxin system (10, 23). The activation of the intracellular antioxidant system can be important, especially to prevent the replication of viral infections. For example, glutathione peroxidase (GPx), a selenoprotein, has been shown to inhibit HIV replication (23-25). In studies showing Nrf2/AREs interaction activated by light OS formed with ozone, it has been reported that ozone application can also increase the levels of strong antioxidant molecules, such as glutathione (GSH), carbon monoxide (CO), and serum bilirubin. Moreover, the effectiveness of ozone therapy can be clarified by the activation of other nuclear transcription factors, such as hypoxia inducible factor-1a (HIF-1a), which is also induced by moderate OS. These concepts have been widely acknowledged recently (22-24).

Leukotriene B4 (LTB4) is a lipid cell mediator synthesized from arachidonic acid and secreted by neutrophils, macrophages, and endothelial cells as a potent chemotactic agent in the

blood. Ozone can have an anti-inflammatory effect in two different ways through enzymes. First, ozone can contribute to anti-inflammatory operation by stimulating the enzyme 12-hydroxyhydrogenase / 15-oxo-prostaglandin 13-reductase (LTB4 12-H / PGR) involved in LTB4 metabolism. Second, ozone can contribute to the inhibition of cytokine mediated inflammation through LTB4 12-HD / PGR enzyme induction (11-14).

Ozone has the capability to release O_2 when aqueous pH rises in the blood as well as protonic environments. The free iron Fe^{+2} load in the lungs may decrease with the increase in the amount of O_3 in the atmosphere or the therapeutic application of ozone gas. On the contrary, an increase in storage iron, that is, the antioxidant molecule ferritin, can be observed. In this way, Fe^{+2}-related cell death, that is, ferroptosis, can be reduced. In this study, the molecule that will reveal the ozone effect and determining factor is water (22-26). Without ozone H_2O, Criegee can react with unsaturated fatty acids to produce ozone. However, if the environment has plenty of water, H_2O, aldehydes, and hydrogen peroxide (H_2O_2) are produced from ozone. Since H_2O is abundant in the lungs, the main reaction with ozone will be the formation of aldehyde and H_2O_2 side-products. As a result, owing to ozonoids, ferritin synthesis, which has antioxidant activity, is induced, and thus the use of free iron and storage of free iron in the synthesis of ferritin inhibit the Fenton reaction (9, 15, 21).

$O_3 + H_2O_2 + Fe^{++}$ ------ → $OH^{\circ} + OH^{-} + Fe^{+++}$

COVID-19 infects the respiratory tract and causes mild-to-severe acute respiratory syndrome, resulting in the overproduction of pro-inflammatory cytokines, including interleukin IL-1 and IL-6. The stimulation of host cell receptors by COVID-19's RNA can lead to the release of pro-IL-1β cleavaged with caspase-1 followed by the production of active mature IL-1β, a mediator of pneumonia and fibrosis. In severe cases of COVID-19, an inappropriate increase in cytokine levels, such as interleukin-6 (IL6), interleukin-10 (IL10), and TNF-α, can be observed. In some cases, these increased cytokine levels create a "cytokine storm" and cause significant damage to the lung tissue (23-26). For example, it should be expected that ozone application may suppress the cytokine production mechanism with NLRP3 inflammasome. Finally, it should be considered that inflammasomes, a cytoplasmic complex that mediates the proceeding and secretion of pro-inflammatory cytokines, are effective in the treatment of COVID-19. The activation of NLRP3 inflammation in monocytes and macrophages can be explained by the pathogenicity of SARS-CoV-1. This inflammasome complex response is the first immunologic process in a viral infection. The IL-1 and IL-6 cytokines were secreted following the activation of inflammasomes, such as NLRP3, which regulate the cells in both the natural and adaptive immune complex and direct subsequent immune responses. Moreover, it can be concluded that ozone can also regulate cytoplasmic inflammasome activity (26-28).

Moreover, DNA repair mechanisms can be activated with ozone, and phagocytic activities can be increased in macrophages. In the severe form of COVID-19, the downregulation at the genetic level of IL-6 and other cytokines can be very valuable. Ozone can be applied as an adjuvant therapy to IL-6 blockers used in the treatment of COVID-19 to prevent cytokine storm. The effect of ozone on the nucleus in the cell results in the reduction of IL-6 and IL-1β after immune modulation is obtained in the Keap1/Nrf2/AREs pathways. These data mean that the cytoprotective effect observed during ozone therapy may contribute to the suppression of the excess immune response caused by COVID-19 (25, 28). All of the discussed mechanisms imply that ozone administration during viral infection is a paradoxical pro-oxidant therapy that initiates an endogenous antioxidant response. All experimental results have shown that O_3 in ex vivo or in vivo therapeutic dosages may activate Nrf2 while inhibiting the NF-κB pathway. The opposite is the NF-κB pathway; TNF-α activates the release of pro-inflammatory cytokines, such as IFN-γ, ILI-γ, IL6, and IL8, as well as the release of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). This effect occurs with pro-inflammatory genes (1-3, 28).

Studies on the genetic effects of ozone in the nucleus have been conducted. Studies have shown that ozone, known to accelerate wound healing, causes an important enhancement in the expression of various genes related to the wound healing process. In these studies, they found that ozone can reduce viral replication. Moreover, studies showing the genetic upregulation of immune system cells are proof that ozone can be much more effective than expected. Increased cell chemotaxis, leukocyte chemotaxis, and granulocyte chemotaxis have been demonstrated with ozone administration. The increased inflammation and excessive cytokine response in COVID-19 suggest that the ozone dose should be carefully adjusted at the hormetic level. Ozone, with its structural feature, can neutralize viruses by its direct oxidation (1, 3, 15, 16).

However, this mechanism may not be valid in vivo, because COVID-19 hides itself in the macrophages in the blood or in $CD4^{+}$ lymphocytes and blood cells. Even worse, since the virus is in the cell in other organs, pneumocytes, hepatocytes, epithelium, and glial and neuronal cells, the direct antiviral effects of ozone become obscure, so the cell's powerful defensive antioxidant system unfortunately protects the viral integrity from itself. Ozone cannot achieve sufficient concentrations in the tissues because the plasma antioxidant capacity protects organ cells and intracellular viruses are inaccessible (1, 18, 20).

Instead of increasing the dose, a low dose may be more appropriate for low-frequency hormetic activity. Thus, a prolonged ozone therapeutic treatment is sufficient for rebalancing the cellular redox condition, which is a basic process for adapting to OS and inhibiting viral replication. In fact, ozone gas permanently stimulates memory in immune cells, after being infused repeatedly during MAH, lymphocytes and monocytes can migrate back into the lymphoid system and over time, the immune system's memory can be stimulated and activate other lymphoid cells (13, 20).

Consequently, at least ozone can be considered a possible adjuvant anti-inflammatory therapy for severe cases of COVID-19. Along with the accepted direct antiviral effects, ozone (partly due to intermediaries) has beneficial biochemical effects: (1) increase in NO; (2) improved erythrocyte rheology; (3) better hemoglobin functionality; (4) increase in 2,3-diphosphoglycerate; (5) improved mitochondrial oxidative phosphorylation; (6) temporary ROS increase; (7) NrF2/AREs pathway activation; (8) increase in ILI0; (9) increase in LTB4 12-HD/PGR enzyme activity; (10) decrease in pro-inflammatory cytokines and TNFa; (11) upregulation of GSH activity and hemeoxygenase pathway (24-28).

Ozone and Hypoxemia

Ozone is a vasodilator, which can induce the production of prostacyclin. In infections that can cause severe hypoxic findings, such as COVID-19, it may cause injuries due to this feature. Ozone may be useful to avoid hypoxemia in viral infections, such as SARS-CoV-1 and COVID-19, since O_3 gas application can lead to the stimulation of 2,3-diphosphoglycerate, resulting in an increased amount of oxygen released into the tissues in erythrocytes. In this context, one of the main effects of ozone gas application is the acceleration of glycolysis, the only energy source of red erythrocytes (1, 3, 20). A basic condition to ensure the continuity of the glycolysis process is the continuous re-oxidation of NADPH, which occurs after ozone exposure. Moreover, ozone can increase ATP production, which will be needed in the organism's protection against infection, by activating the Krebs cycle by increasing the oxidative carboxylation of pyruvate. Studies on the genetic effects of ozone in the nucleus have been conducted. Because ozone can increase these two responses.

Still, a well-calibrated oxidant-stimulating ozone gas can modulate and infect different viral infections by stimulating the endogenous antioxidant system in infected patients. Moreover, the effectiveness of adjuvant ozone administration and correction of organ ischemia reperfusion should be expected as the anti-inflammatory effect of ozone (1, 28-30).

At the same time, the release of some HSP, such as HSP60, HSP70, and HSP90, which stimulate heme-oxygenase -1 (HO-1), a protective enzyme, has been determined by ozone application. HSP and HO-1 are robust activators of the innate immune system and can provoke the monocyte-macrophage system and activation of antigen-presenting cells. Also, ozone therapy increases oxygenation, especially in poorly oxygenated tissues. Ozone gas application stabilizes hepatic metabolism, which could improve hepatic protein synthesis in viral hepatic infections. Moreover, ozone may be beneficial in situations that impair liver enzymes, such as COVID-19. Ozone also activates the Nrf2 pathway, resulting in the induction of an antioxidative enzymatic system, such as HO-1. Ozon gas also suppresses NF-κB activity by eliminating excess ROS that can cause NF-κB activation. It also binds directly to gene promoters by suppressing inflammatory cytokine gene expression (31, 32).

Ozone is ultimately an adjuvant therapy because the infected patient is treated with allopathic medication while undergoing adjuvant therapy. Although ozone has no direct effect on virus infection, it can provide a beneficial clinical effect with the help of the immune system, antioxidative system, and modulation of inflammatory cytokines. However, it should be noted that ozone can lead to an increase in toxic doses, NLRP3, and cytokine. Because ozone is a common oxidant gas in urban weather and ozone exposure can cause OS, it causes airway inflammation and increased respiratory morbidity. It shows that the rapid and wide spread of COVID-19 in northern Italy is highly associated with the measured air pollution of cities (33).

CONCLUSION

Systemic ozone application may be useful as a treatment method for COVID-19. Furthermore, large clinical studies are needed in the future to verify the use of ozone in complementary therapies for COVID-19.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - N.Y.; Design - N.Y.; Supervision - E.E.; Resource - N.Y., C.Ö.; Materials - E.E., C.Ö.; Data Collection and/or Processing - N.Y.; Analysis and/or Interpretation - E.E.; Literature Search - E.E., C.Ö.; Writing - N.Y.; Critical Reviews - N.Y.

Conflict of Interest: Authors have no conflicts of interest to declare.

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

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CYPRUS JOURNAL OF MEDICAL SCIENCES

Letter to the Editor

DOI: 10.5152/cjms.2020.2722

Comment on "Dog Bites and Their Treatment in Federation of Bosnia and Herzegovina"

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Cite this article as: Syrmos N. Comment on "Dog Bites and Their Treatment in Federation of Bosnia and Herzegovina". Cyprus J Med Sci 2020; 5(4): 373.

Dear Editor,

I wish to convey some important information regarding the very interesting manuscript on Dog Bites and their Treatment in the Federation of Bosnia and Herzegovina. In my research work entitled, "Bite wound-related infections in rural areas of Macedonia-Greece: consequences on overall health", we performed a 20 year retrospective analysis on different types of bites (dog, cat, horse, human). The incidence of dog bites was high not only in the elderly (>65 years old), but also among children (<14 years old) further, the incidence was higher in men and was more prevalent during summer time, owing to higher temperature and more humidity (1, 2).

Peer-review: Externally peer-reviewed.

Conflict of Interest: The author has no conflicts of interest to declare.

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

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Received: 26.08.2020
Accepted: 03.09.2020

CYPRUS JOURNAL OF MEDICAL SCIENCES

ERRATUM

DOI: 10.5152/cjms.2021.111

In the article by Sertel et al., entitled "Examination of the Relationship Between Exercise Barriers and Physical Activity, Sleep, and Fatigue in Older Individuals" that was published in the September 2020 issue of Cyprus Journal of Medical Sciences (Cyprus J Med Sci 2020; 5(3): 226-33; DOI: 10.5152/cjms.2020.1534), authors provided incorrect date and number information for the ethics commitee report.

The error was corrected on 09.10.2020 and the updated version of the article is available online.

DOI: 10.5152/cjms.2021.112

In the article by Boyacıoğlu et al., entitled "LGBT+ Individuals' Sexual and Mental Health: A Comparison with Hetereosexual Group" that was published in the September 2020 issue of Cyprus Journal of Medical Sciences (Cyprus J Med Sci 2020; 5(3): 189-95; DOI: 10.5152/cjms.2020.864), incorrect institution information for the first author was published due to an author error.

The error was corrected on 05.01.2021 and the updated version of the article is available online.

DOI: 10.5152/cjms.2021.113

In the article by Rezaie et al., entitled "A Comparative Study on the Effect of Using Three Maternal Positions on Postpartum Bleeding, Perineum Status and Some of the Birth Outcomes During Lathent and Active phase of the Second Stage of Labor" that was published in the March 2020 issue of Cyprus Journal of Medical Sciences (Cyprus J Med Sci 2020; 5(1): 57-65; DOI: 10.5152/cjms.2020.790), author affiliations were published incorrectly.

The errors were corrected on 07.01.2021 and the updated version of the article is available online.

"The best of people are those that bring most benefit to the rest of mankind."

Koşulsuz destekleri için Kıbrıs Vakıflar İdaresi Müdürlüğü'ne teşekkür ederiz.