ISSN 2149-7893 • EISSN 2536-507X

Indexed in Web of Science

Official Journal of
Cyprus Turkish Medical Association

# CYPRUS JOURNAL OF MEDICAL SCIENCES

VOLUME 6 • ISSUE 4 • DECEMBER 2021

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES



AVES

Publisher
İbrahim KARA

Publication Director
Ali ŞAHİN

Editorial Development
Gizem KAYAN TEKAÜT

Finance and Administration
Zeynep YAKIŞIRER ÜREN

Deputy Publication Director
Gökhan ÇİMEN

Publication Coordinators
İrem SOYSAL
Arzu YILDIRIM
Deniz KAYA
Bahar ALBAYRAK
Gamze BİLGEN
Irmak BERBEROĞLU
Elif Bahar BAKIRCI
Ebru BOZ

Project Coordinators
Sinem KOZ
Doğan ORUÇ
Emre KARA

Graphics Department
Ünal ÖZER

Contact
Address: Büyükdere Cad. 105/9 34394 Mecidiyeköy, Şişli, İstanbul, TURKEY
Phone: +90 212 217 17 00
E-mail: info@avesyayincilik.com

Publication Type
Local periodical

Printed Date
September 2021

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

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

## 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).

Cyprus Journal of Medical Sciences is indexed in Web of Science-Emerging Sources Citation Index, TUBITAK ULAKBIM TR Index, EBSCO, and Gale.

Processing and publication are free of charge with the journal. No fees are requested from the authors at any point throughout the evaluation and publication process. All manuscripts must be submitted via the online submission system, which is available at www.cyprusjmedsci.com. The journal guidelines, technical information, and the required forms are available on the journal's web page.

All expenses of the journal are covered by the Cyprus Turkish Medical Association. Potential advertisers should contact the Editorial Office. Advertisement images are published only upon the Editor-in-Chief's approval.

Statements or opinions expressed in the manuscripts published in the journal reflect the views of the author(s) and not the opinions of the Cyprus Turkish Medical Association, editors, editorial board, and/or publisher; the editors, editorial board, and publisher disclaim any responsibility or liability for such materials.

All published content is available online, free of charge at www.cyprusjmedsci.com. Printed copies of the journal are distributed to the members of the Cyprus Turkish Medical Association, free of charge.



**Publisher: AVES**
Address: Büyükdere Cad. 105/9 34394 Mecidiyeköy, Şişli, İstanbul, Turkey
Phone: +90 212 217 17 00
Web page: avesyayincilik.com
E-mail: info@avesyayincilik.com

**Material Disclaimer**
Statements or opinions expressed in the manuscripts published in The Cyprus Journal of Medical Sciences reflect the views of the author(s) and not the opinions of the editors, the editorial board and the publisher; the editors, the editorial board and the publisher disclaim any responsibility or liability for such materials.

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

## Instruction to Authors

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 quarterly 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.

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

Originality, high scientific quality, and citation potential are the most important criteria for a manuscript to be accepted for publication. Manuscripts submitted for evaluation should not have been previously presented or already published in an electronic or printed medium. The journal should be informed of manuscripts that have been submitted to another journal for evaluation and rejected for publication. The submission of previous reviewer reports will expedite the evaluation process. Manuscripts that have been presented in a meeting should be submitted with detailed information on the organization, including the name, date, and location of the organization.

**Peer Review Process**
Manuscripts submitted to Cyprus Journal of Medical Sciences will go through a double-blind peer-review process. Each submission will be reviewed by at least two external, independent peer reviewers who are experts in their fields in order to ensure an unbiased evaluation process. The editorial board will invite an external and independent editor to manage the evaluation processes of manuscripts submitted by editors or by the editorial board members of the journal. The Editor in Chief is the final authority in the decision-making process for all submissions.

**Ethical Procedures**
An approval of research protocols by the Ethics Committee in accordance with international agreements (World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects," amended in October 2013, www.wma.net) is required for experimental, clinical, and drug studies and for some case reports. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning experimental research on humans, a statement should be included that shows that written informed consent of patients and volunteers was obtained following a detailed explanation of the procedures that they may undergo. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. Information on patient consent, the name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manuscript. It is the authors' responsibility to protect the patients' anonymity carefully.

For photographs that may reveal the identity of the patients, signed releases of the patient or their legal representative should be enclosed, and the publication approval must be provided in the Materials and Methods section.

**Plagiarism**
Cyprus Journal of Medical Sciences is extremely sensitive about plagiarism. All submissions are screened by a similarity detection software (iThenticate by CrossCheck) at any point during the peer-review and/or production process. Even if you are the author of the phrases or sentences, the text should not have unacceptable similarity with the previously published data.

When you are discussing others' (or your own) previous work, please make sure that you cite the material correctly in every instance.

Official Journal of
Cyprus Turkish Medical Association

CYPRUS JOURNAL OF MEDICAL SCIENCES

In the event of alleged or suspected research misconduct, e.g., plagiarism, citation manipulation, and data falsification/fabrication, the Editorial Board will follow and act following COPE guidelines.

**Preprint**
Authors must provide the journal with the pre-print server deposition of their article accompanying its DOI during initial submission.

Cyprus Journal of Medical Sciences does not consider preprint publications as prior publication. In other words, authors are allowed to present and discuss their findings on a non-commercial preprint server before submission to the journal.

If the article is published in Cyprus Journal of Medical Sciences, it is the responsibility of the authors to update the archived preprint and link it to the published version of the article.

**Authorship**
Each person listed as an author should fulfill the authorship criteria recommended by the International Committee of Medical Journal Editors (ICMJE - www.icmje.org). The ICMJE recommends that authorship is based on the following four criteria:

I. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
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.

In addition to being accountable for the parts of the work he/she has done, an author should be able to identify which co-authors are responsible for specific other parts of the work. Also, authors should have confidence in the integrity of the contributions of their co-authors.

All those designated as authors should meet all four criteria for authorship, and all who meet the four criteria should be identified as authors. Those who do not meet all four criteria should be acknowledged in the title page of the manuscript.

Cyprus Journal of Medical Sciences requires corresponding authors to submit a signed and scanned version of the Copyright Agreement and Acknowledgement of Authorship form (available for download www.cyprusjmedsci.com) during the initial submission process to act appropriately on authorship rights and to prevent ghost or honorary authorship. If the editorial board suspects a case of "gift authorship," the submission will be rejected without further review. As part of the submission of the manuscript, the corresponding author should also send a short statement declaring that he/she accepts to undertake all the responsibility for authorship during the submission and review stages of the manuscript.

**Declaration Of Interest**
Cyprus Journal of Medical Sciences requires and encourages the authors and the individuals involved in the evaluation process of submitted manuscripts to disclose any existing or potential conflicts of interests, including financial, consultant, and institutional, that might lead to potential bias or a conflict of interest. Any financial grants or other support received for a submitted study from individuals or institutions should be disclosed to the Editorial Board. To disclose a potential conflict of interest, the ICMJE Potential Conflict of Interest Disclosure Form should be filled in and submitted by all contributing authors. The journal's Editorial Board resolves cases of a potential conflict of interest of the editors, authors, or reviewers within the scope of COPE and ICMJE guidelines.

The Editorial Board of the journal handles all appeal and complaint cases within the scope of COPE guidelines. In such cases, authors should get in direct contact with the editorial office regarding their appeals and complaints. When needed, an ombudsperson may be assigned to resolve claims that cannot be resolved internally. The Editor in Chief is the final authority in the decision-making process for all appeals and complaints.

**Copyright And License**
Cyprus Journal of Medical Sciences requires each submission to be accompanied by a Copyright Agreement and Acknowledgement of Authorship form (available for download www.cyprusjmedsci.com). When using previously published content, including figures, tables, or any other material in both print and electronic formats, authors must obtain permission from the copyright holder. Legal, financial

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

and criminal liabilities in this regard belong to the author(s). By signing this form, authors retain the copyright of their work and agree that the article, if accepted for publication by the Cyprus Journal of Medical Sciences will be licensed under a Creative Commons Attribution 4.0 International License (CC BY).

**Disclaimer**
Statements or opinions expressed in the manuscripts published in Cyprus Journal of Medical Sciences reflect the views of the author(s) and not the opinions of the editors, the editorial board, or the publisher; the editors, the editorial board, and the publisher disclaim any responsibility or liability for such materials. The final responsibility regarding the published content rests with the authors.

**Manuscript Preparation**
The manuscripts should be prepared in accordance with ICMJE-Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (updated in December 2019 - http://www.icmje.org/icmje-recommendations.pdf). Authors are required to prepare manuscripts in accordance with the CONSORT guidelines for randomized research studies, STROBE guidelines for observational original research studies, STARD guidelines for studies on diagnostic accuracy, PRISMA guidelines for systematic reviews and meta-analysis, ARRIVE guidelines for experimental animal studies, and TREND guidelines for non-randomized public behavior.

Manuscripts can only be submitted through the journal's online manuscript submission and evaluation system, available at www.cyprusjmedsci.com. Manuscripts submitted via any other medium and submissions by anyone other than one of the authors will not be evaluated.

Manuscripts submitted to the journal will first go through a technical evaluation process where the editorial office staff will ensure that the manuscript has been prepared and submitted in accordance with the journal's guidelines. Submissions that do not conform to the journal's guidelines will be returned to the submitting author with technical correction requests.

Authors are required to submit the following:
- Copyright Agreement and Acknowledgement of Authorship Form, and
- ICMJE Potential Conflict of Interest Disclosure Form (should be filled in by all contributing authors) during the initial submission. These forms are available for download at www.cyprusjmedsci.com.

**Preparation of the Manuscript**
**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

Official Journal of
Cyprus Turkish Medical Association

CYPRUS JOURNAL OF MEDICAL SCIENCES

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 1 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 1 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 |

Official Journal of
Cyprus Turkish Medical Association

CYPRUS JOURNAL OF MEDICAL SCIENCES

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.

**Letters to the Editor**: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

**Tables**

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)"

Official Journal of
Cyprus Turkish Medical Association

CYPRUS JOURNAL OF MEDICAL SCIENCES

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.

While citing publications, preference should be given to the latest, most up-to-date publications. Authors are responsible for the accuracy of references If an ahead-of-print publication is cited, the DOI number should be provided. Journal titles should be abbreviated in accordance with the journal abbreviations in Index Medicus/MEDLINE/PubMed. When there are six or fewer authors, all authors should be listed. If there are seven or more authors, the first six authors should be listed followed by "et al." In the main text of the manuscript, references should be cited using Arabic numbers in parentheses. The reference styles for different types of publications are presented in the following examples.

**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.

**Revisions**
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.

Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

Official Journal of
**Cyprus Turkish Medical Association**

# CYPRUS JOURNAL OF MEDICAL SCIENCES

## Contents

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.2877

# Analysis of Peroperative Lateral Femoral Cortical Burst Complication Related to ToggleLoc with ZipLoop in Anterior Cruciate Ligament Reconstruction

**Deniz Aydın, Mehmet Yalçınozan**

**Near East University Medical Faculty, Department of Orthopedics and Traumatology, Nicosia, Cyprus**

**ORCID iDs of the authors: D.A. 0000-0003-4148-1421; M.Y. 0000-0002-2772-1137.**

**Cite this article as: Aydın D, Yalçınozan M. Analysis of Peroperative Lateral Femoral Cortical Burst Complication Related to ToggleLoc with ZipLoop in Anterior Cruciate Ligament Reconstruction. *Cyprus J Med Sci.* 2021; 6(4): 279-284.**

**BACKGROUND/AIMS**
ToggleLoc adjustable loop suspension device is a widely used implant in anterior cruciate ligament (ACL) reconstruction, which offers several advantages. However, peroperative problems related to these devices are not clear. The objective of this study was to report and analyze the peroperative lateral femoral cortical burst complication of ToggleLoc adjustable loop suspension device in ACL reconstruction.

**MATERIALS AND METHODS**
Fifty-two patients who underwent ACL reconstruction with ToggleLoc device were reviewed for peroperative lateral femoral cortical burst complication. The relation between lateral femoral cortical burst and tunnel position on lateral femoral cortex (condylar--epicondylar) was investigated from patients' records. A case-based analysis was done. Preoperative and postoperative Lysholm scores were also noted.

**RESULTS**
Peroperative lateral femoral cortical burst complication was observed in four of 52 patients. In five of the 52 cases, ToggleLoc button was placed over the condylar region, and four of the cases with condylar button placement had the complication. The mean preoperative and postoperative Lysholm scores were 37.98 (min: 26, max: 62) and 91.73 (min: 81, max: 100), respectively.

**CONCLUSION**
ToggleLoc button structure and distal loading of tensioning sutures cause increased risk of lateral femoral cortical burst during ACL reconstruction. As a precaution, condylar placement of the femoral tunnel lateral cortical aperture should be avoided.

**Keywords:** Anterior cruciate ligament, femoral fixation, suspension device, adjustable loop

## INTRODUCTION

Adjustable loop cortical suspension devices have become increasingly popular in femoral fixation of quadrupled hamstring grafts in anterior cruciate ligament (ACL) reconstruction. ToggleLoc (ToggleLoc™ Device with ZipLoop® Technology) is an widely used adjustable cortical suspensory device in ACL reconstruction. These devices are aimed to provide several advantages: single implant with adjustable technique fits all femoral socket-tunnel measurements, extraspace is not necessary in femoral socket for flipping button over cortex so longer graft placed in femoral socket, decreased distance between button and graft may reduce the bungee effect, and also re-tensioning on femoral side is possible after tibial fixation.[1,2] On the other hand, some concerns are raised about these devices. As the similar complications like fixed loop devices could be expected, another problem of loosening in adjustable loop is questioned in literature.[2,3] In a technical report about ToggleLoc, the complication of lateral femoral cortical breakage was mentioned in a 45-years-old male, and it is suggested to place suspensory button proximally to avoid thin and weak distal metaphyseal area, especially in older patients.[4]

Lateral femoral cortical burst and metaphyseal escape of suspensory button are unusual complications in ACL reconstruction. In this paper, we report this peroperative complication related to ToggleLoc with ZipLoop device and

**Corresponding Author:** Deniz Aydın
**E-mail:** deniz.aydin@neu.edu.tr

**Received:** 17.09.2020
**Accepted:** 26.11.2020

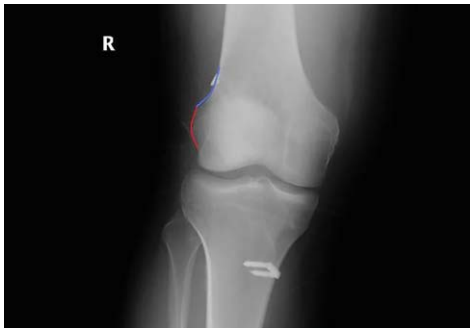


**Figure 1.** Anteroposterior knee X-ray. Condylar region is marked with red and epicondylar region with blue.

hypothesized that it is more common with condylar placement of the suspensory button. We discuss the mechanism of this complication to present the reasons and technical pitfalls for prevention.

## MATERIALS AND METHODS

Hospital records of 52 patients who underwent ACL reconstruction with ToggleLoc between January 2016 and March 2017 in a single institution were evaluated retrospectively. Cases with anatomic single bundle reconstruction through accessory medial portal with quadrupled hamstring autograft were included in the study. ACL reconstructions with transtibial femoral tunnel preparation, femoral fixation technique other than ToggleLoc device, and grafts other than quadrupled hamstring autograft were excluded from the study. All the surgeries were done by the two surgeons with experience more than 5 years. Age, sex, side, injury mechanism, and associated injuries were recorded. Tunnel position on lateral femoral cortex (condylar–-epicondylar) was analyzed by using postoperative anteroposterior knee X-rays. Condylar and epicondylar regions were shown in Figure 1. Distance between lateral cortex to graft (femoral tunnel length – femoral socket depth difference) and graft size was investigated with operation notes. Preoperative and postoperative Lysholm scores were recorded for all patients.

Operation notes and videos were reviewed to document cases with peroperative complications. Four cases were reached. A case-based analysis of peroperative complications for possible reasons and salvage methods was done.

This study was approved by Near East University Ethics Review Board on 20.04.2017 with project number YDU/2017/46-395. All the patients gave written informed consent for the publication of their individual data.

**Main Points**

- Femoral fixation of quadrupled hamstring autograft with ToggleLoc Ziploop system in anterior cruciate ligament reconstruction provides satisfactory results with good postoperative Lysholm scores.
- Lateral femoral cortical burst during ACL reconstruction with ToggleLoc button is not a rare peroperative complication.
- ToggleLoc button structure and distal loading of tensioning sutures cause increased risk of lateral femoral cortical burst during ACL reconstruction.
- As a precaution, surgeons should avoid condylar placement of the femoral tunnel lateral cortical aperture and femoral tunnel-hamstring graft size mismatch.

### Surgical Technique

Patients were operated in supine position under tourniquet with legs swinged from operating table. Diagnostic arthroscopy was done to correlate ACL rupture and evaluate associated intra-articular injuries. Hamstring tendons were harvested, prepared, and placed to ToggleLoc device in the usual manner. Accessory medial portal was created to prepare femoral tunnel. Guide pin was placed through the accessory portal over the anatomic femoral footprints of ACL, and the knee was flexed to 100-120° while advancing the guide pin anterolaterally. The knee hyperflexion was provided by combined flexion of hip in some cases, and the technique provides a more horizontal and anterior directed femoral tunnel to avoid posterior wall blow-out. The knee was kept in the same position to create button tunnel with 4.5 mm drill and the femoral socket with drill size equal to prepared graft. The length of button tunnel was measured. Femoral socket was created 25 mm depth, routinely. Tibial tunnel was drilled in the same size with the graft over a guide wire placed from medial tibial cortex to the center of ACL tibial footprint. Length of the femoral button tunnel was marked on ToggleLoc loops, depth of the femoral socket was marked on quadrupled graft, and tensioning sutures were signed to distinguish from suspensory loops. Passing suture attached to the one side of ToggleLoc button was moved from tibial tunnel to femoral tunnel and passed through anterolateral surface of leg. ToggleLoc button was brought to the femoral lateral cortex with the help of passing suture. It was cautioned to pull ToggleLoc button smooth surface laterally. Passing suture was pulled medially to flip ToggleLoc button, while the marks on loop reach to the femoral tunnel intra-articular aperture. The graft was moderately tensioned distally during all procedure. The presigned tensioning sutures were passed to the medial portal. The graft was pulled from the tibial tunnel to the femoral socket by the force applied distally to the tensioning sutures. Once the marked line over the graft reached to the femoral tunnel intra-articular aperture, tensioning was stopped. The knee was cycled 10 times with manual loading for pretensioning, and then the system was retensioned for possible displacement. Tibial fixation was done in 20° knee flexion under manual tension. The tibial side was fixed with tunnel sized diameter and 20 mm length bioabsorbable screw, which was reinforced with staple. Graft tension, knee stability, and range of motion without impingement were checked before ending the operation.

## RESULTS

Fifty-two patients were evaluated in the study. Four were female and 48 were male. The age of patients ranged from 18 to 44 (mean 28.7, median 28). Measured graft sizes and corresponding tunnel diameters were 7 mm in two, 8 mm in 46, and 9 mm in four patients. Femoral socket depth was standardized to 25 mm. Minimum and maximum femoral tunnel lengths created with 4.5 mm drill were 37 and 52 mm, respectively. So, the minimum distance between ToggleLoc button and graft was 12 mm, and maximum distance was 27 mm (mean 18.1, median

**Table I.** Demographic Characteristics of Patients with Peroperative Complications

| | Case 1 | Case 2 | Case 3 | Case 4 |
|---|---|---|---|---|
| Age | 44 | 23 | 28 | 20 |
| Sex | Male | Male | Male | Male |
| Side | Right | Right | Right | Left |
| Associated injuries | Patellofemoral arthritis<br>Degenerative medial meniscal tear | None | Lateral meniscus posterior horn tear | None |
| Tunnel placement | Condylar | Condylar | Condylar | Condylar |
| Femoral button tunnel length | 42 | 40 | 39 | 37 |
| Distance between button and graft | 17 | 15 | 14 | 12 |
| Graft size | 8 mm | 8 mm | 8 mm | 8 mm |
| Preoperative Lysholm score | 37 | 34 | 41 | 49 |
| Postoperative Lysholm score | Not reported | 90 | 94 | 86 |

18). In 47 of the cases, femoral button placement was over the epicondylar region, and in five of them, it was condylar.

Lateral cortical burst with metaphyseal migration of the ToggleLoc button was observed in four patients, and no other major peroperative complication was observed. Four of the cases with condylar placement of the button had the lateral cortical burst complication. Demographic characteristics and case analysis of patients with peroperative complication were given in Table I.

The mean preoperative Lysholm score was 37.98 (min: 26, max: 62). Postoperative Lysholm score was available in 42 of the patients with a mean follow-up period of 25 months (14-39 months). Four of the patients with peroperative complication were not included in these 42 patients. The mean postoperative Lysholm score was 91.73 (min: 81, max: 100). Reconstruction failure was observed in two of the 42 patients.

**Case 1**

A 44 year-old-male was injured 11 months before operation. Injury mechanism was twisting of the knee while landing from 1 m height. Mild patellofemoral arthritis and degenerative posterior medial meniscal tear were associated. ACL reconstruction was done. was done. Graft was measured as 8 mm, 4.5 mm femoral button tunnel was 42 mm, and 25 mm femoral socket was created. Lateral cortical burst was distinguished at the end of surgery after tibial fixation while moving the knee in full range of motion for testing. Tibial fixation was removed, and graft with ToggleLoc system was taken out. It was observed that there is no distance between graft and loop. As it was assumed that button was not seated over the lateral cortex, a new device was used for suspension and checked with fluoroscopy for appropriate seating of device over the lateral cortex. However, button was migrated again even with initial loading on tensioning sutures. Lateral cortical burst was diagnosed. Lateral incision was used to approach lateral cortex, and screw washer was placed between ToggleLoc button and lateral cortex. The graft was pulled into planned position and fixed. Postoperative X-rays showed condylar placement of button. Standard rehabilitation was applied to the patient with no complication on the third month, but he missed the later follow-ups, so his clinical and radiological results are unknown.

**Case 2**

A 23 year-old-male was injured during football game. No associated intra-articular lesion was existed. Reconstruction was done 11 months after initial injury for recurrent giving way complaints of the knee. Graft diameter was 8 mm, femoral tunnel was 40 mm, and femoral socket was 25 mm. Lateral cortical burst was not understood peroperatively, as the knee was stable, and graft was tensioned during peroperative evaluation. However, postoperative x-rays showed metaphyseal migration of button (Figure 2a). Lateral aperture of femoral tunnel was on condylar region. He was out of complaint in his 2 year follow-up with stable knee and no further displacement of button (Figure 2b). His preoperative and postoperative Lysholm scores were 34 and 90, respectively.

**Case 3**

A 28-year-old male reported that he fell while he is running on an uneven surface. ACL rupture and lateral meniscus posterior horn tear were diagnosed. He was operated 1 month after injury. A 8 mm diameter hamstring graft was used with 39 mm femoral tunnel and 25 mm femoral socket. Lateral cortical burst was diagnosed during graft passage from tibial tunnel to femoral tunnel. Sudden loosening of the tensioning sutures with a crack voice was observed. ToggleLoc system was replaced by Endobutton fixed loop device. Postoperative x-rays revealed condylar placement of button. He was out of complication in his 2-year follow-up with return to sports. His preoperative and postoperative Lysholm scores were 41 and 94, respectively.

**Case 4**

A 20-year-old male was injured 6 months before operation during a football game. No associated intra-articular lesion was existed. Graft diameter, femoral tunnel, and socket lengths were 8, 37, and 25 mm, respectively. Lateral cortical burst was diagnosed during graft passage through the tibial tunnel. Sudden loosening of the tensioning sutures with a crack voice was observed. The ToggleLoc system was replaced by the Endobutton fixed loop device. However, the graft was again stucked in tibial tunnel. The graft diameter was measured again. As the tunnels were 8 mm, the graft was hardly passed from that diameter on the measurement guide. Tunnel diameters were increased to 8.5 mm, and later, the graft passage and endobutton fixation were easily provided. Postoperative radiographies showed that the button was placed distally over the condylar region cortex. No complaint or radiological problem was reported in his 15 month follow-up. His preoperative and postoperative Lysholm scores were 49 and 86, respectively.

**DISCUSSION**

In this study, it was found that lateral cortical burst and metaphyseal escape of button are not rare peroperative

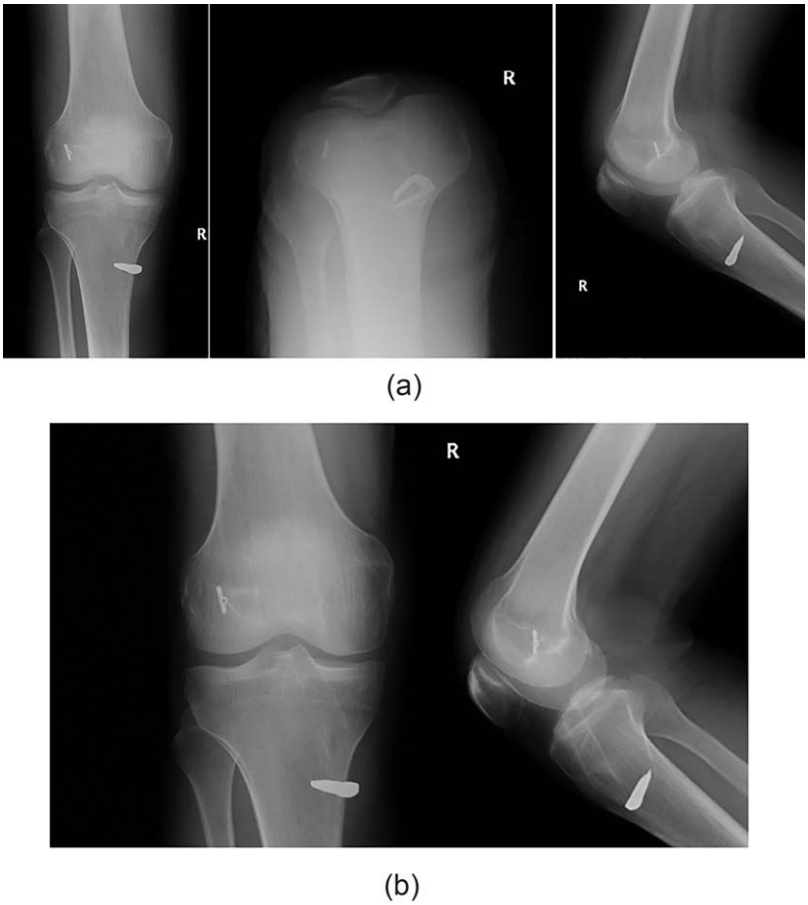


**Figure 2.** (a) Early postoperative knee X-rays of the case 2. It shows metaphyseal migration of ToggleLoc button. (b) Knee X-rays show that the button is not further displaced during the 2-year follow-up.

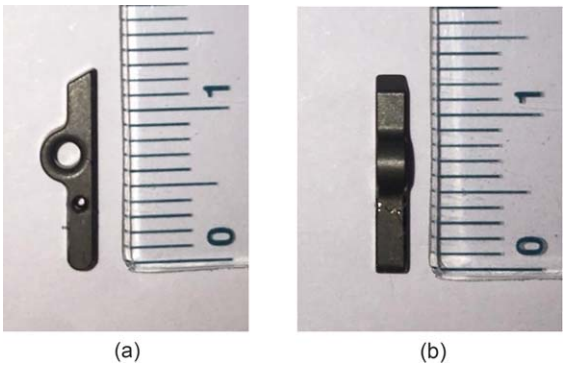


**Figure 3.** (a) Lateral picture of ToggleLoc button. (b) Undersurface picture of ToggleLoc button. It has an asymmetric structure, and the undersurface of the button has a noncentered ring for adjustable loop.

complications of ToggleLoc device in ACL reconstruction. Surgeons are not familiar with this problem as it is not usual by using other suspensory fixation devices.

ToggleLoc device has some unique properties, but these may cause undesired problems. The width of the ToggleLoc button is narrower than the other suspensory devices. The button has an asymmetric structure, and the undersurface of the button has a ring for adjustable loop, which is not centered (Figure 3). In other suspensory devices (Smith & Nephew Endobutton CL, Arthrex RetroButton, Linvatec XO Button), there are two holes on a symmetric button structure.[5] Vertically applied forces to the button should be evenly spread over the lateral femoral cortical surface. Unevenly affecting forces between button and cortex will increase pressure on bone with same load acting on a decreased area. An object with symmetrically centered multiple acting points like two holed suspensory devices is more balanced. ToggleLoc button has an uneven load distribution with one acting point, which is not centered. Figure 4 shows that ToggleLoc button arms are unevenly seated on sides of drill hole. In theory, ToggleLoc button structure could contribute lateral femoral cortical failure with increased forces on a smaller area cause of narrower width, asymmetric shape, and single noncentered suspension point. However, in a biomechanical study, cortical failure of fresh frozen porcine femur bone was reported in only 10% of constructs, which is same for Endobutton and XO Button, and ultimate failure load is also close to them.[6]

The zipping system of ToggleLoc adjustable loop is also unique. The tensioning sutures are pulled distally to place the graft into the femoral tunnel. ToggleLoc button acts like a pulley system. The applied force to the tensioning sutures is equal to the force created on the adjustable loop sutures. The sum of these two forces or twice the force we applied to the

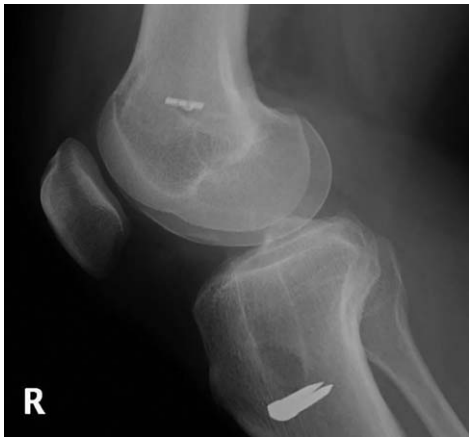


**Figure 4.** ToggleLoc button arms are unevenly seated on sides of drill hole.

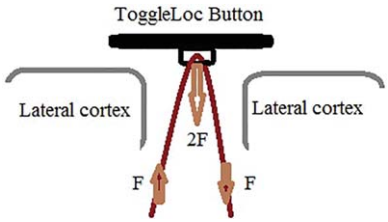


**Figure 5.** The force acting over the lateral femoral cortex by ToggleLoc button is twice the force applied to the tensioning sutures as the button works like a pulley system.

tensioning sutures is the load over the ToggleLoc button. So, the force acting over the lateral femoral cortex by ToggleLoc button is twice the force necessary to bring the graft from tibial tunnel to the femoral tunnel (Figure 5). In anatomic ACL reconstruction, the axis of these two tunnels is different. As a result, the necessary force to pull the graft from tunnels is increased. Any obstacle in graft passage will increase the load on tensioning sutures. Unprepared tibial tunnel intra-articular aperture with ACL remnants or failure to advance drill for appropriate preparation of articular orifice may cause stucking of graft during passage. Also, graft-tunnel size mismatch (a larger graft in a smaller tunnel) will cause big forces over lateral femoral cortex. Measurement mistake or swelling of the graft (while preparing the tunnels, the prepared graft is kept in a saline soaked gauze to prevent from drying) could be the reason for graft-tunnel size mismatch. Graft-tunnel size mismatch is a very serious problem, and it is irreversible if the graft is stucked in the tibial tunnel. In this case, all the system is squeezed, and there is no room for flipping the button reversely. Excessive forces are applied in this situation, which may result with passage of the graft or breakage of the lateral femoral cortex. Graft-tunnel mismatch was observed in I of our complicated cases. We believe that graft swelling after measurement was the reason for mismatch. The problem was solved by enlarging the tunnels' diameter, and Endobutton CL was used for femoral side fixation.

In our study, all the four cases with peroperative complication had the metaphyseal placement of the ToggleLoc button. Certain loads are applied over the lateral femoral cortex during implantation of the graft. So, we suggest that the ToggleLoc button should be placed on a stronger area as far as possible. As the diaphyseal cortex is much more stronger than metaphyseal area, more proximal placement of femoral tunnel external aperture provides stronger support for button. Vertical tunnels are necessary for higher placement of femoral tunnel external aperture. Independent femoral tunnel drilling from an accessory medial portal cause more horizontal tunnel placement than transtibial femoral tunnel drilling.[7] In anatomic reconstructions through the accessory medial portal, the angle between drill and lateral notch wall could be decreased to provide a higher femoral tunnel external aperture placement. However, decreased angle between drill and lateral notch wall could cause cartilage damage on posterior articular surface of lateral femoral condyle and posterior wall blowout.[8]

In the case of lateral cortical burst with ToggleLoc, many salvage procedures are possible. However, revision from the same tunnel with a new ToggleLoc device is useless. In our first complication, we did not recognize the problem in the beginning and tried on revision with a new ToggleLoc device. As it was unsuccessful, we used lateral approach to define the problem. We used screw washer between bone and button cause of lateral cortical breakage. Although no problem was observed in the patient's 3-month follow-up, this kind of fixation is not advisable cause of button slippage risk. In the next two complications, we met peroperatively, we used Endobutton CL fixation as it covers a wider surface over the lateral femoral cortex. In a study, Hammond et al. reported that three femoral cortical suspensory fixation devices (Linvatec XO Button, Arthrex RetroButton and Smith & Nephew Endobutton CL) provide satisfactory fixation strength even in the case of lateral femoral cortical breach with 8 mm or smaller diameter drill.[5]

## LIMITATIONS

There are several limitations in this study. It presents small number of patient, and it reflects the experiences of a single institution. So, this study is unable to estimate the true prevalence of the peroperative lateral cortical burst complication related to ToggleLoc system. Another week point is the lackness of a biomechanical study to compare the ultimate failure load of condylar and epicondylar regions with ToggleLoc button fixation. Statistical analysis was not used as the number of complicated cases, and cases with condylar button placement are very low.

## CONCLUSION

ToggleLoc button structure and distal loading of tensioning sutures cause increased risk of lateral cortical burst during ACL reconstruction. As a precaution, condylar placement of the femoral tunnel lateral cortical aperture should be avoided. Also, graft size should be remeasured just before implantation, and tunnels should be well prepared to prevent catching of the graft in the tunnels. Surgeon should be ready for alternative fixation methods in the case of lateral cortical burst.

**Ethics Committee Approval:** Ethical committee approval was received from the Near East University Ethics Review Board (YDU/2017/46-395)(20.04.2017).

**Informed Consent:** Written informed consent was obtained from all participants who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - D.A., M.Y.; Design - D.A., M.Y.; Supervision - M.Y.; Resources - M.Y.; Materials - D.A.; Data Collection and/or Processing - D.A., M.Y.; Analysis and/or Interpretation - D.A.; Literature Search - M.Y.; Writing Manuscript - D.A., M.Y.; Critical Review - D.A.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES

1. Nye DD, Mitchell WR, Liu W, Ostrander RV. Biomechanical comparison of fixed-loop and adjustable-loop cortical suspensory devices for metaphyseal femoral-sided soft tissue graft fixation in anatomic anterior cruciate ligament reconstruction using a porcine model. *Arthroscopy*. 2017;33(6):1225-1232. [CrossRef]
2. Choi NH, Yang BS, Victoroff BN. Clinical and radiological outcomes after hamstring anterior cruciate ligament reconstructions: Comparison between fixed-loop and adjustable-loop cortical suspension devices. *Am J Sports Med*. 2017;45(4):826-831. [CrossRef]
3. Houck DA, Kraeutler MJ, McCarty EC, Bravman JT. Fixed-versus adjustable-loop femoral cortical suspension devices for anterior cruciate ligament reconstruction: A systematic review and meta-analysis of biomechanical studies. *Orthop J Sports Med*. 2018;6(10):2325967118801762.
4. Kubota M, Ohno R, Sato T, Yamada D, Fukusato S, Kaneko K. Pearls and pitfalls of the ToggleLoc with ZipLoop for anterior cruciate ligament reconstruction. *Arthrosc Tech*. 2018;7(8):e863-e867. [CrossRef]
5. Hammond KE, Dierckman BD, Potini VC, Xerogeanes JW, Labib SA, Hutton WC. Lateral femoral cortical breach during anterior cruciate ligament reconstruction: A biomechanical analysis. *Arthroscopy*. 2012;28(3):365-371. [CrossRef]
6. Petre BM, Smith SD, Jansson KS, et al. Femoral cortical suspension devices for soft tissue anterior cruciate ligament reconstruction: A comparative biomechanical study. *Am J Sports Med*. 2013;41(2):416-422. [CrossRef]
7. Clockaerts S, Van Haver A, Verhaegen J, et al. Transportal femoral drilling creates more horizontal ACL graft orientation compared to transtibial drilling: A 3D CT imaging study. *Knee*. 2016;23(3):412-419. [CrossRef]
8. Zantop T, Haase AK, Fu FH, Petersen W. Potential risk of cartilage damage in double bundle ACL reconstruction: Impact of knee flexion angle and portal location on the femoral PL bundle tunnel. *Arch Orthop Trauma Surg*. 2008;128:509-513. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.1823

# Comparison of Custom-Made 3D Printed Bio-Degradable Plates and Titanium Anatomical Plates at Fracture Treatment: A Biomechanical Study

**Mehmet Yalçınozan[1], Emil Mammadov[2]**

[1]Department of Orthopaedics and Traumatology, Near East University, Faculty of Medicine, Mersin, Turkey
[2]Department of Paediatric Surgery, Near East University, Faculty of Medicine, Nicosia, Cyprus

**ORCID iDs of the authors: M.Y. 0000-0002-2772-1137; E.M. 0000-0001-8143-1643.**

**Cite this article as: Yalçınozan M, Mammadov E. Comparison of Custom-Made 3D Printed Bio-Degradable Plates and Titanium Anatomical Plates at Fracture Treatment. *Cyprus J Med Sci.* 2021; 6(4): 285-289.**

**BACKGROUND/AIMS**
The aim of this study is to evaluate the custom-made three dimensional (3D) printed bio-degradable implants in osteosynthesis of fractures and compare them with widely used titanium implants.

**MATERIALS AND METHODS**
Custom 3D printed bio-degradable poly-L-lactic acid (PLLA) implants were compared with titanium plates. The tibial fracture models were obtained from ankle computerized tomography (CT) images. 3D model files obtained from 10 patients ankle CT images with medial malleolar fractures were used for anatomical reduction and 3D implant modeling. The PLLA and titanium plates were tested on 3D printed bone models. Fracture reduction quality was evaluated by drawing an imaginary line between the proximal and distal cortices of the fracture. A break in this imaginary line was defined as low quality of reduction. Maximum load and compression strengths were evaluated by the mechanical test system. Results were statistically evaluated with independent sample t-test ($P < .05$).

**RESULTS**
The maximum load and compression strength values of the titanium plates were significantly higher than the PLLA plates. The reduction quality was perfect in all custom-made plate fixed fracture models; however, in six of 10 fracture models fixed with titanium plates, the reduction was found in low quality.

**CONCLUSION**
The results of the study revealed that full anatomical custom plates can be manufactured using 3D printer. The 3D design provided better reduction quality at in vitro fracture models. These bio-degradable implants may be eligible for implantation at fracture stabilization at non-weight bearing areas. The improvement of bio-degradable materials and 3D printing techniques may allow these systems to be eligible for the treatment of fractures.

**Keywords:** Fracture healing, bio-degradable, custom implant, 3D printer, osteosynthesis

## INTRODUCTION

Implants are a wide variety of materials applied for the treatment of fractures. Among the common implant types, plate-screw systems that are directly applied on the fracture site to achieve a stable fixation in the means of better healing are manufactured of various metals. Although the use of metals in implant production brings many benefits, there are still disadvantages such as implant irritation, implant-related infection, and difficulties with radiologic imaging, which may bring necessity of additional surgical interventions for implant removal.

Bio-degradable polymers are biocompatible materials that are absorbed with enzymatic reaction over time, without releasing any toxic products for the human body.[1] These polymers have been used in the medical field for a long time in various ways, especially in orthopedics and traumatology.[2] In this regard, while these implants do not exist permanently, its use in orthopedics and traumatology has advantages like the low risk of implant reaction, not effecting the imaging methods, no removal surgery requirements, and the ability to be used as carrier systems for drugs, growth factors, and

**Corresponding Author:** Mehmet Yalçınozan
**E-mail:** mehmet.yalcinozan@med.neu.edu.tr

**Received:** 04.07.2020
**Accepted:** 29.09.2020

cells. However, the use of biodegradable plastics as a plate-screw system is not widely available. The plate-screw system to be evaluated in this study is planned to be produced from bio-degradable polymers and will be the first in the literature in terms of use in the field of orthopedics and traumatology. Studies about the usage of 3D printers in the medical field are available in the literature.[3–10] In addition, it has been shown in various publications that the 3D models of the fracture created by using the 3D computed tomography images of the patient before the operation can be used for the planning of the operation. The existing bone plates can be shaped and adapted to these models before the operation, thereby shortening the operation time.[5] In other publications, titanium, etc., prepared by either 3D molding or direct production for bone defects or fracture models created in the same ways.[11–18] However, there is no study in the literature on the application of 3D printed bio-degradable implants according to fracture reduction on 3D reformatted CT images.

In this study, it was aimed to simulate osteosynthesis of fracture by custom-made 3D printed bio-degradable implants, measure the compressive strengths of these implants, and compare them with the widely used titanium implant systems. We hypothesized that custom-made plates may allow better reduction, but the strength of custom-made bio-degradable plates made with current 3D printing techniques is not enough for fracture treatment of weight bearing areas.

## MATERIAL AND METHODS

Twenty tibial medial malleol fracture models were formed for the evaluation and comparison of 3D custom-made bio-degradable and titanium anatomical plates. Tibial medial malleol fracture models were taken from CT images obtained for surgical planning due to ankle fracture of 10 patients between April 2017 and August 2019. Volume creation and partitioning were done using the Fujifilm Synapse 3D software (Fujifilm Corp, JP). 3D model files were transferred to the Meshmixer software (Autodesk Inc, USA), and anatomical reduction was performed on the same software. In addition, one model of each anatomically reduced tibia model has been created for each patient with a 1 cm horizontal defect starting 1 cm proximal to the tibial joint surface (Figure 1).

Ten of the tibial medial malleol fracture models were fixed by custom-made bio-degradable plates, and the other 10 were fixed with titanium medial tibial anatomic plates, as two groups. Standard titanium medial tibial anatomic plates used in this study were provided by a local distributor, and bio-degradable PLLA-based implants were produced with a 3D printer custom for the patient. The titanium plates evaluated in this study were precontoured anatomical plates, which are, in particular, designed for distal medial tibial fractures. Custom-made plates were produced by using the reducted fracture model. After the reduction procedure of the fracture model, the patient-specific plate was drawn and converted into a solid 3D object. The length of custom plates was defined by the fracture configuration and the planned screw count and placement positions. The width and thickness of these plates were made similar to the titanium plates evaluated in this study. The total lengths of the custom plates were between 76 and 82 mm with three proximal and three distal screws in all plates. All custom plates were printed with 4 mm thickness and 12 mm width. The screw locations suitable for 3 mm screws were determined on the plate, and holes were made on the plate models using the Boolean removal method. As this is an in vitro study, no surface smoothing process was done to improve the implant design.

**FIGURE 1.** 3D printed distal tibia model with anatomical reduction on CT images of the patient.

**Main Points**

- Orthopaedics and Traumatology is a developing surgical specialty in cooperation with various branches of science, especially biomechanical engineering.
- The 3D image processing and usage for manufacturing custom implants will be helpful for better outcomes of Orthopaedic surgeries.
- With the perfection of biodegradable materials 3D implant printing will be a promising technology.

3D printing was done after transferring the model files to the MakerBot Desktop software in the stereolithography (.stl) file format (MakerBot Industries, LLC, USA). Plates and bone models of reduced fractures are manufactured with PLLA filament at 210 °C and 55 mm $s^{-1}$ printing speed, in 3D in MakerBot Replicator 3D Generation (MakerBot Industries, LLC, USA). The evaluated plates were anatomically fixed to these bone defect models (Figure 2). The fracture reduction quality was evaluated by drawing an imaginary line between the proximal and distal cortices around the defect site. A break in this imaginary line after the fixation of the plates was defined as low quality of reduction. In all fixations of both titanium and custom-made plates, 3.5 mm steel screws were used.

After the reduction and fixation with both plate types, maximum load and compressive strength values were measured using a mechanical test system (Llyod-Ametek EZ-50 Material Testing Machine, UK) with 50000-N transducer (Figure 3). Measurement data were collected using the Nexygen ver 4.5.1 issue 3 software. Results were statistically evaluated using independent samples t-test ($P < .05$).

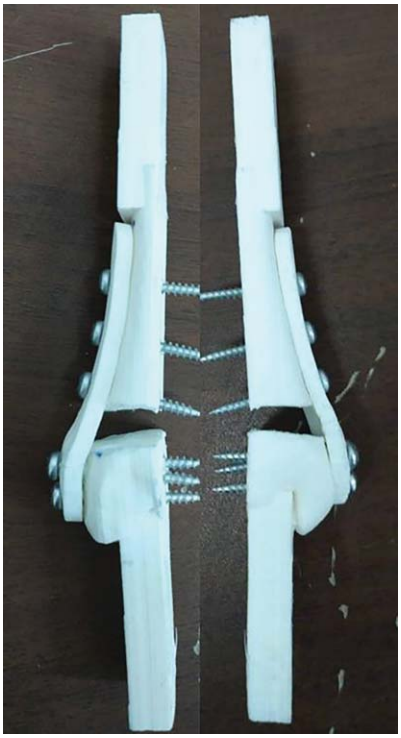

**FIGURE 2.** The implanted PLLA plate on the bone model with full contact. (a) Posterior view; (b) anterior view.

**FIGURE 3.** Tibia model with implanted plate on Llyod-Ametek EZ-50 Material Testing Machine.

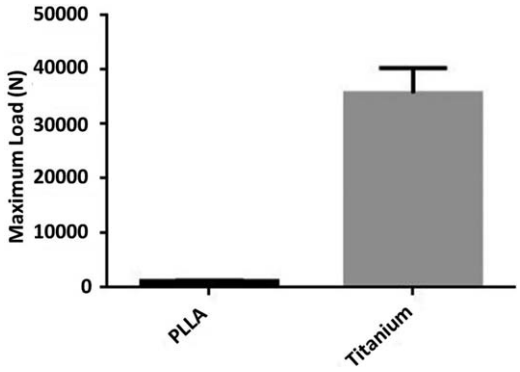


**FIGURE 4.** Mean maximum load values of PLLA-based and titanium plates.

## RESULTS

CT evaluation images of 10 patients with medial malleolar fractures were used to create 10 tibial medial malleolar fracture model and 10 custom-made bio-degradable plates based on these fracture models. Additionally, 1 cm width bone defect was created in every fracture model at the same site. Each formed tibial medial malleolar fracture model with the defect was fixed by a custom plate and a titanium plate. The quality of reduction was evaluated as perfect in all custom-made plate fixed fracture models. However, in six of the titanium plate fixed models, the achieved reduction was defined as low quality.

During the study, all PLLA-based and titanium plates were broken during compressive loading tests. Average maximum load and compressive strength values of PLLA-based plates were determined as 1,172 N and 15.64 MPa. Mean maximum load and compressive strength values of titanium plates were determined as 35,709 N and 1,190 MPa. When both groups were compared in terms of maximum load and compressive strength, titanium plates were found to be statistically stronger ($P < .001$) (Graphs 1 and 2). It was determined that the custom-made plates were broken at the level of the distal screw holes during the study (Figure 4). On the other hand, there was a wide variation at the titanium plates breakage sites.

## DISCUSSION

To the best of our knowledge, there are no studies in the literature related to the 3D printed custom-made bio-degradable plates. This study proved the hypothesis that the full anatomic shape of the custom-made plates may allow better reduction compared to standard titanium plates with a better surface compatibility. In this study, it was observed that the compressive strength of PLLA-based plates was significantly lower than the titanium plates. However, the uniform breaking pattern of 3D printed plates may be related to the plate design, which can be prevented by improving the plate design to increase the compressive strength.

The average maximum load and compressive strength values of titanium plates were found similar to the previous studies in the literature (Figures 5 and 6).[19,20] The strength of the evaluated PLLA-based plates was significantly lower than the titanium plates. The results of this study support that the mechanical strength of 3D printed custom bio-degradable

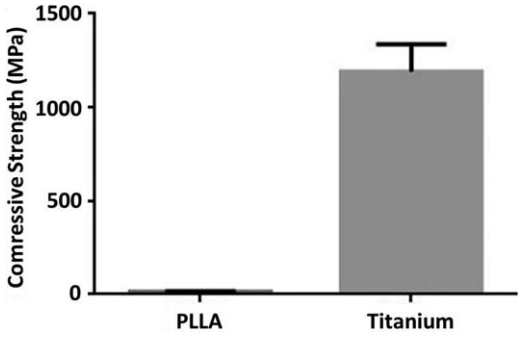


**FIGURE 5.** Mean compressive strength values of PLLA-based and titanium plates.

plates is not enough for fracture treatment of weight bearing areas. Different manufacturing techniques for custom made plates and improved mechanical properties may increase the strength of the implants as shown in other studies.[21,22] Further studies are needed, which are designed closer to a fracture surgery scenario. In our study, we used 3.5 mm steel screws for the fixation of PLLA plates on the fracture model. It could be questioned as a reason for plate failure. B bio-degradable screws like magnesium, etc. may be considered for the fixation of custom-made PLLA plates. Different plate designs and different screw materials may increase the compressive strength of these custom-made plates.

The results of this study showed that the compressive strength of the bio-degradable plates is not enough for fracture fixation of load bearing areas. However, these plates may be used in low- or non-weight bearing areas with external supportive orthoses. Although upper extremity fractures like extra-articular distal radius fractures where the fracture line is facing different force directions than compression, like bending or shear stresses, may cause loss of reduction, these custom-made bio-degradable implants may be useful to support the stability with an additional external orthosis.[23]

In the current literature, different types of bio-degradable materials were mentioned in production of orthopedic bio-absorbable materials. Polyhydroxybutyric acid, polylactic acid, polyglycolic acid, polydioxanon, polyorthoesther, lactic acid, and glycolic acid copolymers are some of these materials.[24] To the best of our knowledge, there are no studies comparing the compressive strength of 3D printed custom-made orthopedic implants based on these materials. In the recent years, many in vitro and in vivo mechanical studies have been made on bio-degradable polymers, and PLLA is the most studied polymer.[21–24] Its mechanical properties and degradation characteristics are superior than other bio-degradable polymers.[25] In this study, we used PLLA as it is the most widely used and bio-compatible bio-degradable polymer in orthopedic practice.[26] The studies to reveal polymers with superior mechanical characteristics may improve the effectivity of bio-degradable custom-made plate usage in daily practice.

Further improvements to increase the compressive strength of the bio-degradable plates are necessary. As the PLLA plates had a uniform failure mechanism, some changes in implant design may also help to increase the compressive strength of these plates. With the improvement of material and 3D implant printing techniques, the bio-degradable materials will have a chance of usage at manufacturing patient-specific fixation systems. In addition, the plates produced using patient anatomy may allow complete anatomical reduction of the fracture, increase the success of the plate-screw system on stability, and reduce surgery time by easier implant application.

There are several limitations of this in vitro experimental mechanical study. First of all, this study does not completely mimic a fracture fixation scenario, in which the fracture site is affected under different directional forces and loads. Steel screws were used for the fixation of custom-made plates, and it may be effective on compressive strength of these plates. Also, this study is designed as an experimental laboratory study, which does not evaluate the clinical usage and the in vivo mechanical degradation and mechanical properties of the custom-made implants.

## CONCLUSION

Further laboratory and clinical studies are needed for the evaluation of fracture fixation with bio-degradable plates. The results of this study revealed that full anatomical custom plates can be manufactured using a 3D printer. The full anatomical design of 3D custom plates provided better reduction quality at

**FIGURE 6.** Samples showing the breakage zones of the 3D printed plates.

in vitro fracture models. These bio-degradable plate-screw systems may be eligible for implantation at fracture stabilization at non-weight bearing areas. With the improvement of bio-degradable materials and 3D printing technique and equipment, these systems can be expected to be eligible for the treatment of fractures.

**Ethics Committee Approval:** As this is an experimental biomechanical study Ethics Committee Approval is not applicable.

**Informed Consent:** All patients informed consents are routinely being taken before radiologic imaging.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - E.M.; Design - M.Y., E.M.; Data Collection and/or Processing - M.Y.; Analysis and/or Interpretation - M.Y.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES


1. Pekşen C, Doğan A. Implant permanence. *TOTBID Dergisi*. 2011;10(2):122-128.
2. Ceyhan T, Köse N. The use of biomaterials in orthopedics: General information and definitions. *TOTBID Dergisi*. 2011;10(2):79-82.
3. Heller M, Bauer HK, Goetze E, et al. Applications of patient-specific 3D printing in medicine. *Int J Comput Dent*. 2016;19(4):323-339.
4. Tack P, Victor J, Gemmel P, Annemans L. 3D-printing techniques in a medical setting: A systematic literature review. *BioMed Eng OnLine*. 2016;15:115. [CrossRef]
5. Zeng CJ, Xing WR, Wu ZL, Huang HJ, Huang WH. A combination of three-dimensional printing and computer-assisted virtual surgical procedure for preoperative planning of acetabular fracture reduction. *Injury*. 2016;47(10):2223-2227. [CrossRef]
6. Chou YC, Lee D, Chang TM, et al. Development of a three-dimensional (3D) printed biodegradable cage to convert morselized corticocancellous bone chips into a structured cortical bone graft. *Int J Mol Sci*. 2016;17(4):595. [CrossRef]
7. Liu A, Xue GH, Sun M, et al. 3D printing surgical implants at the clinic: A experimental study on anterior cruciate ligament reconstruction. *Sci Rep*. 2016;6. Article no. 21704. [CrossRef]
8. Radenkovic D, Solouk A, Seifalian A. Personalized development of human organs using 3D printing technology. *Med Hypoth*. 2016;87:30-33. [CrossRef]
9. Wyatt MC. Custom 3D-printed acetabular implants in hip surgery-innovative breakthrough or expensive bespoke upgrade? *Hip Int*. 2015;25(4):375-379. [CrossRef]
10. Frame M, Leach W. DIY 3D printing of custom orthopaedic implants: A proof of concept study. *Surg Technol Int*. 2014;24:314-317.
11. Liang H, Huang W, Chen X, et al. Reconstruction with 3D-printed pelvic endoprostheses after resection of a pelvic tumour. *Bone Joint J*. 2017;99-B(2):267-275. [CrossRef]
12. Kim D, Lim JY, Shim KW, et al. Sacral reconstruction with a 3D-printed implant after hemisacrectomy in a patient with sacral osteosarcoma: I-Year follow-up result. *Yonsei Med J*. 2017;58(2):453-457. [CrossRef]
13. Phan K, Sgro A, Maharaj MM, et al. Application of a 3D custom printed patient specific spinal implant for CI/2 arthrodesis. *J Spine Surg*. 2016;2(4):314-318. [CrossRef]
14. Bertol LS, Schabbach R, dos Santos LAL. Dimensional evaluation of patient-specific 3D printing using calcium phosphate cement for craniofacial bone reconstruction. *J Biomater Appl*. 2017;31(6):799-806. [CrossRef]
15. Suojanen J, Leikola J, Stoor P. The use of patient-specific implants in orthognathic surgery: A series of 32 maxillary osteotomy patients. *J Cranio-Maxillofac Surg*. 2016;44(12):1913-1916. [CrossRef]
16. Arabnejad S, Johnston B, Tanzer M, et al. Fully porous 3D printed titanium femoral stem to reduce stress-shielding following total hip arthroplasty. *J Orthop Res*. 2017;35(8):1774-1783. [CrossRef]
17. Rodriguez RU, Kemper N, Breathwaite E, et al. Demineralized bone matrix fibers formable as general and custom 3D printed mold-based implants for promoting bone regeneration. *Biofabrication*. 2016;8(3):035007. [CrossRef]
18. Park EK, Lim JY, Yun IS, et al. Cranioplasty enhanced by three-dimensional printing: Custom-made three-dimensional-printed titanium implants for skull defects. *J Craniofac Surg*. 2016;27(4):943-949. [CrossRef]
19. Schulze C, Weinmann M, Schweigel C, et al. Mechanical properties of a newly additive manufactured implant material based on Ti-42Nb. *Materials (Basel)*. 2018;11(1):124. [CrossRef]
20. Tarzimoghadam Z, Sandlöbes S, Pradeep KG, et al. Microstructure design and mechanical properties in a near-a Ti–4Mo alloy. *Acta Mater*. 2015;97:291-304. [CrossRef]
21. Sadeghi-Avalshahr AR, Khorsand-Ghayeni M, Nokhasteh S, et al. Physical and mechanical characterization of PLLA interference screws produced by two stage injection molding method. *Prog Biomater*. 2016;5:183-191. [CrossRef]
22. Liu Y, Shao J, Sun J, et al. Improved mechanical and thermal properties of PLLA by solvent blending with PDLA-*b*-PEG-*b*-PDLA. *J Polym Degrad Stab*. 2014;101:10-17. [CrossRef]
23. Meena S, Sharma P, Sambharia AK, et al. Fractures of distal radius: An overview. *J Family Med Prim Care*. 2014;3(4):325-332. [CrossRef]
24. Daniels AU, Lui MKC, Andriano KP. Mechanical properties of biodegradable polymers and composites for internal fixation of bone. *J App Biomater*. 1990;1(1):57-78. [CrossRef]
25. Tunc DC. A high strength absorbable polymer for internal bone fixation. *Trans Soc Biomater*. 1983;6:47.
26. Narayanan G, Vernekar VN, Kuyinu EL, et al. Poly(lactic acid)-based biomaterials for orthopaedic regenerative engineering. *Adv Drug Deliv Rev*. 2016;107:247-276. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.62-99

# Treatment and Management of Complications in Pediatric Forearm Fractures

**Abbas Tokyay, Sezai Özkan, Necip Güven, Tülin Türközü, Cihan Adanaş, Mehmet Ata Gökalp**

**Department of Orthopedics and Traumatology, Yüzüncü Yıl University, Faculty of Medicine, Van, Turkey**

**ORCID iDs of the authors: A.T. 0000-0002-3914-1906; S.Ö. 0000-0003-4444-6939; N.G. 0000-0002-0363-1524; T.T. 0000-0002-0966-9080; C.A. 0000-0002-3652-6077; M.A.G. 0000-0003-0317-7365.**

**Cite this article as: Tokyay A, Özkan S, Güven N, Türközü T, Adanaş C, Gökalp MA. Treatment and Management of Complications in Pediatric Forearm Fractures. *Cyprus J Med Sci.* 2021; 6(4): 290-294.**

**BACKGROUND/AIMS**
Forearm diaphyseal fractures are common in children. While closed reduction and casting are sufficient in most patients, surgical treatment is required in some cases. The aim of this study is to determine the possible causes of complications occurring in patients with intramedullary fixation with nail (IMN), as well as to evaluate the management of complications and their final results.

**MATERIAL and METHODS**
Between January 2017 and January 2020, 53 children under the age of 16 who had forearm fractures and surgically treated with IMN were included in the study. Demographic data of the patients, type of surgery, surgical technique, postoperative care, complications, and treatment modalities of complications were evaluated.

**RESULTS**
The mean age of 53 patients (41 boys and 12 girls) included in this study was 10.5 ± 2.4 years. The average follow-up period of all our patients was 23.4 months (12-34 months). Complications in our study were seen in 14 (26.4%) patients. These complications included pin-track infection in six (11.3%), refracture in four (7.5%), injury of superficial branch of radial nerve in two (3.7%), extensor pollicis longus rupture in one (1.8%), and pin migration in one (1.8%) patient.

**CONCLUSION**
As a result of the treatment and management of these complications, mild hypesthesia persisted along the superficial branch of the radial nerve in only one case, and all other complications were fully recovered. Successful management of complications can be achieved with close follow-up and appropriate treatment.

**Keywords:** Complications, forearm, pediatric fractures, surgery, treatment

## INTRODUCTION

Forearm diaphyseal fractures in children are frequently seen and constitute approximately 5.4-14.9% of all childhood fractures.[1–3] Due to the high potential for union and remodeling in children, closed reduction and immobilization with plaster cast are considered to be the first successful treatment in most patients. Surgical treatment is recommended in cases that cannot be reduced closed, in unstable fractures, open fractures, and refractures.[4,5] Although plate-screw and intramedullary fixation with nails (IMNs) are used in the surgical treatment of forearm fractures, intramedullary fixation is currently more often preferred in forearm fractures.[6] Kirschner-wires (K-wires), rushrods, Steinmann pins, and elastic stable intramedullary nails (ESINs) are used in intramedullary fixation.

The incidence rates of postoperative complications for pediatric forearm fractures range from 8.9 to 67%.[7–9] The most important of these complications are pin track infections, refracture, pin migration, extensor policislongus (EPL) tendon injury, radial nerve superficial branch injury, nonunion, malunion, compartment syndrome, osteomyelitis, and synocytosis. Our hypothesis is that surgical complications are seen at a high rate in pediatric forearm fractures, but these complications are almost completely healed with appropriate approaches. Our aim in this study is to determine the complications

**Corresponding Author:** Abbas Tokyay
**E-mail:** dr.abbas_tokyay@hotmail.com

**Received:** 25.03.2021
**Accepted:** 21.07.2021

and possible causes that occur in patients who underwent IMN for forearm fractures in our clinic, as well as to evaluate the management of complications and their final results.

## MATERIAL and METHODS

Patients who were operated in the clinic of our tertiary health center for forearm fractures between January 2017 and January 2020 were included in this study. The data about the patients were obtained from the automation records of our hospital using the ICD Codes (International Statistical Classification of Diseases and Related Health Problems). Patients younger than 16 years of age who underwent surgical treatment with IMN and were followed-up for at least 1 year were included in this study. Patients with pathological fractures, cases followed-up for less than 1 year, those with multiple traumas, and those previously had undergone plate-screw osteosynthesis were not included in this study. A total of 53 patients were included in this study based on inclusion and excision criteria.

In determining the fracture site, three regions as proximal, middle, and distal 1/3 were determined as described by Mehlman and Wall.[10] In this study, in addition to the demographic data of the patients, the type of surgery, surgical technique, complications, and treatment modalities of complications were also evaluated.

### Surgical Technique

After the first-generation cephalosporin was administered to all patients as surgical prophylaxis, they were placed on the table in the supine position, and a pneumatic tourniquet was wrapped around their forearms. In most of the cases, surgical intervention was priorly started from the radius. Approximately 2 cm proximal to the radial physis, a small skin incision was made on the dorsolateral aspect of the forearm to reach the distal radius by preserving the EPL and the superficial branch of the radial nerve.

Intramedullary nail was advanced through the entry hole previously opened through an appropriate site for the reduction of the fracture under the guidance of fluoroscopy. For the ulna, a mini-incision was made on the lateral edge of the olecranon, and the nail was directed from proximal to distal. Both approaches for radial and ulna fractures were performed as previously described in the literature.[11–13] In cases where closed reduction cannot be applied on the fracture line, the pneumatic tourniquet was inflated, and through a mini open incision, the fracture line was reduced, and IMN was applied.

We took care not to force the pins beyond the epiphyseal lines, and we ensured that the diameter of the pin filled more than half of the diameter of the diaphysis. In some patients, the pins were bent appropriately and left buried under the skin, while in others, they were left unburied on the skin.

### Postoperative Care

Postoperatively, a long-arm splint was applied for 2 weeks, and then a short arm splint for 3-6 weeks. Joint movements were initiated after the splint was removed. The patients were followed-up clinically and radiologically at 2, 4, 6, and 12 weeks. Union of the fracture was deemed to be achieved when the formation of visible callus on the fracture line was noted on radiograms, and pain, tenderness, and pathological movements disappeared.

### Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences version 22.0 (IBM SPSS Corp.; Armonk, NY, USA). Normality of gender distribution between the two groups was assessed using two-sample proportion test, and the normality of age distribution was assessed using independent two-sample t-test.

**Main Points**

- Forearm fractures are common in children. Nonsurgical approaches are used for most of these fractures.
- Surgical intervention is required in displaced fractures, and reduction cannot be achieved with a conservative method.
- Many surgical methods have been described in these patients. In addition to the success of these surgical methods, complications and their management are also extremely important.
- In this study, we discussed the complications of surgically treated pediatric forearm fractures and their management.

## RESULTS

The mean age of 53 patients (41 boys and 12 girls) included in this study was 10.5 ± 2.4 years. The average follow-up period of all our patients was 23.4 months, and pins were removed on an average of 126 days in those with buried pins and on 67 days in those with unburied pins. The demographic data of our patients are given in Table 1. It can be seen from the table that our patients were mostly men (n = 41, 77.4%) and fractures of the left extremities (n = 30, 56.6%) were encountered. Mostly fractures of the middle 1/3 of the forearm were seen. In 34 of our patients, fixation with IMN was achieved using K-wires,

**Table 1.** Demographic Data of Patients

| Characteristics | n (%) |
|---|---|
| **Gender** | |
| Male | 41 (77.4) |
| Female | 12 (22.6) |
| **Side** | |
| Right | 23 (43.4) |
| Left | 30 (56.6) |
| **Fracture location** | |
| Proximal | 8 (15.09) |
| Middle 1/3 | 32 (60.37) |
| Distal | 13 (24.5) |
| **Type of İMN** | |
| K-wire | 34 (64.2) |
| ESIN | 19 (35.8) |
| **Pin status** | |
| Buried | 31 (58.5) |
| Unburied | 22 (41.5) |
| **Complications** | |
| Pin tract infection | 6 (11.3) |
| Refracture | 4 (7.5) |
| Radial nerve superficial branch injury | 2 (3.7) |
| Ekstansör pollicis longus rupture | 1 (1.8) |
| Pin migration | 1 (1.8) |

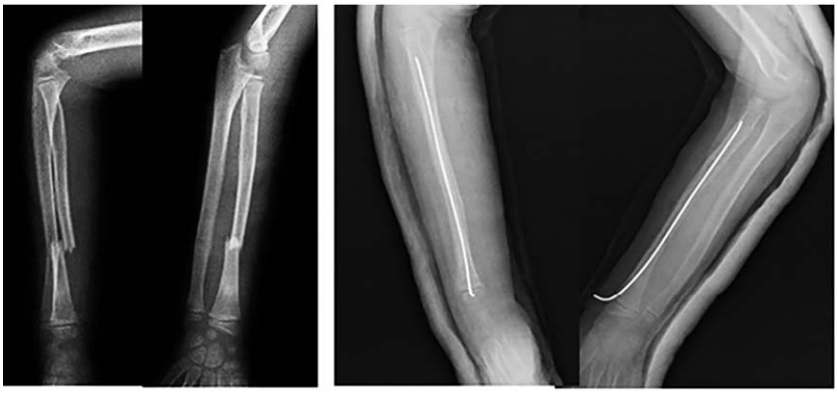

**Figure 1.** Pre- and postoperative radiographs of a 9-year-old patient with displaced radius fracture and nondisplaced ulna fracture.

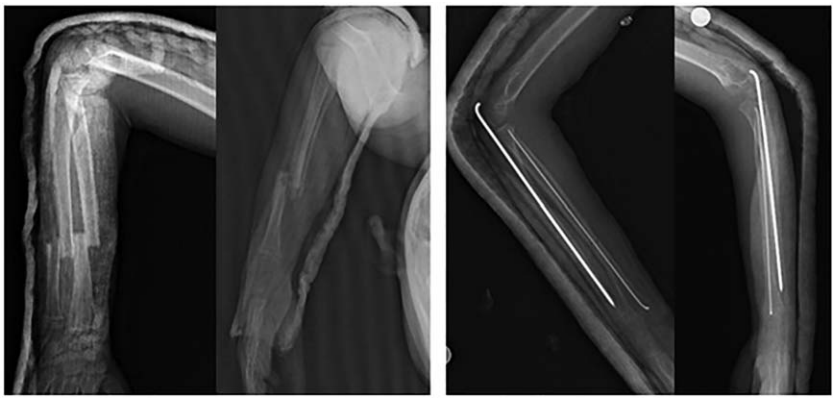

**Figure 2. In** the same patient, the refracture occurred 5 months later, and the IMN was applied.

and in the remaining 19 patients, ESINs method was used for fixation. The number of cases with embedded pins were more numerous than those with unburied pins. In our study, complications were seen in 14 (26.4%) patients.

The most common complication was pin track infection in six (11.3%) patients, five of which occurred in patients with unburied pin tips. Refracture, another important complication in these fractures, was seen in our four (7.5%) patients. It was found that in three of these four patients, the pinheads were unburied, and pins had been pulled out on postoperative 54, 62, and 72 days. Refractures had occurred after spontaneous fall of pins on the postoperative 8, 12, and 17 days after the pins were pulled out. In the case whose pin was buried and pulled out after the 5th month, refracture occurred after a fall from a height of about 1 m 12 days after the pin was pulled out. These refractures occurred in the middle one-third of the forearm in three and in its distal one-third in the other patient (Figures 1 and 2).

Another important complication was the injury of the superficial branch of the radial nerve in two of our patients. In their follow-up, one completely recovered in the sixth month, and in the other patient, mild hypesthesia still persisted in the postoperative 14th month.

EPL rupture was detected in one of our patients during pin removal. Tendon repair was performed in the patient with EPL rupture, and the patient recovered completely without any sequelae. Pin migration was detected in the follow-up of a patient whose pin tip was embedded in the radius. After healing of the fracture was achieved completely, the cortical window was opened where the pin tip and the pin were removed.

## DISCUSSION

In this study, different complications were observed in 14 (26.4%) cases. The most common complication was pin track infection in six (11.3%) patients. As another complication, refracture was observed in four (7.5%) patients. Three of our refractures were seen in the middle 1/3 of the forearm. In our study, EPL rupture was found in only one patient (1.8%), which occurred during the removal of IMN. In two (3.7%) patients, damage to the sensory branch of the radial nerve was detected. There are many methods for the treatment of pediatric forearm fractures, and conservative methods such as closed reduction and plaster casting often provide successful treatment outcomes. The presence of thick periosteal tissue and high potential for remodeling increase the success of conservative treatment.[14,15]

The remodeling potential decreases as the fracture location approaches proximally and with aging.[16,17] In cases where closed reduction cannot be achieved and reduction cannot be maintained, surgical treatment is performed. As surgical

treatment alternatives plate-screw, osteosynthesis and IMN are practiced. Recently, it has been observed that there is a trend toward surgery among the authors due to difficulties such as maintaining reduction of fracture in conservative treatments and higher refracture rates.[6,18,19] With advantages of IMN, such as its being a mini-invasive procedure, easy applicability, lower rates of complications, and cosmetic problems, IMN has been preferred more frequently.[6,9,20] In addition, fixation with an intramedullary nail allows micromovements in the fracture site and earlier call us formation.[21]

In our study, it was found that forearm bone fractures in children were mostly seen in males, on the left upper extremity and mostly in the middle 1/3 of the forearm. These results are similar to the literature.[6] A wide range of complications up to 60% are seen in the surgical treatment of these fractures.[9] The diagnosis, treatment, and management of these complications greatly affect the outcome of the treatment.

In this study, different complications were observed in 14 (26.4%) cases. The most common complication was pin track infection in six (11.3%) patients. Tsukamoto et al.[6] reported that pin track infection rates were 11.7% in a study they conducted on fracture complications. Meriç et al.[22] treated their patients using IMN treated, unlike our findings pin track infection was seen in 22.2% of their patients. In our patients who developed pin track infection, a swab sample was obtained from the pin site, and their treatments were rearranged according to the culture results. Complete healing was achieved within 2-3 weeks in five patients with unburied pin tips. In one of our patients, whose pin tip was buried under the skin, the implants were removed, and the wound debridement was performed when the infection did not regress despite 2 weeks of oral antibiotic treatment, and sufficient callus tissue was detected in the radiograms. Full recovery was achieved after 3 weeks of antibiotherapy in the patients with a long arm splint. Pin track infections seen in these patients are generally superficial, and good results can be obtained with medical treatment. However, in cases that do not respond to the treatment of infection, satisfactory results can be obtained with early intervention, removal of the pins, and debridement of the wound site.

As another complication, refracture was observed in our four (7.5%) patients. Three of our refractures were seen in the middle 1/3 of the forearm. In the literature, the rates of refracture in forearm fractures vary between 4 and 8%, and they are often seen in midshaft fractures. It has been suggested that higher muscle mass percentage in the proximal part of the forearm better protects the forearm, which explains lower rates of refractures involving these regions.[6,9,13] Cullen et al.[23] reported only one refracture in a series of 20 cases, in which they applied intramedullary K-wires. Refractures are reported to occur mostly in males, younger ages, and thin individuals.[9,24] Three of our four refracture cases were male, and refractures were seen in those whose pin tips were unburied. We think that unburied pin tips tend to be removed within a short time. ESIN was performed as revision surgery in the treatment of our three cases of refracture. In our fourth case, osteosynthesis with plate-screws was performed because the intramedullary region was closed, and IMN could not be sent through. In such cases of revision, it is recommended to have the plate screw set ready together with the IMN, as an implant may be required. Removal of the pins in the fracture line without achievement of complete union was thought to play a role in the development of refractures. In the literature, refractures have been also observed more frequently in fractures whose pins were removed prematurely.[24,25] In our study, the main reasons for the early removal of the pins were the patient's frequent requests to remove the pin due to the unburied pin irritating the area, creating a risk for infection and the uneasiness given to the patient. In these cases, we think that the application of the fracture treatment protocol and communication with the patient are important factors rather than the patient's demand.

Surgeons may have different preferences about exposing or buried the pin tips. As a matter of fact, different surgeons in our study either unburied of buried pin tips. Here, during the follow-up of our patients, we especially observed that infection occurred less frequently in patients whose pin tips were buried, and that joint movements were initiated much more earlier in these patients. Some studies have demonstrated that the burying of the pins under the skin and retaining them for at least four or six months prevents infection and reduction loss and can initiate earlier mobility of the extremity.[4,12,13,24,25] In our study, the pin tips were left buried in 31 and unburied in 22 cases. We have observed that our tendency is to leave the pin tips under the skin at an increasing rate. In line with the data we detected in our study, we think that the buried of the pins is safer for preventing development of complications such as infection and refracture.

In forearm fractures, EPL injury is one of the complications that occur while the nail is being implanted or removed.[26,27] In our study, EPL rupture was found in only one patient (1.8%), which occurred during the removal of IMN. In this patient, intraoperative EPL repair was performed, and by wearing a short-arm splint with thumb support for three weeks, recovery was achieved. Kruppa et al.[13] found EPL damage at a rate of 1.5% in their study. Flynn et al.[28] detected tendon rupture in 1.9% of their patients. The EPL complication rate we obtained was similar to the literature findings.

Another complication is the damage to the sensory branch of the radial nerve, which is an important structure, when access through the Lister's tubercle is selected for the management of a radial bone fracture at the wrist level. In our study, this complication developed in two (3.7%) patients. While one of them recovered in the 6th month with close follow-up, the other patient still had mild hypesthesia in the postoperative 14th month. This latter case did not receive any treatment related to hypesthesia, and the patient is being followed-up. In the literature, it has been reported that most of the nerve injuries seen in the treatment of forearm fractures tend to heal spontaneously.[18,29]

Following the treatment of all complications that developed after at least one year of, all of our patients recovered without any sequelae, except for one patient who developed mild hypoesthesia due to the injury of superficial branch of the radial nerve. As our first result in forearm fractures, despite the wide range of complications seen in patients undergoing surgical treatment with IMN, good follow-up and treatment with appropriate surgical technique seriously affect the final result of the treatment. The second result is that the buried of the pins reduces the risk of infection and indirectly prevents development of refracture from as it tendency for late removal.

Our study have some limitations. The most important of these is the small number of patients and the single-center study. Another limitation was its retrospective nature. One of the reasons for the low number of patients may be that they were operated by us and followed-up in other centers.

In conclusion, although the first treatment in pediatric forearm fractures is usually closed reduction and casting, we believe that in cases treated surgically, complications that develop in cases can be completely healed with timely and appropriate interventions.

**Ethics Committee Approval:** Ethical committee approval was received from local Ethics Committee (approval date: December 4, 2020, approval number: 2020/09-17).

**Informed Consent:** Written informed consent was obtained from all participants who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - A.T., S.Ö., C.A., M.A.G.; Design - C.A., M.A.G.; Supervision - A.T., S.Ö., N.G., M.A.G.; Resources - A.T., M.A.G.; Materials - A.T., S.Ö.; Data Collection and/or Processing - S.Ö., N.G., T.T., C.A.; Analysis and/or Interpretation - A.T., S.Ö., N.G., T.T., M.A.G.; Literature Search - S.Ö.; Writing Manuscript - A.T., S.Ö.; Critical Review - A.T., N.G., T.T., C.A.

**Acknowledgments:** Thanks to Dr. Remzi Erten and Dr. Hamit Hakan Alp for the design and statistical support of our study.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES


1. Rennie L, Court-Brown CM, Mok JYQ, Beattie TF. The epidemiology of fractures in children. *Injury*. 2007;38:913-922. [CrossRef]
2. Cheng JC, Ng BK, Ying SY, Lam PK. A 10-year study of the changes in the pattern of 6,493 fractures. *J Pediatr Orthop*. 1999;19(3):344-350.
3. Kokubo Y, Yamasaki T, Shiba T, Sato S. Statistical study on children fractures. *Seikeigeka*. 2004;55(12):1621-1626.
4. Shoemaker SD, Comstock CP, Mubarak SJ, Wenger DR, Chambers HG. Intramedullary Kirschner wire fixation of open or unstable forearm fractures in children. *J Pediatr Orthop*. 1999;19(3):329-337.
5. Calder PR, Achan P, Barry M. Diaphyseal forearm fractures in children treated with intramedullary fixation: Outcome of K-wire versus elastics table intramedullary nail. *Injury*. 2003;34(4):278-282. [CrossRef]
6. Tsukamoto N, Mae T, Yamashita A, et al. Refracture of pediatric both-bone diaphyseal forearm fracture following intramedullary fixation with Kirschner wires is likely to occur in the presence of immature radiographic healing. *Eur J Orthop Surg Traumatol*. 2020;30(7):1231-1241. [CrossRef]
7. Salonen A, Salonen H, Pajulo O. A critical analysis of postoperative complications of antebrachium TEN-nailing in 35 children. *Scand J Surg*. 2012;101:216-221. [CrossRef]
8. Fernandez FF, Langendorfer M, Wirth T. Failures and complications in intramedullary nailing of children's forearm fractures. *J Child Orthop*. 2010;4:159-167. [CrossRef]
9. Poutoglidou F, Metaxiotis D, Kazas C, Alvanos D, Mpeletsiotis A. Flexible intramedullary nailing in the treatment of forearm fractures in children and adolescents, a systematic review. *J Orthop*. 2020;20:125-130. [CrossRef]
10. Mehlman CT, Wall EJ. Injuries to the shaft of the Radius and ulna. In Beaty JH, Kasser JR (eds.): *Rockwood and Wilkins' Fractures in Children*, 7th ed. Philadelphia: Lippincott Williams & Wilkins, 2010: 347-344.
11. Yung SH, Lam CY, Choi KY, Ng KW, Maffulli N, Cheng JCY. Percutaneous intramedullary Kirschner wiring for displaced diaphyseal forearm fractures in children. *J Bone Joint Surg*. 1998;80-B:91-94. [CrossRef]
12. Lascombes P, Prevot J, Ligier JN, Metaizeau JP, Poncelet T. Elastic stable intramedullary nailing in forearm shaft fractures in children: 85 cases. *J Ped Orthop*. 1990;10:167-171.
13. Kruppa C, Bunge P, Schildhauer TA, Dudda M. Low complication rate of elastic stable intramedullary nailing (ESIN) of pediatric forearm fractures: A retrospective study of 202 cases. *Medicine (Baltimore)*. 2017;96(16):e6669. [CrossRef]
14. Kay S, Smith C, Oppenheim WL. Both-bone midshaft forearm fractures in children. *J Pediatr Orthop*. 1986;6:306-310. [CrossRef]
15. Jones K, Weiner D. The management of forearm fractures in children: Aplea for conservatism. *J Pediatr Orthop*. 1999;19:811-815.
16. Larsen E, Vittas D, Trop-Pedersen S. Remodeling of angulated distal forearm fractures in children. *Clin Orthop*. 1988;237:190-195.
17. Stern PJ, Drury WJ. Complications of plate fixation of forearm fractures. *Clin Orthop Relat Res*. 1983;175:25-29.
18. Sinikumpu JJ, Lautamo A, Pokka T, Serlo W. The increasing incidence of paediatric diaphyseal both-bone forearm fractures and their internal fixation during the last decade. *Injury*. 2012;43:362-366. [CrossRef]
19. Kosuge D, Barry M. Changing trends in the management of children's fractures. *Bone Joint J*. 2015;97-B:442-448. [CrossRef]
20. Han B, Wang Z, Li Y, Xu Y, Cai H. Risk factors for refracture of the forearm in children treated with elastic stable intramedullary nailing. *International Orthopaedics (SICOT)*. 2019;43(9):2093-2097. [CrossRef]
21. Qidwai SA. Treatment of diaphyseal forearm fractures in children by intramedullary kirschner wire. *J Trauma*. 2001;50(2):303-307. [CrossRef]
22. Meriç G, Kişin B, Gem M, Şentürkİ, UysalA. Çocuk önkol kırıklarının cerrahi tedavisinde kanal-içi elastik çivileme; 23 hastanın geriye dönük analizi. *Balıkesir Sağlık Bilimleri Dergisi*. 2012;1:43-484l.
23. Cullen MC, Roy DR, Giza E, Crawford AH. Complications of intramedullary fixation of pediatric forearm fractures. *J Pediatr Orthop*. 1998;18(1):14-21.
24. Rousset M, Mansour M, Samba A, Pereira B, Canavese F. Risk factors for re-fracture in children with diaphyseal fracture of the forearm treated with elastics table intramedullary nailing. *Eur J Orthop Surg Traumatol*. 2016;26(2):145-152. [CrossRef]
25. Makki D, Kheiran A, Gadiyar R, Ricketts D. Refractures following removal of plates and elastic nails from paediatric forearms. *J Pediatr Orthop B*. 2014;23(3):221. [CrossRef]
26. Pugh DM, Galpin RD, Carey TP. Intramedullary Steinmann pin fixation of forearm fractures in children. Long-term results. *Clin Orthop Relat Res*. 2000;376:39-48. [CrossRef]
27. Griffet J, El Hayek T, Baby M. Intramedullary nailing of forearm fractures in children. *J Pediatr Orthoped B*. 1999;8:88-89.
28. Flynn JM, Jones KJ, Garner MR, Goebel J. Eleven years experience in the operative management of pediatric forearm fractures. *J Pediatr Orthop*. 2010;30(4):313-319. [CrossRef]
29. Lyman A, Wenger D, Landin L. Pediatric diaphyseal forearm fractures: Epidemiology and treatment in an urban population during a 10-year period, with special attention to titanium elastic nailing and its complications. *J Pediatr Orthop B*. 2016;25(5):439-446. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021-166-232

# Menstrual Practice Needs Scale (MPNS): Reliability and Validity of the Turkish Version

**Pınar Irmak Vural, Yeliz Varışoğlu**

**Department of Nursing, İstanbul Medipol University, Faculty of Health Science, İstanbul, Turkey**

**ORCID iDs of the authors: P.I.V. 0000-0002-8070-2840; Y.V. 0000-0002-6350-7218.**

**Cite this article as: Vural PI, Varışoğlu Y. Menstrual Practice Needs Scale (MPNS): Reliability and Validity of the Turkish Version. *Cyprus J Med Sci.* 2021; 6(4): 295-302.**

**BACKGROUND/AIM**
Menstrual practices have actions undertaken to manage menstrual bleeding, including accessing, storing, and transporting acceptable menstrual materials, changing and disposing of used materials, washing and drying of reusable materials, and cleaning the hands, genitals, and body. This study aims to conduct the reliability and validity study of the Turkish version of the Menstrual Practice Needs Scale (MPNS), which determines the menstrual practices and needs.

**MATERIAL and METHODS**
This methodological study was conducted with 446 university students in Istanbul in Turkey from June to July 2020.

**RESULTS**
In this study, the mean age of the participants was 19.93 ± 2.34. The confirmatory factor analysis showed a good fit. Cronbach's α of this scale was 0.78. The test–retest reliability coefficient was $r = .661$. The content validity index was calculated as 0.89. In this study, the Kaiser Meyer Olkin value was 0.819, which is considered very good. The result of Bartlett's Test of Sphericity was $\chi^2 = 4,894.399$, $P < .001$ for MPNS. Item-total correlation factor loadings varied from 0.24 to 0.94. According to the item-total correlation, one item was removed.

**CONCLUSIONS**
The findings suggest that the Turkish language version of the MPNS is valid and reliable.

**Keywords:** Menstrual Practice Needs Scale, nursing, reliability, validity

## INTRODUCTION

Menstruation is a physiological process that is experienced from the ages of menarche to menopause.[1] Women's strategies to cope with menstrual period needs and menstrual practices may vary by age and culture. Menstrual hygiene management is influenced by the educational, socioeconomic, and cultural status of women. The challenges encountered by menstruating women and adolescent girls in low-resource environments indicated adverse effects on many areas of life, such as health status, education, employment, and also wellness.[2]

Girls usually manage menstruation with methods that could be unhygienic or inconvenient, especially in poorer environments. Girls in low- and middle-income countries have challenges coping with menstrual needs due to a lack of money or social support. Thus, in this case, girls take suboptimal care or investigate improper ways of sourcing menstrual materials. Menstrual needs become more challenging to cope with water, hygiene, and sanitation problems. Thus seen in women and young girls, gynecological infections, such as vaginal secretion and itching associated with poor hygiene conditions.[3–5] There are very few empirical studies in the literature that have measured the extent and intensity of difficulties in managing the menstrual needs of young girls, which has remained under-researched. Few studies have examined causal relationships to confirm the effectiveness of attempts to manage menstrual hygiene to maintain health and attend school.[3] Young girls undertake a variety of actions and use a range of environments to manage their menstruation. They also vary in their satisfaction and concerns about these practices. Menstrual practices are the actions undertaken to manage menstrual bleeding, such as accessing, storing, and transporting acceptable menstrual materials (e.g.,

**Corresponding Author:** Pınar Irmak Vural
**E-mail:** pinar.irmak@windowslive.com

**Received:** 25.06.2021
**Accepted:** 20.10.2021

cloth and pads), changing and disposing of used materials, washing and drying of reusable materials, and cleaning the hands, genitals, and body. In assessing menstrual practices, it is also important to capture the environments women may use to undertake these practices, that is, the spaces women use to change materials, dispose of them, and clean their bodies and materials. There are other actions that women and girls may undertake to care for their bodies during menstruation, such as pain relief or accessing information about the menstrual cycle. However, pain and pain management experiences and menstrual experience of knowledge and social support are separate concepts.[6] Perceptions of menstrual practices, positive or negative, may reflect the practices themselves but are also dictated by women's perspectives and past experiences, and their context and the expectations of others in their community. In some cultures, menstruation is seen as taboo, and women are labeled as "dirty," forced into seclusion or not sent to school, not participating in some of the daily activities, such as not cooking, making it difficult to manage menstrual practices.[7] The menstrual practices were undertaken by women and girls, that is, the behaviors they undertake to manage their menstrual bleeding, which are one of the most frequently assessed outcomes in menstrual health research and program monitoring. Most of the studies and practices aimed to investigate menstrual needs, health, and hygiene have focused on improving menstrual practices of women and girls.[8–10] The Menstrual Practice Needs Scale (MPNS) was developed using a holistic and woman-centered approach. It offers a way to capture women's and girls' perceptions of how they manage their period. That is, if they felt their practices and environments met their needs. The MPNS provides a way to objectively measure menstrual experiences and test if interventions achieved this desired effect.[6] There was not any tool for measuring menstrual experiences in Turkish. Therefore, this study aims to evaluate the reliability and validity of the Turkish version of the MPNS in university girl students.

## MATERIAL and METHODS

### Research Type

This present study had a methodological design.

### Data Collection and Tools

This study was conducted on first and second-year students in the nursing department of a university in Istanbul in Turkey between June and July 2020. Data collection tools were designed using a google form. In the collection of the data, two forms were used, the participant information form, which was prepared by the researchers in light of the literature and similar works and the MPNS.

**Main Points**

- The Turkish version of the MPNS is a valid and reliable measurement scale in the assessment of self-perceived menstrual hygiene needs and practices.
- MPNS measures the extent to which respondents' menstrual management practices and environments were perceived to meet their needs during their last period.
- MPNS items ask about perceptions of comfort, satisfaction, adequacy, reliability, and worries and concerns during the last menstrual period.
- MPNS provides a way for researchers and practitioners to understand if the menstrual management and environmental needs of their population are being met.

### Participant Information Form

The participant information form consisted of 15 questions questioning descriptive and gynecological characteristics of participants.[2,4,11]

### Menstrual Practice Needs Scale

MPNS, designed by Hennegan et al.,[11] consists of 36 items, including materials, hygiene, and practices used in menstrual cycle management. The scale focuses on the experience of participants in women's last menstrual period and explores the experiences of the practices and the environments used to manage their menstrual period. Scale items include comfort, satisfaction, adequacy, reliability, and concerns in the last menstrual period.[11]

The MPNS contains 36 items in total, 28 items that can be applied to all participants, and the remaining eight items for those with washing and drying experience for reuse of menses. Subscales and total scores are calculated as average scores to support the accessibility of girls. The subscales are listed as follows: "material and home-environment needs" (11 questions, $\alpha = 0.79$), "transportation and school environment needs" (five questions, $\alpha = 0.66$), "material reliability concerns" (three questions, $\alpha = 0.51$), "change and disposal distrust" (nine questions, $\alpha = 0.74$), "reuse needs" (five questions, $\alpha = 0.66$), and "reuse distrust" (three questions, $\alpha = 0.47$). Whether the products are home or school-based was relevant in managing the menstrual period. Higher scores from MPNS mean more positive experiences, and the scores are associated with the probability of being absent from school during the menstrual period (95% CI 1.52 to 4.50, OR = 2.62). As a reliability test, test–retest results were moderate in the original form of MPNS (total score of intraclass correlation coefficient = 0.69). Scoring on a "4-point Likert type scale": never, sometimes, usually, and always (0-3 points) for positively and negatively encoded items, the reverse was calculated.[11]

### Participants and Sample Size

Larger sample sizes in studies increase the generalizability of results reached through factor analysis.[12] A reasonable 'observations to variables' rate is 10:1, given that this scale had 28 items, and the expected sample size was 280 participants. This study was conducted with 446 young girls aged 18-25 who volunteered to participate and spoke Turkish.

### Data Statistics

The data collected from the young girls by google form were analyzed using the Statistical Package for the Social Sciences (SPSS) version 18.0 (IBM SPSS Corp.; Armonk, NY, USA) and AMOS 23 (SPSS Inc.; Chicago, IL, USA) programs. In the reliability analysis, the Pearson correlation coefficient was assessed using a test–retest test method in the evaluation of time invariance. For internal consistency assessment, the item-total correlation coefficient and internal consistency coefficient were calculated. In this context, the Pearson correlation coefficient and Cronbach's $\alpha$ reliability coefficient were tested. About the content validity of this scale, the Lawshe technique was used in evaluating expert opinions, and confirmatory

**Table I.** Mean, Standard Deviation, and Distributional Characteristics of Individual's Menstrual Practice Need Scale Subscales (n = 446)

| MPNS Subscales | Mean (SD) | Min-Max | Skew | Kurtosis | SE | Cronbach's α |
|---|---|---|---|---|---|---|
| Material and home environment needs | 2.65 (0.29) | 0-3 | –1.706 | 2.328 | 0.11 | 0.80 |
| Transport and school environment needs | 2.17 (0.62) | 0-3 | –0.523 | –0.272 | 0.22 | 0.75 |
| Material reliability concerns | 1.54 (0.75) | 0-3 | –0.018 | –0.812 | 0.27 | 0.67 |
| Change and disposal insecurity | 2.41 (0.46) | 0-3 | –1.357 | 2.754 | 0.02 | 0.72 |
| Total score | 2.19 (0.35) | 0-3 | –0.288 | 0.234 | 0.01 | 0.78 |

*Abbreviations*: SD, standard deviation; SE, standard error of the mean.

factor analysis (CFA) was applied in evaluating structure validity. The statistical significance level was determined as $P < .05$.

Before data analysis, a pilot study was conducted (n = 35). According to the pilot study, the items of the scale related to the subscales of "reuse needs" and "reuse distrust" were determined. The present study was followed on 28 items of the MPNS that were applied to all participants.[11]

It was deemed appropriate to conduct an exploratory factor analysis (EFA) to test the Turkish cultural validity. The content validity check was examined to determine whether all items could be included in the Turkish version of the scale. In addition, the skewness and kurtosis indices were calculated to test the normality of the distribution.[13] Descriptive characteristics of each MPNS subscale are indicated in Table I and revealed no indication of extreme skew or kurtosis based on Kline's thresholds of 3 (skew) and 10 (kurtosis).

### Ethics

Ethical committee approval was received from the Istanbul Medipol University Noninvasive Clinical Research Ethics Committee (approval number: 471-10.06.2020 and institutional permission was obtained to conduct this study. In addition, the girls who volunteered to participate in the present study were informed about the aim of this study. A written informed consent was obtained from all individual participants included in this study.

## RESULTS

### Participants

Data were collected from 446 participants at the scale validity and reliability phase. The mean age of the participants was 19.93 ± 2.34, the mean age of menarche was 13.11 ± 1.29, and the duration of menstruation was 5 days. The findings obtained in this study showed that 90.8% of the participants experienced pain during menstruation and 78.2% took painkillers.

### Validity

**Language validity:** To evaluate the Turkish cultural appropriateness of the scale and the understandability of each item, the translated version of the scale was submitted to a group of 10 academicians consisting of nurses and midwives using an e-mail. The content validity criterion was specified to be 0.62 in the literature.[14] Based on the Lawshe technique, according to the feedback of these 10 academic experts, it was determined that none of the items of the scale was less than 0.62. No item was removed at this stage.

**Content validity:** The Davis technique was used to evaluate validity while determining content validity, which is another criterion. After the translation process from Turkish into English, the scale was presented to academic experts for content validity. Each item in the MPNS was evaluated on a 4-point scale: the item was scored appropriately to not appropriate (4-1). Based on the Davis technique according to the literature, it is appropriate to have the content validity index (CVI) above 0.80 according to the academic experts who provide opinions.[15] The determination of CVI 0.89 for this scale demonstrates that content validity is adequate.

**Construct validity:** Two analyses were conducted to test the MPNS construct validity; these are EFA and CFA. When the Kaiser–Meyer–Olkin (KMO) value of this scale was tested, it was determined to be 0.819, and this result indicates that it is very good for factor analysis. Besides, the result of Bartlett's test of Sphericity was $\chi^2 = 4{,}894.399$, $P < .001$ for MPNS.

**Confirmatory factor analysis:** CFA is a process for creating factors based on observed variables through a preconstructed model. CFA has been tested for the compatibility of the Turkish language factors. The Chi-square value is the most basic measurement used to test the fit of the model. The goodness of fit indexes and the Chi-square continuity correction (2ℵ†/df) test were applied to the subscales established in the model to test the model. In this study, with CFA, the fit indices were chi-square/degree of freedom (CMIN/DF) = 2.63, comparative fit index (CFI) = 0.88, the goodness of fit index (GFI) = 0.89, root mean square error of approximation (RMSEA) = 0.06, and standardized root mean square residual (SRMR) = 0.044 (Table 2). With these test results, the four-factor structure was confirmed. Item-total correlation factor loadings of the scale (path coefficients) varied from 0.24 to 0.94 (Figure 1). The path diagram of the established model is presented in Figure 1.

### Reliability

**Internal consistency:** Cronbach's α reliability coefficient was calculated to test the reliability of the scale. A Cronbach's α value within 0.70-0.90 indicates the most suitable internal validity.[16] In this study, Cronbach's α value was determined as 0.76, which was acceptable for internal validity. The corrected item-total correlation of this scale was analyzed, which ranged from 0.14 to 0.68 and decided to be an acceptable level (Table 3). According to this result, the 28th item ("When at school, I worried that someone would harm me while I was changing my menstrual materials.") was removed because the total correlation value of this item was under 0.30.

After the 28th item was removed, item-total correlation values of the items in the scale varied from 0.313 to 0.683, and Cronbach's α value increased to 0.78. The statistical analysis of the

**Table 2.** CFA Results of the Fit Index for Menstrual Practice Needs Scale (n = 446)

| Indexes | Good Fit | Acceptable Fit | Model Results | Decisions |
|---|---|---|---|---|
| CMIN/DF | $\chi^2/df < 3$ | $\chi^2/df < 5$ | 2.63 | Good fit |
| GFI | >0.90 | >0.85 | 0.89 | Acceptable fit |
| CFI | >0.95 | >0.90 | 0.88 | – |
| RMSEA | <0.05 | <0.08 | 0.06 | Good fit |
| SRMR | <0.05 | <0.08 | 0.044 | Good fit |

*Note*: $\chi^2$: Chi-square fit test; df: degree of freedom.
*Abbreviations*: CMIN/DF: Chi-square/degree of freedom; CFA: confirmatory factor analysis; CFI: comparative fit index; GFI: goodness of fit index; RMSEA: root mean square error of approximation; SRMR: standardized root mean square residual.

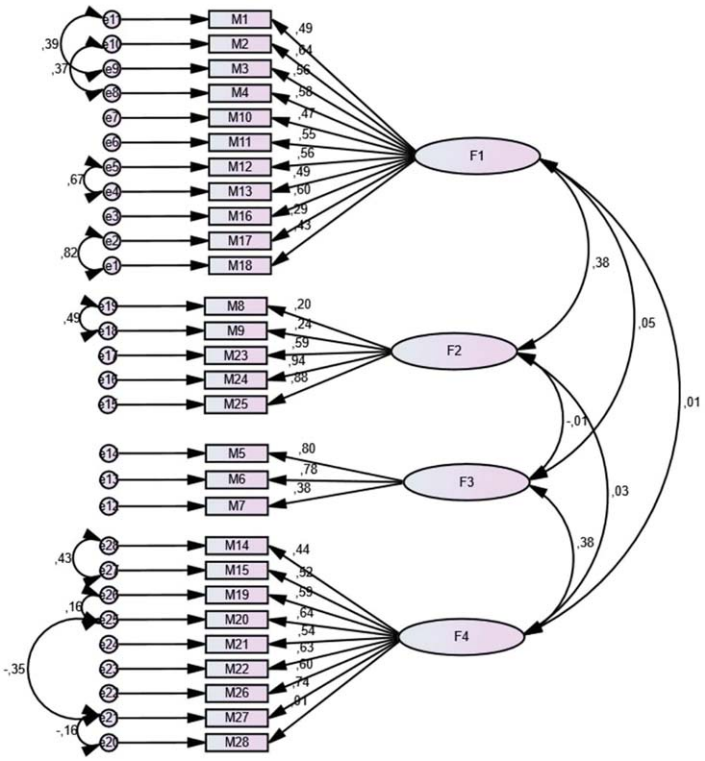


**FIGURE I.** Confirmatory factor analysis of MPNS: path coefficients and error variances (N = 446). *Abbreviations*: M, item; F, factor. *P*-value: .00.

corrected item-total correlations and Cronbach's α if item deleted are given in Table 4.

**Test–retest reliability:** This scale's test–retest reliability was performed. Test–retest analysis shows that a scale applied in the reliability study of the scale is determined to be invariant over time by readministering it to the same group in a certain time interval (between 2 and 4 weeks).[17,18] Thereby, the correlation value between the first and second application scores of the MPNS was determined to be r = 0.661, with a significant difference with a $P < .001$ level. This finding implies that the first and last application scores results applied with a 4-week interval were similar (Table 5).

## DISCUSSION

The adaptation of the MPNS into Turkish and the findings of the Turkish version of the scale were discussed in this section. The findings of this study were discussed in the following headings, discussion of the results on the reliability and validity of the MPNS.

### Discussion of the Results on the Reliability of the MPNS

Reliability is a concept that reveals the consistency of all items with each other in a measurement tool and their homogeneity in measuring the formation under consideration. Internal consistency evaluates the reliability of a scale, which is one of the common methods. Usually, Cronbach's α coefficient should be calculated to evaluate the internal consistency of Likert-type scales.[19] In this study, Cronbach's α coefficient, item total correlation, and test–retest analysis were used to determine the reliability of the MPNS. Cronbach's α coefficient of the MPNS was 0.78. Also, the subscales' Cronbach's α coefficients of the "Material and home environment needs" was 0.73, "Transport and school environment needs" was 0.76, "Material reliability concerns" was 0.69, and "Change and disposal insecurity" was 0.69. Hennegan et al.[11] stated that Cronbach's α coefficient was

**Table 3.** Item Subscale and Total Score Correlations of the Menstrual Practice Needs Scale Total and Subscales (n = 446)

| Subscales and items of the Menstrual Practice Needs Scale | Mean ± SD | Item-Total Score Correlation Coefficient | |
|---|---|---|---|
| | | r | *P* |
| **Material and home environment needs** | | | |
| 1. My menstrual materials were comfortable | 2.4 ± 0.68 | 0.464 | .000 |
| 2. I had enough of my menstrual materials to change them as often as I wanted to | 2.7 ± 0.49 | 0.583 | .000 |
| 3. I was satisfied with the cleanliness of my menstrual materials | 2.7 ± 0.55 | 0.561 | .000 |
| 4. I could get more of my menstrual materials when I needed to | 2.7 ± 0.59 | 0.506 | .000 |
| 10. I felt comfortable storing (keeping) my leftover or cleaned menstrual materials until my next period | 2.7 ± 0.61 | 0.395 | .000 |
| 11. I was able to wash my hands when I wanted to | 2.9 ± 0.28 | 0.474 | .000 |
| 12. I was able to immediately dispose of my used menstrual materials | 2.8 ± 0.45 | 0.549 | .000 |
| 13. I was able to dispose of my used materials in the way that I wanted to | 2.8 ± 0.53 | 0.465 | .000 |
| 16. When at home, I was able to change my menstrual materials when I wanted to | 2.9 ± 0.32 | 0.513 | .000 |
| 17. When at home, I was satisfied with the place I used to change my menstrual materials | 2.9 ± 0.57 | 0.313 | .000 |
| 18. When at home, I had a clean place to change my menstrual materials | 2.9 ± 0.38 | 0.482 | .000 |
| **Transport and school environment needs** | | | |
| 8. I felt comfortable carrying spare menstrual materials with me outside my home | 2.6 ± 0.71 | 0.335 | .000 |
| 9. I felt comfortable carrying menstrual materials to the place where I changed them | 2.4 ± 0.83 | 0.378 | .000 |
| 23. When at school, I was able to change my menstrual materials when I wanted to | 2.2 ± 0.86 | 0.588 | .000 |
| 24. When at school, I was satisfied with the place I used to change my menstrual materials | 1.7 ± 1.04 | 0.683 | .000 |
| 25. When at school, I had a clean place to change my menstrual materials | 1.7 ± 1.03 | 0.621 | .000 |
| **Material reliability concerns** | | | .000 |
| 5. I worried that my menstrual materials would allow blood to pass through to my outer garments | 1.1 ± 0.97 | 0.592 | .000 |
| 6. I worried that my menstrual materials would move from place while I was wearing them | 1.1 ± 1.02 | 0.577 | .000 |
| 7. I worried about how I would get more of my menstrual material if I ran out | 2.4 ± 0.89 | 0.584 | .000 |
| **Change and disposal insecurity** | | | |
| 14. I worried about where to dispose of my used menstrual materials | 2.3 ± 0.97 | 0.489 | .000 |
| 15. I was concerned that others would see my used menstrual materials in the place I disposed of them | 1.8 ± 1.12 | 0.535 | .000 |
| 19. When at home, I worried that I would not be able to change my menstrual materials when I needed to | 2.7 ± 0.67 | 0.534 | .000 |
| 20. When at home, I worried that someone would see me while I was changing my menstrual materials | 2.5 ± 0.84 | 0.514 | .000 |
| 21. When at home, I worried that someone would harm me while I was changing my menstrual materials | 2.9 ± 0.33 | 0.442 | .000 |
| 22. When at home, I worried that something else would harm me while I was changing my menstrual materials (e.g., animals, insects, and unsafe structure) | 2.8 ± 0.43 | 0.479 | .000 |
| 26. When at school, I worried that I would not be able to change my menstrual materials when I needed to | 2.2 ± 0.99 | 0.514 | .000 |
| 27. When at school, I worried that someone would see me while I was changing my menstrual materials | 2.7 ± 0.64 | 0.500 | .000 |
| 28. When at school, I worried that someone would harm me while I was changing my menstrual materials | 0.8 ± 1.02 | 0.014 | .000 |

*Abbreviations*: SD: standard deviation.

0.77 for the MPNS. Subscales Cronbach's α coefficients of the "Material and home environment needs" was 0.79, "Transport and school environment needs" was 0.66, "Material reliability concerns" was 0.51, and "Change and disposal insecurity" was 0.74 in the original version.[11]

As another test, the item-total score correlation was used to evaluate internal consistency. The average of the item total score correlation coefficients gives the reliability of the scale. This method explains the relationship between the scores obtained from the scale items and the total score of the test. The total score correlation of an item of scales should be at least 0.30, according to the literature.[17] The findings obtained in this study showed that the total item score correlation of MPNS was within 0.014 to 0.683 (Table 3). Items with low coefficients should be removed from the scale. According to this result, the 28th item was seen under 0.30 and removed.

Another consistency criterion is test–retest reliability. This provides the capability of measuring the results of the time difference. The scale is applied under the same conditions on the same subjects at two different times (2-4 weeks or 10-20 days). The correlation between observations at two different times will be an indicator of reliability. Eventually, this correlation coefficient is wanted to be positive and high. Besides, in the literature, it is deemed appropriate to have at least 30 participants in these tests.[17,20] Based on the literature, in this study, the scale was reapplied four weeks later to 44 participants. The test–retest correlation value result of the scale was r = .66, and also a significant relationship was found at $P < .001$ significance level (Table 4). The findings obtained in this study indicated

**Table 4.** Explanatory Factor Analysis Results for Menstrual Practice Needs Scale (n = 446)

| | Scale Mean if Item Deleted | Scale Variance if Item Deleted | Corrected Item-Total Correlation | Cronbach's α if Item Deleted |
|---|---|---|---|---|
| Item 1 | 63.1533 | 56.191 | 0.464 | 0.775 |
| Item 2 | 62.7811 | 56.464 | 0.583 | 0.773 |
| Item 3 | 62.8574 | 56.536 | 0.561 | 0.774 |
| Item 4 | 62.8843 | 55.782 | 0.506 | 0.772 |
| Item 5 | 64.5547 | 54.244 | 0.592 | 0.775 |
| Item 6 | 64.5323 | 54.496 | 0.577 | 0.778 |
| Item 7 | 63.1825 | 55.12 | 0.584 | 0.777 |
| Item 8 | 62.9202 | 56.155 | 0.335 | 0.776 |
| Item 9 | 63.1601 | 55.501 | 0.378 | 0.776 |
| Item 10 | 62.8394 | 57.025 | 0.506 | 0.778 |
| Item 11 | 62.6175 | 58.564 | 0.395 | 0.779 |
| Item 12 | 62.7385 | 57.313 | 0.474 | 0.776 |
| Item 13 | 62.7811 | 57.05 | 0.549 | 0.776 |
| Item 14 | 63.281 | 53.776 | 0.505 | 0.773 |
| Item 15 | 63.6673 | 52.552 | 0.579 | 0.773 |
| Item 16 | 62.6376 | 57.929 | 0.513 | 0.777 |
| Item 17 | 62.7587 | 58.326 | 0.313 | 0.783 |
| Item 18 | 62.6892 | 58.119 | 0.482 | 0.779 |
| Item 19 | 62.8325 | 55.644 | 0.545 | 0.773 |
| Item 20 | 63.0516 | 54.978 | 0.546 | 0.774 |
| Item 21 | 62.6251 | 58.201 | 0.439 | 0.778 |
| Item 22 | 62.7108 | 57.465 | 0.498 | 0.776 |
| Item 23 | 63.3394 | 53.551 | 0.588 | 0.768 |
| Item 24 | 63.882 | 53.139 | 0.683 | 0.773 |
| Item 25 | 63.8708 | 53.628 | 0.621 | 0.774 |
| Item 26 | 63.3554 | 54.049 | 0.515 | 0.775 |
| Item 27 | 62.8344 | 55.845 | 0.543 | 0.773 |

**Table 5.** Comparison of Test and Retest Mean Scores of the Menstrual Practice Needs Scale and Subscales and Correlations (n = 44)

| Subscales | First Test Mean (SD) | Second Test Mean (SD) | t | *P* | r | *P* |
|---|---|---|---|---|---|---|
| Material and home environment needs | 2.44 (0.41) | 2.48 (0.21) | −0.616 | .541 | 0.563 | .000 |
| Transport and school environment needs | 2.09 (0.63) | 2.16 (0.57) | −0.539 | .592 | 0.637 | .000 |
| Material reliability concerns | 1.65 (0.75) | 1.46 (0.85) | 1.137 | .262 | 0.557 | .000 |
| Change and disposal insecurity | 2.38 (0.49) | 2.51 (0.33) | −0.946 | .354 | 0.647 | .000 |
| Total score | 2.11 (0.31) | 2.18 (0.36) | −0.695 | .495 | 0.661 | .000 |

*Abbreviation*: SD: standard deviation.

that the test and retest results of MPNS were similar. Also, the test–retest results of the original version of the MPNS were stated as moderate (r = .69).[11] Briefly, this scale showed good internal consistency and acceptable test–retest reliability the findings obtained from the analysis of the reliability.

**Discussion of the Results on the Validity of the MPNS**

In this study, to have content validity, the Davis technique was used. Based on specialists' opinions, the CVI value was 0.89, and it was determined that there was a consensus among the academic experts. In the pilot study, the questions that the participants had difficulty understanding were rearranged, and the scale was made easier to understand. Also, pilot implementation was applied to 35 students, with the draft form created finally. Therefore, in this process, no items were removed from this scale based on content validity.

The KMO test measures sampling adequacy and deals with sample size. It is an index that compares the size of the observed correlation coefficients with the size of the partial correlation coefficients. It can be said that the higher the ratio, the better the data set is for factor analysis. The KMO value of 0.90 is accepted as excellent, 0.80 indicates a very good value, and the lower values mean weaker.[12] KMO value of this study was 0.819, which is very good. In the original version of MPNS, the KMO value was found 0.72.[11] Bartlett's test of sphericity is an indicator of suitability for factor analysis. According to the Chi-square value in Bartlett's test of sphericity, the data were correlated with each other, and also, the result of Bartlett's test of sphericity was tested to be $\chi^2 = 4{,}894.399$, $P < .001$ for MPNS. As a result of these tests, the correlation matrix, sample size, and data were appropriate for factor analysis in this study.

CFA, which is used in scale adaptation studies, is the best way to test whether a scale whose construct validity has been tested preserves this structure in the language and culture to be adapted. While it is expected that the Chi-square value is not significant for a model to be acceptable, it is seen that it generally means in practice. This is because this value is very

sensitive to the sample size. Instead, when the Chi-square value is divided by the degrees of freedom, the resulting value is two or less, and five or less indicates that the model has an acceptable goodness of fit. The CFA explains that the measures of RMSEA, SRMR, CFI, GFI, and the 2ℵ†/df value were at wanted levels.[21] In this study, the value obtained by dividing the Chi-square value by the degrees of freedom according to the CFA results was 2.63; this value showed that the model has a goodness of fit. The other fit index values were RMSEA = 0.06, CFI = 0.88, SRMR = 0.044, and GFI = 0.089. In the original version of MPNS, CFA supported a good model fit for the 28 items (RMSEA = 0.028-0.029, CFI = 0.957-0.959).[11] The results of this study showed that the factor loading and interpretation variance were strong, consistent with EFA results, and had a good factor structure. In summary, the findings from this study demonstrated that the MPNS is a suitable instrument for evaluating practices and needs of menstruation for Turkish university girl students.

The MPNS measures menstrual hygiene experiences and prioritizes participant perceptions of capacity above the researcher determined sufficient menstrual period practices. A scale enables the individual to evaluate the status of her perceived menstrual hygiene and needs. Using this scale, the relationship between menstrual practices in cross-sectional and longitudinal studies with education, health, well-being, and social support can be determined.[11] This study contributes to the literature through its Turkish version of the MPNS to measure the menstrual hygiene experience of university students. With MPNS, young girls' needs regarding menstrual hygiene and practices will be determined, and nursing planning can be done in line with the results of the scale. Using this scale in further research will contribute to its effectiveness.[22,23]

### Limitations

One of the limitations was that this study was conducted in only one university in Istanbul in Turkey. Because our study was conducted at a single university in the city center, it cannot be generalized to the general population. We should note that there is no scale with similar content in Turkish, so this scale could not be correlated and discussed with another scale.

### Results and Recommendations

MPNS is a special self-report scale that helps young girls assess their ability to manage menstruation and to what extent they meet their needs in the environment. We aimed to provide Turkish literature with a scale to obtain reliable, consistent, and valid data with this study. The Turkish version of the MPNS is a valid and reliable measurement scale in the assessment of self-perceived menstrual hygiene needs and practices. It is recommended that nurses contribute to the elimination of the deficiencies in this issue by providing training on menstruation to both adolescent girls and their mothers, and given that not only knowledge but also attitudes stemming from traditions and customs are effective on the practice, nurses taking part in health education are recommended to consider these issues. Also, it is recommended to test the validity and reliability of this scale in different languages in different countries to investigate the intercultural differences in young girls. Differences in other cities in Turkey can also be researched.

**Ethics Committee Approval**: Ethical committee approval was received from the Istanbul Medipol University Noninvasive Clinical Research Ethics Committee (approval number:471-10.06.2020).

**Informed Consent**: Written informed consent was obtained from all participants who participated in this study.

**Peer-review**: Externally peer-reviewed.

**Author Contributions**: Concept - P.I.V., Y.V.; Design - P.I.V., Y.V.; Supervision - P.I.V., Y.V.; Resources - P.I.V., Y.V.; Materials - P.I.V., Y.V.; Data Collection and/or Processing - P.I.V., Y.V.; Analysis and/or Interpretation - P.I.V., Y.V.; Literature Search - P.I.V., Y.V.; Writing Manuscript - Y.V.; Critical Review - P.I.V., Y.V.

**Acknowledgments**: We would like to thank all those who participated in this study.

**Conflict of Interest**: The authors have no conflicts of interest to declare.

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

## REFERENCES

1. Hilary OD, Critchley HO, Babayev E, et al. Menstruation: Science and society. *Am J Obstet Gynecol*. 2020;223(5):624-664. [CrossRef]
2. Hennegan J, Shannon AK, Rubli J, Schwab KJ, Melendez-Torres G. Women's and girls' experiences of menstruation in low- and middle-income countries: A systematic review and qualitative metasynthesis. *Plos Med*. 2019;16(5):E1002803. [CrossRef]
3. Miiro G, Rutakumwa R, Nakiyingi-Miiro J, et al. Menstrual health and school absenteeism among adolescent girls in Uganda (MENISCUS): A feasibility study. *BMC Women's Health*. 2018;18(1):4. [CrossRef]
4. Yadav RN, Joshi S, Poudel R, Pandeya P. Knowledge, attitude, and practice on menstrual hygiene management among school adolescents. *J Nepal Health Res Council*. 2018;15(3):212-216. [CrossRef]
5. Xu T, Tomokawa S, Gregorio ER, Jr, Mannava P, Nagai M, Sobel H. School-based interventions to promote adolescent health: A systematic review in low- and middle-income countries of WHO Western Pacific Region. *PloS One*. 2020;15(3):E0230046. [CrossRef]
6. Menstrual Practice Measures. Available at https://www.menstrualpracticemeasures.org/mpns-36/.
7. Dundar T, Ozsoy S. Menstrual hygiene and visually impaired women. *J Educ Res Nurs*. 2018;15(3):192-195.
8. Hennegan J, Dolan C, Wu M, Scott L, Montgomery P. Measuring the prevalence and impact of poor menstrual hygiene management: A quantitative survey of schoolgirls in rural Uganda. *BMJ Open*. 2016;6(12):e012596. [CrossRef]
9. Girod C, Ellis A, Andes KL, Freeman MC, Caruso BA. Physical, social, and political inequities constraining girls' menstrual management at schools in informal settlements of Nairobi, Kenya. *J Urban Health*. 2017;94(6):835-846. [CrossRef]
10. Nabwera HM, Shah V, Neville R, et al. Menstrual hygiene management practices and associated health outcomes among school-going adolescents in rural Gambia. *PloS One*. 2021;16(2):e0247554. [CrossRef]
11. Hennegan J, Nansubuga A, Smith C, Redshaw M, Akullo A, Schwab KJ. Measuring menstrual hygiene experience: Development and validation of the menstrual practice needs scale (MPNS-36) in Soroti, Uganda. *BMJ Open*. 2020;10:e034461. [CrossRef]
12. DeVellis RF. *Scale Development: Theory and Applications*. Thousand Oaks, CA: SAGE Publications, 2017.
13. Orçan F. Exploratory and confirmatory factor analysis: Which one to use first? *J Meas Eval Educ Psychol*. 2018;9(4):414-421. [CrossRef]
14. Chapelle CA. Validity in language assessment. In Loewen S, Sato M (eds.): *The Routledge Handbook of Second Language Acquisition and Language Testing*. New York: Taylor & Francis, 2020.

15. Esin MN. Data collection methods and tools & reliability and validity of data collection tools. In Erdoğan S,Nahcivan N,Esin MN (eds.): *Research Process, Practice and Critical in Nursing*. Vol. 3. Istanbul, Turkey: Nobel Tıp Kitabevleri, 2018.
16. Wu ML. *SPSS Operation—Questionnaire Statistical Analysis of Practice*. Taipei: Wunan, 2012.
17. Güngör D. Psychological measuring instruments development and customization guide. *Turk Psychol Artic*. 2016;19(38):104-112.
18. Büyüköztürk Ş. *Sosyal Bilimler İçin Veri Analizi El Kitabı*. Ankara: Pegem Akademi Yayınları, 2008.
19. Karagöz Y. *SPSS 23 and AMOS 23 Applied Statistical Analysis*. Ankara, Turkey: Nobel Akademik Publishing, 2016.
20. Kula Kartal S, Mor Dirlik E. Historical development of the concept of validity and the most preferred technique of reliability: Cronbach alpha coefficient. *J Abant İzzet Baysal Univ Educ Fac*. 2016;6(4):1865-1879.
21. Schweizer K, Reiß S, Ren X, et al. Speed effect analysis using the CFA framework. *Front Psychol*. 2019;10:1-11. **[CrossRef]**
22. Ibrahim H, Sabar Ismail M. The effect of an educational booklet about menstruation on female nursing students' knowledge, practices and beliefs. *IOSR J Nurs Health Sci*. 2019;8:17-29.
23. Kuhlmann AS, Henry K, Wall LL. Menstrual hygiene management in resource-poor countries. *Obstet Gynecol Surv*. 2017;72(6):356-376. **[CrossRef]**

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.104.149

# Apoptotic and Antiproliferative Effect of Gingiva Mesenchymal Stem Cells on Acute Leukemia T Cells

**Deniz Genç[1,2], Merve Sezer Kürkçü[2], Serhat Sezgin[3]**

[1]Muğla Sıtkı Koçman University, Faculty of Health Sciences, Muğla, Turkey
[2]Muğla Sıtkı Koçman University, Research Laboratories Center, Muğla, Turkey
[3]Muğla Sıtkı Koçman University, Faculty of Dentistry Muğla, Turkey

**ORCID iDs of the authors: D.G. 0000-0003-0351-2805; M.S.K. 0000-0003-0947-2912; S.S. 0000-0001-7899-8171.**

**Cite this article as: Genç D, Kürkçü MS, Sezgin S. Apoptotic and Antiproliferative Effect of Gingiva Mesenchymal Stem Cells on Acute Leukemia T Cells. *Cyprus J Med Sci.* 2021; 6(4): 303-310.**

**BACKGROUND/AIMS**
The aim of this study was to investigate the antiproliferative and apoptotic effect of gingiva-derived mesenchymal stem cells (GMSCs) on the Jurkat cells as t-cell acute lymphoblastic leukemia cell line.

**MATERIAL and METHODS**
The Jurkat cells were cocultured with GMSCs or alone at 37°C 5% $CO_2$ humidified atmosphere with different culture periods and concentrations. The Jurkat cells were subjected to flow cytometry analysis for proliferation, apoptosis, and necrosis by staining the cells with Annexin V and 7AAD antibodies. Intracellular IL-2 secretion in the Jurkat cells was analyzed to determine the proliferative cytokine secretion. CD4+CD25+FoxP3+ cells were analyzed to determine the regulatory T cell population. TNFRI and TNFR2 expressions were analyzed for cell death signaling pathways.

**RESULTS**
GMSCs significantly reduced the proliferative response of the Jurkat cells in 48 hours of culture period in 1:1, 1:2, and 1:5 (GMSC:Jurkat) ratios. The minimum inhibitory effect on the proliferative response was found to be in 1:5 ratios. GMSCs significantly increased the rate of early apoptosis and necrosis of Jurkat cells in 1:5 (GMSC:Jurkat) ratios. Intracellular IL-2 secretion of the Jurkat cells significantly reduced with GMSCs ($P < .05$). GMSCs tended to increase CD4+CD25+FoxP3+Tcell population in the Jurkat cells in 24 and 48 hours of culture periods, but no significant difference was observed ($P > .05$). TNFR2 expression on the Jurkat cells significantly increased within the culture periods when cultured with GMSCs.

**CONCLUSION**
This study demonstrated that GMSCs can response to acute leukemia T cells and can modulate the proliferative response by increasing the apoptosis and necrosis and TNFR2 expression and by decreasing IL-2 secretion. Further in vitro or in vivo studies can be performed to investigate the molecular mechanisms or suppressive effects of GMSCs on acute leukemia T cell cells.

**Keywords:** Gingiva mesenchymal stem cells, T-cell acute lymphoblastic leukemia, apoptosis

## INTRODUCTION

T-cell acute lymphoblastic leukemia, which are aggressive proliferations of transformed T-cell progenitors, account for 10-15% of T-cell acute lymphoblastic leukemia cases in children and 25% of adult T-cell acute lymphoblastic leukemia cases.[1] In addition to current T-cell acute lymphoblastic leukemia treatment, it is important to develop a new strategy to support the apoptosis of lymphoma cells. Most apoptosis inducers currently used in the treatments for T-cell acute lymphoblastic leukemia contain large amounts of heavy metals and, therefore, have many side effects.[2] Therefore, it is necessary to develop new cell-based therapies with lower toxicity.

Gingiva tissue-derived mesenchymal stem cells (GMSCs) are easily accessible multipotent stromal cells originated from oral cavity, which have both anti-inflammatory and anticancer effects as well as its regenerative effects on tissue damages.[3,4] Although the effects of mesenchymal stem cells (MSCs) on tumor prognosis are uncertain, it has been demonstrated by previous studies that their inhibitory effects on tumor growth processes have been observed, and they have

**Corresponding Author:** Deniz Genç
**E-mail:** denizgenc@mu.edu.tr

**Received:** 07.07.2021
**Accepted:** 10.08.2021

been shown to be targeted anticancer agents that can inhibit tumor growth by blocking various tumor processes by regulating the growth of many tumor cells through paracrine mechanisms.[5]

The immunosuppressive properties of MSCs play an important role in preventing cancer progression. MSCs derived from various sources such as bone marrow, adipose tissue, or umbilical cord have been shown to be able to regulate the immune response by affecting the activation, maturation, proliferation, differentiation, and effector function of immune system cells.[6]

Although MSCs have the same morphological features, they develop different immune responses depending on the tissue from which they originate and the inflammatory niche. To date, most studies on the modulatory effect of MSCs on acute lymphoblastic leukemia have mostly been performed with bone marrow MSCs.[7] However, the diverse immunosuppressive mechanisms of MSCs isolated from different tissues, uncovering new sources of MSCs that may have higher immunosuppressive capacity will contribute to the expansion of cellular therapy options. Therefore, we investigated the antiproliferative and apoptotic effects of gingiva MSCs on the Jurkat cells as T-cell acute lymphoblastic leukemia cell line by evaluating the antiproliferative and apoptotic responses, FoxP3 expressing T regulatory cell frequency, and IL-2 secretion by culturing acute T cell lymphoma cells in vitro for the first time.

## MATERIAL and METHODS

### Cell Lines

The Jurkat cell line (ATCC, Clone E6-1) was used as acute T cell leukemia cell line and obtained from the Muğla Sıtkı Koçman University Research Laboratories Center culture isolates. GMSCs from five healthy donors in the third passage were obtained from culture isolates of the same center. The ethical approval for GMSC isolation was previously obtained from the Muğla Sıtkı Koçman University Clinical Research Ethics Committee (10/VII 01.10.2020).

### The Analysis of GMSCs for the Cell Surface Markers

GMSCs from five donors in the third passage were analyzed for the positive (CD29, CD73, and CD105) and negative (CD3, CD28, and HLA-DR) cell surface markers for MSCs. In brief, frozen cells were thawed at 37°C, washed with phosphate buffered saline (PBS) (Sigma–Aldrich, Germany) twice, and centrifuged at 1,500 rpm for 5 minutes. The remaining cell pellet was stained with anti-CD29 (APC), anti-CD90 (PerCp), anti-CD105 (FITC), anti-HLA-DR (APC), anti-CD3 (PerCp), and anti-CD28 (PE) and incubated at +4°C for 30 minutes. All antibodies were purchased from BD Biosciences, USA. Cells were analyzed via flow cytometry for the mean fluorescent index % (MFI%) on the Accuri C6 Plus software (BD Biosciences, USA).

**Main Points**

- Gingiva mesenchymal stem cells (GMSCs) downregulate the proliferative response of T-cell acute lymphoblastic leukemia cells by decreasing IL-2 production.
- GMSCs have an apoptotic effect on T-cell acute lymphoblastic leukemia cell line.
- The activation and proliferation of T-cell acute lymphoblastic leukemia cells can be reduced with GMSCs depending on the cell ratio.

### Culture Conditions

GMSCs were separately seeded in 24-well plates with the amount of $5 \times 10^4$ cells per well in Dulbecco's modified Eagle's medium (Pan Biotech, Germany) supplemented with 10% fetal bovine serum (FBS) (Pan Biotech, Germany) and 1% pencillin/streptomycin (100 IU $mL^{-1}$, 100 µg $mL^{-1}$) (Thermofisher, USA) 48 hours before the coculture. The Jurkat cells were cultured alone or with GMSCs with the ratio of 1:1, 1:2, 1:5, and 1:10 (GMSCs:Jurkat cells) suspended in RPMI 1640 medium (Pan Biotech, Germany) supplemented with 10%FBS (Pan Biotech, Germany) and 1% pencillin/streptomycin (100 U $mL^{-1}$, 100 µg $mL^{-1}$) (Thermofisher, USA) in 24-well plates at 37°C and 5%$CO_2$ incubator for time periods of 6, 12, 24, and 48 hours. T cell stimulation was done with anti-CD3 and anti-CD28 (Thermofisher, USA) 10 and 2 µg $mL^{-1}$, respectively. At the end of each culture periods, the Jurkat cells were collected and analyzed for apoptosis, necrosis, proliferation rate, IL-2 secreting CD3+ cells, FoxP3 expressing CD4+CD25+ T regulatory cells, and tumor necrosis factor receptor I (TNFRI) and tumor necrosis factor receptor 2 (TNFR2) expressions via flow cytometry, as described in the analysis sections. The minimum antiproliferative response was determined by observing the significant decrease in the proliferation ratio in the cocultured cells compared to the Jurkat cell line cultures alone.

### Coculture of the Jurkat Cells with GMSCs

After determining the minimum concentration of GMSCs:Jurkat cells, we cultured the Jurkat cells in the presence and absence of GMSCs or GMSCs with healthy mononuclear cells suspended in RPMI 1640 medium (Pan Biotech, Germany) supplemented with FBS (Pan Biotech, Germany) and 1%pencillin/streptomycin (Thermofisher, USA) with the specific T lymphocyte stimulation with anti-CD3 and anti-CD28 (Thermofisher, USA) 10 and 2 µg $mL^{-1}$, respectively, in 24-well plates at 37°C and 5%$CO_2$ incubator for time periods of 6, 12, 24, and 48 hours.

### Analysis of Apoptosis of the Jurkat Cells

To analyze the apoptosis or necrosis of the Jurkat cells, the collected cell suspension was stained using Annexin V (PE) and 7AAD (BD Biosciences, USA) in the room temperature in the dark for 15 minutes. Cells were analyzed via flow cytometry and data recorded as MFI%. The Jurkat cells were gated from total cell population and gated for $CD3^+$ cells. Four quadrants were analyzed as follows: lower left quadrant for cell survival, lower right quadrant for early apoptosis, upper right quadrant for late apoptosis, and upper left quadrant for necrosis.

### Analysis of Proliferation of the Jurkat Cells

The Jurkat cells were labeled using Carboxyfluorescein succinimidyl ester (CFSE) labeling kit (Thermofisher, USA) at the beginning of the culture period. Briefly, cells were washed with PBS and centrifuged at 1,500 rpm for 5 minutes. Remaining cell pellet was resuspended in 1 mL of PBS, and 5 µM of CFSE solution was added in the cell suspension and incubated at 4°C for 20 minutes in the dark. Cells were then washed twice with RPMI medium supplemented with 10%FBS. Thereafter, culture period cells were analyzed via flow cytometry in the FITC channel. The proliferation of the Jurkat cells was detected by gating $CD3^+$ cell population and analyzed for CFSE signaling

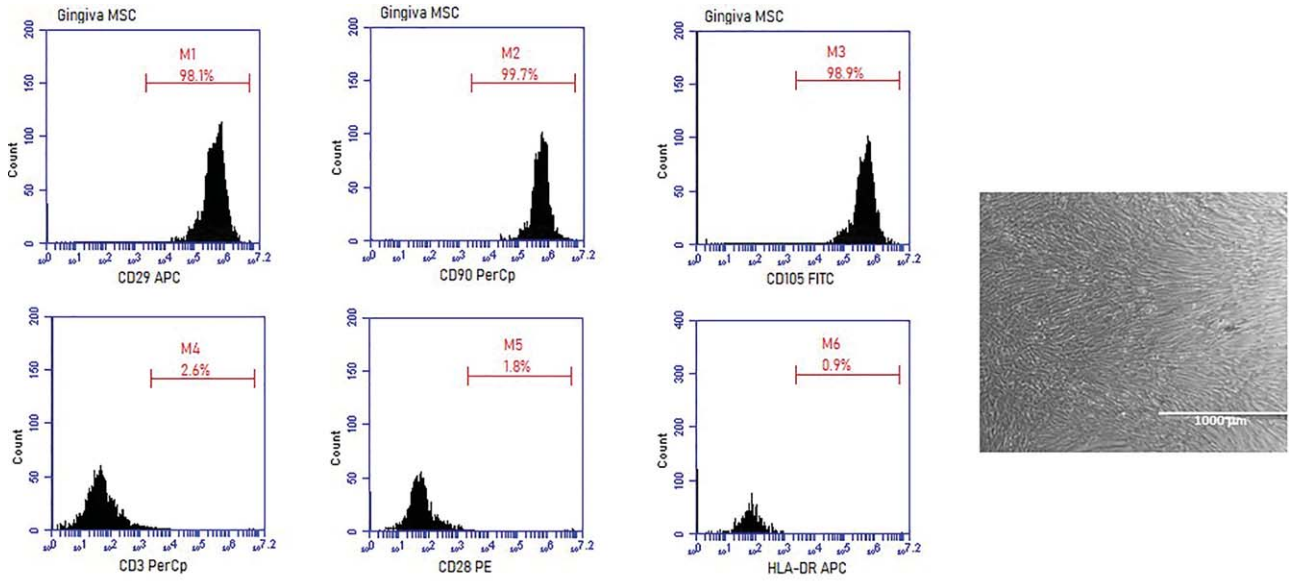


**Figure 1.** Characterization of GMSCs. Flow cytometry analysis for the positive cell surface expressions (CD29, CD90, and CD105) and negative cell surface expressions (CD3, CD28, and HLA-DR) for mesenchymal stem cells. GMSCs expressed positive cell surface markers over 95% and lack the expressions of negative markers. GMSCs showed fibroblast-like colonies in the third passage.

via flow cytometry (BD Biosciences, USA). Histogram analysis was performed for proliferation ratio.

### Analysis of CD4+CD25+FoxP3+ T Cells

T regulatory cell population is a suppressive cell type in immune responses. Therefore, we analyzed CD4+CD25+FoxP3+ T regulatory cells after culture periods. The Jurkat cells were washed twice with PBS, cell surface staining was performed using anti-CD4 (FITC) and anti-CD25 (APC) at 4°C for 30 minutes, and intracellular staining was performed using anti-FoxP3 (PE) at 4°C for 20 minutes. Cells were analyzed via flow cytometry, and CD25 cells were gated to analyze CD4+FoxP3+ cells. The data were recorded as MFI% in the dot plot analysis.

### Intracellular IL-2 Analysis

The Jurkat cells were analyzed for intracellular cytokine secretion for IL-2, which induces T cell proliferation. The cells were first stained using anti-CD3 (PerCp) for the cell surface marker of T lymphocytes and incubated at 4°C for 30 minutes in the dark. After washing the cells using PBS, permeabilization buffer was added and incubated for 20 minutes at 4°C. The permeabilized cells were then stained with anti-IL-2 (APC) (BD Biosciences, USA) antibody for 30 minutes at 4°C and analyzed for intracellular IL-2 secreting T lymphocytes. All antibodies were purchased from BD Biosciences, USA.

### Analysis for TNFRI and TNFR2 Expression

TNFRI and TNFR2 signaling pathways are key regulatory factors that generate apoptotic cell death signals in many of the cells. We analyzed TNFRI and TNFR2 expressions on the Jurkat cells at the end of the culture periods. Cells were stained using anti-CD120α (PE) for TNFRI or anti-CD120b (PE) for TNFR2 expressions. Histogram analysis was performed for the expression of TNFRI or TNFR2 for the Jurkat cells via flow cytometry. Histogram analysis was performed for $CD3^+$ cell population.

### Statistical Analysis

Differences between groups were analyzed using the SPSS program and the Graphpad Prism program version 8.0 (Graphpad Software, Inc., CA, USA). Data were given as mean ± standard deviation (SD) (minimum-maximum) values in each group. Comparison of the data of two groups was done by one-way ANOVA test. $P < .05$ values were considered significant.

## RESULTS

### GMSCs Showed Positive Cell Surface Markers Expression for MSCs and Formed Fibroblast-Like Colonies in the Culture

The third passage GMSCs were analyzed for positive and negative cell surface markers expressions to confirm these cells as MSCs. The cells were expressed as CD29, CD73, and CD105 over 95% and lack the expressions of negative markers. The cells showed fibroblast-like colonies in the culture flasks (Figure 1).

### The Proliferation of the Jurkat Cells Reduced with GMSCs

The proliferation analysis was performed to evaluate the suppressive effect of GMSCs on the Jurkat cells. Five separate cultures were performed for each of GMSCs with the Jurkat cells. CFSE-labeled Jurkat cells were cultured in the presence and absence of GMSCs with the ratio of 1:1, 1:2, 1:5, and 1:10 (GMSCs:Jurkat cells) or Jurkat cells alone, and analysis was done for 6, 12, 24, and 48 hours of the culture periods. The proliferative response of the Jurkat cells significantly increased in 24 hours (24.6 ± 5.7) and 48 hours (43.1 ± 4.8) culture periods compared to 6 and 12 hours of the culture periods (6 hours: 6.4 ± 2.1, 12 hours: 10.8 ± 2.9) ($P < .001$). GMSCs significantly inhibited the proliferation ratio of the Jurkat cells in 1:1, 1:2, and 1:5 cocultures in 48 hours of the incubation period

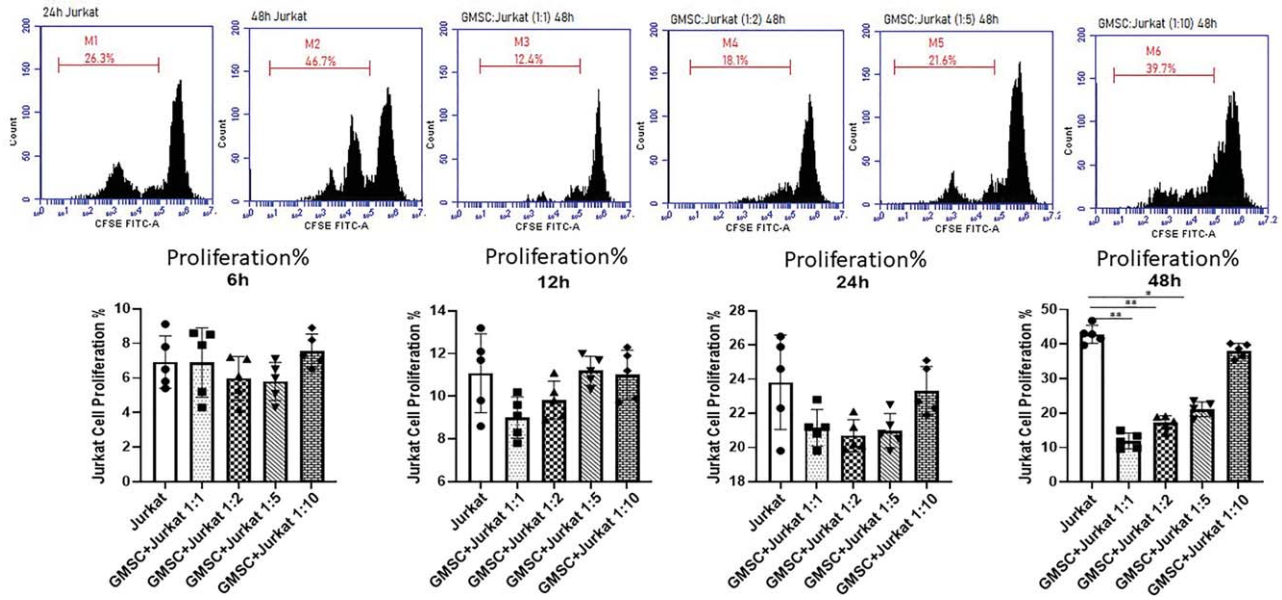


**Figure 2.** Lymphoproliferative response of the Jurkat cells. CFSE-labeled Jurkat cells were stimulated with anti-CD3/anti-CD28 for specific T lymphocyte responses. The Jurkat cells cultured alone showed high proliferation ratio within 24 and 48 hours of culture periods. GMSCs significantly reduced the lymphoproliferative response of the Jurkat cells in 1:1, 1:2, and 1:5 (GMSC:Jurkat) ratios. The minimum suppressive response for the proliferation of Jurkat cells cultured with GMSCs was found to be in 1:5 ratios. Statistical analysis of the proliferation of the Jurkat cells in the presence and absence of GMSCs. The proliferative response of the Jurkat cells with GMSCs did not significantly change in 6, 12, and 24 hours culture periods but tended to decrease in 24 hours. GMSCs significantly decreased proliferation ratio of the Jurkat cells in 48 hours of culture period in 1:1, 1:2, and 1:5 ratios ($P < .01$, $< .01$, and $< .05$, respectively). *$P < .05$, **$P < .01$, ***$P < .005$, and ****$P < .001$.

compared to 48 hours of culture period of the Jurkat cells alone (11.8 ± 2.4, 16.2 ± 4.3, and 21.4 ± 3.7, respectively) ($P < .01$, $< .01$, and $< .05$).

The Jurkat cells cultured with GMSCs in the ratio of 1:10 tended to decrease the proliferation ratio (39.6 ± 4.8) in 48 hours of culture period, but no significant difference was observed when compared with 48 hours of the Jurkat cell cultures alone ($P > .05$) (Figure 2).

The minimum suppressive ratio of the cocultured cells was found to be 1:5 (GMSCs:Jurkat). Therefore, we continued the cell cultures in 1:5 ratio with the culture periods of 6, 12, 24, and 48 hours for the analysis of apoptosis, intracellular IL-2 cytokine secretion, CD4+CD25+FoxP3+ T cell frequency, and TNFRI and TNFR2 expressions.

### GMSCs Increased Early Apoptosis and Necrosis in the Jurkat Cells

The analysis for apoptosis was performed by staining the cells with Annexin V and 7AAD antibodies to analyze early and late apoptosis and necrosis in the Jurkat cells. The Jurkat cells cultured alone showed low ratio of early apoptosis (6 hours: 1.1 ± 0.2, 12 hours: 10.4 ± 0.5, 24 hours: 11.8 ± 0.3, and 48 hours: 11.9 ± 0.2), late apoptosis (6 hours: 3.2 ± 0.3, 12 hours: 0.6 ± 0.2, 24 hours: 2.0 ± 0.4, and 48 hours: 1.7 ± 0.4), and necrosis (6 hours: 1.6 ± 0.1, 12 hours: 4.8 ± 0.4, 24 hours: 3.3 ± 0.5, and 48 hours: 6.6 ± 0.3). GMSCs significantly increased the early apoptosis and necrosis of the Jurkat cells (early apoptosis: 6 hours: 7.5 ± 0.4, $P < .05$, 12 hours: 16.8 ± 0.3, $P < .05$, 24 hours: 55.6 ± 5.7, $P < .001$, and 48 hours: 59.2 ± 4.8, $P < .001$; necrosis: 6 hours: 8.7 ± 0.6, $P < .01$, 12 hours: 13.7 ± 1.9, $P < .01$, 24 hours: 7.8 ± 3.2, $P < .005$, and 48 hours: 15.6 ± 4.2, $P < .01$) compared to those in the Jurkat cells alone (Figure 3).

### Intracellular IL-2 Secretion in Jurkat Cells Reduced with GMSCs

IL-2 is a cytokine that induces activation and proliferation of T cells. In this study, we analyzed intracellular IL-2 secreting T cells to determine the effect of GMSCs on the activation of the Jurkat cells. The Jurkat cells were stained with anti-CD3 for cell surface marker of lymphocytes, and intracellular staining was done with anti-IL-2 antibody. The Jurkat cells cultured alone showed a high ratio of intracellular IL-2 in 24 and 48 hours of culture periods (14.1 ± 1.7, 16.3 ± 0.8, respectively). GMSCs significantly decreased intracellular IL-2 secreting cells in 24 and 48 hours of cocultures, compared to cultures of the Jurkat cells alone (24 hours: 9.2 ± 0.4, $P < .01$, 48 hours: 8.3 ± 0.2, $P < .005$, respectively) (Figure 4).

### Frequency of FoxP3 Expressing CD4+CD25+ Tregulatory Cells in the Jurkat Cell Population Tended to Increase with GMSCs

We evaluated the effect of GMSCs on FoxP3 expressing CD4+CD25+ T regulatory cell population in Jurkat cells. The cultured cells first analyzed for cell surface expression of CD4 and CD25, and intracellular FoxP3 staining was performed to determine CD4+CD25+FoxP3+ T regulatory cell frequency. The cultured Jurkat cells showed low frequency of CD4+CD25+FoxP3+ T cell population when cultured alone in 6, 12, 24, and 48 house of culture periods (6 hours: 1.3 ± 0.2, 12 hours: 2.2 ± 0.1, 24 hours: 2.6 ± 0.6, and 48 hours: 3.3 ± 1.1). GMSCs tended to increase the CD4+CD25+FoxP3+ cell population in 24 and 48 hours cultures, compared to those in Jurkat cells alone cultures (6 hours: 1.6 ± 0.3, 12 hours: 3.1 ± 0.2, 24 hours:

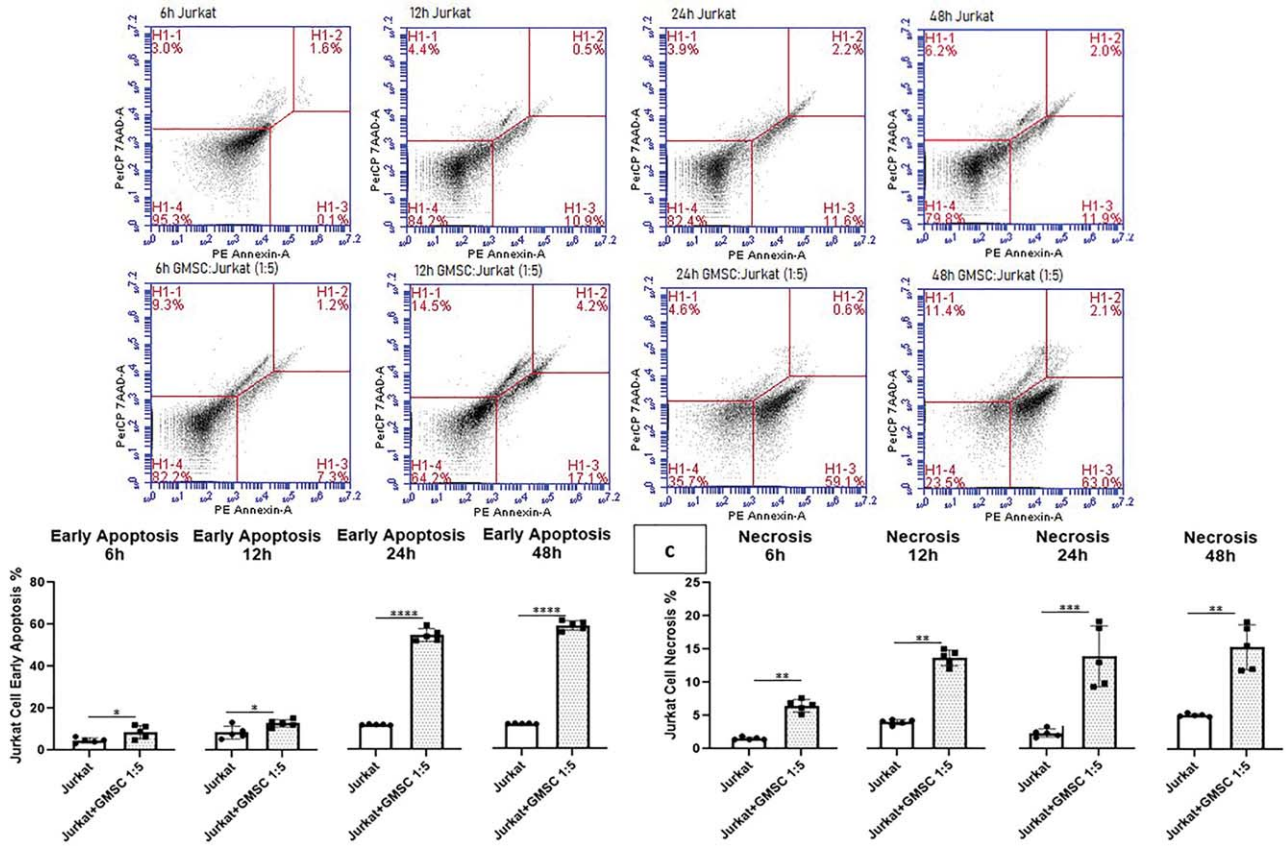


**Figure 3.** Analysis for apoptosis, necrosis, and cell survival of the Jurkat cells in the presence and absence of GMSCs. Representative analysis for Annexin V and 7AAD in the Jurkat cells. Analysis of quadrants was done as following: lower left: cell survival; lower right: early apoptosis; upper right: late apoptosis; upper left: necrosis. Statistical analysis for apoptosis and necrosis. The Jurkat cells showed low ratio of early and late apoptosis and necrosis when cultured alone in 6, 12, 24, and 48 hours of the culture periods. GMSCs significantly increased early apoptosis and necrosis in Jurkat cells compared to the Jurkat cell cultures alone (early apoptosis: 6 hours: 7.5 ± 0.4, $P < .05$, 12 hours: 16.8 ± 0.3, $P < .05$, 24 hours: 55.6 ± 5.7, $P < .001$, and 48 hours: 59.2 ± 4.8, $P < .001$; necrosis: 6 hours: 8.7 ± 0.6, $P < .01$, 12 hours: 13.7 ± 1.9, $P < .01$, 24 hours: 7.8 ± 3.2, $P < .005$, and 48 hours: 15.6 ± 4.2, $P < .01$). *$P < .05$, **$P < .01$, ***$P < .005$, and ****$P < .001$.

3.2 ± 0.1, and 48 hours: 3.6 ± 0.2), but no significant difference was observed ($P > .05$) (Figure 5).

### GMSCs Increased TNFR2 Expression on the Jurkat Cells

We analyzed TNFRI and TNFR2 expressions for the apoptotic signals on the Jurkat cells. The Jurkat cells showed low expression of TNFRI and TNFR2 when cultured alone (6 hours: TNFRI: 1.0 ± 0.2, TNFR2: 1.9 ± 0.5, 12 hours: TNFRI: 1.5 ± 0.3, TNFR2: 2.1 ± 0.4, 24 hours: TNFRI: 2.9 ± 0.8, TNFR2: 2.6 ± 0.1, and 48 hours: TNFRI: 2.1 ± 0.4, TNFR2: 3.0 ± 0.5). TNFR2 expression was not significantly changed in the Jurkat cells in 6 hours culture period with GMSCs, but GMSCs significantly increased TNFR2 expression on the Jurkat cells in 12, 24, and 48 hours of culture periods (6 hours: 3.5 ± 0.7, $P > .05$, 12 hours: 19.7 ± 3.8, $P < .001$, 24 hours: 26.9 ± 4.2, $P < .001$, and 48 hours: 28.9 ± 3.1, $P < .001$, respectively). There was no significant difference in TNFRI expression in cultures of Jurkat cells with GMSCs ($P > .05$) (Figure 6).

## DISCUSSION

T-cell acute lymphoblastic leukemia is one of the most common T lymphocyte-related malignancies, accounting for 10-25% of all cancer cases in children and 25% in adults.[8] Although many treatments have been developed on tumor cells, the effectiveness of the treatments is still not sufficient, and innovative therapeutic approaches are needed. In this study, we investigated the antiproliferative and apoptotic effects of GMSCs on the Jurkat cells as T-cell acute lymphoblastic leukemia cell line. The results showed that GMSCs downregulated proliferative response of the Jurkat cells by enhancing early apoptosis and downregulating IL-2 secretion. In addition, GMSCs upregulated TNFR2 expression on the Jurkat cells; this may be evidence of an apoptotic effect of GMSCs on Jurkat cells through a member of the TNFR family.

GMSCs are the most easily isolated stromal adult stem cells with a high doubling time and high anti-inflammatory effect compared to many other sources. In a previous study, it was demonstrated that GMSCs inhibit the proliferation of oral cancer cells by inducing proapoptotic signals with the soluble factors.[3] In addition, bone marrow or Wharton's jelly MSCs have an anti-tumoral effect on lung cancer cells, colorectal cancer, or bladder tumor cells by inducing macrophages in the regulatory phenotype or inducing apoptosis of cancer cells.[9] We, therefore, in the present study, investigated the effects of GMSCs on the proliferative responses, apoptosis, and T regulatory cell frequency in the Jurkat cells.

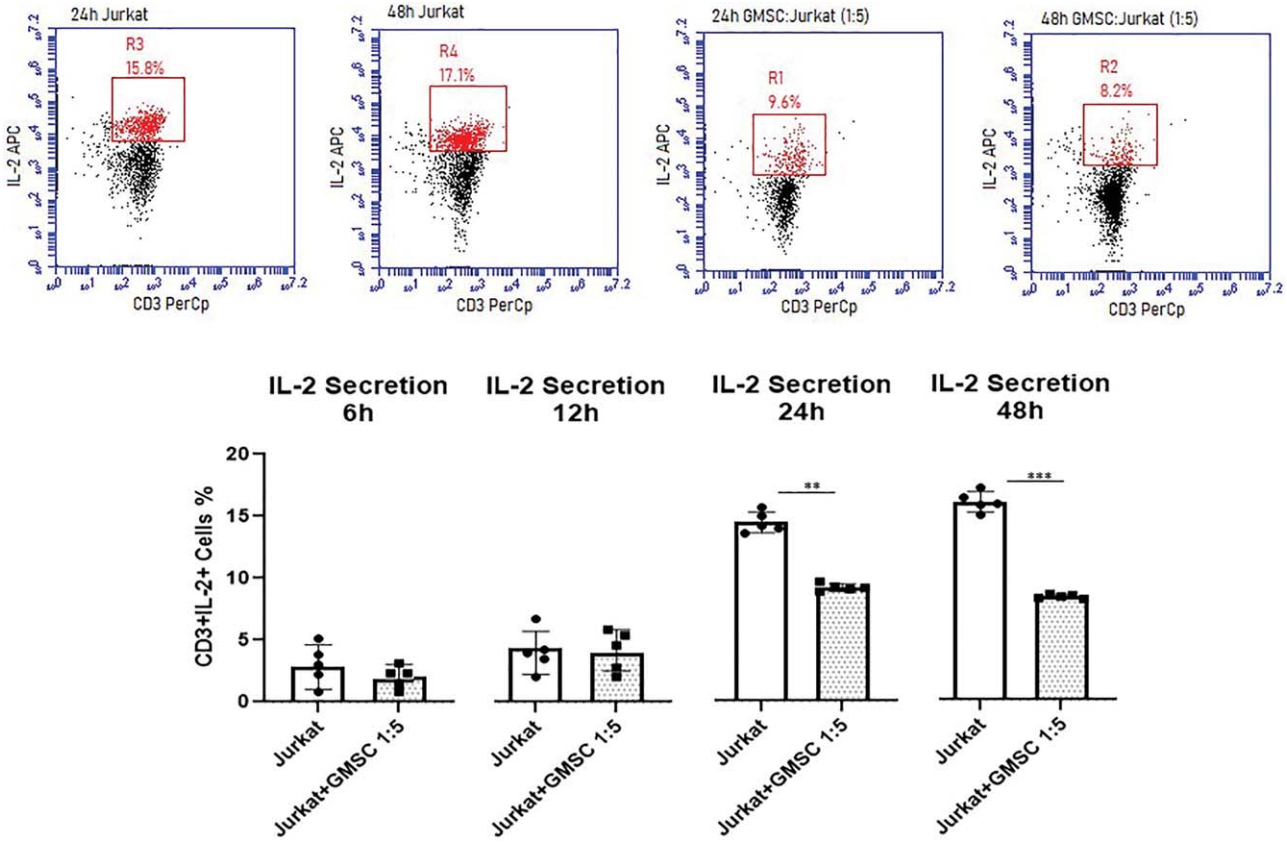


**Figure 4.** Analysis of IL-2 secreting CD3+ cells. The representative analysis for IL-2 secreting Jurkat cells by flow cytometry. The Jurkat cells were gated as CD3+ cell population, and IL-2 secretion of CD3+ cells was analyzed by gating double positive area in the dot plot analysis. Statistical analysis for IL-2 secreting CD3+ cells. IL-2 secretion significantly decreased in Jurkat cells when cultured with GMSCs in 24 and 48 hours of culture periods, compared to those in the Jurkat cell cultures alone (24 hours: $P < .01$ and 48 hours: $P < .005$, respectively). *$P < .05$, **$P < .01$, ***$P < .005$, and ****$P < .001$.

Avoiding apoptosis is an important feature of cancer and is vital for maintaining a balance between proper apoptotic signaling, cell survival, and cell death. The dysregulation of apoptotic pathways in cancer cells not only promotes tumor formation but can also make cancer cells resistant to anticancer agents.[7,10] However, antitumor effects of MSCs are still controversial. Additionally, different results obtained in studies may vary depending on the source of MSCs or the type and stage of cancer. In this study, we demonstrated that increasing concentrations of GMSCs and increased expression of TNFR2 in Jurkat cells, which may be directly involved in cell death. Targeting TNFR2 is crucial in cancer therapy since it controls both immunosuppression and angiogenesis in cancer cells.[11,12] The results showed that the proliferation of the Jurkat cells decreased with GMSCs in concentration-dependent manner. The inhibition of proliferative responses found to be the increasing rate of early apoptosis and necrosis of the Jurkat cells.

Regulatory T cells are known to suppress immune functions through various mechanisms such as the production of IL-10 as an immunosuppressive cytokine. T regulatory cells also provide immunoregulation by consuming the cytokine IL-2 produced by activated T lymphocytes in the inflammatory environment.[13] In the present study, we evaluated the effect of GMSCs on the frequency of CD4+CD25+FoxP3+ T regulatory cells within the Jurkat cells. Although no significant difference was observed, the results showed that GMSCs tended to increase the generation of FoxP3 expressing CD4+CD25+ regulatory T cell population in the Jurkat cell line. One reason for the inhibitory effect of GMSCs on Jurkat cells may be the slight increase in T regulator cells.

IL-2 is a pleiotropic cytokine that promotes the differentiation of both pro- and anti-inflammatory T cells. Additionally, IL-2 supports the proliferative expansion of T lymphocytes and the proliferation of antigen-specific T cell clones.[14] IL-2 transduced MSCs can secrete or produce various antitumor agents or mediators, which could prevent and keep from metastases.[15] Studies to date indicate that MSCs can downregulate IL-2 production from T lymphocytes through induction of CD25 cleavage and have an essential role in the inhibition of T cell proliferative responses.[16–18] Previous studies indicate that IL-2 produced during inflammatory responses in the microenvironment may further enhance the immunosuppressive response effect of MSCs. We investigated the effect of GMSCs on IL-2 production in Jurkat cells and found that GMSCs strongly inhibit IL-2 production in Jurkat cells. The proliferative response of Jurkat cells cocultured with GMSCs may have been inhibited by down-regulation of IL-2 production.

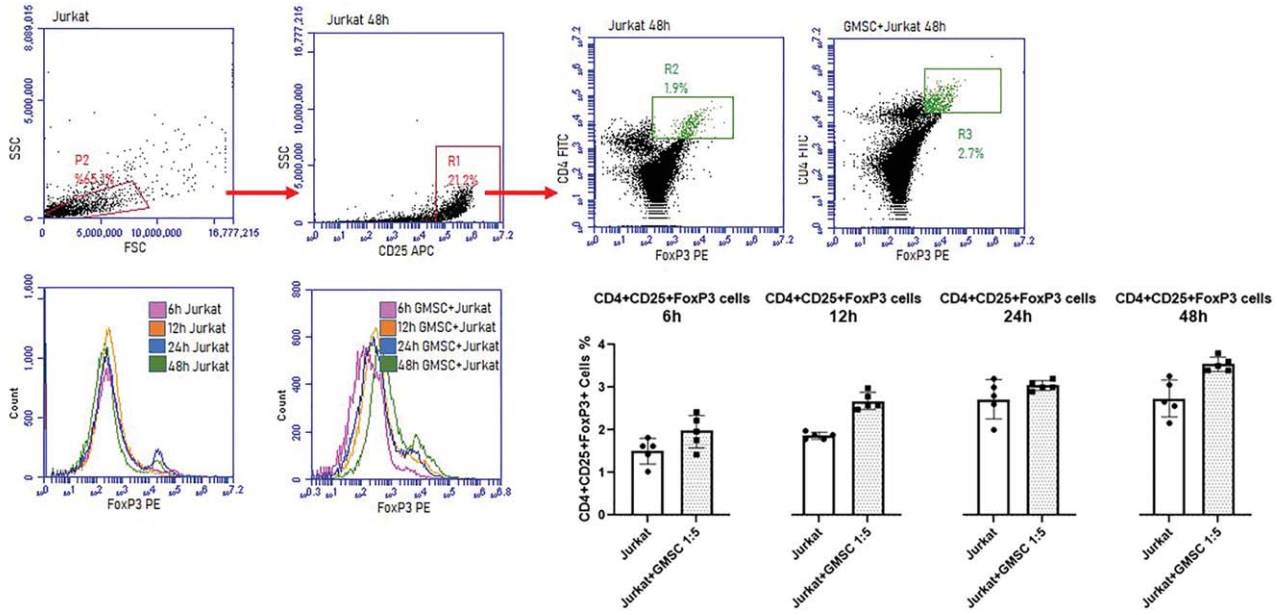


**Figure 5.** FoxP3 expressing CD4+CD25+ T regulatory cells. The Jurkat cells were analyzed for the effect of GMSCs on the generation of T regulatory cells in the Jurkat cell cultures. Gating strategy for flow cytometry analysis of the CD4+CD25+FoxP3+ cell population in the Jurkat cells. Analysis was done by gating CD25+ cells in the total lymphocyte population. CD25+ cells were analyzed for CD25+FoxP3+ cells. Comparative histogram analysis for the Jukat cells in the presence and absence of GMSCs. Statistical analysis of the CD4+CD25+FoxP3+ cells in the Jurkat cells in the presence and absence of GMSCs. GMSCs tended to increase T regulatory cell population in the Jurkat cells, but no significant difference was observed when compared with Jurkat cell cultures alone ($P > .05$).

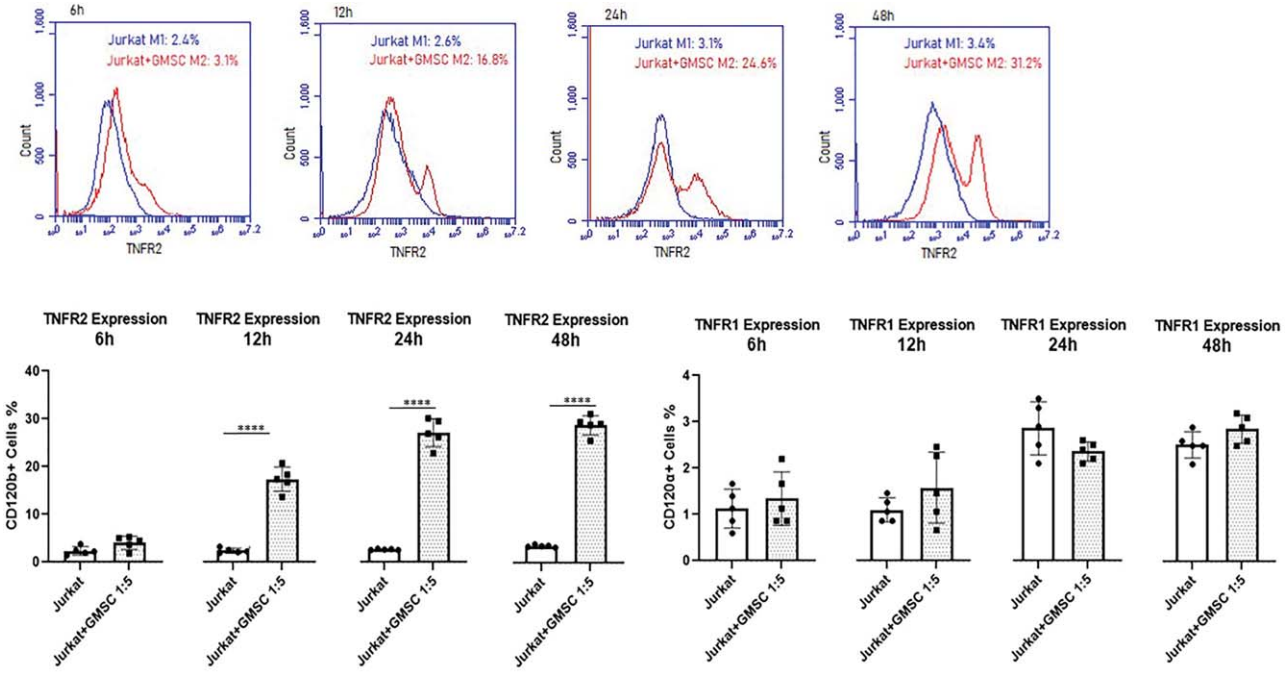


**Figure 6.** TNFRI and TNFR2 expressions on the Jurkat cells. One of the apoptotic pathways was analyzed for the Jurkat cells cultured with or without GMSCs. The comparative histogram analysis for TNFR2 expression on the Jurkat cells. Statistical analysis for TNFRI or TNFR2 expressions of Jurkat cells in the presence and absence of GMSCs. GMSCs significantly increased TNFR2 expression on the Jurkat cells in 12, 24, and 48 hours of culture periods (12 hours: 19.7 ± 3.8, $P < .001$, 24 hours: 26.9 ± 4.2, $P < .001$, and 48 hours: 28.9 ± 3.1, $P < .001$). ****$P < .001$.

In conclusion, results showed that GMSCs downregulate the proliferative response of the Jurkat cells by increasing apoptosis and necrosis and reducing IL-2 production by the Jurkat cells. In addition, GMSCs upregulate the expression of TNFR2 on the Jurkat cells, which, in turn, may promote apoptosis of these cells. Further in vitro or in vivo studies can be conducted with GMSCs for the cell-based treatment of T-cell acute lymphoblastic leukemia.

**Ethics Committee Approval:** Ethical committee approval was received from the Muğla Sıtkı Koçman University Clinical Research Ethics Committee (10/VII 01.10.2020).

**Informed Consent:** Informed consent was obtained from all participants.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Design - D.G.; Data Collection and/or Processing - D.G., M.S.K., S.S.; Analysis and/or Interpretation - D.G., M.S.K.

**Acknowledgment:** The present study was funded by the Muğla Sıtkı Koçman University Research Projects (Project No: 20/106/02/3/4).

**Conflict of Interest:** The authors have no conflicts of interest to declare.

**Financial Disclosure:** The present study was funded by Muğla Sıtkı Koçman University Research Projects (Project No: 20/106/02/3/4).

## REFERENCES

1. Yang T, Yao S, Zhang X, Guo Y. Andrographolide inhibits growth of human T-cell acute lymphoblastic leukemia Jurkat cells by down-regulation of PI3K/AKT and upregulation of p38 MAPK pathways. *Drug Des Dev Ther*. 2016;10:1389-1397. [CrossRef]
2. Zhu XL, Jiang L, Qu F, Wang ZY, Zhao LM. Inhibitory effect of Embelin on human acute T cell lymphoma Jurkat cells through activation of the apoptotic pathway. *Oncol Lett*. 2015;10(2):921-926. [CrossRef]
3. Ji X, Zhang Z, Han Y, et al. Mesenchymal stem cells derived from normal gingival tissue inhibit the proliferation of oral cancer cells in vitro and in vivo. *Int J Oncol*. 2016;49(5):2011-2022. [CrossRef]
4. Fawzy El-Sayed KM, Dörfer CE. Gingival mesenchymal stem/progenitor cells: A unique tissue engineering gem. *Stem Cells Int*. 2016;2016:1-16. [CrossRef]
5. Lin W, Huang L, Li Y, et al. Mesenchymal stem cells and cancer: Clinical challenges and opportunities. *Biomed Res Int*. 2019;2019:1-12. [CrossRef]
6. Chang AI, Schwertschkow AH, Nolta JA, Wu J. Involvement of mesenchymal stem cells in cancer progression and metastases. *Curr Cancer Drug Targets*. 2015;15(2):88-98. [CrossRef]
7. Lee MW, Ryu S, Kim DS, et al. Mesenchymal stem cells in suppression or progression of hematologic malignancy: Current status and challenges. *Leukemia*. 2019;33(3):597-611. [CrossRef]
8. Baghbani E, Khaze V, Sadreddini S, et al. PTPN22 silencing in human acute T-cell leukemia cell line (Jurkat cell) and its effect on the expression of miR-181a and miR-181b. *Adv Pharm Bull*. 2018;8(2):277-282. [CrossRef]
9. Wang Q, Li T, Wu W, Ding G. Interplay between mesenchymal stem cell and tumor and potential application. *Human Cell*. 2020;33:444-458. [CrossRef]
10. Plati J, Bucur O, Khosravi-Far R. Apoptotic cell signaling in cancer progression and therapy. *Integr Biol (Camb)*. 2011;3(4):279-296. [CrossRef]
11. Naserian S, Abdelgawad ME, Afshar Bakshloo M, et al. The TNF/TNFR2 signaling pathway is a key regulatory factor in endothelial progenitor cell immunosuppressive effect. *Cell Commun Signal*. 2020;18(1):94. [CrossRef]
12. Depuydt B, van Loo G, Vandenabeele P, Declercq W. Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors. *J Cell Sci*. 2005;118(Pt 3):497-504. [CrossRef]
13. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci*. 2019;110(7):2080-2089. [CrossRef]
14. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes. *Annu Rev Immunol*. 2018;36:411-433. [CrossRef]
15. Chulpanova DS, Solovyeva VV, James V, Arkhipova SS, et al. Human mesenchymal stem cells overexpressing interleukin 2 can suppress proliferation of neuroblastoma cells in co-culture and activate mononuclear cells in vitro. *Bioengineering (Basel)*. 2020;7(2):59. [CrossRef]
16. Park MJ, Shin JS, Kim YH, Hong SH, et al. Murine mesenchymal stem cells suppress T lymphocyte activation through IL-2 receptor α (CD25) cleavage by producing matrix metalloproteinases. *Stem Cell Rev Rep*. 2011;7(2):381-393. [CrossRef]
17. Yoo HS, Lee K, Na K, et al. Mesenchymal stromal cells inhibit CD25 expression via the mTOR pathway to potentiate T-cell suppression. *Cell Death Dis*. 2017;8(2):e2632-e2632. [CrossRef]
18. Davies LC, Heldring N, Kadri N, Le Blanc K. Mesenchymal stromal cell secretion of programmed death-1 ligands regulates T cell mediated immunosuppression. *Stem Cells*. 2017;35(3):766-776. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.1470

# The Smoking Status and Attitudes of Medical School Students in a University of Nicosia

**Ozen Asut, Songul Vaizoglu, Gulifeiya Abuduxike, Sanda Cali**

**Department of Public Health, Faculty of Medicine, Near East University, Nicosia, Cyprus**

**ORCID iDs of the authors: O.A. 0000-0002-9604-4037; S.V. 0000-0001-9279-1740; G.A. 0000-0002-9798-7459; S.C. 0000-0002-9929-2637.**

**Cite this article as: Asut O, Vaizoglu S, Abuduxike G, Cali S. The Smoking Status and Attitudes of Medical School Students in a University of Nicosia. *Cyprus J Med Sci.* 2021; 6(4): 311-318.**

**BACKGROUND AND AIMS**
The smoking prevalences of physicians and medical students continue to preserve their high levels around the world. This study is the 2018 survey of a broader intervention started in 2017, aiming to monitor the smoking status of medical students in a multinational university of Northern Cyprus.

**MATERIAL and METHODS**
The universe of this cross-sectional study was all of the 1,461 medical students registered at the medical school for the academic year 2018-2019. The data were collected by a self-administered questionnaire applied under direct observation. Of the students, 1,304 responded to the questionnaire with a response rate of 89.3%. The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 18.0 (IBM SPSS Corp.; Armonk, NY, USA), with $P < .01$ evaluated as significant.

**RESULTS**
The participants were from 31 counties, the majority being citizens of the European-Central Asian country group. The overall smoking frequency was 33.7%, with 42.1% for males and 26.1% for females. While 26.8% consumed tobacco products other than cigarettes, 50.8% of smokers had starting smoking after registered to medical school. Sub-Saharan African country citizens smoked least by 6.0%, and the European-Central Asian group smoked most by 35.7%. Nonsmokers displayed significantly more positive attitudes for tobacco regulations and doctors being role models, compared to smokers.

**CONCLUSION**
The results of this survey revealed a high tobacco use frequency in spite of a medical curriculum including a specific tobacco program. Stronger educational and other behavioral interventions are needed to alter the smoking behaviors of medical students, and thus, medical education should be modified accordingly.

**Keywords:** Smoking, tobacco use frequency, attitudes, medical students, Northern Cyprus

## INTRODUCTION

Tobacco use is a major public health problem globally and specifically in developing countries, resulting in more than seven million deaths per year according to the World Health Organization (WHO). More than one billion people of our planet are smokers.[1]

Health professionals and particularly doctors are in a position to curb health problems related to tobacco use by community approaches. Attitudes and behaviors of doctors and their role model functions are influential on increasing the willingness of their patients to quit smoking, as well as in affecting other lifestyle behaviors.[2–5] A number of studies conducted during the last decades of the previous century and up to the present time have revealed the efficacy of physician's role in assisting individuals for smoking cessation, and doctors with good lifestyle behaviors have a better chance for this end.[3–5] Yet, smoking among doctors and medical students continues to stay high in many countries, in spite of the WHO and World Medical Association efforts to assist medical doctors globally and to guide national medical associations for effective measures.[2]

**Corresponding Author:** Ozen Asut
**E-mail:** ozen.asut@neu.edu.tr

**Received:** 04.11.2019
**Accepted:** 30.01.2020

The tobacco use attitudes and behaviors of doctors and medical students are well documented by ample research worldwide.[5–10] For example, a study of 2008-2012 from Brazil found the level of agreement of the students with the WHO recommendations as high. The prevalence of cigarette smoking was 5.2%, but 43.8% of the students reported experimenting water-pipe,[11] whereas a study among medical students of Saudi Arabia reported a 13% smoking frequency among male students.[12] According to an international review of 2007 on tobacco smoking among medical students, the prevalence ranged from 3% in the United States to 58% in Japan.[13] In general, the attitudes and behaviors of medical students in this respect closely reflect the community they are a part of, just as the medical doctors and other health professionals.

Turkey is one of the countries with a considerable number of studies on the tobacco issue conducted during recent decades. On the average, the frequencies of smoking among medical students were found in the range of 30-40% in Turkey during late 1990s and early 2000s.[14–16]

Smoking frequency is consistently higher for male medical students than females, and the smoking rates increase with the advancing years of medical education[14–17] in Turkey. In one study, smoking frequency was 34.5% in the first year rising up to 44.2% in the sixth year of medical school.[17]

Comprehensive tobacco control education in the medical curriculum is crucial for preventing the initiation of the smoking habit or lowering the prevalence of smoking among medical students and assisting the students to identify themselves as nonsmoking role models for the community.[5,13]

This study is part of an intervention study designed to follow-up the students in a medical school of the Turkish Republic of Northern Cyprus (TRNC). The aim is to monitor the students as to the changes in trends of smoking attitudes and behaviors in relation to the impact of educational interventions.

**Main Points**

- The smoking status was found higher in this study than most medical student studies, probably related to the high smoking atmosphere in Northern Cyprus; smoking frequency is significantly higher among male students with 42.1% for males and 26.1% for females.
- Exposure to various forms of passive tobacco smoke was stated by %80.7 of the participants in our study, particularly in cafes and restaurants but in the university campus and facilities as well.
- More students have started smoking after registering to the medical school (50.8%) and smoking status increased with advancing years of medical education, revealing the necessity of early and more comprehensive tobacco education throughout the medical curriculum.
- In addition to increasing education on tobacco, it is crucial to fully implement all tobacco control measures in medical school including banning sales of tobacco products in the university campuses.
- All campuses should be smoke-free and the rules and regulations regarding tobacco use should be strictly supported and enforced.

### Objectives

The objective of this study is to determine the tobacco use prevalence and attitudes regarding tobacco use among medical students in a university of Northern Cyprus for the year 2018.

The broader and long-term objectives of this study include monitoring tobacco use prevalence among medical students of the Near East University between 2017 and 2021 and to follow-up the changes in smoking behaviors of the students after integrating comprehensive tobacco education into the NEU medical curriculum.

## MATERIAL and METHODS

The university where the study was conducted is located in Nicosia, Northern Cyprus, and accepts citizens of countries from various regions of the world. The current study was designed as a cross-sectional survey with the aim of accessing all the 1,461 medical students registered at the Turkish and English programs of the Medical School in December 2018.

The data were collected by a questionnaire of 12 questions developed by the researchers and applied under direct observation. The questionnaire included four questions on sociodemographic features and eight questions on smoking behaviors and attitudes, which were developed in compliance with WHO adult questionnaires for Global Adult Tobacco Survey.[18–20] The questions were revised, so that the best input from the participants would be attained. Besides, some adaptations were performed relevant to the local conditions, i.e., the grouping of number of cigarettes per day. The questionnaire has been attached as Appendix I.

### Statistical Analysis

The data collected were analyzed by Statistical Package for the Social Sciences (SPSS) version 18.0 (IBM SPSS Corp.; Armonk, NY, USA) program. Chi-square and Fisher's exact tests were used for determining the differences of the groups, and $P < .01$ was evaluated as significant.

### Ethics Approval and Consent to Participate

An informed consent of the participants was requested, and anonymity of the responses was guaranteed. Permission from the dean of the Faculty of Medicine and the approval of the Ethics Committee of the university were provided (Report No. YDU/2017/52-482 for the English medical program students and YDU/2018/54-516 for the Turkish medical program students, dated November 22, 2018).

### Terms and Definitions

The following terms and definitions based on WHO guidelines were used in accordance with the context of the study[20–22]:

Current smoker: Percentage of adults who currently smoke tobacco.

Daily smoker: Percentage of adults who currently smoke tobacco daily.

Occasional smoker: Percentage of adults who currently smoke tobacco less than daily.

**Table I.** Some Sociodemographic Characteristics and Smoking Features of the NEU Medical Students (Nicosia, December 2018) (N = 1,304)

| Sociodemographic Characteristics | n | % |
|---|---|---|
| **Age Group (years) (n = 1,292)** | | |
| ≤17 | 24 | 1.9 |
| 18–24 | 1,180 | 91.3 |
| ≥25 | 88 | 6.8 |
| Mean ± SD = 21.3 ± 2.3; minimum = 16; maximum = 39 | | |
| **Sex (n = 1,304)** | | |
| Male | 623 | 47.8 |
| Female | 681 | 52.2 |
| **Lifetime Smoking (n = 1,304)** | | |
| Never | 541 | 41.5 |
| Only tried | 214 | 16.4 |
| Former smoker | 109 | 8.4 |
| At least one cigarette daily | 337 | 25.8 |
| Less than one cigarette daily | 103 | 7.9 |
| **Use of Noncigarette Tobacco Products (n = 1,060)** | | |
| Never | 776 | 73.2 |
| Water pipe (Narghile) | 249 | 23.5 |
| Electronic cigarettes | 15 | 1.4 |
| Cigar | 11 | 1.0 |
| Pipe | 9 | 0.9 |
| Male students (n = 503) | 194 | 38.6 |
| Total | 284 | 26.8 |
| **Current Cigarette Smoking Status (n = 1,304)** | | |
| Nonsmoker | 865 | 66.3 |
| Smoker | 439 | 33.7 |
| **Number of Daily Cigarettes Consumed by Smoker Students (n = 435)** | | |
| Occasional (less than 1 daily) | 70 | 16.1 |
| 1-5 | 88 | 20.2 |
| 6-10 | 95 | 21.9 |
| 11-20 | 128 | 29.4 |
| Over 20 | 54 | 12.4 |
| **Duration of Smoking (n = 578)*** | | |
| One year or less | 136 | 23.5 |
| 2-5 years | 317 | 54.8 |
| 6-10 years | 109 | 18.9 |
| More than 10 years | 16 | 2.8 |
| **Initiation of Smoking in Relation to Starting Medical School (n = 478)** | | |
| Before | 235 | 49.2 |
| After | 243 | 50.8 |

*Includes exsmokers as well.

Former smoker: Percentage of adults who are ever daily and occasionally tobacco smokers and currently do not smoke tobacco.

Cigarette smoker: Percentage of adults who currently smoke cigarettes.

Current tobacco user: Percentage of adults who currently use tobacco (current daily and less than daily tobacco smokers and/or smokeless tobacco users).

## RESULTS

The total number of students studying at the Turkish and English programs of the Medical School was 1,461 in December 2018. The number of students who responded to the questionnaire was 1,304, comprising a response rate of 89.3%. The majority of the Turkish medical program students (97.5%) are citizens of Turkey and TRNC. Most of the English medical program students (75.6%) are from five countries, Turkey, Nigeria, Syria, Jordan, and TRNC, in descending order.

Table I shows some sociodemographic characteristics, and the cigarette and noncigarette tobacco product use features of the participants.

Of the participants, 57.9% are lifetime nonsmokers and 33.7% are current smokers, while 76.4% of the smokers are daily smokers and 12.4% smoke more than 20 cigarettes per day. Of the smokers and former smokers together, 54.8% reported a smoking duration of 2-5 years. Notably, 50.8% of the smokers stated they started smoking after admittance to medical school.

Consumers of noncigarette tobacco products comprise 26.8% of the total participants. 17.7% of the nonsmokers and 44.2% of the smokers consume noncigarette tobacco products. Cigarette smokers consume noncigarette tobacco products at significantly higher levels than nonsmokers ($\chi^2 = 86.0$, $P < .001$). 38.6% of the male students and 16.2% of the females consume noncigarette tobacco products; the difference between the genders being significant as well.

**Table 2.** Smoking Status of NEU Medical Students in Relation to Some Sociodemographic Features and to Their Countries of Origin Classified as WB Country Groups by Region and by Income Groups* (Nicosia, December 2018) (N = 1,304)

| | Smoking Status | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Nonsmoker | | Smoker | | Total | | | |
| **Sociodemographic Feature** | **n** | **%** | **n** | **%** | **n** | **%** | $\chi^2$ | ***P*** |
| Sex (n = 1,304) | | | | | | | 36.9 | <.001 |
| Male | 361 | 57.9 | 262 | 42.1 | 623 | 100 | | |
| Female | 503 | 73.9 | 178 | 26.1 | 681 | 100 | | |
| **Age Group (n = 1,292)** | | | | | | | 1.1 | .292 |
| <25 | 805 | 66.9 | 399 | 33.1 | 1,204 | 100 | | |
| ≥25 | 54 | 61.4 | 34 | 38.6 | 88 | 100 | | |
| **Grade of Medical School (n = 1,304)** | | | | | | | | |
| First grade | 182 | 71.1 | 74 | 28.9 | 256 | 100 | | |
| Second grade | 176 | 68.0 | 83 | 32.0 | 259 | 100 | | |
| Third grade | 149 | 62.6 | 89 | 37.4 | 238 | 100 | | |
| Fourth grade | 206 | 65.0 | 111 | 35.0 | 317 | 100 | | |
| Fifth grade | 91 | 64.5 | 50 | 35.5 | 141 | 100 | | |
| Sixth grade | 60 | 64.5 | 33 | 35.5 | 93 | 100 | | |
| **Country Group (Region) (n = 1,290)** | | | | | | | | |
| Middle East and North Africa | 124 | 66.0 | 64 | 34.0 | 188 | 100 | | |
| Europe and Central Asia | 642 | 64.3 | 357 | 35.7 | 999 | 100 | | |
| Latin America and Caribbeans | – | – | 1 | 100 | 1 | 100 | | |
| Sub-Saharan Africa | 78 | 94.0 | 5 | 6.0 | 83 | 100 | | |
| South Asia | 13 | 76.5 | 4 | 23.5 | 17 | 100 | | |
| North America | 1 | 50.0 | 1 | 50.0 | 2 | 100 | | |
| **WB Income Group (n = 1,282)** | | | | | | | 31.7 | <.001 |
| High income | 135 | 70.7 | 56 | 29.3 | 191 | 100 | | |
| Higher middle income | 564 | 62.6 | 337 | 37.4 | 901 | 100 | | |
| Lower middle income | 99 | 87.6 | 14 | 12.4 | 113 | 100 | | |
| Low income | 56 | 72.7 | 21 | 27.3 | 77 | 100 | | |

*World Bank Country and Lending Groups. 2019. https://datahelpdesk.worldbank.org/knowledgebase/articles/906519.

On the other hand, 80.7% of the students indicated being exposed to some form of secondhand smoke. Notably, 39.5% of the passive smokers were exposed at cafes and restaurants. Other locations of exposure included homes, polluted either by smoker household members (22.9%) or by visitors (19.3%) and indoor places other than home (34.7%) (not shown in tables).

Table 2 shows the smoking status of the participants in relation to some sociodemographic features and their countries of origin classified as World Bank (WB) country groups by region and by income.

Male students smoke more than females at significantly higher rates ($\chi^2 = 36.9$, $P < .001$): 42.1% of the male students and 26.1% of the female students smoke. Third-grade students smoke most with 37.4%, followed by fifth- and sixth-grade students with 35.5% each.

Sub-Saharan country citizens smoke least by 6.0%, while European and Central Asian region members smoke most with 35.7%. Smoking is highest among citizens of higher-middle income countries with 37.4%. Smoking frequency among lower-middle income country citizens (12.4%) is significantly lower than citizens of high income, higher-middle income, and low-income countries ($P < .001$) (Table 2).

The comparison of the attitudes of smoker and nonsmoker medical students regarding their attitudes on support of indoor smoking bans and their opinions on doctors being nonsmoking role models is presented in Table 3.

91.9% of the nonsmokers as compared to 73.9% of smokers are in support of smoking bans indoors. Nonsmokers support smoking bans at significantly higher levels than smokers ($\chi^2 = 76.9$, $P < .001$). Besides, nonsmokers adopt the role model function of doctors at significantly higher levels than smokers at 87.7% for nonsmokers and 54.3% for smokers.

Smoking status of all students according to their nationalities is indicated in Table 4, country by country.

Overall, citizens of Turkey, comprising 63% of the participants, smoke at a higher frequency by 37.5% than the total average of 33.7%.

The analyses of medical students of various country income groups in regard to the use of noncigarette tobacco products revealed that low-income countries of sub-Saharan Africa consume these products at the lowest level with 7.2% (not shown tables). The consumption of these products in all other regions is above 25%. Surprisingly, consumption is highest among students of low-economy countries with 31.3%, while 23.1% for high-income countries and 28.9% for upper middle income countries. The students from lower middle-income countries have the lowest frequency in this respect with 12.5%, which is significantly lower than other country groups.

**Table 3.** The Comparison of the Attitudes of Smoking and Nonsmoking NEU Medical Students Regarding Support of Indoor Smoking Bans and Doctors Being Nonsmoking Role Models (Nicosia, December 2018) (N = 1,304)

| | Attitudes Regarding Indoor Smoking Bans (n = 1,290) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Supporting | | Not Supporting | | Total | | | |
| | n | % | n | % | n | % | $\chi^2$ | P |
| **Smoking Status** | | | | | | | 76.9 | <.001 |
| Nonsmoker | 784 | 91.9 | 69 | 8.1 | 853 | 100 | | |
| Smoker | 323 | 73.9 | 114 | 26.1 | 437 | 100 | | |
| Total | 1,107 | 85.8 | 183 | 14.2 | 129 | 100 | | |
| | Attitude on doctors being nonsmoking role models (n = 1,291) | | | | | | | |
| | | | | | | | 179.5 | <.001 |
| Nonsmoker | 751 | 87.7 | 105 | 12.3 | 856 | 100 | | |
| Smoker | 236 | 54.3 | 199 | 45.7 | 435 | 100 | | |
| Total | 987 | 76.5 | 304 | 23.5 | 129 | 100 | | |

**Table 4.** Smoking Status According to Country of Origin of NEU Medical Students (Nicosia, December 2018) (N = 1,304)

| Smoking Status (n = 1,293) | Nonsmoker | | Smoker | | Total | |
|---|---|---|---|---|---|---|
| Country | n | %* | n | %* | n | % |
| Turkey | 509 | 62.5 | 305 | 37.5 | 814 | 63.0 |
| TRNC | 125 | 72.5 | 48 | 27.7 | 173 | 13.4 |
| Nigeria | 62 | 96.9 | 2 | 3.1 | 64 | 4.9 |
| Syria | 44 | 68.8 | 20 | 31.3 | 64 | 4.9 |
| Jordan | 31 | 56.4 | 24 | 43.6 | 55 | 4.3 |
| Egypt | 15 | 75.0 | 5 | 25 | 20 | 1.5 |
| Bangladesh | 9 | 75.0 | 3 | 25 | 12 | 0.9 |
| Iraq | 6 | 54.5 | 5 | 45.5 | 11 | 0.9 |
| Libya | 7 | 77.8 | 2 | 22.2 | 9 | 0.7 |
| Palestine | 4 | 50 | 4 | 50 | 8 | 0.6 |
| Oman | 6 | 85.7 | 1 | 14.3 | 7 | 0.5 |
| Sudan | 5 | 71.4 | 2 | 28.6 | 7 | 0.5 |
| Pakistan | 4 | 66.7 | 2 | 33.3 | 6 | 0.5 |
| Lebanon | 4 | 80 | 1 | 20 | 5 | 0.4 |
| Yemen | 4 | 100 | – | – | 4 | 0.3 |
| Tanzania | 3 | 75 | 1 | 25 | 4 | 0.3 |
| Germany | 2 | 50 | 4 | 50 | 4 | 0.3 |
| Kenya | 3 | 100 | – | – | 3 | 0.2 |
| Somalia | 3 | 100 | – | – | 3 | 0.2 |
| Saudi Arabia | 1 | 33.3 | 2 | 66.7 | 3 | 0.2 |
| USA | 1 | 50 | 1 | 50 | 2 | 0.2 |
| Iran | 1 | 50 | 1 | 50 | 2 | 0.2 |
| Sweden | 1 | 50 | 1 | 50 | 2 | 0.2 |
| Kosovo | 1 | 50 | 1 | 50 | 2 | 0.2 |
| Turkmenistan | 2 | 100 | – | – | 2 | 0.2 |
| Uganda | 2 | 100 | – | – | 2 | 0.2 |
| Other† | 4 | 80 | 1 | 20 | 5 | 0.4 |

*Row percentages.
†Algeria, Armenia, Bulgaria, Romania, and Venezuela.

Similar to the cigarette smoking data, 74.8% of noncigarette tobacco users compared to 89.2 of nonconsumers support the ban in indoor public places, the difference being significant ($\chi^2 = 34.0$, $P < .001$). Similar results are valid for the opinions of the participants about the role model function of the physician.

## DISCUSSION

The status of tobacco use among medical students and their attitudes on the tobacco issue were studied in this cross-sectional study. The smoking frequency was 33.7% in general, 37.5% among Turkish citizens, and 27.7% among Northern Cyprus citizens. The smoking prevalences are higher in our study than most medical student studies in Turkey.[16,17,23] However, the smoking frequency was much higher (39%) in a study among students of a private medical school in Turkey.[24]

Recent studies among university students and particularly medical students in the Middle East and African countries have found lower prevalences compared to the results of our study in general. However, two studies in Nigeria found higher frequencies compared to our Nigerian group; lifetime tobacco use was 9.6%[25] and 10.5%[26] in these surveys. The Nigerian medical students in our study showed a lower frequency as 3.1%.

A 2007 study in Syria on medical students found the cigarette smoking prevalence to be 10.9%,[27] much lower than our general result of 33.7% and the Syrian students in particular, who had a smoker rate of 31.3% in our study.

A study in Jordan among medical students found the smoking prevalence as 26% for male and 7% for female students.[28] On the contrary, the findings of the Jordanian students in our study showed a much higher frequency as 43.6%. Syrian and Jordanian results in the current study may be a reflection of the social status of our students, as well as the prosmoking atmosphere of Cyprus grounds.

According to an international review, the prevalence of smoking among medical students ranged from 3% in the United States to 58% in Japan, with 29.5% for Italy, 29% for Saudi Arabia, and 10-10.5% for Nigeria.[13] Medical students are subject to similar impacts of their social environment and present behaviors as other members of their society, in spite of the fact that they are studying medicine. Thus, the higher smoking rates displayed by the present study may be attributed to the high smoking atmosphere of Northern Cyprus.

Smoking frequency was significantly higher among male students than female students in our study as expected. Furthermore, smokers were higher for both sexes among Turkish medical program students with 43.9% for men and 29.1% for women. These results are similar to but higher than the results of some medical schools of Turkey, which show a range of 8.2-17.3% for female students and 25.1-35.0% for males.[23,24]

The finding that male students smoke more than female students is consistent with other studies' findings.[29] The study among medical students in Jordan revealed the prevalences as 26% for male and 7% among female students.[28]

Contrary to other research, a study from China found that medical students have higher rates of smoking than other students, but most of the smokers were occasional smokers.[30] On the other hand, a study in Greece found smoking rates of medical students as 35.3%, while 50.2% for other students.[31]

Smoking frequency is 28.9% among first year students and 35.5% for sixth-grade students in the current study, while it is highest in the third year with 37.4%. Smoking prevalences range from 8 to 18% among first year medical students and 21-39% among 6th year students according to various studies conducted in Turkey.[16,32]

Our results show higher frequencies for almost all parameters. In a study comparing the students of seven medical schools and the same grades of other schools of science in Turkey, a significant difference between the medical and other students was found in the first grades, with lower frequencies for medical students. However, the difference was not statistically significant for last grade students, although smoking rates were lower for medical students.[32]

Of the participants in our study, 26.8% have stated using noncigarette tobacco products, of which water pipe (narghile) is the leading product with 23.5%. According to a 2011 study among medical students in Turkey, 17% of the first-year students and 14% of the sixth-year students were using water pipe.[33]

Exposure to various forms of passive tobacco smoke was stated by 80.7% of the participants in our study. Although the law regulating tobacco use has been enforced since 2008 in Northern Cyprus, these results display the noncompliance regarding indoor bans. On the other hand, the students are highly in favor of the indoor bans regardless of their smoking status. Still the rate of supporters is significantly higher among nonsmokers.

The majority of the students are in the opinion that doctors should be nonsmoking role models for the community. Nonsmokers share this view at significantly higher levels than smokers (87.7% of nonsmokers vs 54.3% of smokers). The 1994 study from Turkey had displayed a much lower frequency for this parameter; 44% of the smokers and 70% of the never smokers were in support of the role model function of doctors at that time.[32] The difference between the two studies may be evaluated as an indicator of progress of the community and particularly of the medical students on the tobacco control issue during the years in between. Interestingly, 26.1% of smoker students and 8.1% of nonsmokers do not support legal measures for tobacco control in the current study, the difference of the groups being highly significant. In comparison, these rates were 24 and 6%, respectively, in the seven-university study of 1994.[32] These results reveal the role of smoking on attitudes of doctors and the importance of nonsmoking behaviors.

More students have started smoking after registering to the medical school (50.8%) than those who initiated smoking before their medical education period (49.2%). These findings indicate the importance of early tobacco control education in the medical school for prevention of picking up the habit. The findings of the present study are similar to the results of the first baseline study in our university[34] and indicative of inadequate tobacco control interventions, including current content of the medical education.

The role of medical education on the smoking trends of doctors has been a subject of research in recent years. According to global surveys, the tobacco education in medical curricula varies widely among countries and medical schools.[13,35]

An international study among medical students of 42 countries in Europe, Asia, and Africa found significant deficiencies in medical education on tobacco and recommended urgent changes in the medical curricula.[35] The content, program, and methods of the tobacco education curriculum continue to be topics of discussion in the context of medical education.

**The Restrictions of the Study**

The study was conducted in one medical school of Northern Cyprus. Thus, the results of our study can neither be representative of all the medical schools of Cyprus nor be associated with students studying in other schools of universities.

The questions of the study questionnaire were limited as for a descriptive study to obtain baseline data with the intention of accessing more students and more responses. Therefore, neither in depth and detailed analyses of the data could be performed in this study, nor we could derive a wider variety of results from the data obtained.

## CONCLUSION

This study about the smoking status of medical students revealed high smoking frequencies for both male and female students, compared to medical students of other countries, probably related to the high smoking atmosphere of Northern Cyprus. More than half of the smokers started smoking after entering the medical school, and smoking status increased with advancing years of medical education, all showing the necessity of more tobacco control measures in the university and more tobacco education throughout the medical curriculum.

In addition to increasing education on tobacco, it is crucial to fully implement all tobacco control measures in medical schools. University campuses should be smoke-free, and the rules and regulations regarding tobacco use should be strictly supported and enforced. Related educational and other activities about tobacco control should be available for all medical students throughout all the years and content of medical education.

Medical education should be restructured to include the skills for tobacco cessation and other relevant subjects. Tobacco control and cessation techniques education in the curriculum of medical education are one of the intervention methods, which is in the agenda of our medical faculty in the near future.

**Ethics Committee Approval:** Ethical committee approval was received from the Near East University (Report No. YDU/2017/52-482 for the English medical program students and YDU/2018/54-516 for the Turkish medical program students, dated November 22, 2018).

**Informed Consent:** Written informed consent was obtained from all participants who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - O.A., S.V., G.A., S.C.; Design - O.A., S.V., G.A., S.C.; Supervision - O.A., S.V., G.A., S.C.; Resources - O.A., S.V., G.A., S.C.; Materials - O.A., S.V., G.A., S.C.; Data Collection and/or Processing - O.A., S.V., G.A., S.C.; Analysis and/or Interpretation - O.A., S.V., G.A., S.C.; Literature Search - O.A., S.V., G.A., S.C.; Writing Manuscript - O.A.; Critical Review - S.V., G.A., S.C.

**Acknowledgments:** We thank the following medical students for their assistance in data collection and analyses phases of the study: Ersen Ceylan, Ali Göçer, Cemre Kahya, Ahmet Karaosman, Kemal Kararlı, Ali Kardüz, Zınar Ozmen, Ezgi Pala, and Y. Roza Şahin.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

**Availability of Data and Material:** The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## REFERENCES

1. Tobacco Breaks Hearts. World Health Organization, 2018. Available at https://apps.who.int/iris/bitstream/handle/10665/272675/WHO-NMH-PND-18.4-eng.pdf?ua=1&ua=1.
2. Tobacco. Health effects of tobacco secondhand smoke. World Medical Association, 2019. Available at https://www.wma.net/what-we-do/education/tobacco/.
3. Canadian Medical Association. The role of physicians in prevention and health promotion. *CMAJ*. 1995;153:208.
4. Frank E, Segura C, Shen H, et al. Predictors of Canadian physicians' prevention counseling practices. *Can J Public Health*. 2010;101:390-395. [CrossRef]
5. Pipe A, Sorensen M, Reid R. Physician smoking status, attitudes toward smoking, and cessation advice to patients: An international survey. *Patient Educ Couns*. 2009;74:118-123. [CrossRef]
6. Melani A, Verponziani W, Boccoli E, et al. Tobacco smoking habits, attitudes and beliefs among nurse and medical students in Tuscany. *Eur J Epidemiol*. 2000;16(7):607-611. [CrossRef]
7. Omair A, Kazmi T, Alam SE. Smoking prevalence and awareness about tobacco related diseases among medical students of Ziauddin Medical University. *J Pak Med Assoc*. 2002;52(9):389-392.
8. Jp M, Akshava KM. Association between the duration of stay in medical colleges and the smoking behaviour among medical students. *Indian J Community Med*. 2017;42(4):193-196.
9. Vakeflliu Y, Argjiri D, Peposhi I, et al. Tobacco smoking habits, beliefs, and attitudes among medical students in Tirana, Albania. *Prev Med*. 2002;34(3):370-373. [CrossRef]
10. La Torre G, Kirch W, Bes-Rastrollo M, et al. Tobacco use among medical students in Europe: Results of a multicentre study using the global health professions student survey. *Public Health*. 2012;126(2):159-164. [CrossRef]
11. Martins SR, Paceli RB, Bussacos MA, et al. Effective tobacco control measures: Agreement among medical students. *J Bras Pneumol*. 2017;43(3):202-207. [CrossRef]
12. Al-Turki YA. Smoking habits among medical students in Central Saudi Arabia. *Saudi Med J*. 2006;27(5):700-703.
13. Smith DR, Leggat PA. An international review of tobacco smoking among medical students. *J Postgrad Med*. 2007;53(1):55. [CrossRef]
14. Akvardar Y, Demiral Y, Ergor G, et al. Substance use among medical students and physicians in a medical school in Turkey. *Soc Psychiatry Psychiatr Epidemiol*. 2004;39(6):502-506. [CrossRef]
15. Ogus C, Ozdemir T, Kara A, et al. Smoking habits of grade I and VI medical students at Akdeniz university medical faculty. *Turkiye Klinikleri Arch Lung*. 2004;5(3):139-142 (in Turkish).
16. Vatan I, Ocakoglu H, Irgil E. Determining smoking prevalence in Uludag university faculty of medicine. *TAF Prev Med Bull*. 2009;8(1):43-48.
17. Kocabas A. Smoking prevalence and features of tobacco use in Turkey. *Solunum Hastaliklari*. 1994;5:133-147 (in Turkish).
18. WHO Tobacco Questionnaire. World Health Organisation. Available at https://www.who.int/tobacco/surveillance/6_GATS_CoreQuestionnairewithOptionalQuestions_v2.1_FINAL_13June2014.pdf?ua=1.
19. Global Adult Tobacco Survey Collaborative Groups. *Tobacco Questions for Survey: A Subset of Key Questions from the Global Adult Tobacco Survey (GATS)*. 2nd ed. Atlanta, GA: Centers for Disease Control and Prevention, 2011.
20. WHO Report on the Global Tobacco Epidemic. *Implementing Smoke-Free Environments*. Geneva: World Health Organization, 2009.
21. Global Adult Tobacco Survey (GATS). Fact sheet template version 2.1, 2012. Available at https://www.paho.org/hq/dmdocuments/2017/18-GATS-AnalysisPackage-12June2014.pdf.
22. Global Adult Tobacco Survey (GATS) Indicator Guidelines. Definition and syntax final edition, 2009. Available at https://www.who.int/tobacco/surveillance/en_tfi_gats_indicator_guidelines.pdf.
23. Oktay G, Hodoglugil NS, Agca E, et al. Smoking behaviour and attitudes of 6th year medical students of Hacettepe University. In *International Public Health Congress Health 21 in Action*. Istanbul, Turkey, October 2000: 149.
24. Gunes G, Pehlivan E, Egri M, et al. The frequency of smoking among physicians, nurses and medical students of Turgut Ozal

health center [turgut ozal tip merkezi hekim, hemsire ve tip ogrencilerinde sigara icme sikligi]. *J Turgut Ozal Med Cent*. 1997;4(4):407-411 (in Turkish).
25. Makanjuola AB, Daramola TO, Obembe AO. Psychoactive substance use among medical students in a Nigerian university. *World Psychiatry*. 2007;6(2):112.
26. Dania MG, Ozoh OB, Bandele EO. Smoking habits, awareness of risks, and attitude towards tobacco control policies among medical students in Lagos, Nigeria. *Ann Afr Med*. 2015;14(1):1. [CrossRef]
27. Almerie MQ, Matar HE, Salam M, et al. Cigarettes and water pipe smoking among medical students in Syria: A cross-sectional study. *IJTLD*. 2008;12(9):1085-1091.
28. Merrill RM, Madanat HN, Cox E, et al. Perceived effectiveness of counseling patients about smoking among medical students in Amman. *East Mediterr Health J*. 2009;15(5):1180-1191.
29. Hitchman SC, Fong GT. Gender empowerment and female-to-male smoking prevalence ratios. *Bull World Health Organ*. 2011;89(3):195-202. [CrossRef]
30. Zhu T, Feng B, Wong S, et al. A comparison of smoking behaviours among medical and other college students in China. *Health Promot Int*. 2004;19(2):189-196. [CrossRef]
31. Alexopoulos EC, Jelastopulu E, Aronis K, et al. Cigarette smoking among university students in Greece: A comparison between medical and other students. *Environ Health Prev Med*. 2010;15(2):115-120. [CrossRef]
32. Kocabas A, Burgut R, Bozdemir N, et al. Smoking patterns at different medical schools in Turkey. *Tob Control*. 1994;3:228-235. [CrossRef]
33. Emlek G, Keklikoglu I, Demir N, et al. The opinions on smoking and tobacco use experiences of the first and sixth grade students of Hacettepe University Medical School (Hacettepe Universitesi Tip Fakültesi birinci ve altinci sinif ogrencilerinin tutun urunlerini kullanma deneyimleri ve sigara icmeye yonelik bazi düsünceleri). In *Proceedings of the 5th Tobacco and Health Congress*, 8-11 May, Kocaeli [Besinci Sigara ve Saglik Ulusal Kongre Kitabi, 8-11 Mayıs, Kocaeli], 2011: 114 (in Turkish).
34. Asut O, Cali S, Ozcan A, et al. Tobacco use status among the students of the medical faculty Turkish medical program at a university in Nicosia. *STED*. 2019;28(1):17-27 (in Turkish).
35. Robyn L, Richmond RL, Debono DS, et al. Worldwide survey of education on tobacco in medical schools. *Tob Control*. 1998;7(3):247-252. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.2293

# Proapoptotic and Anticancer Potentials of *Thymus capitatus* Essential Oil on Colon Cancer Stem Cells

**Dudu Özkum Yavuz[1], Eda Becer[2,3], Remziye Kendirci[4], Feyzan Özdal Kurt[5], H. Seda Vatansever[3,4]**

[1]Department of Pharmaceutical Botany, Near East University, Faculty of Pharmacy, Nicosia, Cyprus
[2]Department of Biochemistry, Near East University, Faculty of Pharmacy, Nicosia, Cyprus
[3]Near East University, DESAM Institute, Nicosia, Cyprus
[4]Department of Histology and Embryology, Manisa Celal Bayar University, Faculty of Medicine, Manisa, Turkey
[5]Department of Biology, Manisa Celal Bayar University, Faculty of Sciences and Letter, Manisa, Turkey

**ORCID iDs of the authors: D.Ö.Y. 0000-0001-8743-8862; E.B. 0000-0002-2378-128X; R.K. 0000-0002-6839-8823; F.Ö.K. 0000-0001-6070-3742; H.S.V. 0000-0002-7415-9618.**

**Cite this article as: Yavuz DÖ, Becer E, Kendirci R, Kurt FÖ, Vatansever HS. Proapoptotic and Anticancer Potentials of *Thymus Capitatus* Essential Oil On Colon Cancer Stem Cells. *Cyprus J Med Sci.* 2021; 6(4): 319-324.**

**BACKGROUND/AIMS**
The aim of this study was to investigate the proapoptotic activity of *Thymus capitatus* essential oil in either colon cancer stem (CDI33+ Colo-320) or nonstem (CDI33– Colo-320) cells.

**MATERIAL and METHODS**
*T. capitatus* essential oil was obtained by water distillation and analyzed by GC–MS. Cancer stem cells (CDI33+ Colo-320) were obtained from the Colo-320 cells by the MiniMACS system. Proapoptotic activity of *T. capitatus* essential oil was investigated by immunocytochemistry using antibodies directed against caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

**RESULTS**
Caspase-3 immunoreactivity was significantly increased in 0.5% dilution *T. capitatus* essential oil-treated Colo-320 cells for 48 hours. Moreover, the number of TUNEL positive cells was significantly higher in Colo-320 cells when compared with CDI33+ and CDI33– Colo-320 cells.

**CONCLUSION**
We conclude that *T. capitatus* essential oil increases caspase-3 molecules, which play a crucial role in apoptosis. Interestingly, *T. capitatus* essential oil is found to be more effective in Colo-320 cells than CDI33+ Colo-320 and CDI33– Colo-320 cells in terms of apoptosis.

**Keywords:** *Thymus capitatus*, essential oil, apoptosis, colon cancer

## INTRODUCTION

Colorectal cancer is the fourth leading cause of cancer-associated mortality and composed of heterogeneous cell populations. Metastasis and disease relapse are the critical challenges in the management of colorectal cancer. The cancer stem cells are a group of tumor cells with self-renewal characteristics and multidirectional differentiation potential. Also, they are closely related to tumor metastasis, recurrence after primary treatment, and drug resistance in colorectal cancer.[1] Colorectal cancer stem cells have surface markers that are used for identification such as CDI33. CDI33, a transmembrane glycoprotein, containing colorectal cancer cells (CDI33+) is resistant to radio- and chemotherapy and associated with tumor size.[2]

Apoptosis is a cell suicide pathway for normal cell turnover, managing stress and maintaining tissue homeostasis. The intrinsic (mitochondrial) and extrinsic (death receptor) pathways are important apoptotic pathways. Caspase-3 cleavage is stimulated by both apoptotic pathways. The caspase-3 activation results in inducing DNA fragmentation, cytoskeletal and nuclear proteins degradation, formation of apoptotic bodies, and finally, uptake by phagocytic cells.

**Corresponding Author:** Dudu Özkum Yavuz
**E-mail:** dudu.ozkum@neu.edu.tr

**Received:** 10.07.2020
**Accepted:** 18.09.2020

Apoptosis is a safeguard mechanism against tumorigenesis.[3] However, cancer cells become resistant to apoptosis as a result of epigenetic variations and mutations in genes that control mitosis such as adenomatous polyposis coli (APC6) and P53 in colorectal cancer.[1] In particular, deregulations of apoptotic pathways are shown in colorectal cancer stem cells that are resistant to cancer therapies. In recent years, several researchers have focused on drug discovery and combination therapy that are specific for genetic mutations and selective induction of apoptosis in colorectal cancer. The crucial roles of several plant products such as oils, gums, alkaloids, flavonoids, biomolecules in inhibiting cancer cell activating proteins, enzymes, and signaling pathways with their less toxic effect in adjuvant cancer therapy have been shown in extensive research.[4] For example, essential oil from Libyan *Thymus capitatus* indicated cytotoxicity activities against human cell lines such as MRC-5, HCT 116, and HT-29.[5]

*T. capitatus* is a species of the genus of Lamiaceae, which contains over 300 species of hardy perennial herbaceous plants. It is a native species in the Mediterranean region.[6] Also, *T. capitatus* is economically the most important genera employed by the cosmetic and fragrance industries. In traditional medicine, thyme tea is consumed against gastro-intestinal disorders, and its essential oil is also used for expelling intestinal parasites.[7] Previous studies have shown that *T. capitatus* essential oil has antiseptic, antioxidant, and also antimicrobial properties.[5,8–11] *T. capitatus* has a potential thymol (62.3%) source, thymol chemotype according to the previous results.[12] *T. capitatus* essential oil has a variety of different biological activities. In particular, the anticancer effects of thymol are known, while the antioxidant, anti-inflammatory/immunomodulatory, and antigenotoxicity properties have also been shown.[13]

There are only a few studies associated with the cytotoxic activities of *T. capitatus* essential oil.[5,14] To the best of our knowledge, no work has been carried out on the effects of *T. capitatus* essential oil on colon cancer with in vitro and in vivo studies. The specific effects of *T. capitatus* essential oil with respect to proapoptotic signaling pathway molecules in both colon carcinoma cells and colon cancer stem cells remain undefined. The aims of this study were: (i) to compare the effects of different dilutions of *T. capitatus* essential oil with respect to their proapoptotic activities in Colo-320, CD133+, and CD133– Colo-320 cells and (ii) to determine the proapoptotic effects of *T. capitatus* essential oil via caspase-3 expression in Colo-320, CD133+, and CD133– Colo-320 cells.

**Main Points**

- In this study, our results showed that *Thymus capitatus* essential oil from Northern Cyprus stimulated apoptosis in primary human colon adenocarcinoma cell line (Colo-320).
- It was found that CD133+ Colo-320 cells (cancer stem cells) may be have resistance to *Thymus capitatus* essential oil. However, apoptosis was stimulated in Colo-320 cells which include both CD133+ Colo-320 and CD133- Colo-320 cells.
- Interestingly, apoptosis was stimulated in %0.5 dilution *Thymus capitatus* essential oil treated Colo-320 cells. %1 and %2 dilutions of essential oil were not showed effective proapoptotic properties.

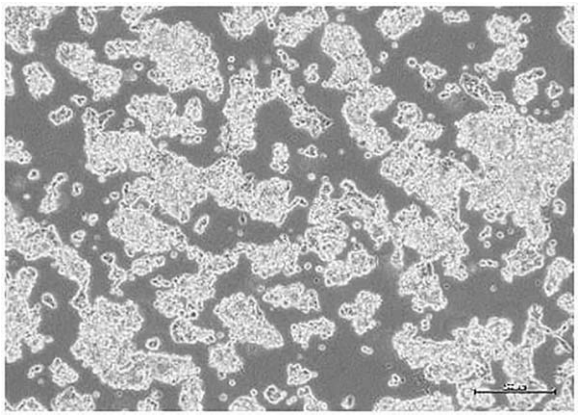

**Figure 1.** Colo-320 cells imaged under the inverted microscope. Scale bar = 200 μm.

## MATERIAL and METHODS

The plant materials, isolation of the essential oil, and GC/MS analysis in this study are in parallel with those used in previous studies.[12]

### Cell Line and Cell Culture

In this study, primary human colon adenocarcinoma cell line (Colo-320 (ATCC: CCL220)) was used, and cells were maintained in RPMI 1640 containing 10% fetal bovine serum (Capricorn Scientific, FBS-HI-11B), 1% L-glutamine (Capricorn Scientific, GLN-B), and 1% penicillin-streptomycin (Capricorn Scientific, PS-B) (Figure 1). Cells were cultured in a humidified atmosphere at 37°C and 5% $CO_2$ culture condition. Cells subcultured when they reached 70-80% confluency.

### Isolation of CD133+ Cells with Immunomagnetic System

Primary human colon adenocarcinoma (Colo-320) CD133+ cancer stem cells were separated from Colo-320 cells using a MiniMACS system (Miltenyi Biotec, Germany). Buffer (Miltenyi Biotec, Germany, 130-100-857) was used for the preparation of Colo-320 cells ($2 \times 10^8$ cells/mL) suspension. Then, FcR blocking reagent (Miltenyi Biotec, Germany, 130-100-857) was added. Cells were incubated in stirring on ice for 30 min after antibody-labeled CD133 microbeads (Miltenyi Biotec, Germany, 130-100-857) adding. Cells washed with buffer and centrifuged for 10 min. Resuspension of cells was performed using buffer after the removal of supernatant. Magnetic field was used for magnetic separation. Column was washed using buffer, and then the CD133 cells were collected in a tube. The column was took out from the magnetic field and washed with buffer to collect the CD133+ Colo-320 cells in another tube. The cells were centrifuged for 10 minutes, and then the buffer was removed. CD133+ and CD133– Colo-320 cells were transferred into a flask and cultured separately.

### Cultivation of Cells with *T. capitatus* Essential Oil

According to their types, cells were disunited into three groups. Colo-320, CD133+ Colo-320, and CD133– Colo-320 cells were our study groups. Also, three cell groups were divided into three subgroups and incubated for 48 hours. These were 0.5%

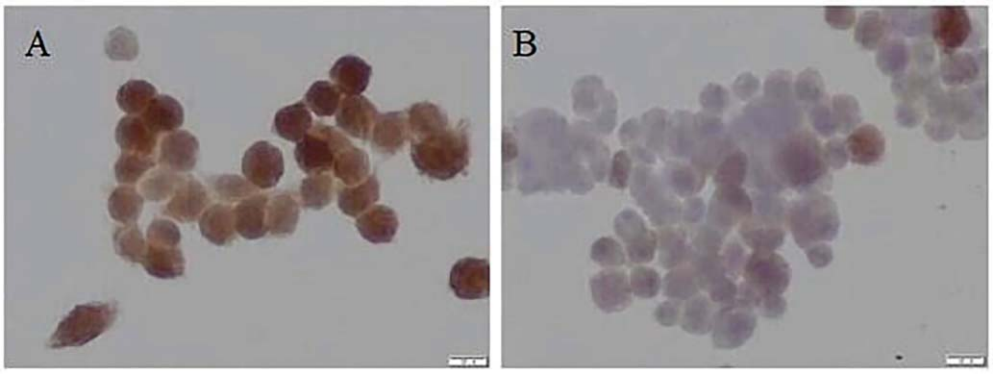


**Figure 2.** Colo-320 CDI33+ cells (A), and CDI33- cells (B) achieved from Colo-320 cell line by MiniMACS. Scale bars = 10 μm.

of *T. capitatus* essential oil treated cell group, 1% *T. capitatus* essential oil treated cell group, and 2% *T. capitatus* essential oil cell group.

### Immunocytochemistry

To evaluate cell responses to *T. capitatus* essential oil, caspase-3 (sc-7272, Santa Cruz Biotechnology, Inc., USA) distribution on the Colo-320, CDI33+ and CDI33– Colo-320 cells was analyzed using previously described indirect immunoperoxidase staining protocol.[15]

H-SCORE was used for graded semi-quantitatively graded of caspase-3 staining. In H-SCORE = Σл (i + 1) formula, i is the intensity of dyeing with a value of 1, 2, or 3 (mild, moderate, or strong, respectively). л is the percentage of cells stained (between 0 and 100%) with each intensity.

### TUNEL Assay

To detect the apoptotic DNA fragmentation, the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used. TUNEL assay was performed as described previously.[15]

### Statistical Analysis

The data were expressed as mean ± standard deviation (SD). The GraphPad Prism 7 software was used for analysis, and group differences were analyzed using the Kruskal–Wallis test. The differences in the mean values of continuous variables in the three genotype subgroups were confirmed by a post hoc Dunn test. A $P < .05$ was regarded as statistically significant.

## RESULTS

### The Immunohistochemical Stain Analysis of CDI33+ Colo-320 and CDI33– Colo-320 Cells

The CDI33+ cells (cancer stem cells) were achieved from the Colo-320 cells using the MiniMACS system. Immunocytochemical characterization of CDI33+, cancer stem cells, was performed using CDI33 antibody cell labeling. After immunostaining, the CDI33+ cells percentage was 88.8 (Figure 2A), and the CDI33+ cells intensity was higher than the CDI33– cells (Figure 2).

### Immunohistochemical Evaluation

Strong caspase-3 immunostaining was detected in 0.5% *T. capitatus* essential oil-treated Colo-320 cells (Figure 3A). Caspase-3 H-SCORE was significantly higher in 0.5% *T. capitatus* essential oil-treated Colo-320 cells than 2% *T. capitatus* essential oil-treated Colo-320 cells ($P = .0012$, Table 1). According to the H-SCORE analysis, caspase-3 immunoreactivity was significantly higher in 0.5% *T. capitatus* essential oil-treated Colo-320 cells (Figure 3A) than CDI33+ Colo-320 cells (Figure 3D) ($P = .017$, Table 1).

Additionally, the immunoreactivity of caspase-3 was weak in 0.5% *T. capitatus* essential oil-treated CDI33– Colo-320 cells (Figure 3G). The H-SCORE value of caspase-3 was significantly lower in 0.5% *T. capitatus* essential oil-treated CDI33– Colo-320 cells in comparison to 0.5% *T. capitatus* essential oil-treated Colo-320 cells ($P < .048$, Table 1).

The immunostaining intensity of caspase-3 was moderate in 0.5%, 1%, and 2% dilution of *T. capitatus* essential oil-treated CDI33+ Colo-320 and CDI33– Colo-320 cells (Figure 3D–F and 3G–I). The immunoreactivity for caspase-3 was similar in all *T. capitatus* essential oil-treated CDI33+ Colo-320 and CDI33– Colo-320 cells ($P < .05$, Table 1).

### TUNEL Assay

A TUNEL assay was used in Colo-320, CDI33+ Colo-320, and CDI33– Colo-320 cells. All cells were incubated with 0.5%, 1%, and 2% *T. capitatus* essential oil for 48 hours. In Colo-320 cells treated with 2% *T. capitatus* essential oil, the number of TUNEL positive cells was significantly lower than 0.5% *T. capitatus* essential oil-treated Colo-320 cells and 1% *T. capitatus* essential oil-treated Colo-320 cells, respectively ($P = .018$, Figure 4A and C, Table 2 and $P = .023$, Figure 4A and B, Table 2, respectively). Moreover, the number of TUNEL positive cells was highly significant in 0.5% *T. capitatus* essential oil-treated CDI33+ Colo-320 cells when compared with 2% *T. capitatus* essential oil-treated CDI33+ Colo-320 cells ($P < .023$, Figure 4D and F, Table 2).

## DISCUSSION

Globally, colorectal cancer is one of the common reasons of morbidity and mortality. Drug resistance, tumor metastasis, and recurrence after primary treatment are related to cancer stem cells in colorectal cancer. Radiotherapy and chemotherapy may relieve solid tumors, but they cannot kill cancer stem cells. In recent years, scientists have been focused on alternative therapies that target and effectively kill cancer stem cells.[2] Among the alternative approaches, varied plant products such

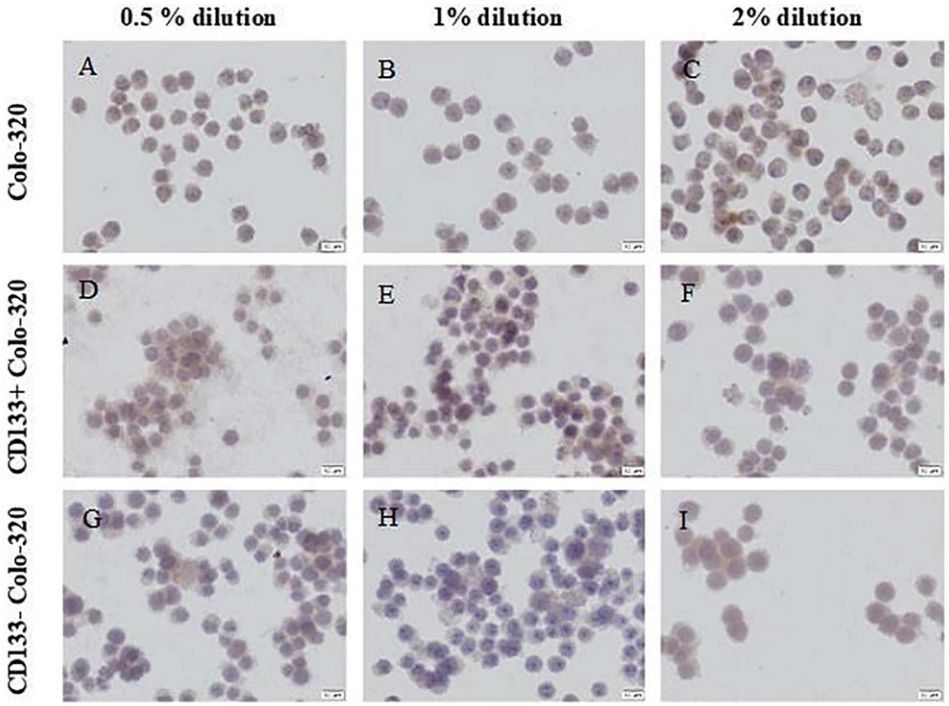


**Figure 3.** Immunoreactivity of caspase-3 in 0.5%, 1% and 2% *Thymus capitatus* essential oil treated Colo-320 (A, B, C), CDI33+Colo320 (D, E, F) and CDI33-Colo-320 (G, H, I) cells for 48 h. Scale bars = 10 μm.

**Table I.** The H-SCORE Values for Caspase-3 in Colo-320, CDI33+ Colo320, and CDI33– Colo-320 Cells Treated with *Thymus capitatus* Essential Oil at 0.5%, 1%, and 2% Dilution for 48 hours

| | Colo-320 Cells | CDI33+ Colo-320 Cells | CDI33– Colo-320 Cells |
|---|---|---|---|
| 0.5% dilution | 257.4 ± 5.49*,†,‡ | 150.8 ± 19.73 | 161.8 ± 11.84 |
| 1% dilution | 233.4 ± 5.655 | 124.6 ± 5.678 | 138.2 ± 16.68 |
| 2% dilution | 161.8 ± 34.45 | 129.5 ± 17.93 | 149.2 ± 21.17 |

*The data were significant when compared with 2% *T. capitatus* essential oil-treated Colo-320 cells ($P = .0012$).
†The data were significant when compared with 0.5% *T. capitatus* essential oil-treated CDI33+ Colo-320 cells ($P = .017$).
‡The data were significant when compared with 0.5% *T. capitatus* essential oil-treated CDI33-Colo-320 cells ($P = .048$).

as essential oils have demonstrated anticancer properties. Also, essential oils have been shown to improve the life quality of the cancer patients by lowering the side effects.[16,17]

Thymol is a natural monoterpene phenol derivative of cymene and a major component of the *T. capitatus* essential oil from Northern Cyprus. From the different experimental model study reports, thymol has been reported to exert anticancer activities through different mechanisms including inducing apoptosis, depolarizing mitochondrial membrane potential, and activating the proapoptotic caspase proteins.[18–20] To date, only the cytotoxic and antimicrobial activities of the *T. capitatus* essential oil from Northern Cyprus have been reported, which is rich in thymol.[20,21] No study has investigated the effects of *T. capitatus* essential oil from Northern Cyprus with respect to its proapoptotic effects in Colo-320, CDI33+ Colo-320, and CDI33– Colo-320 cells. We showed that 0.5% of the *T. capitatus* essential oil is highly effective in activating the apoptosis in Colo-320 and CDI33+ Colo-320 cells.

Apoptotic signaling is important for maintaining balance between cell death and cell survival, and also the evasion of apoptosis is a prominent hallmark of cancer. Apoptosis is controlled by extrinsic and intrinsic mitochondrial pathways. Both pathways converge at caspase-3, an executioner caspase, which can elicit apoptosis, while caspase-3 is a crucial marker of apoptosis. Significant attention has been paid to developing varied experimental anticancer drugs that can target and modulate apoptotic pathways in recent years.[3] Specifically, essential oils containing thymol have been reported to exert anticancer activities through different mechanisms such as inducing apoptosis and activating the proapoptotic proteins. Moreover, recent studies have reported that thymol stimulates apoptotic cell death via extrinsic and intrinsic mitochondrial pathways in different cancer cells.[18–20,22] In our study, we showed that the immunoreactivity of caspase-3 was significantly higher in 0.5% diluted *T. capitatus* essential oil-treated Colo-320 cells than CDI33+ Colo-320 and CDI33– Colo-320 cells. In addition, the caspase-3 immunoreactivity was higher in

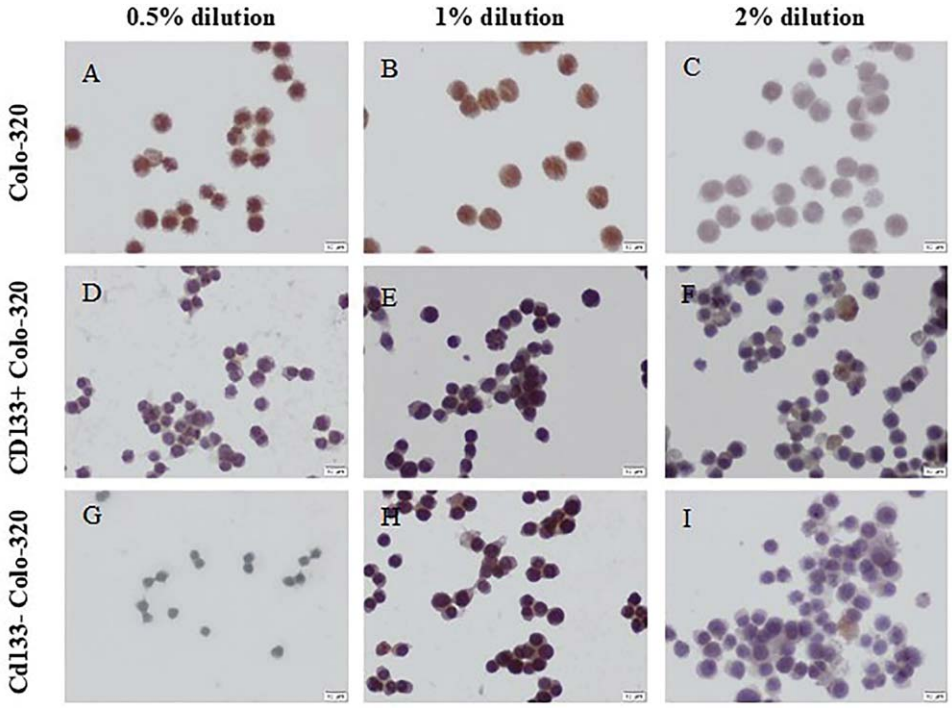


**Figure 4.** Evaluation of TUNEL staining in 0.5%, 1% and 2% *Thymus capitatus* essential oil treated Colo-320 (A, B, C), CDI33+Colo320 (D, E, F) and CDI33-Colo-320 (G, H, I) cells for 48 h (Scale bars = 10 μm).

**Table 2.** The Percentage of TUNEL Positive Colo-320, CDI33+ Colo-320, and CDI33– Colo-320 Cells Treated with 0.5%, 1% and 2% Dilution of *T. capitatus* Essential Oil for 48 hours

| | Colo-320 Cells | CDI33+ Colo-320 Cells | CDI33– Colo-320 Cells |
|---|---|---|---|
| 0.5% dilution | 97.2 ± 2.61 | 19.7 ± 7.18* | 13.4 ± 4.98 |
| 1% dilution | 96.9 ± 4.24 | 10.98 ± 4.27 | 19 ± 7.41 |
| 2% dilution | 48.97 ± 12.39†,‡ | 7.79 ± 1.87 | 12 ± 2.73 |

Data are expressed as means ± SD and were compared by the Kruskal–Wallis test.
*The data were significant when compared with 2% *T. capitatus* essential oil-treated CDI33+ Colo-320 cells ($P = .023$).
†The data were significant when compared with 0.5% *T. capitatus* essential oil-treated Colo-320 cells ($P = .018$).
‡The data were significant when compared with 1% *T. capitatus* essential oil-treated Colo-320 cells ($P = .023$).

CDI33+ Colo-320 cells than CDI33-320 cells. Therefore, primary human colon adenocarcinoma cells treated with 0.5% diluted *T. capitatus* essential oil might be more effective on cancer stem cells. Additionally, the caspase-3 immunoreactivity was significantly higher in 0.5% diluted *T. capitatus* essential oil-treated Colo-320 cells than 2% diluted *T. capitatus* essential oil-treated Colo-320 cells. This result indicates that CDI33– Colo320 cells may have resistance to *T. capitatus* essential oil in the stimulation of apoptosis because of nonseparated Colo-320 cells. We speculate that the high amount of the thymol in *T. capitatus* essential oil may be the main reason for the caspase-3 immunoreactivity upregulation in both Colo-320 cells that exerted anticancer properties. Moreover, we found that 0.5% diluted *T. capitatus* essential oil was more effective than other dilutions of essential oil in apoptosis stimulation in all cell types.

The TUNEL assay is used to detect apoptosis and imply a specificity for apoptosis.[23] In line with the caspase-3 results, our TUNEL assay results showed that *T. capitatus* essential oil was more effective in Colo-320 cells than CDI33+ Colo-320 and CDI33– Colo-320 cells. TUNEL positive cells were significantly higher in 2% and 1% diluted *T. capitatus* essential oil-treated Colo-320 cells than 0.5% diluted *T. capitatus* essential oil-treated Colo-320 cells. Also, the TUNEL positive cell number was significantly higher in 0.5% diluted *T. capitatus* essential oil-treated CDI33+ Colo-320 cells compared to 2% diluted *T. capitatus* essential oil-treated CDI33+ Colo-320 cells. The meaning of the TUNEL positive cells is that they were triggered to cell death with different pathways, which were controlling either apoptosis or necrosis or necroptosis or other types of the cell death. However, the level of the caspase-3 was indicated to apoptotic cells death. Therefore, our results indicated that *T. capitatus* essential oil is more effective in both Colo-320 cells and CDI33+ Colo-320 cells in terms of apoptotic DNA fragmentation.

In conclusion, we have demonstrated the proapoptotic and anticancer effects of *T. capitatus* essential oil in Colo-320, CDI33+ Colo-320, and CDI33– Colo-320 primary colon adenocarcinoma cell lines using various dilutions. Also, we compared the proapoptotic effects of three different dilutions of the *T.*

*capitatus* essential oil in Colo-320, CD133+ Colo-320, and CD133- Colo-320 cells. Interestingly, 0.5% dilution of the *T. capitatus* essential oil elevated caspase-3 intensity and TUNEL positive cell number in Colo-320 cells. In order to verify the main proapoptotic and anticancer activities of *T. capitatus* essential oil on colon cancer cells, further assessment with different multiple signaling pathway molecules that include all possible apoptosis and cancer progression mechanisms is necessary.

**Ethics Committee Approval:** The study did not need Ethics Committee approval.

**Informed Consent:** N/A.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - D.Ö.Y., H.S.V.; Design - D.Ö.Y., H.S.V.; Supervision - D.Ö.Y., H.S.V., F.Ö.K.; Resource - E.B., F.Ö.K., R.K., H.S.V., D.Ö.Y.; Materials - F.Ö.K., R.K., H.S.V., D.Ö.Y.; Data Collection and/or Processing - E.B., H.S.V., D.Ö.Y.; Analysis and/or Interpretation - E.B., F.Ö.K., R.K., H.S.V., D.Ö.Y.; Literature Search - E.B., D.Ö.Y.; Writing - E.B., H.S.V., D.Ö.Y; Critical Reviews - E.B., F.Ö.K., R.K., H.S.V., D.Ö.Y.

**Acknowledgments:** The authors wish to thank Prof. Dr. Betül Demirci (Anadolu University, Faculty of Pharmacy, Pharmacognosy Department) for her valuable contribution to the isolation and analysis of the essential oil compositions and Yard. Doç. Dr. Azmi Hanoğlu (Near East University, Faculty of Pharmacy, Pharmacognosy Department) and Yard. Doç. Dr. Duygu Yiğit Hanoğlu (Near East University, Faculty of Pharmacy, Pharmaceutical Botany Department) for kindly providing the essential oil.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES

1. Dalerba P, Dylla SJ, Park IK, et al. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA*. 2007;104(24):10158-10163. [CrossRef]
2. Bu Y, Cao D. The origin of cancer stem cells. *Front Biosci (Schol Ed)*. 2012;4:819-830. [CrossRef]
3. Cotter TG. Apoptosis and cancer: The genesis of a research field. *Nat Rev Cancer*. 2009;9(7):501-507. [CrossRef]
4. Amin A, Gali-Muhtasib H, Ocker M, Schneider-Stock R. Overview of major classes of plant-derived anticancer drugs. *Int J Biomed Sci*. 2009;5(1):1-11.
5. Džamić AM, Nikolić BJ, Giweli AA, et al. Libyan *Thymus capitatus* essential oil: Antioxidant, antimicrobial, cytotoxic and colon pathogen adhesion-inhibition properties. *J Appl Microbiol*. 2015;119(2):389-399. [CrossRef]
6. Morales R. The genus Thymus L. in the Western Mediterranean region (Lamiaceae). *Biocosme Mesogeen*. 1989;6:205-211.
7. Figueiredo AC, Barroso JG, Pedro LG, Salgueiro L, Miguel MG, Faleiro ML. Portuguese *Thymbra and thymus* species volatiles: Chemical composition and biological activities. *Curr Pharmaceut Des*. 2008;14(29):3120-3140. [CrossRef]
8. Cosentino S, Tuberoso CI, Pisano B, et al. Palmas in-vitro antimicrobial activity and chemical composition of *Sardinian thymus* essential oils. *Lett Appl Microbiol*. 1999;29(2):130-135. [CrossRef]
9. Al-Mustafa AH, Al-Thunibat OY. Antioxidant activity of some Jordanian medicinal plants used traditionally for treatment of diabetes. *Pakistan J Biol Sci*. 2008;11(3):351-358. [CrossRef]
10. Mkaddem MG, Romdhane M, Ibrahim H, et al. Essential oil of *Thymus capitatus* Hoff. et link. from Matmata, Tunisia: Gas chromatography-mass spectrometry analysis and antimicrobial and antioxidant activities. *J Med Food*. 2010;13(6):1500-1504. [CrossRef]
11. Russo M, Suraci F, Postorino S, Serra D, Roccotelli A, Agosteo GE. Essential oil chemical composition and antifungal effects on Sclerotium cepivorum of *Thymus capitatus* wild populations from Calabria, Southern Italy. *Braz J Pharmacog*. 2013;23(2):239-248. [CrossRef]
12. Hanoglu A, Hanoglu DY, Demirci B, Özkum Yavuz D. Chemical composition of essential oil of the aerial parts of wild growing Thymus capitatus (L.) Hoffm. & Link Species collected from three different locations in Northern Cyprus. *J. Essent Oil Bear Pl*. 2017;20(2):546-551. [CrossRef]
13. Islam MT, Khalipha AB, Bagchi R, et al. Anticancer activity of thymol: A literature-based review and docking study with emphasis on its anticancer mechanisms. *IUBMB Life*. 2019;71(1):9-19. [CrossRef]
14. Yavuz DÖ, Mavis M, Ateş G, et al. Identification of potential therapeutic role of *thymus capitatus* essential oil using cellular imaging. *Procedia Comput Sci*. 2017;120:961-966. [CrossRef]
15. Soykut G, Becer E, Calis I, Yucecan S, Vatansever S. Apoptotic effects of *Corchorus olitorius* L. leaf extracts in Colon adenocarcinoma cell lines. *Prog Nutr*. 2018;20:689-698.
16. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. *J Ethnopharmacol*. 2005;100(1-2):72-79. [CrossRef]
17. Bhalla Y, Gupta VK, Jaitak V. Anticancer activity of essential oils: A review. *J Sci Food Agric*. 2013;93(15):3643-3653. [CrossRef]
18. De La Chapa JJ, Singha PK, Lee DR, Gonzales CB. Thymol inhibits oral squamous cell carcinoma growth via mitochondria-mediated apoptosis. *J Oral Pathol Med*. 2018;47(7):674-682. [CrossRef]
19. Deb DD, Parimala G, Devi SS, Chakraborty T. Effect of thymol on peripheral blood mononuclear cell PBMC and acute promyelotic cancer cell line HL-60. *Chem Biol Interactions*. 2011;193(1):97-106. [CrossRef]
20. Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Pichardo S, et al. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line caco-2. *Food Chem Toxicol*. 2014;64:281-290. [CrossRef]
21. Guvenir M, Hanoglu D, Hanoglu A, et al. Antimicrobial activity of the essential oil of *Thymus capitatus* against *Helicobacter Pylori*. *Acta Poloniae Pharmaceutica*. 2020;77(1):155-160. [CrossRef]
22. Li Y, Wen JM, Du CJ, et al. Thymol inhibits bladder cancer cell proliferation via inducing cell cycle arrest and apoptosis. *Biochem Biophys Res Commun*. 2017;491(2):530-536. [CrossRef]
23. Loo DT. In situ detection of apoptosis by the TUNEL assay: An overview of techniques. In *InDNA Damage Detection In Situ, Ex Vivo, and In Vivo*. Totowa, NJ: Humana Press, 2011: 3-13.

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.84-113

# Coronavirus Disease 2019 Outbreak: Use of a Chest X-ray Scoring System and Evaluation of the Radiologic Findings

**Aslı Tanrıvermiş Sayit[1], Çetin Çelenk[1], Şeyma Genç[2]**

[1]Department of Radiology, Ondokuz Mayıs University Faculty of Medicine, Samsun, Turkey
[2]Department of Public Health, Ondokuz Mayıs University Faculty of Medicine, Samsun, Turkey

**ORCID iDs of the authors: A.T.S. 0000-0003-2861-156X; Ç.Ç. 0000-0002-6078-7525; S.G. 0000-0002-2885-584X.**

**Cite this article as: Sayit AT, Çelenk Ç, Genc S. Coronavirus disease 2019 outbreak: Use of a Chest X-ray Scoring System and Evaluation of the Radiologic Findings. *Cyprus J Med Sci.* 2021; 6(4): 325-330.**

**BACKGOUND/AIMS**
Our objective was to identify the chest X-ray (CXR) features of the patients with coronavirus disease 2019 (COVID-19) and to evaluate the relationship between CXR scores and age and gender. Also, we aimed to detect the sensitivity, positive predictive value (PPV), and negative predictive value (NPV) of CXRs in patients with COVID-19 pneumonia.

**MATERIAL and METHODS**
A total of 35 patients who underwent CXR and computed tomography (CT) and had a positive real-time reverse transcriptase-polymerase chain reaction test result were included in the study. The initial CXRs of all patients were evaluated and scored using the Brixia scoring system. Then, chest CT scans were assessed the presence of pneumonia.

**RESULTS**
Of the 35 patients, CXRs of 13 patients (6 male and 7 female) were normal, and 22 patients had unilateral or bilateral opacities, which was considered to indicate pneumonia. The sensitivity, PPV, NPV, and accuracy of the CXRs in the detection of pneumonia were 81.4%, 100%, 61.5%, and 85.7%, respectively. The median Brixia score of patients without pneumonia was less than that of the patients with pneumonia (4 [1-6] vs 7 [4-12]; $P < .01$).

**CONCLUSION**
This scoring system might be useful for identifying the highest-risk patients at an early stage and determine who requires early medical management in the pandemic service or intensive care unit.

**Keywords:** SARS-CoV-2, COVID-19, chest radiography, pneumonia, BRIXA score

## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus that was first detected in Wuhan, Hubei Province of China, in December 2019.[1] The disease was named as coronavirus disease 2019 (COVID-19) and affected a large number of people in a short time. COVID-19 disease was announced as a global pandemic on March 11, 2020 by the World Health Organization.[2] By January 30, 2021, more than 100 million cases and 2 million deaths have been reported around the world.[3]

The precise diagnostic test for COVID-19 disease is a reverse transcription-polymerase chain reaction (RT-PCR) assay.[4] However, the RT-PCR results may be negative due to an inadequate nasopharyngeal or oropharyngeal swab sample. In addition, the RT-PCR results are usually available within 1–2 days. Therefore, radiological imaging methods have become critical in the early detection and treatment, especially patients with suspected pneumonia. Although its sensitivity to detect pneumonia is not very high, chest radiography is the first radiological imaging method that should be performed, especially children and young adults with suspected pneumonia. The most commonly reported chest X-ray (CXR) features of the COVID-19 pneumonia are bilateral pulmonary infiltration and consolidation, often involving the lower lobes.[5] Wong et al.[6] conducted a study including 64 patients that evaluated the frequency and pattern of CXR features and reported the sensitivity of CXR in the detection of SARS-CoV-2 pneumonia, which was 69% [95% CI: 56-80%]. Evaluating

**Corresponding Author:** Aslı Tanrıvermiş Sayit
**E-mail:** draslitanrivermissayit@gmail.com

**Received:** 22.03.2021
**Accepted:** 18.08.2021

the CXR can be confusing, especially in patients with chronic disease such as heart failure and chronic lung disease. The Brixia scoring system, which is a semiquantitative scoring method, was established to evaluate the severity of COVID-19 on a CXR due to the high virulence and mortality of the disease, especially in patients with comorbid diseases, such as hypertension, diabetes mellitus, and cardiovascular and oncological diseases.[7] This scoring system might help to determine who is under high risk and who require specific treatment modality.[7]

Due to the close relationship between pulmonary involvement and mortality, we aimed to (i) explain the CXR findings in patients with SARS-CoV-2 infection who had a positive RT-PCR test finding, (ii) evaluate the relationship between CXR scores and age and gender, and (iii) determine the sensitivity, positive predictive value (PPV), and negative predictive value (NPV) of CXR in patients with SARS-CoV-2 pneumonia.

## MATERIAL and METHODS

This retrospective study was approved by the institutional review board of our hospital, and a written informed consent was waived due to the outbreak of COVID-19 disease (2020/450).

### Patients

Between March 11 and June 10, 2020, we identified 100 patients who had a positive nasopharynx or oropharynx RT-PCR test result and admitted to the emergency service with complaints of fever, dyspnea, dry cough, and weakness. A total of 35 patients who underwent CXR and chest CT were included in the study. Of these 35 patients, 15 (42.9%) were female, and 20 (57.1%) were male. The age, gender, medical history, and presence of comorbidities were retrospectively evaluated with data from the electronic medical record of our hospital. This is a blinded study. The initial CXRs of all patients were evaluated and scored by one radiologist (Ç.Ç.) experienced in thoracic radiology. Then, the other radiologist (A.T.S.) reviewed all chest CT scans for the presence of pneumonia.

### CXR Scoring System

Posteroanterior (PA) CXRs were obtained by having the patient stand in the upright position with full inspiration. Anteroposterior (AP) CXRs were acquired in patients who were unable to stand when using a mobile chest radiograph machine. All CXRs were acquired as computed or digital radiographs following usual local protocols in the emergency department. Sometimes, CXRs may have relatively low quality due to emergency service circumstances. All isolation rules against contamination of a possible infection were obeyed in the radiology unit. The radiologist described the presence, localization, and characterization of infiltrations in the PA or AP radiographs without the knowledge of the CT findings.

**Main Points**

- SARS-CoV-2 is a newly identified $\beta$-coronavirus that leads to an outbreak of unusual viral pneumonia.
- The precise diagnostic test for COVID-19 disease is a RT-PCR test.
- In the presence of suspicion of pneumonia, CXR should be done first.
- The BRIXA scoring system can be used in patients with infiltration on CXR, which can estimate the course of the disease and the prognosis of the patient.

We used the Brixia scoring system, which was defined by Borghesi and Maroldi, to score the patients' CXRs.[4] We ranked pulmonary involvement on an 18-point severity scale according to the extent and characteristics of lung abnormalities.

The Brixia scoring system includes two steps. In the first step, the lungs are divided into six regions as follows:

Upper zones (A and D): above the inferior wall of the aortic arch.

Middle zones (B and E): below the inferior wall of the aortic arch and above the inferior wall of the right inferior pulmonary vein.

Lower zones (C and F): below the inferior wall of the right inferior pulmonary vein.[4]

In the second step, we gave point (from 0 to 3) for each side based on lung abnormalities identified on PA or AP radiographs as follows:

- Score 0: no lung abnormalities
- Score 1: interstitial infiltrates
- Score 2: interstitial and alveolar infiltrates (interstitial predominance)
- Score 3: interstitial and alveolar infiltrates (alveolar predominance)[4]

Then, all scores of the six regions were summed to obtain a total score between 0 and 18. After defining and scoring the CXR findings, chest CT scans of all patients were assessed for the presence of pneumonia.

### CT Imaging

Noncontrast-enhanced chest CT scans (Canon, Aquilion Prime SP, Canon Medical Systems, Japan) were performed for patients who were evaluated with CXRs who had a pathology on their CXR or a normal CXR with suspected pneumonia during physical examination. All the chest CT scans acquired as a noncontrast-enhanced volumetric scans with a low dose technique (tube voltage, 120 kV; tube current, standard (reference mAs, 60-120) to low dose (reference mAs, 30) with automatic exposure control; slice thickness, 1.0 mm) in the emergency department, which was specifically separated in the isolation area. Chest CT was performed in a supine position and full inspirium. They described the presence, localization, shape, density, and borders of all lesions. The lesions were defined as patchy, confluent, or nodular according to their shape. Patchy lesions were non-nodular isolated focal lesions, and confluent lesions were large lesions with multiple segment involvement that tended to merge with each other. The pattern of lesions from patchy to confluent was classified as pure ground-glass opacity (GGO), mixed GGO and consolidation, consolidation, and crazy-paving pattern. The density of nodular lesions was defined as pure GGO, part-solid, and solid. The

**Table 1.** Demographic Findings of the Patients

| | | Female (n = 15) | Male (n = 20) | *P* |
|---|---|---|---|---|
| **Age (years)** | | **45.8 ± 24.2** | **46.05 ± 19** | **.98** |
| Symptoms | Fever | 1 | 5 | |
| | Cough | 10 | 8 | |
| | Chest pain | 2 | 1 | |
| | Dyspnea | 0 | 4 | |
| | Asymptomatic | 2 | 2 | |
| Treatment | Pandemic service | 13 | 14 | |
| | Intensive care unit | 2 | 6 | |

**Table 2.** Comorbidities of the Female and Male Patients

| Comorbidities | Female, n | Male, n |
|---|---|---|
| Asthma | 1 | 0 |
| Hypertension | 3 | 2 |
| Diabetes mellitus type II | 3 | 0 |
| Medical history of previous carcinoma | 4 | 2 |
| Coronary artery disease | 1 | 2 |
| Hyperlipidemia | 1 | 1 |
| Chronic renal disease | 0 | 1 |
| Chronic obstructive lung disease | 0 | 1 |
| Cerebrovascular disease | 1 | 1 |

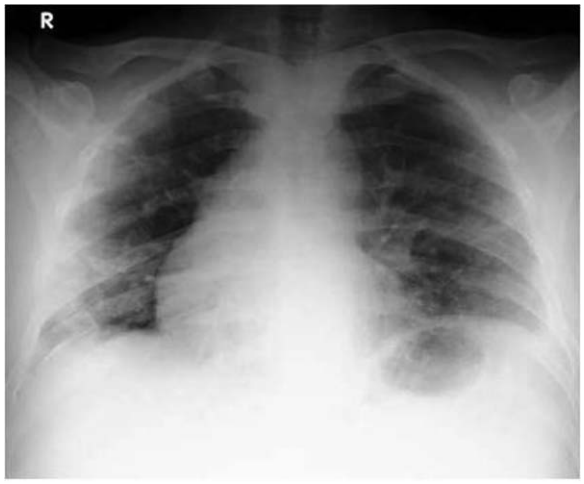


**Figure 1.** A chest X-ray (CXR) showed multifocal patchy peripheral consolidations in the middle and lower zones of both lungs, especially in the right lung. The Brixia score of the CXR was 14. (The right lung score was 7, and the left lung score was 7.)

borders of the infiltrations were described as well-demarcated or ill-demarcated. In addition, the presence of an air bronchogram, a reverse halo, and a tree-in-bud appearance was described.[8]

### Statistical Analysis

Statistical Package for the Social Sciences (SPSS) version 21 (IBM SPSS Corp.; Armonk, NY, USA) was used to perform all statistical calculations. The normality of distribution for the variables was examined using the Shapiro–Wilk test. Descriptive analysis results were expressed as the mean and standard deviation for normally distributed variables. The median (minimum-maximum) was used for non-normally distributed variables. Categorical variables are expressed as percentages. The student t-test was used for normally distributed variables, and the Mann–Whitney U test was used for non-normally distributed variables. The sensitivity, specificity, PPV, NPV, and accuracy of the CXR were compared to reference test, which was chest CT. A value of $P < .05$ was considered significant in all statistical analyses.

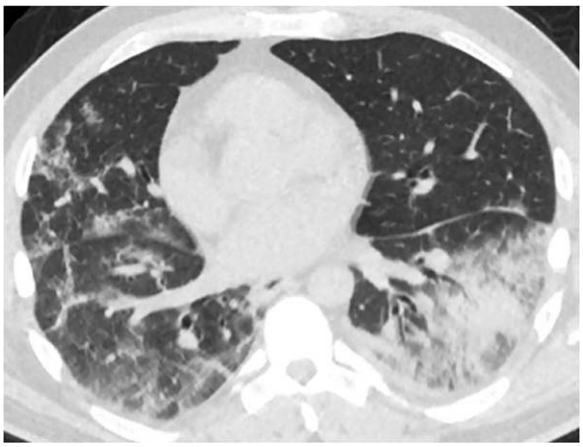

**Figure 2. An** axial nonenhanced chest computed tomography (CT) image showed patchy ground-glass opacities (GGOs) in the right lung with consolidative lesions in the left lower lobe.

## RESULTS

### Patients

Of the 35 patients (45.97 ± 21 years), 15 were female (45.87 ± 24.27 years), and 20 were male (46.05 ± 19.01 years) (Table 1). No statistically significant differences were detected in age between the female and male patients ($P = .98$). Of the 35 patients, 27 (77.1%) were treated in the pandemic service and discharged from the hospital without any complications. Eight patients (22.9%) received intensive care. Three of them died, and five of them were discharged from the hospital. The age of the patients who received intensive care (65.7 ± 16.9 years) was significantly higher than that of those treated in the pandemic service (40.11 ± 18.6 years) ($P = .001$). The comorbidities of the patients are shown in Table 2.

### CXR Features and Scoring System

The CXRs of 13 patients were normal (6 males and 7 females), and the CXRs of 22 patients demonstrated unilateral or bilateral opacities (15 males and 7 females), which indicated pneumonia. The CXR interval of patients was 3 ± 2.5 days during the

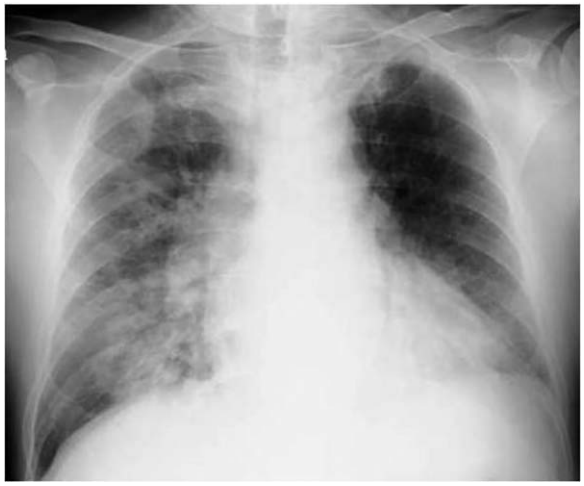

**Figure 3.** A CXR showed multifocal patchy consolidations in the upper, middle, and lower zones of the right lung and slightly increased opacity in the left lower zone. The Brixia score of the CXR was 16. (The right lung score was 12, and the left lung score was 4.)

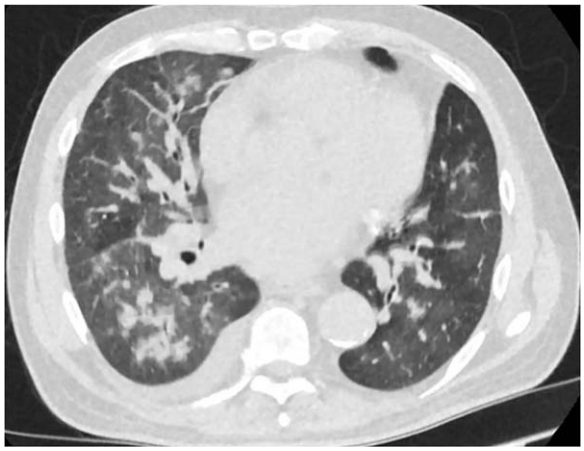

**Figure 4. An** axial nonenhanced chest CT showed slight GGOs in both lungs with rounded GGOs in the right lung.

first admission before CT examination. The sensitivity, PPV, NPV, and accuracy of chest radiography in the detection of pneumonia were 81.4%, 100%, 61.5%, and 85.7%, respectively. The Brixia score in the CXR reports ranged from 1 to 12. The patients were classified into two groups with and without pneumonia as regards to CXR results. The Brixia score of patients without pneumonia was 4 (1–6), and the Brixia score of those with pneumonia was 7 (4–12) (Figures 1 and 2). There was a statistically significant difference in the Brixia scores between patients with and without pneumonia ($P < .01$) (Table 3). The Brixia score of the 27 patients who were treated with medication in the pandemic service was 5 (1–11), and the Brixia score of the eight patients who were treated in the intensive care unit was 8 (5–12) ($P = .004$) (Figures 3 and 4 and Table 3).

### Chest X-ray Correlation with CT

The low-dose chest CT results of all patients were evaluated for the presence of pneumonia. The mean radiation dose was 2.22 ± 1.85 mSv. Of the 35 patients, eight (24.5 ± 18.4 years) had normal thorax CT images, and 27 (52.3 ± 17.4 years) had characteristic features of COVID-19 pneumonia. Of these 27 patients, three had unilateral (right) pneumonia, and 24 had bilateral lung parenchymal abnormalities, which were considered SARS-CoV-2 pneumonia. The chest CT imaging features are shown in Table 4. When the chest CT scans and CXRs were retrospectively evaluated, opacities were visualized on chest CT but not CXRs in five of the 27 patients (Figures 4 and 5). The CXRs of these patients were reevaluated for the Brixia score by dividing them into two groups as patients with or without pneumonia according to chest CT features. The Brixia score of patients with pneumonia (6 [1–2]) was significantly higher than those without pneumonia (2 [1–6]) ($P = 0.001$).

## DISCUSSION

In here, the Brixia score of patients with pneumonia was significantly higher than the patients without pneumonia. Also, the sensitivity, PPV, and accuracy of the CXRs in the detection of pneumonia were found high in our study. CXR is primarily the preferred imaging method for evaluating the pneumonia due to its wide availability and low radiation exposure. Also, it is the least expensive imaging method. However, the sensitivity of CXRs in the diagnosis of pneumonia is not as high as that of CT. They may lead to relatively low quality of the images due to emergency service circumstances. CXRs may be more useful

**Table 3.** Per-Lesion Analysis of Chest X-Ray and Computed Tomography (CT) Findings

| Chest X-Ray Findings | | n |
|---|---|---|
| Laterality | Right lung | 8 |
| | Left lung | 1 |
| | Bilateral | 13 |
| | Normal chest X-ray | 13 |
| Density | Consolidation | 2 |
| | Diffüz opacity | 33 |
| Pneumonia | Patients with pneumonia | 22 |
| | Normal chest X-ray | 13 |
| BRIXA score | Patients with pneumonia | 7 (4-12) |
| | Normal chest X-ray | 4 (1-6) |
| BRIXA score | Patients who were treated in the pandemic service | 5 (1-11) |
| | Patients who were treated in the intensive care unit | 8 (5-12) |
| BRIXA score | Patients with pneumonia in the thorax CT | 6 (1-12) |
| | Patients with normal thorax CT | 2 (1-6) |

**Table 4.** Imaging Characteristic on the Thorax CT in 27 Patients

| Opacity Characteristics | n |
|---|---|
| Peripheral multifocal ground-glass opacities | 17 |
| Multifocal rounded ground glass opacities | 6 |
| "Crazy-paving" pattern | 0 |
| Alveolar consolidation with ground glass opacities | 3 |
| "Reverse halo" sign | 1 |
| **Airways** | |
| Bronchial wall thickening | 1 |
| Bronciectasis | 0 |
| **Underlying Lung Disease** | |
| Pulmonary emphysema | 1 |
| Pulmonary fibrosis | 1 |
| **Other Findings** | |
| Pleural effusion | 6 |
| Pericardial effusion | 2 |
| Atelectasis | 2 |
| Thoracic lympadenopathy | 3 |

*Abbreviation*: CT: computed tomography.

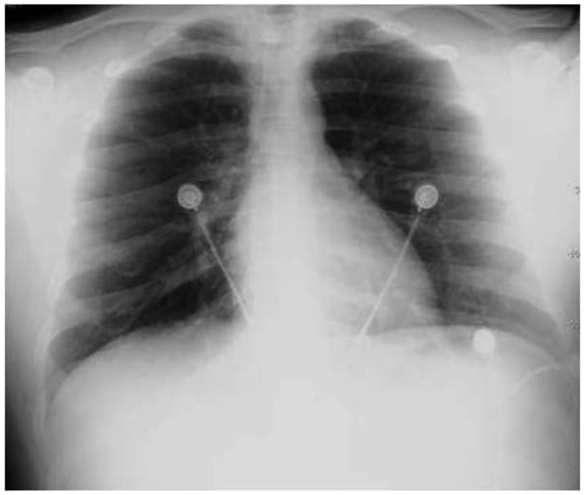

**Figure 5.** The CXR was normal. There was no pneumonic infiltration or pleural effusion. The Brixia score of the CXR was 4. (The right lung score was 2, and the left lung score was 2.)

and sensitive in the diagnosis of pneumonia in children and young adults.[1] The BRIXA scoring system might help identify the highest-risk patients at an early stage and determine who requires early medical management in the pandemic service or intensive care unit.

Recent studies have shown that the distribution of CXR features and lung abnormalities shows a variable appearance.[6] During the SARS-CoV-2 outbreak in 2003, bilateral disease and infiltration of more than two zones were reported on CXRs and related to worse outcomes.[9,10] Similar imaging findings have been identified in various other pneumonias.[11,12] Although CXR is generally accepted as the reference standard, a recent Cochrane review identified two studies that suggested that routine CXR did not affect outcomes in patients with lower respiratory tract infections.[13]

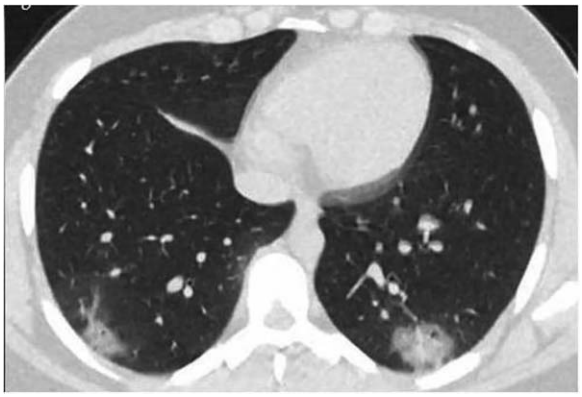

**Figure 6. An** axial nonenhanced chest CT image showed rounded GGOs in the peripheral regions of the lower lobes.

Wong et al.[6] revealed that the sensitivity of an initial RT-PCR analysis (91% [95% CI: 83-97%]) was higher than that of a baseline CXR (69% [95% CI: 56-80%]) ($P = .009$). They also reported that six patients showed CXR abnormalities before eventually testing positive with RT-PCR. In their study, all patients with positive CXRs also had positive thorax CT, and only one patient (1/4, 25%) had a falsely negative CXR when compared to thorax CT.[6] In our study, the sensitivity, PPV, NPV, and accuracy of chest radiography were 81.4%, 100%, 61.5%, and 85.7%, respectively, for detecting COVID-19 pneumonia. All patients with positive CXRs also had positive chest CT, and only five patients had a false negative CXR when made a comparison with chest CT.

The most frequent CXR and CT features of SARS-CoV-2 pneumonia are bilateral, peripheral, and basilar opacities/densities.[1] However, CXRs can be normal in some patients. There may be two reasons for this situation. First, GGOs may not have been detected on the CXR, which may not have had sufficient density (Figure 6). Second, lesions located in basal and retrocardiac areas are very difficult to evaluate.[14] In our study, 22 of the 35 patients had increased opacity on their CXRs, which indicated pneumonia, and a positive RT-PCR test. Of the 35 patients, 13 had normal CXRs. However, five of the 13 patients had GGOs on chest CT, which was considered pneumonia. Peripheral and basal zone distribution were the most frequent locations, and most had bilateral involvement, which is compatible with the findings reported in the literature. There was bilateral involvement in 13 patients, right lung involvement in eight patients, and left lung involvement in one patient. However, when the chest CT scans of these patients were evaluated, only three patients had right lung involvement, while 24 had bilateral involvement. The other chest CT scans were normal.

Toussie et al.[1] evaluated the prognostic value of a CXR severity scoring system for younger (21-50-year-old) patients with COVID-19. CXRs were divided into three zones per lung, and severity score was scored according to the presence (1 point) or absence (0 point) of opacity in each region. They reported that a CXR score $\geq 3$ was an independent predictor of intubation ($n = 28$) ($P = .002$) at the hospital site. They also reported no significant difference in primary outcomes across race/ethnicity, history of tobacco use, asthma, or diabetes mellitus type II.[1]

Borghesi et al.[7] reported that the CXR score was significantly higher in males than in females only in those aged 50-79 years. They showed that males aged ≥50 years and females aged ≥80 years with SARS-CoV-2 infection had the highest CXR score (median ≥8). They concluded that the Brixia score can help identify the highest-risk patients at an early stage and identify who needs specific treatment and who needs protection from the infection.[7]

In our study, the Brixia score was significantly higher in patients with pneumonia than in patients without pneumonia. Also, Brixia score was significantly higher in patients who were treated in the intensive care unit than those treated in the pandemic service.

Using the Brixia score is easy and quick for showing the severity index of lung infiltration. It can assist early diagnosis, treatment, and supplies the relevant information about lung infiltration. CXR is an inexpensive, low-cost, easily accessible, and low-radiation imaging method to evaluate lung abnormalities and is especially preferred in children and younger adults. According to the Fleischner Society, chest CT is not recommended for patients who have mild symptoms or are asymptomatic.[15,16] Therefore, chest CT should be performed in patients who had a suspected moderate-to-severe clinical features of the SARS-CoV-2 infection, additional comorbidities, or opacities on a CXR. In addition, patients with features of pneumonia on thorax CT can be followed up with a CXR because of its low radiation dose and low rate of infectiousness due to its portability. However, in patients with a high Brixia score based on the CXR and suspected progression of pneumonia, chest CT should be performed to evaluate the lung parenchyma. In addition, the use of low-dose CT scans was recently started to prevent patients from receiving high doses of radiation. In our study, the lowest radiation dose was 0.15 mSv, and the highest radiation dose was 7.67 mSv.

Small number of patients is the limitation of our study. The reason for the small sample size is that most patients with suspected pneumonia directly undergo chest CT without an initial CXR. Studies with a larger sample of patients can contribute to the literature. Since no false positive case for CXRs, it is not possible to calculate specificity. So, not to have specificity result is the another limitation.

The Brixia score was significantly higher in patients with pneumonia than in patients without pneumonia. Also, it was significantly higher in patients who were treated in the intensive care unit than those treated in the pandemic service. This scoring system might contribute to identification of the highest-risk patients at an early stage and determination of who requires early medical management in the pandemic service or intensive care unit.

**Ethics Committee Approval:** The ethical approval was obtained from the Ethical Committee of Ondokuz Mayis University Faculty of Medicine (2020/450).

**Informed Consent:** Written informed consent was obtained from all participants who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - A.T.S., Ç.Ç., S.G.; Design - A.T.S.; Supervision - A.T.S., Ç.Ç., S.G.; Resources - A.T.S.; Materials - A.T.S., S.G.; Data Collection and/or Processing - A.T.S., Ç.Ç., S.G.; Analysis and/or Interpretation - A.T.S.; Literature Search - A.T.S., Ç.Ç.; Writing Manuscript - A.T.S.; Critical Review - A.T.S.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES


1. Toussie D, Voutsinas N, Finkelstein M, et al. Clinical and chest radiography features determine patient outcomes in young and middle age adults with COVID-19. *Radiology*. 2020;297(1):E197-E206. [CrossRef]
2. Li Q, Guan X, Wu P, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus–infected pneumonia. *N Engl J Med*. 2020;382:1199-1207. [CrossRef]
3. COVID-19 Map. Johns Hopkins coronavirus resource center. 2016. Available at https://coronavirus.jhu.edu/map.html.
4. Borghesi A, Maroldi R. COVID-19 outbreak in Italy: Experimental chest X-ray scoring system for quantifying and monitoring disease progression. *Radiol Med*. 2020;125:509-513. [CrossRef]
5. Chen SG, Chen JY, Yang YP, et al. Use of radiographic features in COVID-19 diagnosis: Challenges and perspectives. *J Chin Med Assoc*. 2020;83:644-647. [CrossRef]
6. Wong HYF, Lam HYS, Fong AH, et al. Frequency and distribution of chest radiographic findings in patients positive for COVID-19. *Radiology*. 2020;296(2):E72-E78. [CrossRef]
7. Borghesi A, Zigliani A, Masciullo R, et al. Radiographic severity index in COVID-19 pneumonia: Relationship to age and sex in 783 Italian patients. *Radiol Med*. 2020;125(5):461-464. [CrossRef]
8. Hansell DM, Bankier AA, MacMahon H, McLoud TC, Muller NL, Remy J. Fleischner society: Glossary of terms for thoracic imaging. *Radiology*. 2008;246(3):697-722. [CrossRef]
9. Hui DS, Wong KT, Antonio GE, et al. Severe acute respiratory syndrome: Correlation between clinical outcome and radiologic features. *Radiology*. 2004;233(2):579-585. [CrossRef]
10. Antonio GE, Wong KT, Tsui EL, et al. Chest radiograph scores as potential prognostic indicators in severe acute respiratory syndrome (SARS). *AJR*. 2005;184(3):734-741. [CrossRef]
11. Lim WS, Macfarlane JT. Defining prognostic factors in the elderly with community acquired pneumonia: A case controlled study of patients aged ≥75 yrs. *Eur Respir J*. 2001;17:200-205. [CrossRef]
12. Leroy O, Devos P, Guery B, et al. Simplified prediction rule for prognosis of patients with severe community-acquired pneumonia in ICUs. *Chest*. 1999;116(1):157-165. [CrossRef]
13. Cao AMY, Choy JP, Mohanakrishnan LN, Bain RF, van Driel ML. Chest radiographs for acute lower respiratory tract infections. *Cochrane Database Syst Rev*. 2013;2013(12):CD009119.
14. Li B, Li X, Wang Y, et al. Diagnostic value and key features of computed tomography in coronavirus disease 2019. *Emerg Microbes Infect*. 2020;9(1):787-793. [CrossRef]
15. Rubin GD, Ryerson CJ, Haramati LB, et al. The role of chest imaging in patient management during the COVID-19 pandemic: A multinational consensus statement from the fleischner society. *Chest*. 2020;158(1):106-116. [CrossRef]
16. Rubin GD, Ryerson CJ, Haramati LB, et al. The role of chest imaging in patient management during the COVID-19 pandemic: A multinational consensus statement from the fleischner society. *Radiology*. 2020;296(1):172-180. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Original Article

DOI: 10.5152/cjms.2021.1863

# Effect of Different Obturation Techniques on Dentinal Tubule Penetrations of MTA Fillapex and AH Plus

**Fatma Kermeoğlu[1], Meltem Küçük[1], Umut Aksoy[1], Kaan Orhan[2]**

[1]Department of Endodontics, Near East University, Faculty of Dentistry, Nicosia, Cyprus
[2]Department of Dentomaxillofacial, Ankara University, Faculty of Dentistry, Ankara, Turkey

**ORCID iDs of the authors: F.K. 0000-0001-9238-5312; M.K. 0000-0001-5073-4289; U.A. 0000-0001-7281-508X; K.O. 0000-0001-6768-0176.**

**Cite this article as: Kermeoğlu F, Küçük M, Aksoy U, Orhan K. Effect of Different Obturation Techniques on Dentinal Tubule Penetrations of MTA Fillapex and AH Plus. *Cyprus J Med Sci.* 2021; 6(4): 331-336.**

**BACKGROUND/AIMS**
The aim of this in vitro study was to evaluate three different obturation techniques with two different root canal sealers for penetration into dentinal tubules using confocal laser scanning microscopy (CLSM).

**MATERIAL and METHODS**
Sixty root canals were prepared and divided into six groups (n = 10): AH Plus + single-cone technique, AH Plus + lateral compaction, AH Plus + continuous-wave technique, MTA Fillapex + single-cone technique, MTA Fillapex + lateral compaction, and MTA Fillapex + continuous wave technique. Following the obturation of the root canals, the specimens were horizontally sectioned, and the sealer penetration percentage, depth, and area were measured at the apical and middle root areas using CLSM analysis.

**RESULTS**
The single-cone obturation technique exhibited lower penetration values than the continuous wave and lateral compaction techniques in the AH Plus groups ($P < .05$). No significant differences were observed between different obturation techniques in MTA Fillapex groups ($P > .05$). MTA Fillapex exhibited significantly higher penetration values than AH Plus in both the apical and the middle third area in terms of depth, area, and percentage ($P < .05$).

**CONCLUSION**
Within the limitations of this in vitro study, MTA Fillapex penetration into the dentinal tubules was not affected by the obturation technique. Using the lateral compaction and continuous wave techniques provided enhanced AH Plus penetration compared to the single-cone technique.

**Keywords:** Root canal obturation, AH plus, MTA fillapex, obturation techniques, sealer penetration

## INTRODUCTION

The removal of microorganisms and microbial by-products from the root canal system and the prevention of reinfection is the primary objectives of endodontic therapy.[1] The root canal filling is the final step in the classic triad of endodontic therapy: cleaning, shaping, and obturation. The degree of disinfection and integrity of the root canal obturation greatly depend on the cleaning and shaping processes. However, complete eradication of all microorganisms in the radicular space is infeasible or practically almost impossible due to the intricate anatomy of the root canal system and components such as the lateral canals, deltas, isthmuses, and dentinal tubules.[2] It is well established that many species seen in endodontic infections, such as anaerobic/facultative bacteria and fungi, can easily infiltrate the dentinal tubules.[3,4] Thus, a hermetic root canal filling has a significant positive effect on clinical outcomes, as it entombs and restricts the surviving microorganisms and microbial by-products within the root canal space.[5]

The most frequently used obturation core material inside the root canal is gutta-percha. Its downside is that it has no adhesive penetration to root canal dentin regardless of the obturation technique used.[6] Sealers plug the gaps between the dentin wall and the core material. They also function as lubricants, thus facilitating the filling of the root canal. The sealers may spread into anatomical irregularities, small and inaccessible areas of the root canal system, and even tubules in the dentin.[7]

**Corresponding Author:** Fatma Kermeoğlu
**E-mail:** fatma.kermeoglu@neu.edu.tr

**Received:** 19.03.2020
**Accepted:** 29.04.2020

In endodontic treatment, spreading sealers inside the dentinal tubules is potentially beneficial and, thus, desirable. It provides a reduced interface between the dentin wall and gutta-percha, which can enhance the sealing capacity. The obturation material's retention may also be improved as a result of the mechanical joining. Sealers inside the dentinal tubules can also entomb any remaining viable microorganisms, and the chemical constituents of many sealers have antimicrobial properties.[8–10]

AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany) is a paste–paste type material based on epoxy resin. It has been extensively used in endodontic practice. AH Plus has good manipulation characteristics and favorable physical properties, including adhesion to dentin and adequate sealing capability.[11,12] It has been used as a control material in most research on sealers because it has been tested in numerous studies and is considered the gold standard.[13,14]

MTA Fillapex (Angelus Dental Solutions, Londrina, PR, Brazil) root canal sealer is a calcium silicate-based obturation material. It was developed to take advantage of the excellent properties of mineral trioxide aggregate, such as superior biocompatibility, antimicrobial action, and sealing capability. MTA Fillapex consists of mineral trioxide aggregate, salicylate, diluted and natural resins, nanoparticulated silica, and bismuth oxide.[15]

Several techniques have been proposed to achieve hermetic three-dimensional obturation, including lateral compaction (warm or cold), warm vertical compaction (continuous wave), and single-cone obturation.[7,16,17] The choice of the obturation technique plays an essential role in enhancing the seal, preventing microleakage and filling irregularities within the root canal system. It has been observed that the obturation technique affects the rate of sealer penetration into dentinal tubules.[7,16] However, few studies have examined the tubule penetration properties of MTA Fillapex, and, to the best of our knowledge, none of them have evaluated its use with different obturation methods in comparison to AH Plus. Thus, this study aimed to investigate the dentinal tubule penetration of a calcium silicate-based MTA Fillapex sealer compared with AH Plus using three different obturation methods. The null hypothesis was that the obturation method would not affect the dentinal tubule penetration properties of MTA Fillapex or AH Plus.

**Main Points**

- AH Plus sealer penetration into the dentinal tubules was significantly affected by the root canal obturation techniques ($P < .05$), whereas MTA Fillapex sealer penetration rates were not significantly affected by obturation techniques.
- Regardless of the sealer used, the middle thirds had greater tubule penetration values compared to the apical thirds, in terms of all evaluated parameters.
- The greatest sealer penetration values were found in MTA Fillapex + Continuous Wave group in the middle third area, and the lowest sealer penetration values were obtained in AH Plus + Single Cone group in apical third area.

## MATERIAL and METHODS

Approval for the study protocol was obtained from the Animal Ethics Committee of the Near East University (2019/66/755).

### Preparation of the Teeth

Sixty extracted mandibular premolar teeth with closed apices were used in this study. Periapical radiographs were obtained from two different aspects (mesiodistal and buccolingual) to verify the presence of a single canal. A dental operating microscope was used to examine the teeth. Teeth with cracks, root fractures, or caries were eliminated. The selected teeth were stored in vials containing thymol solution (0.1%) until the experimental procedure was initiated. The vertical size of the teeth from the apex was standardized to 14 mm using a cylindrical diamond bur under water cooling. The working length was determined using a #10 K file (VDW, Munich, Germany) inserted into the root canal until its tip was extruded from the apical foramen and then positioned 1 mm short. The root canal of each specimen was instrumented using ProTaper Universal rotary files (Dentsply Maillefer, Ballaigues, Switzerland) to a size of F4 (40/06). A 2 mL NaOCl (5.25%) solution was used to irrigate the root canals after each instrumentation. After instrumentation, all root canals were rinsed with 5 mL ethylenediaminetetraacetic acid (EDTA; 17%) to eliminate the residual smear layer. Saline (5 mL) was used as a final irrigation solution, and sterile F4-sized paper points were used in all root canals. Two layers of nail polish were applied on the outer surface of the specimens, and their apices were sealed with wax.

### Experimental Design

The instrumented root specimens were then randomly divided into six main groups of 10 samples each with the following combinations of obturation materials and techniques:

Group 1: AH Plus + single-cone technique

Group 2: AH Plus + lateral compaction

Group 3: AH Plus + continuous-wave technique

Group 4: MTA Fillapex + single-cone technique

Group 5: MTA Fillapex + lateral compaction

Group 6: MTA Fillapex + continuous-wave technique

The AH Plus and MTA Fillapex sealers were mixed labeled with fluorescent rhodamine B isothiocyanate (0.01%; Merck, Darmstadt, Germany) to allow confocal laser scanning microscopy (CLSM) evaluation. The labeled sealer materials were then inserted into the root canals with a paste carrier (Lentulo Spiral Filler #35, Malleifer, Baillagues, Switzerland) operating at 300 rpm for 5 seconds.

In the single-cone technique, F4-sized gutta-percha points were coated with AH Plus or MTA Fillapex and placed in each root canal.

In the lateral compaction technique, size 40 master gutta-percha points were coated with AH Plus or MTA Fillapex and placed in each root canal. Then, cold lateral compaction was performed using size 20 accessory gutta-percha points and finger spreaders (Thomas, Bourges, France). The procedure was continued until the instrument could not be positioned more than 2 mm from the coronal orifice.

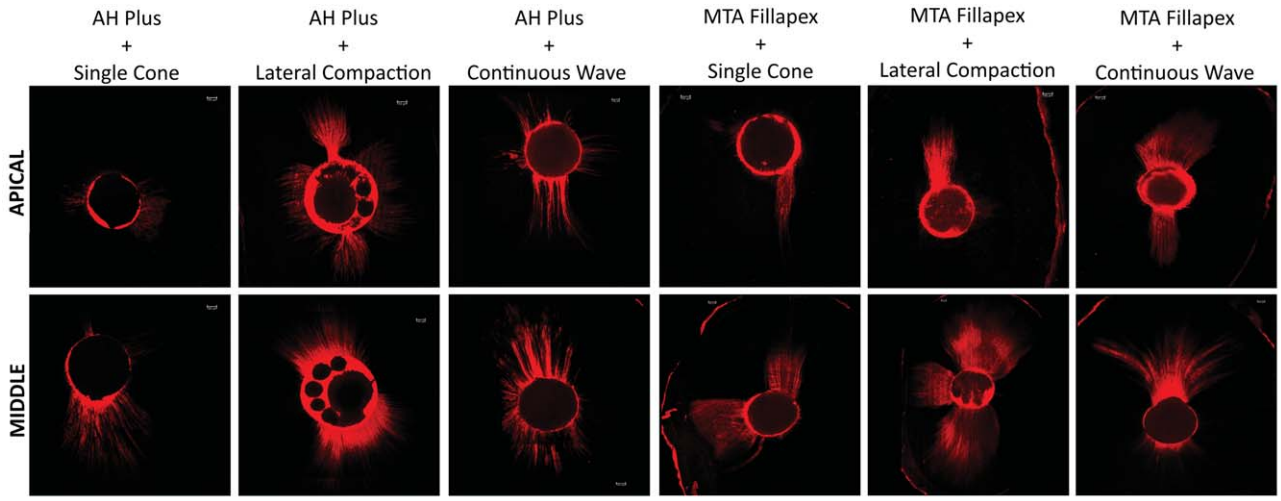


**Figure 1.** Representative CLSM images from each experimental group at the apical third and middle third of the root canal.

In the continuous wave technique, root canal obturations were performed with labeled AH Plus or MTA Fillapex and gutta-percha using a Calamus Dual 3D Obturation Device (Dentsply Tulsa Dental, Tulsa, OK, USA) at a temperature of 160°C and 60% flow rate, according to the manufacturer's operation specifications. The master gutta-percha point with the sealer was adapted as in the single-cone technique and removed at the level of the orifice. Then, appropriate sizes of Calamus Electrical Heat Pluggers were used in one continuous motion to remove the gutta-percha 4 mm short of the working length. The remaining gutta-percha in the root canal was condensed using manual pluggers to obtain a uniformly dense mass at the apical third. A Calamus Flow Obturation Delivery System was used to perform obturation by backfilling the rest of the canal, followed by compaction with a manual plugger. A temporary filling material (Cavit-G, 3M ESPE, Neuss, Germany) was used to seal the specimens' coronal openings. The success of the root canal obturation procedures was confirmed with periapical radiographs. Specimens were then incubated for 2 weeks in 100% humidity at 37°C for a complete setting.

### Sectioning and Confocal Laser Scanning Microscopic Analysis of the Roots

After 2 weeks, the root specimens were vertically mounted on acrylic blocks. Each specimen was transversally cleaved at the middle and apical thirds (5 and 3 mm from the apex, respectively), and a section approximately 1 ± 0.1 mm thick was collected with a low-speed IsoMet saw (0.3 mm blade; Buehler, Lake Bluff, IL, USA) under water cooling. Silicon carbide abrasive papers were used to polish the coronal surfaces of the sample slices to remove debris formed during sectioning. The apical surface of each sample slice was mounted onto a sliding glass. The slides were examined using CLSM (Leica TCS SP2, Leica Microsystems, L'Hospitalet de Llobregat, Spain) at 5× magnification under Ar/HeNe laser excitation with a wavelength of 543 nm.

The ImageJ software (National Institutes of Health) was used to perform sealer penetration area, percentage, and depth measurements in digital CLSM images by two blinded operators. The percentage of sealer penetration was calculated by outlining and measuring the parts of the canal circumference, in which sealer penetration was seen and dividing it by the total circumference of the canal wall. The total dentinal penetration area was calculated by measuring the entire root canal and sealer-penetrated areas and subtracting the canal area from the value. The results were recorded in square millimeters ($mm^2$). The penetration depth was calculated by measuring the point of maximum penetration from the canal wall and recorded in micrometers ($\mu m$).

### Statistical Analysis

The data were analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM SPSS Corp.; Armonk, NY, USA). The Kruskal–Wallis H test and T-test were used for overall group comparison at each level. A value of $P < .05$ was considered statistically significant.

## RESULTS

The results are illustrated in Figure 1. Representative CLSM images from each experimental group are shown in Figure 2. In the AH Plus groups, the dentinal tubule penetration depth, area, and percentage were significantly affected by the root canal obturation techniques ($P < .05$). The single-cone obturation technique exhibited significantly lower penetration values than the continuous wave and lateral compaction techniques ($P < .05$). In the MTA Fillapex groups, the continuous wave technique had the highest penetration depth, area, and percentage values both in the apical and in the middle third area, although the difference was not statistically significant ($P > .05$). Regardless of the obturation technique used, MTA Fillapex exhibited significantly greater penetration values than AH Plus in both the apical and the middle third area in terms of depth, area, and percentage ($P < .05$).

A statistically significant association was found between the different root thirds and the dentinal tubule sealer penetrations ($P < .05$). The middle third had greater sealer penetration values than the apical third in all evaluated parameters ($P < .05$).

## DISCUSSION

Several techniques, such as light microscopy,[16,17] scanning electron microscopy (SEM),[9,18,19] CLSM,[20–25] and microcomputed

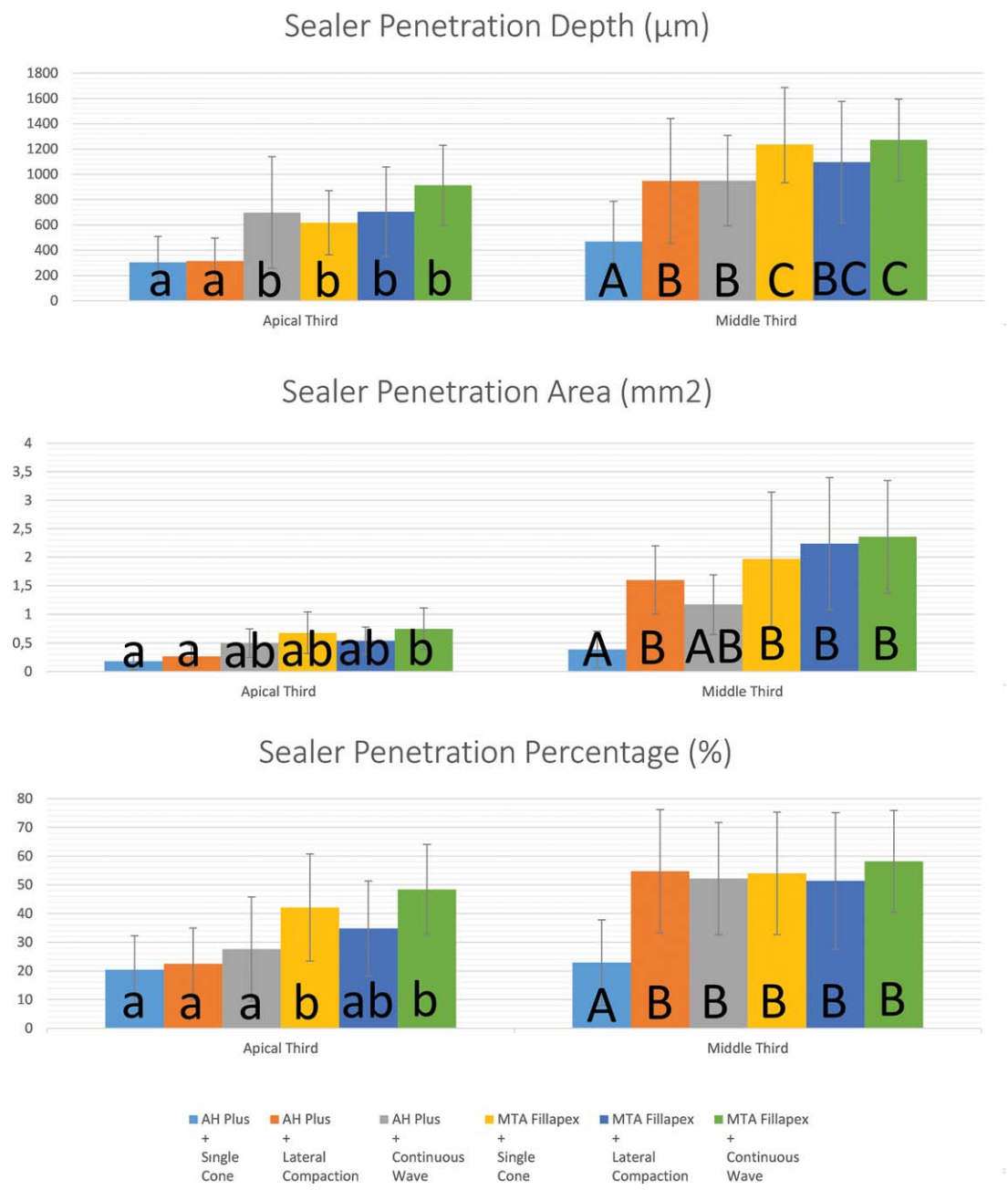


**Figure 2.** The penetration depth (µm), penetration area ($mm^2$), and penetration percentage (%), of AH Plus and MTA Fillapex in different groups at apical third and middle third regions. Different uppercase and lowercase letters in each column indicate statistically significant differences at apical third and middle third ($P < .05$).

tomography (micro-CT),[26] have been employed to study sealer penetration in dentinal tubules. SEM imaging has several advantages in exploring the adaptation of sealer materials. It can effectively visualize the dentinal tubules and their contents as well as the dentin-sealer interface, which cannot be achieved with any other method.[27] On the other hand, CSLM offers several advantages over other conventional methods (SEM and light microscopy) for the study of dental materials.[28] It allows effective monitoring and analysis of the inward layers under the smear formation without requiring destructive sample processing, thus eliminating the need for technical artifacts due to the loss of tooth structure and sealer material.[7,16] For this reason, CLSM was selected for this study.

The sealer's penetration capability depends on various factors, including in vivo conditions (smear layer, dentinal tubule density, and diameter, root canal dimensions), as well as such physicochemical properties, such as surface tension, viscosity, solubility, and particle size.[8,9] The smear layer is a critical factor for penetration capability. Smear formation obstructs the penetration of sealers, irrigation solutions, and intracanal medication agents into dentinal tubules and should, therefore, be eliminated with chelating agents.[8] Previous research has particularly focused on the effect of various irrigation materials and protocols on the penetration ability of sealers.[13,29-31] Since the effect of smear formation was not the main focus of our study, smear layers were removed from all specimens using the same

NaOCl and EDTA protocols to increase dentinal permeability. As a result, high penetration values were observed in all groups.

The impact of the various obturation techniques on sealer penetration has received relatively little attention in the literature. Hence, this study aimed to determine the effect of obturation techniques on the penetration capability of two different sealers. The results indicate significant differences in maximum sealer penetration percentage, depth, and area between AH Plus groups, and consequently, the null hypothesis tested is partially rejected. These findings seem to be consistent with some earlier studies, which found that penetration was affected by both the obturation technique and sealer material.[10,21] However, contrary results have also been reported. Weis et al.[17] found that tubule penetration was not affected by different obturation techniques, including continuous wave, cold lateral compaction, and two different carrier-based techniques. Kuçi et al.[21] observed higher tubule penetration when using MTA Fillapex with the cold lateral compaction technique and AH26, the predecessor of AH Plus, with the warm vertical compaction technique. These findings suggest that the effect of the obturation technique on penetration is related to the sealer type. Our results regarding AH Plus reveal that the continuous wave condensation technique exhibits significantly greater values of maximum penetration depth, area, and percentage than the lateral compaction and single-cone techniques, which provides further support to the hypothesis that the effect of the obturation technique on penetration depends on the type of sealer. In contrast to AH Plus, MTA Fillapex groups exhibit similar sealer penetration values when obturated with different techniques. It can be assumed that this difference is due to the behavior of the sealer materials under different physical conditions such as pressure and temperature. Greater AH Plus tubule penetration is possibly related to a higher flow of the material under pressure and/or its transformation from a paste to a fluid consistency upon warm application during the obturation process, which enables it to penetrate deeper into the dentin tissue.

Regarding the sealer penetration assessment with CLSM, three parameters have been measured in the literature: the percentage of sealer penetration, the maximum and mean penetration depth, and the dentinal tubule penetration area. Most studies have explored the first two parameters based on the method described by Gharib et al.[20] However, these methods have some limitations. Multiple or single measurements have been performed for the calculation of the deepest sealer penetration. A single or a few measurements for depth may not accurately reflect the actual penetration ability of a sealer. Similarly, assessing the penetration percentage may be insufficient, as it ignores the penetration depth and thickness. To overcome these limitations, recent studies have measured the total dentinal tubule penetration area.[13,32,33] In this study, the depth and percentage measurements performed in addition to area measurements were aimed at comparing the compatibility between the parameters. The findings suggest that depth, percentage, and area values are largely consistent with each other. For instance, MTA Fillapex exhibits significantly higher penetration values than AH Plus in all three measurements.

The results of this study show that the sealers' dentinal tubule penetration is greater in the middle third section compared with the apical section, regardless of the sealer and technique used. This is in line with previous studies that investigated various sealers and obturation techniques.[20,21] A few explanations can be proposed for this observation. First, it is known that dentinal tubule diameters are smaller in the apical area and that the tubule density also decreases toward the apex.[34] Second, more sclerotic dentin is more present at the apical level of the root.[35] A third factor is a difficulty eliminating the smear layer in the apical area.[36] These factors affect the dentin's permeability and, consequently, the sealer's penetration rate. The interesting difference observed in the penetration ratios between buccolingual direction and mesiodistal direction may be explained by the effect of increased dentinal sclerosis on the lateral sides (distal and mesial) of the root dentin. This has been termed the "butterfly effect" because its shape resembles a butterfly, as seen in cross-section images of root dentin.[21,27] The findings of the present study are clinically significant owing to the fact that all evaluated parameters that are the tubule penetration depth, percentage, and area by sealers may affect filling quality and impermeability of the root canal system and prevent reinfection.[8,22] However, the sealer penetration into dentinal tubules was reported not to be directly related to the apical seal in a recent study by De-Deus et al.[37] A limitation of the current study was the impossibility of standardization of the amount and distribution of sclerotic dentin despite the careful sample selection.[38] Irregular secondary dentin may influence sealer penetration.

Clinicians' understanding of the features of the endodontic sealer materials improves the treatment outcome. The continuous wave and lateral compaction techniques are more effective than the single-cone technique with AH Plus. The tubular penetration of MTA Fillapex is not affected by different obturation techniques. Regardless of the technique used, MTA Fillapex penetrates the dentinal tubules significantly better than AH Plus, suggesting that, in terms of filling durability, it might be beneficial to obturate the root canal with MTA Fillapex.

**Ethics Committee Approval:** Ethical committee approval was reccieved from the Animal Ethics Committee of the Near East University (2019/66/755).

**Informed Consent:** N/A

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - F.K., M.K., U.A., K.O.; Design - F.K., M.K., U.A., K.O.; Supervision - F.K., U.A., K.O.; Resources - F.K., M.K.; Materials - F.K., M.K.; Data Collection and/or Processing - M.K., X.X.; Analysis and/or Interpretation - M.K., U.A.; Literature Search - U.A., K.O.; Writing Manuscript - U.A., K.O.; Critical Review - U.A., K.O.

**Acknowledgments:** The authors would like to thank Ms. Gulsen Ozduran for assisting with the statistical analysis. This study was not supported by fundings.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES

1. Zehnder M. Root canal irrigants. *J Endod.* 2006;32(5):389-398. [CrossRef]
2. Peters LB, Van Winkelhoff AJ, Buijs JF, Wesselink PR. Effects of instrumentation, irrigation and dressing with calcium hydroxide on

infection in pulpless teeth with periapical bone lesions. *Int Endod J*. 2002;35(1):13-21. [CrossRef]
3. Love RM. Enterococcus faecalis—A mechanism for its role in endodontic failure. *Int Endod J*. 2001;34(5):399-405. [CrossRef]
4. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. *Crit Rev Oral Biol Med*. 2002;13(2):171-183. [CrossRef]
5. Whitworth J. Methods of filling root canals: Principles and practices. *Endod Top*. 2005;12(1):2-24. [CrossRef]
6. Evans JT, Simon JH. Evaluation of the apical seal produced by injected thermoplasticized gutta-percha in the absence of smear layer and root canal sealer. *J Endod*. 1986;12(3):100-107.
7. Jeong JW, DeGraft-Johnson A, Dorn SO, Di Fiore PM. Dentinal tubule penetration of a calcium silicate-based root canal sealer with different obturation methods. *J Endod*. 2017;43(4):633-637. [CrossRef]
8. Kokkas AB, Boutsioukis AC, Vassiliadis LP, Stavriaos CK. The influence of the smear layer on dentinal tubule penetration depth by three different root canal sealers: An in vitro study. *J Endod*. 2004;30(2):100-102. [CrossRef]
9. Mamootil K, Messer HH. Penetration of dentinal tubules by endodontic sealer cements in extracted teeth and in vivo. *Int Endod J*. 2007;40(1):873-881. [CrossRef]
10. Chandra SS, Shankar P, Indira R. Depth of penetration of four resin sealers into radicular dentinal tubules: A confocal microscopic study. *J Endod*. 2012;38(10):1412-1416. [CrossRef]
11. Marin-Bauza GA, Rached-Junior FJA, Sousa-Gabriel AE, et al. Physicochemical properties of methacrylate resin-based root canal sealers. *J Endod*. 2010;36(9):1531-1536. [CrossRef]
12. Marciano MA, Guimarães BM, Ordinola-Zapata R, et al. Physical properties and interfacial adaptation of three epoxy resin-based sealers. *J Endod*. 2011;37(10):1417-1421. [CrossRef]
13. Akcay M, Arslan H, Durmus N, Mese M, Capar ID. Dentinal tubule penetration of AH Plus, iRoot SP, MTA fillapex, and guttaflow bioseal root canal sealers after different final irrigation procedures: A confocal microscopic study. *Lasers Surg Med*. 2016;48(1):70-76. [CrossRef]
14. El Hachem R, Khalil I, Le Brun G, et al. Dentinal tubule penetration of AH Plus, BC Sealer and a novel tricalcium silicate sealer: A confocal laser scanning microscopy study. *Clin Oral Invest*. 2019;23(4):1871-1876. [CrossRef]
15. Torabinejad M, Parirokh M. Mineral trioxide aggregate: A comprehensive literature review—Part II: Leakage and biocompatibility investigations. *J Endod*. 2010;36(2):190-202. [CrossRef]
16. De Deus GA, Gurgel-Filho ED, Maniglia-Ferreira C, Coulinho-Filho T. The influence of filling technique on depth of tubule penetration by root canal sealer: A study using light microscopy and digital image processing. *Aust Endod J*. 2004;30(1):23-28. [CrossRef]
17. Weis MV, Parashos P, Messer HH. Effect of obturation technique on sealer cement thickness and dentinal tubule penetration. *Int Endod J*. 2004;37(10):653-663. [CrossRef]
18. Kouvas V, Liolios E, Vassiliadis I, Parissis-Messimeris S, Boutsioukis A. Influence of smear layer on depth of penetration of three endodontic sealers: An SEM study. *Endod Dent Traumatol*. 2007;14(4):191-195. [CrossRef]
19. Balguerie E, van der Sluis L, Vallaeys K, Gurgel-Georgelin M, Diemer F. Sealer penetration and adaptation in the dentinal tubules: A scanning electron microscopic study. *J Endod*. 2011;37(11):1576-1579. [CrossRef]
20. Gharib SR, Tordik PA, Imamura GM, Baginski TA, Goodell GG. A confocal laser scanning microscope investigation of the epiphany obturation system. *J Endod*. 2007;33(8):957-961. [CrossRef]
21. Kuçi A, Alaçam T, Yavaş O, Ergul-Ulger Z, Kayaoglu G. Sealer penetration into dentinal tubules in the presence or absence of smear layer: A confocal laser scanning microscopic study. *J Endod*. 2014;40(10):1627-1631. [CrossRef]
22. Moon YM, Shon WJ, Baek SH, Bae KS, Kum KY, Lee W. Effect of final irrigation regimen on sealer penetration in curved root canals. *J Endod*. 2010;36(4):732-736. [CrossRef]
23. Kok D, Da Rosa RA, Barreto MS, et al. Penetrability of AH plus and MTA fillapex after endodontic treatment and retreatment: A confocal laser scanning microscopy study. *Microsc Res Tech*. 2014;77(6):467-471. [CrossRef]
24. Amoroso-Silva PA, Guimarães BM, Marciano MA, et al. Microscopic analysis of the quality of obturation and physical properties of MTA Fillapex. *Microsc Res Tech*. 2014;77(12):1031-1036. [CrossRef]
25. Gunes B, Yeter KY, Terlemez A, Seker B, Altay Y. Dentinal tubule penetration of endodontic sealers after nonthermal plasma treatment: A confocal laser scanning microscopy study. *Microsc Res Tech*. 2019;82(6):903-908. [CrossRef]
26. Huang Y, Orhan K, Celikten B, Orhan AI, Tufenkci P, Sevimay S. Evaluation of the sealing ability of different root canal sealers: A combined SEM and micro-CT study. *J Appl Oral Sci*. 2018;15(26):e20160584.
27. Russell A, Friedlander L, Chandler N. Sealer penetration and adaptation in root canals with the butterfly effect. *Aust Endod J*. 2018;44(3):225-234. [CrossRef]
28. Moodley D, Grobler SR, Rossouw RJ, Oberholzer TG, Patel N. in vitro evaluation of two adhesive systems used with compomer filling materials. *Int Dent J*. 2000;50(6):400-406. [CrossRef]
29. Tuncer AK, Tuncer S. Effect of different final irrigation solutions on dentinal tubule penetration depth and percentage of root canal sealer. *J Endod*. 2012;38(6):860-863. [CrossRef]
30. Aksel H, Küçükkaya Eren S, Puralı N, Serper A, Azim A. Efficacy of different irrigant protocols and application systems on sealer penetration using a stepwise CLSM analysis. *Microsc Res Tech*. 2017;80(12):1323-1327. [CrossRef]
31. Aksel H, Arslan E, Puralı N, Uyanık O, Nagaş E. Effect of ultrasonic activation on dentinal tubule penetration of calcium silicate-based cements. *Microsc Res Tech*. 2019;82(5):624-629. [CrossRef]
32. Akcay M, Arslan H, Mese M, Durmus N, Capar ID. Effect of photon-initiated photoacoustic streaming, passive ultrasonic, and sonic irrigation techniques on dentinal tubule penetration of irrigation solution: A confocal microscopic study. *Clin Oral Invest*. 2017;21(7):2205-2212. [CrossRef]
33. Aydın ZU, Ozyürek T, Keskin B, Baran T. Effect of chitosan nanoparticle, QMix, and EDTA on TotalFill BC sealers' dentinal tubule penetration: A confocal laser scanning microscopy study. *Odontology*. 2019;107(1):64-71. [CrossRef]
34. Carrigan PJ, Morse DR, Furst ML, Sinai IH. A scanning electron microscopic evaluation of human dentinal tubules according to age and location. *J Endod*. 1984;10(8):359-363. [CrossRef]
35. Ribeiro RG, Marchesan MA, Silva RG, Sousa-Neto MD, Pécora JD. Dentin permeability of the apical third in different groups of teeth. *Braz Dent J*. 2010;21(3):216-219. [CrossRef]
36. Blank-Gonçalves LM, Nabeshima CK, Martins GH, de Lima Machado ME. Qualitative analysis of the removal of the smear layer in the apical third of curved roots: Conventional irrigation versus activation systems. *J Endod*. 2011;37(9):1268-1271. [CrossRef]
37. De-Deus G, Brandão M, Leal F, et al. Lack of correlation between sealer penetration into dentinal tubules and sealability in non-bonded root fillings. *Int Endod J*. 2012;45:642-651. [CrossRef]
38. Jardine AP, Da Rosa RA, Santini MF, et al. The effect of final irrigation on the penetrability of an epoxy resin-based sealer into dentinal tubules: A confocal microscopy study. *Clin Oral Invest*. 2016;20:117-123. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Review

DOI: 10.5152/cjms.2021.1565

# p97/VCP and Inclusion Body Myopathy with Early-Onset Paget Disease and Frontotemporal Dementia (IBMPFD)

**Yalçın Erzurumlu**

**Department of Biochemistry, Faculty of Pharmacy, Süleyman Demirel University, Isparta, Turkey**

**ORCID iD of the author: Y. E. 0000-0001-6835-4436.**

**Cite this article as: Erzurumlu Y. p97/VCP and Inclusion Body Myopathy with Early-Onset Paget Disease and Frontotemporal Dementia (IBMPFD). *Cyprus J Med Sci.* 2021; 6(4): 337-344.**

Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD), which is an autosomal dominant inheritance multisystem disease, is rare, occurring in adulthood, progressive progression, and often results in the death of patients. Clinically, IBMPFD is characterized by limb-girdle muscle dystrophy syndrome sighted proximal and distal muscle weakness, early-onset bone Paget's disease, and premature frontotemporal dementia. First, in 2004, the IBMPFD disease locus was mapped at the p21-p12 locus of chromosome 9 and was associated with mutations observed in the p97/VCP gene. Biologically, p97/VCP has been shown to have a regulatory and catalyzing role in many cellular processes, including postmitotic homotypic membrane fusion, nuclear sheath regeneration and packaging, cell cycle regulation, programed cell death, endoplasmic reticulum-associated degradation, organelle biogenesis, regulation of transcription factors, Endoplasmic reticulum membrane fusion, stimulation of B and T cells, and inhibition and separation of protein aggregates. IBMPFD has been associated with mutations observed in the p97/VCP gene. Currently, the number of p97/VCP mutations identified in IBMPFD patients is 28. The molecular mechanisms of only a few of these mutations in IBMPFD pathogenesis were understood. In this review, all of the p97/VCP mutations associated with IBMPFD are discussed, and the molecular mechanisms of p97/VCP in disease process are examined.

**Keywords:** p97/VCP, inclusion body myopathy with early-onset Paget disease and frontotemporal dementia, endoplasmic reticulum-associated degradation

## INTRODUCTION

### Inclusion Body Myopathy with Early-Onset Paget Disease and Frontotemporal Dementia

Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD; OMIM #605382) is rare, occurring in adulthood, gradually progression, and often results in the death of patients. IBMPFD has autosomal dominant inheritance and onset in adults between 20 and 40 years of age. IBMPFD was mapped at 9p21.1-p12 locus of chromosome 9. This locus, which is very rich in gene diversity, is characterized as a multiple disease locus with different myopathies. IBMPFD is observed with myopathy, Paget's disease, and dementia in various penetrations.[1,2]

Clinically, IBMPFD is characterized by limb-girdle muscle dystrophy syndrome sighted proximal and distal muscle weakness, early-onset bone Paget's disease (PDB), and premature frontotemporal dementia (FTD). The first symptoms of the disease are observed in the brain and bone tissue.[3] The muscle weakness observed in the patients gradually progressed, affecting the muscles of the other limbs and respiratory tract. It has also been reported that deformation and enlargement of long bones and occasionally pathological fractures of the bones could be seen in some patients. Currently, dilated cardiomyopathy, amyotrophic lateral sclerosis (ALS), and Parkinson's disease are known to be part of the spectrum of findings associated with IBMPFD.[4]

IBMPFD was first reported in 2004 due to mutations in the gene encoding valosin-containing protein (VCP and p97/VCP) located at p13-p12 locus of chromosome 9.[1,2] p97/VCP is known as a molecular chaperone that plays an active role in many cellular processes.[5] The first symptoms of IBMPFD are observed in brain and bone tissue.[3] Typically, clinical muscle phenotype seen in IBMPFD patients is characterized by weakness in the lower extremities and upper proximal, often with axial myopathy, wing-like extensions of the shoulder blades, and variable involvement of the distal muscles. It

**Corresponding Author:** Yalcin Erzurumlu
**E-mail:** yalcin.erzurumlu@gmail.com, yalcinerzurumlu@sdu.edu.tr

**Received:** 05.12.2019
**Accepted:** 14.02.2020

has been observed that respiratory failure and cardiomyopathy findings are encountered in some patients due to myopathy.[2]

Inclusion body myopathy (IBM) with autosomal dominant inheritance exhibits a heterogeneous course profile, in which clinical reflections and genetic disorders are observed. IBM is characterized by late-onset shoulder and pelvic weakness in patients and creatine kinase (CK) levels that are clinically above the desired range. The reflection of myopathic changes in patients' cells is observed as cytoplasm, and more rarely, vacuoles observed in the nucleus of cells.[6]

PDB is more common in male individuals than in women. The pathology of the disease is characterized by increased resorption of bone by osteoclasts and subsequent reshaping of bone formation. Although it is generally asymptomatic, it is characterized by abnormal, overactive osteoclasts, bone thickening, and usually large, excessively large nuclei and often paramyxovirus-like inclusions. Ubiquitinated cytoplasmic and nuclear inclusions were observed in pagetoid osteoclasts. PDB was found to occur at similar ages with myopathy in 50% of IBMPFD patients.[3] In PDB patients, clinical symptoms are observed, including bone deformation, spine, hip and skull pain, and bone fractures. Increased alkaline phosphatase enzyme levels were also determined in patients.[2]

FTD in IBMPFD patients is an important part of primary degenerative dementia before the age of 65. FTD is observed as frontal lobe functions associated with disproportionately weakened behavioral changes. It is observed that memory, visual, and spatial abilities are partially preserved in the early stages of the disease, but the symptoms strongly support the diagnosis of FTD. The underlying cause of all these symptoms is due to the changes observed in the frontal lobe. Patients also have localized atrophy of the frontal and anterior temporal lobes. 38-45% of all FTD cases have a strong hereditary transmission, and approximately 80% of this group has an autosomal dominant inheritance.[6] In IBMPFD patients, the penetrance of FTD was determined to be 30%, and it was determined that the onset of FTD occurred much later than myopathy.[3]

Further studies show that cytosolic and intranuclear sections of IBMPFD tissue were found to be significantly ubiquitinated inclusions.[7–9] In the studies that healthy muscle samples were used as a control group, it was observed that p97/VCP was localized in the endomysial capillary structure, which is mostly the structure of the ligament layer in muscle by staining with polyclonal primer antibody. The formation of ubiquitinated small inclusions and vacuolar structures in the cytosolic segments of muscle fibers of IBM patients was determined. In the same examples, p97/VCP was found to be strongly involved in endomysial inflammatory cells surrounding muscle fibers. Besides, p97/VCP levels increased in the regenerated muscle fibers, and p97/VCP was found to be involved in wide focal inclusions of muscle fibers belonging to IBMPFD patients.[10]

When IBMPFD was examined in terms of central nervous system pathology, it was observed that these cells were negative for tau protein and positive for ubiquitin, consistent with ubiquitinated inclusions and frontotemporal lobar degeneration.[11] IBMPFD is characterized by disturbances in the degradation of the accumulation of proteins, and ubiquitinated protein aggregates targeted by p97/VCP.[9,12–14] Cellular degeneration and ubiquitinated protein inclusions combine the pathology of these three separate tissues (bone, muscle, and brain) in IBMPFD. It is known that p97/VCP is a multifunctional protein and plays a major role as a molecular chaperone in the transfer of proteins that unfolded and misfolded proteins in the ER.[10] Furthermore, the pathogenicity exhibited by p97/VCP is emphasized by p97/VCP positive protein aggregates observed in neurons and skeletal muscle cells located in the central nervous system of IBMPFD patients.[7] It has been reported that p97/VCP positive inclusions are mainly concentrated in the nucleus of neuron cells in IBMPFD patients, and only in cytoplasmic aggregate foci in skeletal muscle cells.[9]

Nuclear inclusions containing ubiquitin were found to be colocalized with p97/VCP in various neurodegenerative disorders such as Huntington's disease, Lewy body disease, Parkinson's disease, spinocerebellar ataxia type III (Machado-Joseph disease), and ALS.[1,9] In studies conducted by different groups, these aggregates, which were positive for p97/VCP, were not reported to be specific to IBMPFD pathology. Similarly, different proteins such as ubiquitin, $\beta$-amyloid, apolipoprotein E, and phospho-Tau in the cytoplasmic aggregates in the muscles of IBMPFD patients were found to accumulate in the brain cells of Alzheimer's patients.[12]

The phenotypically related features of IBMPFD are also found in 90% of patients and are often characterized in the 40s, which is the adulthood of individuals. Atrophy associated with proximal and distal muscle weakness is observed in these individuals. In skeletal muscles with IBMPFD pathology, amorphous vacuoles (rimmed vacuoles), myonuclear, and sarcoplasmic inclusions have been reported. These inclusions have been reported to be congophilic and immunoreactive for TAR DNA binding protein-43 (TDP-43) in some cases.[3]

In the molecular pathogenesis of IBMPFD, p97/VCP is critical due to the role in targeting the proteasome of unfolded and misfolded proteins that may cause proteotoxicity by means of endoplasmic reticulum (ER)-associated degradation (ERAD). Therefore, it is of great importance to understand the biological role of p97/VCP and associating it with the pathogenesis of IBMPFD.

**Valosin Containing Protein (p97/VCP)**

p97/VCP is encoded in the p13.3 locus of chromosome 9 and localized in the negative (–) strand of the genome. The 17 exons p97/VCP are encoded by a 3859 bp transcript. Consisting of 806 amino acids, p97/VCP has a molecular weight of about 89,322 Da. The calculated basal isoelectric point is known to be 5.14.[15]

The p97/VCP protein was first identified as the protein responsible for cell division control in yeast under the name CDC48, while it was reported as provalosin in metazoans. The sequence analysis revealed that the yeast and human VCP gene had a similarity of 69%. In the evolutionary process, p97/VCP was found to be highly conserved between diverse species. In sequence homology studies, it was observed that p97/VCP showed advanced homology among different species. p97/VCP, VAT in Archaebacteria (VCP-like ATPase), CDC48p in Saccharomyces cerevisiae (cell division control protein 48), TER94 (transitional endoplasmic reticulum ATPase) in

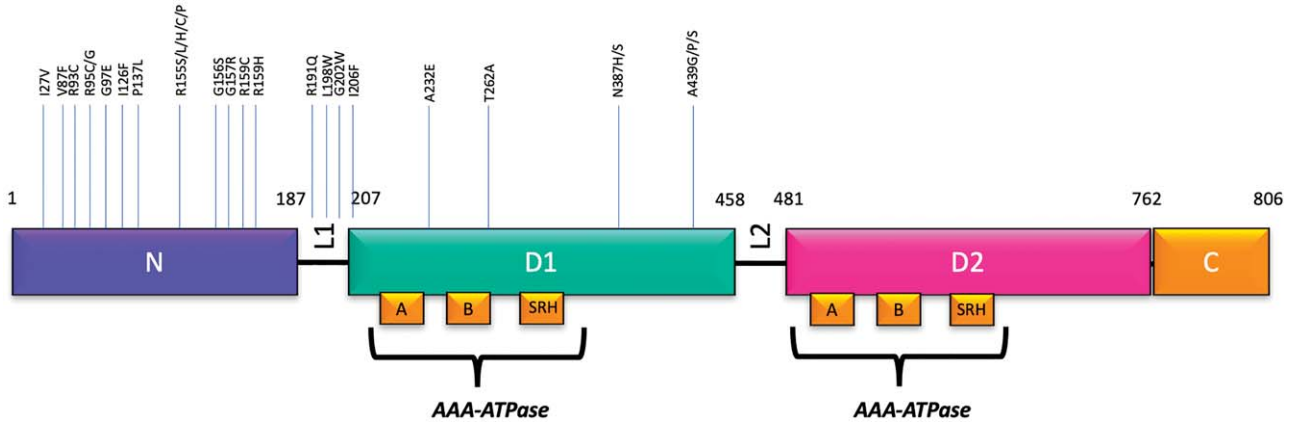


**Figure I.** Protein organization of p97/VCP and localization of IBMPFD-related p97/VCP mutations. Schematic organization of a p97/VCP protein structure showing the three domains: N-terminal N domain and two ATPase domains in DI and D2, and the positions of IBMPFD-related mutations.

Drosophila melanogaster, p97 in Xenopus laevis, and plant and mammalian species orthologs are named as VCP. In Northern blot analyses, it was determined that p97/VCP was expressed in all tissues in the body, including brain tissue in humans.[16] Also, p97/VCP, which constitutes more than 1% of the protein content in the cells, was found to be ubiquitously expressed in cells and be in large amounts of cytosolic compartments.[17] These data highlight the critical role of p97/VCP in living systems.

Biologically, p97/VCP has been shown to have a regulatory and catalyzing role in many cellular processes, which include postmitotic homotypic membrane fusion, nuclear sheath regeneration, packaging, cell cycle regulation, programed cell death, ERAD, organelle biogenesis, mitotic spindle separation, regulation of transcription factors, ER membrane fusion, stimulation of B and T cells, inhibition and separation of protein aggregates, DNA repair, and autophagy.[18]

Each unit of p97/VCP, which consists of six identic subunits with each other and exhibits a homo-hexameric structure, has four different domains known to be important for maintaining its functionality. These are N-domain (1-187), DI domain (209-460), D2 domain (481-761), C-terminal domain (762-806), N-DI linker LI region (188-208), and flexible DI-D2 linker L2 region (461-480).[19,20] Electron micrography studies have shown that p97/VCP exhibits two homo-hexameric structures organized as barrels. Also, it has been indicated in crystallographic studies that the p97/VCP hexamer exhibits a flattened hourglass or mushroom-like structure.[20]

A good understanding of protein organization is essential for comprehending the role of p97/VCP in biological systems. The amino (N) and carboxyl (C) terminal domains of p97/VCP mediate interaction with various adapter and accessory proteins. It has been shown that the N-terminal domain is critical for interaction with several cofactors for cellular position and activity and also for substrate interactions.[21] The C-terminal domain of p97/VCP has flexible acidic amino acid residues. Defects in the C-terminal domain have been shown to cause changes in cellular responses due to the change in binding of cofactors that provide substrate modifications. Furthermore, the C-terminal domain contains a major tyrosine phosphorylation residue (Tyr805) having a regulatory role.[22] Phosphorylation in this region regulates protein interactions by inhibiting cofactor binding to p97/VCP.[23] Also, the ATPase cassettes required for the biological activity of p97/VCP are located in the domains DI and D2, respectively.[22]p97/VCP is a member of the ATPase superfamily of type II AAA + (ATPases-associated with diverse Activities) involved in multiple cellular pathways. AAA + proteins are categorized into two classes based on the number of conserved ATP-binding cassettes contained in the protein structure. While class I AAA + ATPase family member proteins have one AAA + cassette in their structure, class II members have two AAA + cassettes. p97/VCP, a member of the Class II superfamily, performs the mechanical role in biological events by the energy obtained from the hydrolysis of ATP using ATPase cassettes.[23] In homology studies, each AAA + domain of p97/VCP has a high sequence similarity among different species. This supports that AAA + proteins, including p97/VCP, are extremely important structural elements in biological systems for survival.[24]

The AAA+ domains of ATP binding cassettes are well protected in the evolutionary process. AAA+ cassettes consist of Walker A and Walker B motif, sensors I and 2, the second region of homology (SRH), and the pore loop. Walker A motifs having the conserved GxxxxGK(T/S) sequence are necessary for nucleotide binding. Walker B motifs having (R/K)xxxGxxx(L/V)hhh(D/E) have been determined to have the conserved sequence. It has been reported that the ATPase cassette of the Walker B and SRH regions are necessary for efficient ATP hydrolysis. It has been reported that mutations in Sensor I cause defects in hydrolysis function. In addition, a conserved arginine residue of Sensor 2 directly interacts with the g-phosphate group of ATP via the neighboring subunit, and this interaction is necessary for ATP binding and hydrolysis. It has been indicated that the SRH region contains the critical arginine fingers, which is important in interaction, and the pore loop region is required for binding to and processing of the substrate (Figure I).[25]

Allosteric interactions are very essential for the function of p97/VCP, which has a multimeric structure. ATP binding to the ATPase cassette in the DI domain of p97/VCP was found to accelerate the reassociation of p97/VCP monomers. This

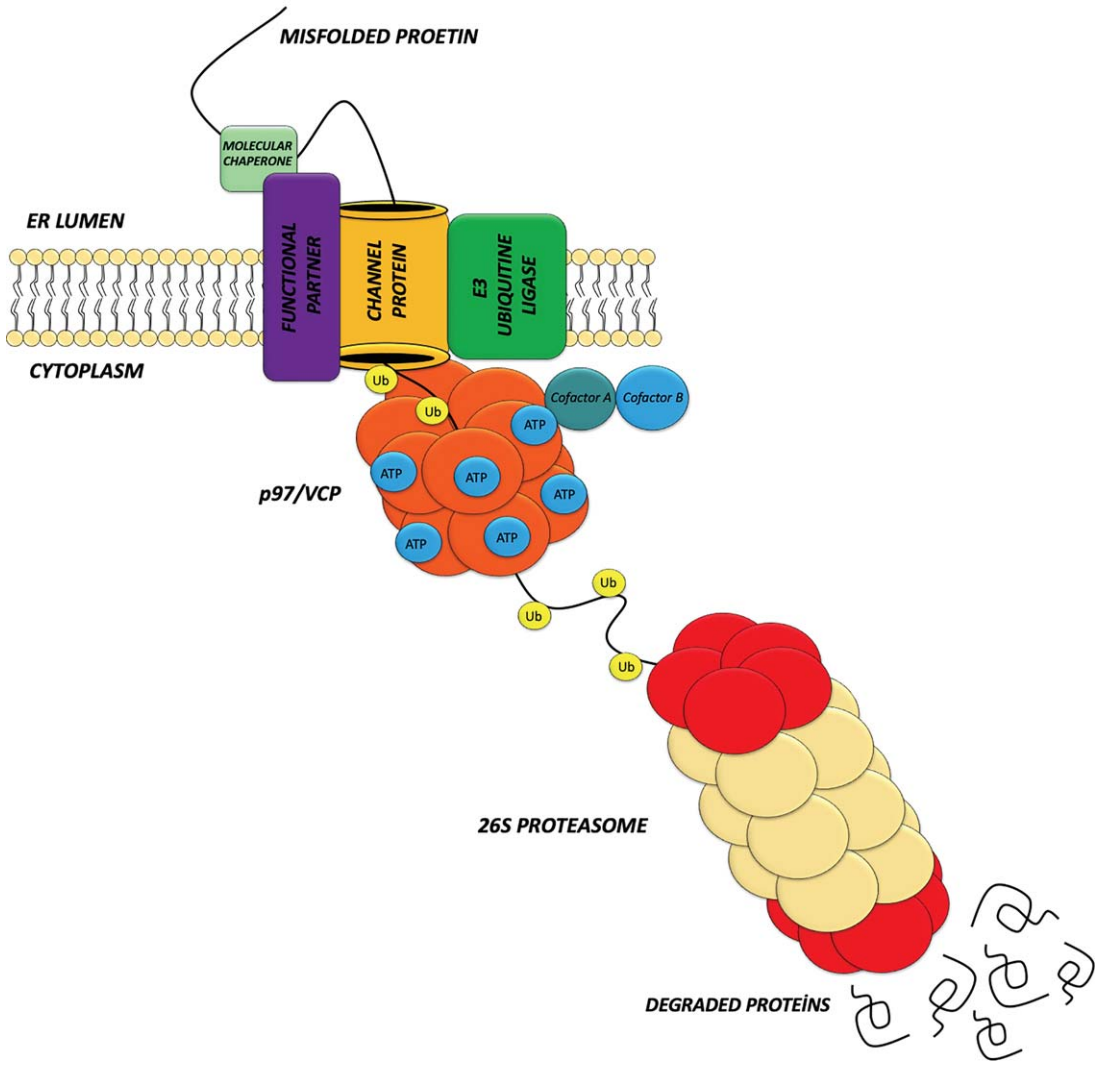


**Figure 2.** A model for the direction of misfolded proteins in ER to 26S proteasome. Primarily, misfolded proteins are recognized by luminal molecular chaperone complex and targeted to a putative channel in the membrane. The partially unfolded substrate is dislocated across the ER lumen simultaneously; ubiquitylation occurs through membrane-bound substrate-specific E3 ubiquitin ligases, facilitating recruitment of p97/VCP. p97/VCP, with its ATPase activity, provides the transfer of misfolded protein from the channel protein to the cytosolic face of ER. The misfolded protein is then directed to different protein complexes the 26S proteasome and degraded.

result suggests that DI domain is an important tool for hexamerization. DI domain has a major role in oligomerization of protein, whereas D2 domain has a minor effect in this process. The formation of the hexameric structure is very important for the biological functions of p97/VCP, especially critical for its role in the ubiquitin-proteasome system.[20] The mutations seen in Walkers A and B motifs in D2 domain of p97/VCP were predominantly fatal in individuals, and mutations in DI domain did not have such an effect.[24] Functional distortions in p97/VCP trigger dysregulated processes, such as impaired protein quality control, mitochondrial dysfunction, oxidative stress, and apoptotic cell death resulting in cell death.[26]

p97/VCP is a key molecule involved in the retrotranslocation step of ERAD.[27] Unfolded, incorrectly oligomerized and misfolded proteins are potentially proteotoxic to the cells. Newly synthesized proteins and synthesized proteins cannot reach their mature forms due to various reasons, such as genetic mutation, viral infection, temperature changes, and oxidative stress. Evolutionary, mammalian cells have developed quality-control systems for the removal of these abnormal proteins.[28] These systems only allow proteins that reach their final conformational state to have them move to their sites. Newly synthesized proteins in the ER are continuously monitored by the various quality control system. Unwanted abnormal protein forms are targeted to 26S proteasome by ERAD mechanism.[28] ERAD also functions to regulate endogenous levels of diverse proteins, including cholesterol synthesis-associated enzyme 3-Hydroxy-3-Methylglutaryl-CoA Reductase and tumor suppressor KAII. Thus, ERAD may regulate the cellular homeostasis and adapt the cells to changing physiological conditions.

The ubiquitin molecule is conjugated to the misfolded proteins in the ER by ubiquitin ligase enzymes. p97/VCP interacts with ubiquitinated proteins on the cytosolic surface of the ER lumen, performing retrotranslocation of these proteins from ER to cytosol (Figure 2).[27] Mutations observed in p97/VCP and the factors impairing its functionality prevent the removal of these

**Table I.** IBMPFD-Related p97/VCP Mutations. The Amino Acid, Base Change, and Location on the Protein Domain Observed in 28 Mutations Associated with IBMPFD Are Represented in the Table

| | Mutation | Location of Mutation on Protein Domain | Base Changes | References |
|---|---|---|---|---|
| 1 | I27V | N-terminal | 79A>G | 29, 30, 31 |
| 2 | V87F | N-terminal | 259G>T | 32 |
| 3 | R93C | N-terminal | 277C>T | 9, 33 |
| 4 | R95G | N-terminal | 283C>G | 34, 35 |
| 5 | R95C | N-terminal | 283C>T | 36 |
| 6 | G97E | N-terminal | 290G>A | 37, 38 |
| 7 | I126F | N-terminal | 376A>T | 39 |
| 8 | P137L | N-terminal | 410C>T | 2, 40 |
| 9 | R155S | N-terminal | 463C>A | 40 |
| 10 | R155L | N-terminal | 464G>T | 41 |
| 11 | R155H | N-terminal | 464G>A | 9, 34, 35, 36, 41 |
| 12 | R155C | N-terminal | 463C>T | 34, 41 |
| 13 | R155P | N-terminal | 464G>C | 34 |
| 14 | G156S | N-terminal | 466G>A | 41 |
| 15 | G157R | N-terminal | 469G>C<br>469G>A | 40, 41 |
| 16 | R159C | N-terminal | 475C>T | 41 |
| 17 | R159H | N-terminal | 476G>A | 41 |
| 18 | R191Q | N-D1 linker | 572G>A | 34, 41 |
| 19 | L198W | N-D1 linker | 593T>G | 34, 41 |
| 20 | G202W | N-D1 linker | 604G>T | 41 |
| 21 | I206F | N-D1 linker | 616A>T | 41 |
| 22 | A232E | D1 domain | 695C>A | 41 |
| 23 | T262A | D1 domain | 784A>G | 41 |
| 24 | N387H | D1 domain | 1159A>C | 34 |
| 25 | N387S | D1 domain | 1160A>G | 41 |
| 26 | A439G | D1 domain | 1316C>G | 41 |
| 27 | A439S | D1 domain | 1315G>T | 40, 41 |
| 28 | A439P | D1 domain | 1315G>C | 41 |

unwanted proteins from the cells. Given that the protein aggregates accumulated in cells are caused by the molecular pathogenesis of IBMPFD, functional losses in p97/VCP will result in the accumulation of ubiquitinated proteins of cells, the formation of protein aggregates, and also the induction of ER stress. When these protein aggregates that cause cellular damage cannot be overcome, programmed cell death is triggered by increased cellular stress.[29] It is known that p97/VCP is associated with many functional disorders. Especially, increasing our knowledge about p97/VCP biology is important for understanding the role of pathogenesis in IBMPFD.

**Functional Role of p97/VCP in IBMPFD Pathogenesis: Molecular view**

The IBMPFD disease locus with multiple system disease was mapped at the p21-p12 locus of chromosome 9 and was associated with mutations observed in the p97/VCP gene. To date, the number of p97/VCP mutations associated with the pathogenesis of IBMPFD in patients has been identified as 28 (Table I).[2,29–41] However, the details of only a few of these mutations have been revealed in the molecular pathogenesis of IBMPFD.

The point mutations associated with IBMPFD were found in three different domains of p97/VCP.

The mutations that are I27V, V87F, R93C, R95C/G, G97E, I126F, P137L, R155H/P/C/S/L, G156S, G157R, and R159H/C in the N-terminal domain, R191Q, L198W, G202W, and I206F in the N-D1 link domain, and A232E, T262A, N387H/S, and A439G/P/S in the D1 ATPase domain were identified in IBMPFD patients. To date, 28 missense mutations associated with IBMPFD have been demonstrated in p97/VCP.[2,29–41] The most common point mutation in IBMPFD patients is the amino acid of arginine at position 155.[3,30,42] Fernández-Sáiz and Buchberger[11] found that IBMPFD-related R95G and R155H mutations lead to conformational changes in the N-terminal domain of p97/VCP, and thus weakened the interaction between the N and D1 domains. It has been reported that these mutations lead to changes in the interaction of p97/VCP with cofactors and are important in the pathology of IBMPFD. In 2007, Issacson et al. identified the interaction pattern between p97/VCP and Npl4, thereby using nuclear magnetic resonance spectroscopy methodology. Some point mutations associated with IBMPFD (R93, R95, and R155) have been reported to be located in the region where p97/VCP interacts with Npl4. As a result of these point mutations, it is shown that functional losses may occur in the ERAD pathway because of the difference or deterioration in the interaction pattern of the Ufd1-Npl4 cofactor complex and p97/VCP.[43] In the studies conducted with R95G mutation in patients, a double $\psi$ barrel structure of p97/VCP was found to be destructured.[34] In cell culture models, it was determined that R95G mutant caused a significant increase in ubiquitin conjugated protein levels. Functionally, these results suggest that the R95G mutant p97/VCP weakens proteasomal degradation of substrates molecules.[12] Furthermore, ubiquitinated proteins and transgenic IBMPFD mouse models were determined to accumulate in cell culture after the suppression of p97/VCP with RNA interference-mediated silencing or pharmacologically inhibition. Similar results have been obtained in

IBMPFD-related R95G, RI55H, LI98W, and A232E p97/VCP mutant protein expressing cells.[3] These results suggest that p97/VCP mutants lead to the disruption of functional ERAD in IBMPFD patients. In the protein structure studies, it was reported that mutations of RI55C, RI55H, and RI55P located at the N-terminus of p97/VCP disrupt the folding of four strands $\beta$ barrel protein of p97/VCP. In the studies conducted with RI9IQ mutant protein, deterioration has been found in the organization of the flexible binding region.[10] The LI98W mutation has been determined stoichiometrically to disrupt the normal movement of the N-terminal of p97/VCP. This mutation causes conformational change at the N-terminal of p97/VCP and leads to changes in protein interactions via this domain.[42] The A232E mutation has been reported to affect the $\alpha$5-helix structure in the a/b subdomains of DI involved in the ATPase domain of p97/VCP. This ATPase domain is responsible for the catalytic activity of p97/VCP and the formation of the hexameric protein form. Therefore, it suggests that the destructive effect of A232E mutation in the pathogenesis of IBMPFD has been stronger.[34]

In the myoblast cell culture (C2CI2) model, after the ectopic expression of RI55H or R95G mutant proteins, steady-state levels of mutant cystic fibrosis transmembrane conductance regulator CFTRΔF508 protein, which is degraded by targeting to the proteasome by ERAD, increased.[3] However, RI55H and A232E p97/VCP mutant proteins did not affect the proteasomal degradation of another well-known ERAD substrate CD3$\delta$.[12] These results suggest that all IBMPFD-associated p97/VCP mutations exhibit specific characteristic patterns in the ERAD or ubiquitin-proteasome system. Therefore, it is important to determine the mechanistic details of all the p97/VCP mutations associated with IBMPFD at the level of protein interactions.

Erzurumlu et al.[44] examined the effect of 12 IBMPFD-associated p97/VCP mutations, including R93C, R95G, PI37L, RI55C, GI57R, RI59C, RI9IQ, LI98W, A232E, T262A, N387H, and A439S on CFTRΔF508 and Tyrosinase C89R of ERAD substrates. The studies indicated that CFTRΔF508 and Tyrosinase C89R substrates accumulated in mutant p97/VCP-expressing cells compared to wild-type p97/VCP.[44] This result suggested that the deterioration of the ERAD pathway was the result of p97/VCP mutations and was a common feature of the molecular pathogenesis of IBMPFD. In the ectopic expression trial, the interaction patterns and subcellular localization of the PI37L mutant have been significantly altered. In the studies carried out with U2OS and C2CI2 cells, it was seen that PI37L mutant showed a profile of abundant small, discrete, and punctate cytoplasmic structures in cells unlike R95C and RI55C mutants. It was also determined that PI37L mutant passed to the insoluble protein phase compared to other p97/VCP mutant proteins. The ability of PI37L mutant to bind with ubiquitin was found to have largely lost. However, the interaction of R95G and RI55C mutants with ubiquitin was found to have enhanced.[44]gp78 is an E3 ubiquitin ligase enzyme located in the ER lumen, which is responsible for the ubiquitination of substrate molecules associated with ERAD. The studies have shown that p97/VCP interacts with the VIM motif of gp78.[45] Similar to the interactions of R95G and RI55C mutants with ubiquitin, their interaction with gp78 and cofactor proteins Ufdl-Npl4 have been reported to have enhanced. Moreover, the interaction of PI37L mutant with gp78 was found to be similar to wild type p97/VCP. It has also been reported that the ability of the PI37L mutant to interact with Ufdl was lost. Similar results were observed in p47 interaction, a well-known cofactor of p97/VCP. PI37L was found to have lost the ability to interact with p47.[44] Johnson et al. showed in vivo studies with RI55H, RI9IQ, and A232E that these mutant proteins disrupt the formation of lysosomal tubules. Impaired lysosomal tubular formation is associated with deformed autophagosome-lysosome fusion, increased cytoplasmic ubiquitin aggregates, damaged mitochondria, and impaired muscle function.[46] In another study, PI37L was reported to be the target of the autophagic process. PI37L was selectively degraded by autophagy due to lysosomal activity under basal cellular conditions. In a study carried out with U2OS and PC-12 cells, PI37L was found to stimulate autophagosome and autolysosome formations. At the same time, this study determined that protein aggregates were created by GI57R mutant-induced autophagy and were eliminated by autophagic degradation. These results suggest that some p97/VCP mutants are a target of the autophagic destruction pathway.[47]

Although the effects of some p97/VCP mutations have been identified in the pathophysiology of IBMPFD at molecular levels, the vast majority of these p97/VCP mutations remain unknown.

## METHODS

A literature review was conducted concerning studies about IBMPFD, p97/VCP, and its mutations related to IBMPFD pathogenesis, and also underlying details of molecular pathogenesis mechanism. The current IBMPFD related mutations and their association with p97/VCP were searched by using National Center for Biotechnology Information (NCBI) and Online Mendelian Inheritance in Man (OMIM) databases.

## CONCLUSION

IBMPFD is a multiple system disease, which three different pathologies are observed simultaneously. The underlying cause of IBMPFD is the mutations observed in the gene called VCP. In the light of the above-mentioned literature, it is clear that p97/VCP mutations associated with IBMPFD promote the pathogenesis process through a variety of molecular properties in cells. Characterizing all interaction patterns of p97/VCP by means of mechanistically approaches is important in order to understand the mechanisms underlying the p97/VCP mutation, which is associated with IBMPFD pathogenesis in patients, and develop new generation treatment approaches.

**Informed Consent:** N/A

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - Y.E.; Design - Y.E.; Supervision - Y.E.; Data collection and processing - Y.E.; Analysis and interpretation - Y.E.; Literature review - Y.E.; Writer - Y.E.; Critical review - Y.E.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES

1. Mengel D, Librizzi D, Schoser B, et al. Inclusion body myopathy, Paget's disease, and fronto-temporal dementia: A VCP-related

multi-systemic proteinopathy. *Fortschr Neurol Psychiatr.* 2018;86(7): 434-438.
2. Palmio J, Sandell S, Suominen T, et al. Distinct distal myopathy phenotype caused by VCP gene mutation in a Finnish family. *Neuromuscul Disord.* 2011;21(8):551-555. [CrossRef]
3. Ju JS, Weihl CC. Inclusion body myopathy, Paget's disease of the bone and fronto-temporal dementia: A disorder of autophagy. *Hum Mol Genet.* 2010;19(1):38-45.
4. Kimonis V. 2019. *Inclusion Body Myopathy with Paget Disease of Bone and/or Frontotemporal Dementia.* Seattle, WA: GeneReviews®.
5. Mérai Z, Chumak N, García-Aguilar M, et al. The AAA-ATPase molecular chaperone Cdc48/p97 disassembles sumoylated centromeres, decondenses heterochromatin, and activates ribosomal RNA genes. *Proc Natl Acad Sci USA.* 2014;111(45):16166-16171. [CrossRef]
6. Panginikkod S, Musa R. *Inclusion Body Myositis.* Treasure Island, FL, StatPearls, 2019.
7. Rodriguez-Ortiz CJ, Hoshino H, Cheng D, et al. Neuronal-specific overexpression of a mutant valosin-containing protein associated with IBMPFD promotes aberrant ubiquitin and TDP-43 accumulation and cognitive dysfunction in transgenic mice. *Am J Pathol.* 2013;183(2):504-515. [CrossRef]
8. Yi L, Donsante A, Kennerson ML, et al. Altered intracellular localization and valosin-containing protein (p97 VCP) interaction underlie ATP7A-related distal motor neuropathy. *Hum Mol Genet.* 2012;21(8):1794-1807. [CrossRef]
9. Hübbers CU, Clemen CS, Kesper K, et al. Pathological consequences of VCP mutations on human striated muscle. *Brain.* 2007;130(2):381-393. [CrossRef]
10. Al-Obeidi E, Al-Tahan S, Surampalli A, et al. Genotype-phenotype study in patients with valosin-containing protein mutations associated with multisystem proteinopathy. *Clin Genet.* 2018;93(1):119-125. [CrossRef]
11. Fernández-Sáiz V, Buchberger A. Imbalances in p97 co-factor interactions in human proteinopathy. *EMBO Rep.* 2010;11(6):479-485. [CrossRef]
12. Guo X, Zhao Z, Shen H, et al. VCP myopathy: A family with unusual clinical manifestations. *Muscle Nerve.* 2019;59(3):365-369. [CrossRef]
13. Janiesch PC, Kim J, Mouysset J, et al. The ubiquitin-selective chaperone CDC-48/p97 links myosin assembly to human myopathy. *Nat Cell Biol.* 2007;9(4):379-390. [CrossRef]
14. Ju JS, Miller SE, Hanson PI, et al. Impaired protein aggregate handling and clearance underlie the pathogenesis of p97/VCP-associated disease. *J Biol Chem.* 2008;283(44):30289-30299. [CrossRef]
15. Erzurumlu Y, Ilhan R, Gozen O, et al. VCP (valosin containing protein). *Atlas Genet Cytogenet Oncol Haematol.* 2013;17(8):550-556.
16. Hirabayashi M, Inoue K, Tanaka K, et al. VCP/p97 in abnormal protein aggregates, cytoplasmic vacuoles, and cell death, phenotypes relevant to neurodegeneration. *Cell Death Differ.* 2001;8(10):977-984. [CrossRef]
17. Woodman PG. p97, a protein coping with multiple identities. *J Cell Sci.* 2003;116:4283-4290. [CrossRef]
18. Al-Tahan S, Al-Obeidi E, Yoshioka H, et al. Novel valosin-containing protein mutations associated with multisystem proteinopathy. *Neuromuscul Disord.* 2018;28(6):491-501. [CrossRef]
19. Bodnar NO, Kim KH, Ji Z, et al. Structure of the Cdc48 ATPase with its ubiquitin-binding cofactor Ufd1-Npl4. *Nat Struct Mol Biol.* 2018;25(7):616-622. [CrossRef]
20. Huang R, Ripstein ZA, Rubinstein JL, et al. Cooperative subunit dynamics modulate p97 function. *Proc Natl Acad Sci USA.* 2019;116(1):158-167. [CrossRef]
21. Haines DS. p97-containing complexes in proliferation control and cancer: Emerging culprits or guilt by association? *Genes Cancer.* 2010;1(7):753-763. [CrossRef]
22. Lucente G, Almendrote M, Ramos-Fransi A, et al. A novel mutation in the valosin-containing-protein gene found in a Spanish family. *J Neurol Sci.* 2018;391:112-113. [CrossRef]
23. Zhang X, Gui L, Zhang X, et al. Altered cofactor regulation with disease-associated p97/VCP mutations. *Proc Natl Acad Sci USA.* 2015;112(14):E1705-E1714. [CrossRef]
24. Bulfer SL, Chou TF, Arkin MR. p97 disease mutations modulate nucleotide-induced conformation to alter protein-protein interactions. *ACS Chem Biol.* 2016;11(8):2112-2116. [CrossRef]
25. DeLaBarre B. Central pore residues mediate the p97/VCP activity required for ERAD. *Mol Cell.* 2006;22:451-462. [CrossRef]
26. Nishimura N, Radwan MO, Amano M, et al. Novel p97/VCP inhibitor induces endoplasmic reticulum stress and apoptosis in both bortezomib-sensitive and -resistant multiple myeloma cells. *Cancer Sci.* 2019;110(10):3275-3287. [CrossRef]
27. Liu Y, Ye Y. Roles of p97-associated deubiquitinases in protein quality control at the endoplasmic reticulum. *Curr Protein Pept Sci.* 2012;13(5):436-446. [CrossRef]
28. Araki K, Nagata K. Protein folding and quality control in the ER. *Cold Spring Harb Perspect Biol.* 2011;3(11):a007526-a007526.
29. Weihl CC, Dalal S, Pestronk A, et al. Inclusion body myopathy-associated mutations in p97/VCP impair endoplasmic reticulum-associated degradation. *Hum Mol Genet.* 2006;15(2):189-199. [CrossRef]
30. Rohrer JD, Warren JD, Reiman D, et al. A novel exon 2 I27V VCP variant is associated with dissimilar clinical syndromes. *J Neurol.* 2011;258(8):1494-1496. [CrossRef]
31. Majounie E, Traynor BJ, Chio? A, et al. Mutational analysis of the VCP gene in Parkinson's disease. *Neurobiol Aging.* 2012;33(209):e201-209.202.
32. Inoue M, Lida A, Hayashi S, et al. Two novel *VCP* missense variants identified in Japanese patients with multisystem proteinopathy. *Hum Genome Var.* 2018;5:9. [CrossRef]
33. Guyant-Maréchal L, Laquerriere A, Duyckae?rts C, et al. Valosin-containing protein gene mutations: Clinical and neuropathologic features. *Neurology.* 2006;67:644-651. [CrossRef]
34. Watts GD, Wymer J, Kovach MJ, et al. Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat Genet.* 2004;36:377-381. [Database] [CrossRef]
35. Kimonis VE, Mehta SG, Fulchiero EC, et al. Clinical studies in familial VCP myopathy associated with Paget disease of bone and frontotemporal dementia. *Am J Med Genet.* 2008b;146A:745-757. [CrossRef]
36. Kimonis VE, Fulchiero E, Vesa J, et al. VCP disease associated with myopathy, Paget disease of bone and frontotemporal dementia: Review of a unique disorder. *Biochim Biophys Acta.* 2008a;1782:744-748. [CrossRef]
37. Gu JM, Ke YH, Yue H, et al. A novel VCP mutation as the cause of atypical IBMPFD in a Chinese family. *Bone.* 2013;52:9-16. [CrossRef]
38. Jerath NU, Crockett CD, Moore SA, et al. Rare manifestation of a c.290 C>T, p.Gly97Glu VCP mutation. *Case Rep Genet.* 2015;2015:1-5.
39. Matsubara S, Shimizu T, Komori T, et al. Nuclear inclusions mimicking poly(a)-binding protein nuclear 1 inclusions in a case of inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia with a novel mutation in the valosin-containing protein gene. *Neuromuscul Disord.* 2016;26:436-440. [CrossRef]
40. Stojkovic T, Hammouda el H, Richard P, et al. Clinical outcome in 19 French and Spanish patients with Valosin-containing protein

myopathy associated with Paget's disease of bone and fronto-temporal dementia. *Neuromuscul Disord*. 2009;19:316-323. [CrossRef]
41. Kwan Tang W, Xia D. Mutations in the human AAA+ chaperone p97 and related diseases. *Front Mol Biosci*. 2016;3:79.
42. Watts GD, Thomasova D, Ramdeen SK, et al. Novel VCP mutations in inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia. *Clin Genet*. 2007;72(5):420-426. [CrossRef]
43. Issacson RL, Pye VE, Simpson P, et al. Detailed structural insights into the p97-Npl4-Ufd1 interface. *J Biol Chem*. 2007;282(29):21361-21369. [CrossRef]
44. Erzurumlu Y, Kose FA, Gozen O, et al. A unique IBMPFD-related P97/VCP mutation with differential binding pattern and subcellular localization. *Int J Biochem Cell Biol*. 2013;45(4):773-782. [CrossRef]
45. Wang Y, Ballar P, Zhong Y, et al. SVIP induces localization of p97/VCP to the plasma and lysosomal membranes and regulates autophagy. *PLoS One*. 2011;6(8):e24478. [CrossRef]
46. Johnson AE, Shu H, Hauswirth AG, et al. VCP-dependent muscle degeneration is linked to defects in a dynamic tubular lysosomal network in vivo. *Elife*. 2015;4:07366.
47. Bayraktar O, Oral O, Kocaturk NM, et al. IBMPFD disease-causing mutant VCP/p97 proteins are targets of autophagic-lysosomal degradation. *PLoS One*. 2016;11(10):e0164864. [CrossRef]

CYPRUS JOURNAL OF MEDICAL SCIENCES

Review

DOI: 10.5152/cjms.2021.1780

# Diagnostic Difficulties in the Natural Rubber Latex Allergy

**Mehmet Ünsel**

**Department of Internal Medicine, Near East University Hospital, Division of Allergy and Immunology, Nicosia, Cyprus**

**ORCID iD of the author: U.M. 0000-0003-2152-3786.**

**Cite this article as: Mehmet U. Diagnostic Difficulties in the Natural Rubber Latex Allergy. *Cyprus J Med Sci.* 2021; 6(4): 345-348.**

Natural rubber latex (NRL) allergy is a serious illness that presents itself with a spectrum of clinical signs and symptoms, including urticaria, allergic rhinitis, asthma, and anaphylaxis. NRL allergy is more commonly encountered among healthcare professionals and patients who undergo frequent surgical procedures because of sipina bifida or urogenital malformation. The ratio may rise to as high as 30% among children who have sipina bifida, while NRL allergy prevalence is just about 5% in healthcare professionals. Moreover, NRL allergy is the second most important cause of perioperative anaphylaxis. In routine practice, diagnosis of NRL allergy is based on specific IgE analysis and skin prick test. Nevertheless, not only is the diagnostic value of each one alone reaches 100%, but also false positive test results are encountered. The precision of the diagnostic methods used for NRL allergy is important because false positive IgE results may negatively influence the patients' quality of life due to the stringent measures required to ensure latex-free environment. For this reason, the search for diagnostic methods, which would confer more sensitive and specific results for a precise diagnosis, has been intensified in the last two decades, and for this purpose, recently recombinant NRL allergens are being used for the diagnosis of NRL allergy. In this article, performances of old and new diagnostic methods used in NRL allergy and what we gained from recombinant NRL allergens have been discussed.

**Keywords:** Natural rubber latex allergy, diagnosis, skin prick test, in vitro tests, provocation test

## INTRODUCTION

Natural rubber latex (NRL) is the sap of the Hevea brasiliensis tree. It is cheap and provides elasticity, durability, and protection to the materials for which it is produced; therefore, it is particularly used in the production of various medical equipment commonly used in the field of health care such as latex gloves, sphygmomanometer muff, branule, and hot water bag. Outside of medical sector, NRL is also used in the production of many goods, such as toys, balloons, feeding bottles, and preventatives, which are used commonly. Due to the widespread use of NRL in the field of health, primarily in healthcare professionals and in patients who undergo frequent surgical procedures connected to sipina bifida and urogenital malformation that cause intense NRL exposure, NRL allergy is more commonly encountered. According to the results of 11 epidemiological research that were conducted using skin prick test (SPT) and/or specific igE analysis, NRL allergy prevalence has been reported as 5.1% in healthcare professionals.[1] The ratio is higher and can even reach as high as 30% in children who have spina bifina.[2] In general public, NRL allergy prevalence is under 1%.[3]

### NRL Allergens

Up until today, 15 latex allergens have been identified (Hev b 1-15). Out of these, Hev b1, 3, 5, and 6 are major, the rest are minor allergens. Hev 1 and 3 in children who have spina bifida and Hev b 5 and 6 in healthcare professionals are the major allergens that are responsible for this kind of allergy.[1]

### Clinical Manifestations of NRL Allergy

NRL causes contact urticaria with skin contact, and allergic rhinitis and/or asthma via inhalation of airborne particles of the powdered latex gloves and anaphylaxis with mucosal (oral, vaginal, and gastrointestinal) or direct contact of inner organs during intraoperative period. The second most common cause of perioperative anaphylaxis is NRL allergy.[4] For the patients who are diagnosed with NRL allergy, their contact with the entire group of products which contain NRL must be avoided for lifelong. Otolaryngological and gynecological examinations that involve direct mucosal contact and

**Corresponding Author:** Unsel Mehmet
**E-mail:** unselmehmet@yahoo.com

**Received:** 12.02.2020
**Accepted:** 04.03.2020

dental treatments, due to risk of anaphylaxis, must be performed with nonlatex gloves. For all surgical procedures of these patients, latex-free hospitals and operating rooms must be provided.

### Diagnosis of NRL Allergy

In routine practice, diagnosis of NRL allergy is based on specific IgE analysis and SPT. Nevertheless, not only the diagnostic value of merely these methods alone is not 100%, but also false positive test results are reported.[2,5] The precision of the diagnostic methods used for NRL allergy is of great importance because false positive IgE results may negatively influence the patients' quality of life due to the stringent measures required to ensure latex-free environment. That is why the diagnostic methods that would be more sensitive and specific for precise diagnosis have been intensified in the last two decades.

### Clinical History

Although clinical history has great importance in diagnosis of NRL allergy, its specificity and predictive values alone are low. In a research studying the value of clinical history in diagnosis of NRL allergy with specific bronchial provocation test, 87% of sensitivity, 14% of specificity, 75% of positive predictive value (PPV), and 50% of negative predictive value (NPV) were determined. In the same study, when the positivity of both clinical history and SPT was accepted together as diagnostic criteria, sensitivity, specificity, and NPV increased (sensitivity of 100%, specificity of 36%, PPV of 76%, and NPV of 71%).[6] Also, in the study conducted by Quirce et al.[7] who utilized bronchial provocation test with NRL, diagnostic value of clinical history (sensitivity of 89%, specificity of 50%, PPV of 77%, and NPV of 71%) was found similar.

**Main Points**

- NRL allergy is a serious illness that presents with a spectrum of clinical signs, including urticaria, allergic rhinitis, asthma, and even anaphylaxis.
- It is more commonly reported among healthcare professionals and patients who undergo frequent surgery.
- The diagnosis of NRL allergy is based on specific IgE analysis and SPT in routine practice. Nevertheless, not only is the diagnostic value of each one alone reaches 100%, but also false positive or negative results are encountered. Provocation tests should be used in patients whose diagnostic test results and clinical history are incompatible.
- Because of conjunctival, nasal, and bronchial provocation tests that have the risk of systemic allergic reaction, new in vitro tests including BAT and specific IgE analysis with recombinant NRL allergens have been started to use in the diagnosis of NRL allergy.
- SPT is still a more sensitive method than all the other in vitro tests in NRL allergy diagnosis. In clinical practice, the combined positivity of history and SPT seems enough in diagnosing NRL allergy most of the time. When clinical history and SPT are incompatible, specific IgE analysis or BAT with recombinant NRL allergens can give reliable results.

### Skin Prick Test

The SPT sensitivity against NRL varies between 65 and 100%. The difference in diagnostic criteria in NRL allergy as well as different allergen extract use might account for the wide-range in SPT sensitivity percentages. In 33 healthcare professionals with allergy history who were exposed to NRL, when NRL-specific IgE positivity (ImmunoCAP, Phadia, Uppsala, Sweden) was accepted as diagnosis criterion, the sensitivity, specificity, PPV, and NPV of SPT performed with five different NRL extracts ranged between 66 and 88%, 91 and 95%, 75 and 88%, and 88 and 95%, respectively.[8] In 42 pediatric patients who displayed suspicion of allergy when the glove use test (provocation test) positivity was accepted as diagnostic criteria, the sensitivity and specificity of SPT performed with three different NRL extracts ranged between 65 and 96% and 88 and 94%, respectively.[9] Similarly, in two other studies in which bronchial provocation test with NRL was accepted as diagnostic criterion, the sensitivity and NPV of SPT were determined as 100%, while specificity and PPV were determined as 20 vs 21% and 70 vs 74%, respectively.[6,7] As a result, when a credible testing such as the bronchial provocation test was accepted as diagnostic criterion, SPT sensitivity with NRL increased; however, specificity and PPV decreased. The reduced specificity and PPV of SPT performed with NRL point out that SPT may yield false positive results in some cases. Although not often, asymptomatic cases might be seen who show positive SPT with NRL. In such cases, provocation methods are warranted to obtain a precise diagnosis. Nevertheless, conducting a qualified provocation tests is not possible based on the facts that not only are these tests are cumbersome, but also test materials are not commercially available. In our previous nasal provocation test (NPT) study in which NRL extract (500 μg/mL NRL protein, ALK-Abello, Madrid, Spain) was used, NPT was found negative in two out of 26 patients who showed positive SPT with NRL. Those two nonhealthcare professional patients had polen allergy, yet they did not have NRL allergy history. Thereby, those two cases were accepted as false positive. It was suggested that this condition may have developed as a cross reaction due to pollen allergy.[10]

### In Vitro Tests

**Specific IgE analysis.** Up until today, various in vitro methods (ImmunoCAP, DPC, AlaSTAT, and Hycor HyTEC) that research specific IgE presence to NRL were applied. The most commonly used one among those is ImmunoCAP (Uppsala, Sweden). Hamilton et al.[11] compared three different latex-specific IgE analyses (ImmunoCAP, DPC AlaSTAT, and Hycor HyTEC). In this study, although the assay gave similar results, the best performance (sensitivity of 76.3%, specificity of 96.7%, PPV of 94.3%, and NPV of 85%) was obtained with immunoCAP when a positive SPT was accepted as the gold standard.[11] Ownby et al.[12] demonstrated that ImmunoCAP had a sensitivity of 79.5%, specificity of 90.2%, PPV of 91.7%, and NPV of 76.4% when concomitant positivity of history and SPT was accepted as the gold standard. In our study, when taking the positivity of NPT and SPT together as the diagnostic criteria, we determined the sensitivity of immunoCAP k82 of 90%, specificity of 72.2%, NPV of 96.3%, and PPV of 50%.[13] These three studies exhibited that the diagnostic value of NRL-specific IgE analyze was lower compared to SPT. Particularly, in patients who have pollen allergy, probability of false positivity of ImmunoCAP k82 can rise up to 30%.[10,13] In these patients, profilins and cross-reactive carbohydrate determinants (CCD) are responsible for

nonclinical-related NRL specific IgE positivity.[14] Hence, in patients allergic to pollen, due to high ratios of false positivity, ImmunoCAP k82 is not a convenient diagnostic method and must not be used in NRL prevalence studies.

Specific IgE analysis with NRL merely shows sensitization and cannot distinguish true NRL allergy or cross-reactivity. This distinction can be performed by means of specific IgE analyses with recombinant NRL allergens and CCD. Because of this, studies that research the diagnostic value of specific IgE analyses by using recombinant NRL allergens have accelerated in recent years. Recombinant NRL allergens and specific IgE analyses are studied with the methods of ImmunoCAP and Immuno Solid-phase allergen Chip (ISAC). ImmunoCAP carries out specific IgE analysis for each recombinant NRL allergen (Hevb 1, 3, 5, 6.01, 6.02, 8, 9, and 11), whereas ISAC (Phadia, Uppsala, Sweden) searches specific IgE response to 103 native/recombinant allergens, in which five recombinant NRL allergens (Hevb 1, 3, 5, 6.01, and 8) are included. Vandenplas et al.[15] studied responses of specific IgE (ImmunoCAP) to 12 recombinant NRL allergens (Hev b, 1, 3, 5, 6.01, 6.02, 7-12, and 15) in 82 patients who had occupational asthma and positive specific inhalation challenges (SIC) and SIC negative 25 cases encompassing the control group. In the patient group, Hev b 5 of 63%, Hev b 6.01 of 78%, and Hev b 6.02 of 78% were determined as positive, while in the control group, Hev b 5 of 12%, Hev b 6.01 of 32%, and Hev b 6.02 of 24% were determined as positive.[15] Ott et al.[16] found diagnostic performance of ImmunoCAP and ISAC similar in 52 NRL allergic and 50 venom allergic patients (for Hev b 5 and 6.02 in ImmunoCAP sensitivity of 50 and 71%, specificity of 100 and 100%, PPV of 100 and 100%, and NPV of 66 and 77%, and in ISAC, sensitivity of 44 and 69%, specificity of 100 and 100%, PPV of 100 and 100%, and NPV of 63 and 76%, respectively). In Vandenplas' study and also in other similar studies, sensitivity and NPV of specific IgE with recombinant NRL allergens were found below 80%.[3,15–18] Besides this, as seen in Vandenplas' study, specific IgE with major allergens such as Hev b 5 and 6 may be determined as positive also in healthy individuals. Following the determination of major recombinant allergens, recombinant allergen NRL Hev b 5 was added to this test kit (Hev b 5-amplified ImmunoCAP k82) to increase diagnostic value of ImmunoCAP k82. Seyfart et al.[18] found sensitivity of Hev b 5-amplified ImmunoCAP k82 more superior than nonamplified ImmunoCAP k82 and ISAC (70, 62.5, and 55%, respectively) in 40 patients who have NRL allergy. As seen in all the studies, sensitivity and NPV of specific IgE analyses (ImmunoCAP and ISAC) with recombinant allergens were determined lower compared to that of ImmunoCAP k82. Despite that, in individuals who have pollen allergy and especially multiple pollen allergy, ratio of false positivity of ImmunoCAP k82 is high. Because significant part (average 40%) of NRL allergic patients is atopic and a significant part (21–60%) of those is pollen sensitive, ImmunoCAP k82 is not a dependable diagnostic method in this patient group.[13,14,19] Therefore, in patients with ImmunoCAP k82 positive who have not NRL allergy history, Hev b 1, 3, 5, 6, 8 (NRL profilin), and CCD specific IgE must be searched in order to determine true NRL allergy or cross-reactivity. Without having major allergen positivity, Hev b 8 and/or CCD specific IgE being positive points out clinically irrelevant sensitization.

**Basophil activation test.** Given the fact that the sensitivity of specific IgE analyses performed with recombinant NRL allergens being lower compared to SPT, specific IgE analysis with NRL not being superior to SPT and due to systemic allergic reaction risk that may develop in provocation tests, basophil activation test (BAT) has been used recently. BAT is a flow cytometric method on measurement of activation markers (CD63 and/or CD203c) that appear on the surface of basophil as a result of basophiles incubation with NRL in vitro environment. Sans et al.[20] determined the sensitivity, specificity, PPV, and NPV of BAT carried out with NRL extract in 43 patients who had NRL allergy as being 90.5, 100, 100, and 100%, respectively. Same researcher, in his later study based on BAT performed with NRL extract and recombinant NRL allergens (Hev b 5, 6, and 6.01) in 23 children who had NRL allergy showed that BAT performed with NRL extract yielded better results (95.6% vs 86%). In the same study when ImmunoCAP and BAT with recombinant NRL allergens used concomitantly, sensitivity (of 95.6%) did not increase compared to the BAT performed with NRL extract.[19] Later, the study enrolling 22 patients who had NRL allergy determined the sensitivity of the BAT with NRL extract below 80%.[17] This study showed that BAT gave better results than specific IgE analysis (ISAC) with recombinant NRL allergens (Hev b 1, 3, 5, and 6).

**Provocation Tests**

In cases when in vitro and in vivo (SPT) diagnostic methods do not be compatible with clinical history or in the setting of negative SPT in spite of positive in vitro test result, provocation tests should be used in order to differentiate asymptomatic sensitization from true NRL allergy. In provocation tests, skin and mucosas (sublingual, conjunctival, nasal, and bronchial) are exposed to NRL and manifesting symptoms and results are scored. Therefore, it is time consuming compared to in vitro tests and poses a risk of systemic allergic reaction. Besides, medications such as steroid, antihistamine, and broncodilatator must be stopped at certain periods prior to the provocation test. There are only a few studies about provocation tests due to difficulties of the procedure, risk of systemic allergic reaction, and absence of standardized commercial kits.[10,21] We successfully applied NPT with NRL extract (500 $\mu g\ mL^{-1}$, ALK-Abello, Madrid, Spain) for the first time in 26 patients who had positive SPT with NRL, 35 atopic, and 30 healthy control subjects, and we did not observe systemic allergic reaction during NPT. Also, we compared the diagnostic performance of NPT versus glove usage test (GUT) with powdered latex gloves and showed that NPT is a more sensitive method compared with GUT (sensitivity of 96 vs 81%, specificity of 100 vs 90%, NPV of 98 vs 75%, and PPV of 100 vs 93%, respectively). Nucera et al.[21] applied GUT, sublingual, conjunctival, nasal, and bronchial provocation tests with NRL extract (500 $\mu g\ mL^{-1}$, ALK-Abello, Madrid, Spain) in 40 patients who had NRL allergy and 20 healthy control subjects. While sensitivity of oral and sublingual provocation test was determined as lower than 50%, sensitivity of GUT, conjunctival, nasal, and bronchial provocation tests was determined above 70%. Sensitivity of bronchial and NPT was even higher (76% and 82%, respectively). While the specificity and PPV of all the tests were 100%, NPV of the NPT and bronchial provocation tests were determined higher (75% and 70%, respectively). In this study, systemic allergic reaction was not observed in any patient during provocation test. According to the results of both studies, NPT seems as a more reliable provocation method. When the difficulties are taken into consideration in other provocation test procedures, GUT whose sensitivity and specificity is close to bronchial and NPT appears as a useful and simple provocation method.[10]

In summary, SPT is still a more sensitive method than all the other in vitro tests in NRL allergy diagnosis. In clinical practice, the combined positivity of history and SPT seems enough in diagnosing NRL allergy most of the time. Yet, in the setting of an absence of pollen allergy and when clinical history and SPT are incompatible, specific IgE analysis with NRL extract (ImmunoCAP) can give reliable results. In patients with pollen allergy, especially those presenting multiple pollen sensitization, specific IgE analysis (ImmunoCAP) with recombinant NRL allergens (Hev b 1, 3, 5, 6, and 8) and CCD will be useful. Due to the fact that sensitivity of specific IgE analysis with recombinant NRL allergens is below 80% when specific IgE with recombinant NRL allergens is found to be negative, BAT known as a more sensitive method should be performed with NRL extract or recombinant allergens. Since recombinant NRL allergens and BAT have been used in the diagnosis of NRL allergy, conjunctival, nasal, and bronchial provocation tests have lost their importance in clinical practice. GUT, which is simple and does not require a device, can be helpful in determining true NRL allergic patients in clinical practice. In the future, nasal or bronchial provocation tests can only be used in scientific studies for the purpose of researching diagnostic performance of in vitro tests. Due to the probability of risk of systemic allergic reaction and difficulties in application, nasal and bronchial provocation tests might be replaced by BAT, which sensitivity, specificity, and predictive values are high. Besides, taking into consideration of ImmunoCAP k82 can give high false positive results in pollen sensitive patients in particular, SPT should be used in NRL allergy prevalence studies.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - U.M.; Resources - U.M.; Materials - U.M.; Data Collection and/or Processing - U.M.; Analysis and/or Interpretation - U.M.; Literature Search - U.M.; Writing Manuscript - U.M.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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

## REFERENCES

1. Vandenplas O, Raulf M. Occupational latex allergy: The current state of affairs. *Curr Allergy Asthma Rep.* 2017;17(3):14. [CrossRef]
2. Pires G, Morais-Almeida M, Gaspar A, et al. Risk factors for latex sensitization in children with spina bifida. *Allergol Immunopathol (Madr).* 2002;30(1):5-13. [CrossRef]
3. Mari A, Scala E, D'Ambrosio C, Breiteneder H, Wagner S. Latex allergy within a cohort of not-at-risk subjects with respiratory symptoms: Prevalence of latex sensitization and assessment of diagnostic tools. *Int Arch Allergy Immunol.* 2007;143(2):135-143. [CrossRef]
4. Ebo DG, Fisher MM, Hagendorens MM, Bridts CH, Stevens WJ. Anaphylaxis during anaesthesia: Diagnostic approach. *Allergy.* 2007;62(5):471-487. [CrossRef]
5. Rendeli C, Nucera E, Ausili E, et al. Latex sensitisation and allergy in children with myelomeningocele. *Childs Nerv Syst.* 2006;22(1):28-32. [CrossRef]
6. Vandenplas O, Binard-Van Cangh F, Brumagne A, et al. Occupational asthma in symptomatic workers exposed to natural rubber latex: Evaluation of diagnostic procedures. *J Allergy Clin Immunol.* 2001;107(3):542-547. [CrossRef]
7. Quirce S, Swanson MC, Fernández-Nieto M, de las Heras M, Cuesta J, Sastre J. Quantified environmental challenge with absorbable dusting powder aerosol from natural rubber latex gloves. *J Allergy Clin Immunol.* 2003;111(4):788-794. [CrossRef]
8. van Kampen V, de Blay F, Folletti I, et al. Evaluation of commercial skin prick test solutions for selected occupational allergens. *Allergy.* 2013;68(5):651-658. [CrossRef]
9. Bernardini R, Pucci N, Azzari C, Novembre E, De Martino M, Milani M. Sensitivity and specificity of different skin prick tests with latex extracts in pediatric patients with suspected natural rubber latex allergy—A cohort study. *Pediatr Allergy Immunol.* 2008;19(4):315-318. [CrossRef]
10. Unsel M, Mete N, Ardeniz O, et al. The importance of nasal provocation test in the diagnosis of natural rubber latex allergy. *Allergy.* 2009;64(6):862-867. [CrossRef]
11. Hamilton RG, Biagini RE, Krieg EF. Diagnostic performance of food and drug administration-cleared serologic assays for natural rubber latex-specific IgE antibody. The multi-center latex skin testing study task force. *J Allergy Clin Immunol.* 1999;103(5 Pt 1):925-930. [CrossRef]
12. Ownby DR, Magera B, Williams PB. A blinded, multi-center evaluation of two commercial in vitro tests for latex-specific IgE antibodies. *Ann Allergy Asthma Immunol.* 2000;84(2):193-196. [CrossRef]
13. Unsel M, Mete N, Ardeniz O, Sin A, Gülbahar O, Kokuludağ A. Diagnostic value of specific IgE analysis in latex allergy. *Int Arch Allergy Immunol.* 2012;158(3):281-287. [CrossRef]
14. Gürlek F, Ünsel M, Ardeniz O, et al. Misleading allergens in the diagnosis of latex allergy: Profilin and cross-reactive carbohydrate determinants. *Int Arch Allergy Immunol.* 2018;176(1):1-7. [CrossRef]
15. Vandenplas O, Froidure A, Meurer U, et al. The role of allergen components for the diagnosis of latex-induced occupational asthma. *Allergy.* 2016;71(6):840-849. [CrossRef]
16. Ott H, Schröder C, Raulf-Heimsoth M, et al. Microarrays of recombinant Hevea brasiliensis proteins: A novel tool for the component-resolved diagnosis of natural rubber latex allergy. *J Investig Allergol Clin Immunol.* 2010;20(2):129-138.
17. Ebo DG, Hagendorens MM, De Knop KJ, et al. Component-resolved diagnosis from latex allergy by microarray. *Clin Exp Allergy.* 2010;40(2):348-358. [CrossRef]
18. Seyfarth F, Schliemann S, Wiegand C, Hipler UC, Elsner P. Diagnostic value of the ISAC(®) allergy chip in detecting latex sensitizations. *Int Arch Occup Environ Health.* 2014;87(7):775-781. [CrossRef]
19. Sanz ML, García-Avilés MC, Tabar AI, et al. Basophil activation test and specific IgE measurements using a panel of recombinant natural rubber latex allergens to determine the latex allergen sensitization profile in children. *Pediatr Allergy Immunol.* 2006;17(2):148-156. [CrossRef]
20. Sanz ML, Gamboa PM, García-Avilés C, et al. Flow-cytometric cellular allergen stimulation test in latex allergy. *Int Arch Allergy Immunol.* 2003;130(1):33-39. [CrossRef]
21. Nucera E, Pollastrini E, Sabato V, et al. Challenge tests in the diagnosis of latex allergy. *Int J Immunopathol Pharmacol.* 2010;23(2):543-552. [CrossRef]