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Journal of Academic Research in Medicine-JAREM, yayın dili Türkçe-İngilizce olan, açık erişimli, bağımsız ve önyargısız çift-kör hakemlik prosedürlerine bağlı olarak yayın yapan uluslararası bir dergidir. Dergide deneysel ve klinik tıp alanlarında yapılan araştırmalar, güncel konularla ilgili derlemeler, editöre mektuplar ve tıp eğitimiyle ilgili yazılar yayınlanır. Dergi, Nisan, Ağustos ve Aralık aylarında olmak üzere yılda 3 sayı yayınlanmaktadır. Derginin finansmanı Sağlık Bilimleri Üniversitesi Gaziosmanpaşa Eğitim ve Araştırma Hastanesi tarafından sağlanmaktadır.

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Journal of Academic Research in Medicine (JAREM) is an open access international journal published in both Turkish and English and complies with independent and unbiased double-blind reviewing procedures. The journal publishes research in the fields of experimental and clinical medicine, reviews on recent topics, letters to the editor, and other manuscripts on medical education. The journal is published three times per year; in April, August, and December. The journal is funded by University of Health Sciences Turkey Gaziosmanpaşa Training and Research Hospital.

The aim of JAREM is to publish research on recent topics at an international level. Moreover, reviews, editor's note and images are also published in the journal. The target audience of readers and authors is composed of educators, academics, researchers, specialists and general practitioners, and all publication process and procedures comply with the standards of ICMJE, WAME and COPE. JAREM is indexed in Web of Science-Emerging Sources Citation Index, TUBITAK ULAKBIM TR Index, EBSCO, Index Copernicus, Gale, CINAHL, J Gate, Türk Medline and CAB International (CABI).

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JOURNAL OF ACADEMIC RESEARCH IN MEDICINE

Yazarlara Bilgi

Journal of Academic Research in Medicine-JAREM, çift-kör hakemli, açık erişimli bir dergi olarak, tıp alanında yapılan deneysel, temel, özgün klinik çalışmaları; mezuniyet sonrası eğitim, tıp tarihi, yayın ve araştırma etiğiyle ilgili yazıları yayınlar. Editörlerin yazı seçiminde temel unsur olarak dikkate alacağı hakemler, yurt içi ve yurtdılında konusunda uzman olan dış bağımsız kişilerden seçilir. Dergi, Nisan, Ağustos ve Aralık aylarında olmak üzere yılda 3 sayı yayınlanmaktadır.

Deneysel, klinik ve ilaç araştırmaları için ilgili uluslararası anlaşmalara uygun etik komisyon raporu gerekmektedir. (Helsinki Declaration of 1975, revised 2008-http://www.wma.net/en/30publications/10policies/b3/index.html, "Guide for the care and use of laboratory animals - www.nap.edu/catalog/5140.html)

Tüm yazarlar bilimsel katkı ve oranlarını ve ilgili sorumluluklarını; ayrıca çıkar çatışması olmadığını bildiren toplu imzaları ile yayına katılmalıdırlar. Araştırmalara kısmi de olsa yapılan nakdi ya da ayni yardımların hangi kurum, kuruluş, ilaç-gereç firmalarınca yapıldığı dip not olarak bildirilmelidir. (ICMJE Potansiyel Çıkar Çatışmaları Bildirim Formu)

Makalelerin formatı ICMJE-Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals (updated in December 2018 - http://www.icmje.org/icmje-recommendations.pdf) kurallarına göre düzenlenmelidir.

Orijinal Araştırmalar ve Derlemeler'in sunumu çalışma bildirimi kılavuzlarına göre düzenlenmelidir: randomize çalışmalar için CONSORT, gözlemsel çalışmalar için STROBE, tanısal değerli çalışmalar için STARD, sistematik derleme ve meta-analizler için PRISMA, hayvan deneyli çalışmalar için ARRIVE, randomize olmayan davranış ve halk sağlığına müdahale çalışmaları için TREND.

Orijinal Araştırma için genel etik kurallar çerçevesinde yayının yapıldığı kurumun yetkililerinin hazırladığı etik kurul onayı ya da eşdeğeri bir kabul yazısının sunulması şarttır. Yazılardaki düşünce ve öneriler tümüyle yazarların sorumluluğunda olup, Editör ve yardımcıların kanaatlerini yansıtmaz.

Dergide basılması amacıyla gönderilen yazılar başka yerde yayınlanmamış olmalıdır. Daha önce bilimsel toplantılarda sunulan 200 kelimeyi geçmeyen özet yayınları, durumu açıklanmak koşulu ile kabul edilebilir.

İşlemleri yürütülüp karar aşamasına yaklaşmış olan yazıların, makul bir neden olmadan geri çekilme talebi "ret" kapsamına girmektedir. Yayına kabul edilen yazılar için birinci yazar, Türkçe ve İngilizce açısından olduğu gibi, metinde temel değişiklik yapmamak kaydı ile düzeltmelerin Editörlerce yapılmasını kabul etmiş sayılır.

Yazıların dergide yayınlanmak üzere kabul edilmesi için; atıf alabilme olasılığı, orijinal ve bilimsel akademik üst düzeyde olması ön koşuldur.

Genel Kurallar

Yazılar sadece derginin çevrimiçi makale kabul sistemi www.jarem.org üzerinden gönderilebilir. Yayına kabul edilmeyen yazılar, sanatsal resimler dışında geriye gönderilmez. Tüm yazılar, Editör başta olmak üzere, Editör danışmanı ve yardımcıları, istatistik danışmanları ve en az iki hakem tarafından incelenir. Yazı konusunun en önde gelen otörü olan, fakat çalışmanın dışında olup yazarlarla ve kurumları ile ilişkisi-bilgisi olmayan üç kişinin ilk yazar tarafından hakem olarak önerilmesi dergi için çok önemlidir.

Editör, hakemlere yazıyı göndermeden önce aşağıda bildirilen biçimsel kurallara uygunluğunu araştırır. Düzeltmeler orijinal metinde değil, düzeltilmesi istenen bölümlerle kısıtlı olmalıdır. Yazılar gönderilmeden önce yazım ve çizim hatalarından tam olarak arındırılmalıdır.

Yazım Kurallarına uygun hazırlanmayan makaleler değerlendirmeye alınmayacaktır.

Araştırma Yazıları

1. Özgün Araştırmalar: Yazının tamamı 5000 kelimeyi geçmemeli ve yalnızca içeriği anlamak için gerekli olan sayı ve içerikte tablo ve grafik desteği olmalıdır. Kaynakların 50'den az olması inandırıcılık için genelde yeterlidir. Özgün Araştırma yazılarının yazar sayısı 5 ile sınırlandırılmıştır. İstisnai durumlarda bu sayı artırılabilir ancak sorumlu yazar tarafından gerekçesi dergiye gönderilmelidir.

1.1 Kapak sayfası: Birinci sayfadır ve ayrı MS Word dosyası olarak düzenlenir. Yazarların tam ve açık isimleri, son aldıkları akademik unvanlar ile 50 karakteri geçmeyecek şekilde yazının başlığı yazılır. Yazarların ilgili oldukları kurum, bölüm ve şehir sıra ile bildirilmelidir. Birden fazla yerde yapılan çalışmalar sembollerle açıklanır. Bu sayfanın altına yazışmaya yetkili ve düzeltmeleri yapacak yazarın açık adı, posta ve e-posta adresi, telefon ve faks numaraları yazılır. Ayrıca çalışma bilimsel toplantıda önceden bildirilen koşullarda tebliğ edildi ya da özeti yayınlandı ise açıklaması yapılır.

1.2 Orijinal araştırma makalesi için bölümlü özet: Özetler 250 kelimeyi aşmayacak şekilde çalışmanın amacını, tipini, çalışmadaki ana bulguları ve kısaca çalışmanın sonucunu içermelidir.

Özetler; Amaç, Yöntemler, Bulgular, Sonuç şeklinde alt başlıklarla düzenlenmelidir.

NLM MESH terimleri ile uyumlu en az , en fazla 6 tane anahtar kelime bölümlü özetin altında verilmelidir (http://www.nlm.nih.gov./mesh/MBrowser.html).

1.3 Metin: Makale Başlığı, Giriş, Yöntemler (alt başlıklı), Bulgular, Tartışma, Çalışma kısıtlamaları ile Sonuçlar ve Kaynaklar kısımlarını içermelidir. Metnin özellikle yöntemler, bulgular ve tartışma kısmının alt başlıklara bölünmesi yararlı olabilir. Metin toplam 5000 kelimeyi geçmemeli ve Times New Roman yazım sitili ile 12 puntoda yazılmalıdır. En son bölüme teşekkür yazılacak ise, ciddi bilimsel katkı dışında araştırmanın yürütülmesine önemli katkıda bulunanlarla, yazının son şeklinin verilmesine yardım edenler yazılır. Bu bilginin e-posta ile gönderilmesi gerekir veya ayrı MS Word dosyasında "Teşekkür Notu" olarak sisteme yüklenir.

1.4 İstatistiksel Analiz: Tıbbi dergilerdeki istatistik verilerini bildirme kurallarına göre yapılmalıdır (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). İstatistiksel analiz için kullanılan yazılım tanımlanmalıdır. Sürekli değişkenlerin karşılaştırılmasında parametrik testler kullanıldığı zaman verilerin ortalama±standart sapma olarak bildirilmesi gerekir. Parametrik olmayan testler için de Medyan (Minimum-Maksimum) veya Medyan (25'inci ve 75'inci persantiller) değerleri olarak bildirilmesi gerekir. İleri ve karmaşık istatistiksel analizlerde, göreceli risk (RR, relative risk), olasılık (OR, odds ratio) ve tehlike (HR, hazard ratio) oranları güven aralıkları (confidence intervals) ve p değerleri ile desteklenmelidir.

1.5 Kaynaklar: Metin içinde geçiş sırasına göre numaralandırılır ve ayrı sayfada yazılır. Kişisel bilgi, yayınlanmamış veriler, "baskıda gibi" ulaşılamayan kaynaklar burada değil, metin içinde parantez ile sunulur. İki yıldan eski özetler kaynakçaya alınmaz; alınanlar parantezde (abstr.) şeklinde verilir. Kaynakların gerçekliğinden yazarlar sorumludur. Atıf yapılırken en son ve en güncel yayınlar tercih edilmelidir. Yazarlar 10 yıldan eski yayınlara atıf yapmamaya özen göstermelidir. Dergimizde eski kaynakların kullanımı %15 ile sınırlı tutulmaktadır.

Dergiler

Dergi isimlerinin kısaltmaları Index Medicus/Medline/PubMed listesine göre yapılır (dergilerin kısaltmaları için NLM tarafından her yıl yayınlanan MEDLINE dergilerin listesine http://www.nlm.nih.gov/tsd/serials/lji.html adresinden ulaşılabilir). Altı ve daha yazarlı makalelerde tüm isimler yazılır. Yedi ve fazla yazarlı olanlarda ilk altı isim yazılır ve "et al." ilave edilir. Yazar isimlerinden sonra, o yazının tam başlığı, yıl, cilt ve sahifeler sıralanır.

Örnek: Müller C, Büttner HJ, Petersen J, Roskomun H. A randomized comparison of clopidogrel and aspirin versus ticlopidine and aspirin after the placement of coronary-artery stents. Circulation 2000; 101: 590-3.

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Yazarlara Bilgi

Kitaplar

Kitap içinde bölüm: Sherry S. Detection of thrombi. In: Strauss HE, Pitt B, James AE, editors. Cardiovascular Medicine. 2nd ed. St Louis: Mosby; 1974. p.273-85.

Tek yazarlı kitap: Cohn PF. Silent myocardial ischemia and infarction. 3rd ed. New York: Marcel Dekker; 1993.

Yazar olarak Editör (ler): Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996.

Toplantıda sunulan makale: 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. P. 1561-5.

Bilimsel veya teknik rapor: Smith P. Golladay K. Payment for durable medical equipment billed during skilled nursing facility stays. Final report. Dallas (TX) Dept. of Health and Human Services (US). Office of Evaluation and Inspections: 1994 Oct. Report No: HHSIGOE 169200860.

Tez: Kaplan SI. Post-hospital home health care: the elderly access and utilization (dissertation). St. Louis (MO): Washington Univ. 1995.

Elektronik formatta makale

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) l995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlEID/cid.htm.

1.6 Şekiller, Tablolar ve Resimler: Şekil ve resimler, hasta, doktor ve kurum isimleri gözükmeyecek şekilde hazırlanmalıdır. Metinden ayrı olarak, metin içinde geçiş sırasına göre numaralandırılarak verilir. Başlık ve altyazılar ayrı bir sayfada sunulur. Grafiklerde yeteri kalınlıkta çizgi kullanılır. Böylece gerekli küçültmelerde kayıplar en aza iner. Genişlikler en fazla 9 ya da 18 cm. olmalıdır. Çizimlerin profesyonellerce yapılması faydalı olacaktır. Gri renkler kullanılmamalıdır. Kullanılan kısaltmalar alt kısımda alfabetik sıra ile mutlaka açıklanmalıdır. Tablo ve Şekil başlıklarında ve tablonun yazı içinde anılmasında Roma rakamları kullanılmamalıdır. Metin, Tablo ve Şekillerde kullanılan ondalık sayılar Türkçe metinlerde virgül İngilizce metinlerde ise nokta ile ayrılmalıdır. Özellikle tablolar metni açıklayıcı ve kolay anlaşılır hale getirmek amacı ile hazırlanmalı ve metnin tekrarı olmamalıdır.

Video Görüntüler

Özgün Görüntüler'de yer alan resimlere ek olarak video/hareketli görüntüler ve ekstra imaj/statik görüntüler aşağıdaki teknik özelliklerde gönderildiği takdirde web sayfamızda yayınlanacaktır.

1. İmaj/statik görüntü formatında sunumlar: JPG, GIF, TIFF, BMP

2. Video/hareketli görüntü formatında sunumlar: MPEG, VMF.

3. Dosya boyutu maksimum 2 MB olmalıdır.

4. Resimlerde ve özellikle video görüntülerinde doktor, kurum, şehir ve hasta tanımlamaları tümü ile silinerek gönderilmelidir.

Makalenizde yer alan tablolar, şekiller ve resimler için orijinal oldukları ayrıca bildirilmelidir. Orijinali dışında ve başka kaynaktan alındıklarında mutlaka alınan kaynağa atıfta bulunmalı ve alınan kaynağın "hardcopy" veya elektronik formatta versiyonları Telif Hakkı sahibinden (yayınevi, dergi veya yazar) alınan izinler ile birlikte Baş Editör ofisine sunulmalıdır. Kaynaklar, şekiller ve tablolar ile ilgili kurallar tüm makale türleri için geçerlidir.

Özel Bölümler

2. Davetli Derlemeler: Editör ofisinin kararıyla davetli yazarlar tarafından hazırlanabilir. Bir bilgi ya da konunun klinikte kullanılması için son vardığı düzeyi anlatan, tartışan, değerlendiren ve ileride yapılacak çalışmalara yön belirleyen düzeyde olmalıdır. Yazarının konusunda otör olması ve atıfta bulunulmuş yazılarının olması gerekir.

Bölümsüz özet: Araştırma makalelerindeki kelime sayıları burada da geçerlidir, sadece bölümlü olmayacaktır. NLM MESH terimleri (http://www.nlm.nih.gov./mesh/MBrowser. html adresinde bulunabilir) ile uyumlu en az , en fazla 6 tane anahtar kelime bölümlü özetin altında verilmelidir. Kelime sayısı 5000, kaynak sayısı 50 ile sınırlıdır.

3. Editöryel Yorum: Dergide çıkan bir araştırmanın o konunun otörü veya iyi değerlendirme yapan hakem tarafından kısaca değerlendirilmesi amacı güder. Sonunda; klinik anlam ve kısa özet bulunur.

4. Bilimsel Mektup: Yeni bilimsel buluş ve verileri duyurmayı amaçlayan, klinik açıdan önemli ancak ön bildiri niteliğinde olan yazılar bilimsel mektup olarak yayına kabul edilir. Bilimsel mektuplar içerik olarak alt başlıksız olup toplam 900 kelimeyi aşmamalıdırlar. Kaynak sayısı 10, tablo ve resim sayısı ise 2 ile sınırlı olmalıdır.

4. Editöre Mektuplar: Derginin temel yayın amaçlarından birini oluşturmaktadır. Yayınlanan bir yazının önemini, gözden kaçan bir yapısını ya da noksanını tartışır. Yazarlar, yayınlanan makaleler hakkında yorum içeren mektuplar dışında da okurlarımızın ilgi alanlarına giren konular veya özellikle eğitici vakalar hakkında da Editöre Mektup formatında yorumlarını sunabilirler. Kaynak sayısı 5, metin ise 500 kelimeyi geçmemelidir, alt başlıkları bulunmaz.

6. Eğitim: Son yıllarda araştırma sonuçları ile kesinleşen, akademik düzeydeki eğitimde yerini alan ve klinik uygulamada yer bulan bilgiler ayrıntıları ile sunulur.

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Section in a book: Sherry S. Detection of thrombi. In: Strauss HE, Pitt B, James AE, editors. Cardiovascular Medicine. 2nd ed. St Louis: Mosby; 1974. p.273-85.

Book with single author: Cohn PF. Silent myocardial ischemia and infarction. 3rd ed. New York: Marcel Dekker; 1993.

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Article presented at a meeting: 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. P. 1561-5.

Scientific or technical report: Smith P. Golladay K. Payment for durable medical equipment billed during skilled nursing facility stays. Final report. Dallas (TX) Dept. of Health and Human Services (US). Office of Evaluation and Inspections: 1994 Oct. Report No: HHSIGOE 169200860.

Thesis: Kaplan SI. Post-hospital home health care: the elderly access and utilization (dissertation). St. Louis (MO): Washington Univ. 1995.

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) l995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlEID/cid.htm.

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İçindekiler / Contents

Özgün Araştırmalar / Original Investigations

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J Acad Res Med 2022;12(2):42-8

Can Urine Sarcosine Predict the Prostate Biopsy Necessity in Patients with Total PSA Value Ranging Between 2.5-10 ng/mL?

Total PSA Değeri 2,5-10 ng/mL Aralığındaki Hastaların İdrar Sarkozin Değerleri Prostat Biyopsisi Gereksinimini Öngörebilir mi?

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ABSTRACT

Objective: The most common oncologic disease in men is prostate cancer. There have been studies for alternative methods for early screening. Over the past years, the interest in sarcosine as a potential marker for prostate cancer has increased. We evaluated the predictability of prostate biopsy necessity by using urine sarcosine for prostate cancer examination during our study.

Methods: The study included 84 male patients aged between 45 and 79 in our hospital between 15.12.2013 and 15.03.2014. After the primary evaluation, standard 12 cores transrectal ultrasonography prostate biopsy was performed by the clinician to the appropriate patients with total prostate specific antigen (PSA) values ranging between 2.5-10 ng/mL. A sarcosine measurement with colorimetric and fluorometric principles was performed on patients' urine samples taken before the prostate biopsy, following the prostate massage.

Results: Statistically significant negative correlation in malignant group and positive correlation in benign group were found between percentage change in PSA values and fluorometric sarcosine measurements ($r=-0.418$; $p=0.042$; $p<0.05$ / $r=0.318$; $p=0.013$; $p<0.05$ respectively).

Conclusion: The correlation between percentage change in PSA values and fluorometric sarcosine measurements can be used in patients with a grey zone PSA (such as PI-RADS 2-3 and low level PSA patients) in order to avoid unnecessary biopsies.

Keywords: Prostate cancer, prostate biopsy, sarcosine, urine, biomarker, prostate cancer screening

ÖZ

Amaç: Prostat kanseri erkeklerde en sık görülen onkolojik hastalıktır. Erken taramanın öneminden dolayı klasik taramalar dışında alternatif belirteçler aranmaktadır. Son yıllarda prostat kanserinde potansiyel marker olarak sarkozine artmış ilgi mevcuttur. Biz çalışmamızda idrar sarkozininin prostat kanseri araştırılmasında prostat biyopsisi gereksinimini öngörebilirliğini değerlendirdik.

Yöntemler: Çalışma 15.12.2013 ve 15.03.2014 tarihleri arasında hastanemizde yaşları 45 ile 79 yıl arasında değişmekte olup, ortalaması 60,49±6,81 olan toplam 84 erkek olgu ile yapılmıştır. Birincil değerlendirme sonucunda çalışma için uygun bulunan hastalara [prostat spesifik antijen (PSA) değerleri 2,5-10 ng/mL] hekim tarafından standart 12 kor transrektal ultrasonografi eşlinde prostat biyopsisi uygulanmıştır. Hastalardan prostat biyopsisi hemen öncesinde prostat masajını takiben alınan idrarlarda kolorimetrik ve florometrik prensiplerle sarkozin ölçümü yapıldı.

Bulgular: PSA değerlerindeki yüzde değişim ile florometrik sarkozin ölçümleri arasında malign grupta istatistiksel olarak anlamlı negatif korelasyon ve benign grupta pozitif korelasyon bulundu ($r=-0.418$, $p=0.042$, $p<0.05$ / $r=0.318$, $p=0.013$, $p<0.05$ sırasıyla).

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Sonuç: Gereksiz biyopsilerden kaçınmak için gri bölge PSA'lı hastalarda (PI-RADS 2-3 ve düşük seviye PSA'sı olan hastalar gibi) PSA değerlerindeki yüzde değişim ile florometrik sarkozin ölçümleri arasındaki korelasyon kullanılabilir.

Anahtar kelimeler: Prostat kanseri, prostat biyopsisi, sarkozin, idrar, biyobelirteç, prostat kanseri taraması

INTRODUCTION

Although the normal total prostate specific antigen (PSA) rate according to the EAU guidelines is still a subject to clarify, the guidelines suggest that the normal levels for young men are <2-3 ng/mL and 10 ng/mL total PSA level is set for the prognostic categorization of prostate carcinoma. Remarkably, it is still not clarified at what age the early screening should be started and between what levels the total PSA should be. However, it's recommended that the total PSA level be checked after the age of 40, and the screening is unnecessary after the age of 70 (1). In clinical practice, management of patients with total PSA level ranging between 2.5-10 ng/mL differs. Although some clinics recommend a transrectal ultrasonographic biopsy (TRUS-biopsy) directly, some clinics postpone the decision and examine a new total PSA level after antibiotic treatment (2).

Among those patients who have undergone a TRUS-biopsy, some of the patients have benign results. Prostate biopsies are found to be painful and stressful for many patients. Many patients refuse the procedure. The group of patients with benign results are exposed to unnecessary invasive manipulation because of the failure of total PSA in the prediction of cancer. On the other hand, some of the patients who refuse the procedure are advantageous because of being exempt from unnecessary biopsies while some of them are disadvantageous because of unawareness of their malignancy. Setting off on a quest for finding an adequate solution to this problem is important. We believe that urologists should provide comfort to the patients while diagnosing with the correct indication and avoiding missing malignant cases among this group. In recent years, the number of studies about prostate cancer screening, diagnosis, and the prediction of progression with the levels of a molecule called sarcosine in urine and serum is increasing (3-8). In our research, we aimed to identify if the sarcosine level in urine would be beneficial in the decision of TRUS-biopsy in patients with PSA level ranging between 2.5-10 ng/mL.

METHODS

The population of the study consisted of male patients who were admitted to Taksim Training and Research Hospital Urology Clinic. This study was a prospective, analytical study aiming to determine the ability of urine sarcosine levels of patients with total PSA level of 2.5-10 ng/mL in the prediction of prostate biopsy necessity. Study was performed between 15.12.2013-15.03.2014 after the Taksim Training and Research Hospital Clinical Research Ethics Committee approval (decision no: 29, date: 04.12.2013). Male patients between the ages of 40 and 79 were included in the study. All patients gave their informed consent for participation. The exclusion criteria were as follows; previous malignancy diagnosis, lower urinary tract surgery in last six months, the presence of active urinary tract infection, symptoms of abnormal digital rectal examination (frozen pelvis, rectal malignancy, etc.), lack of the sarcosine dehydrogenase enzyme, and the presence of sarcosinemia disease. Total PSA (in the first application and one month after the initial application on the day of the biopsy), free PSA, urine examination, uroflowmetry, International Prostate Symptom score, complete blood count, urine sarcosine level, ultrasonography (USG) measurements (prostate volume, residue urine after urinate) were examined. The data were recorded with the patient study registration form. As a result of the primary evaluation, standard 12 cores transrectal USG (TOSHIBA Aplio300) prostate biopsy was performed by the same clinician on the appropriate patients. After differentiating the patients according to the prostate biopsy reports as benign-malignant, the data were evaluated. Statistical comparison was performed concerning patients' other parameters, demographic features, and urine sarcosine levels during the diagnostic process.

Sarcosine Determination

Following the prostate massage before biopsy (after providing enough prostate fluid to pass to urine), the urine samples of patients were kept in -40 °C until the study day. The urine samples were melted in room temperature on study day and centrifuged for 10 minutes in 2000 g. The supernatant was separated.

Sarcosine kit (Sarcosine Assay Kit, Abcam, ab65338) was kept in -20 °C for two months until the study day (Table 1).

The sarcosine tampons and probes of the kit were ready to use. The sarcosine Enzyme Mix was melted in 220 µl sarcosine tampon with the help of an automatic pipette. Sarcosine standard was mixed with 100 µl distillate water, and 100 nmol/µl sarcosine standard was acquired. Sarcosine enzyme kit and standard were aliquoted and prepared to be examined. According to the test method, 46 µl sarcosine tampon, 2 µl sarcosine enzyme, and 2 µl probe were required to be added to each well; a reaction mix pool was created after the calculations of the tampon, enzyme, and probe number necessary for both the control and patient groups and mixed slowly with confounder.

As 10 µl of ready sarcosine standard was mixed with 990 µl sarcosine tampon, 1 nmol/µl standard study solution was prepared. After that, to prepare a standard curve, the standard study solution was pipetted in 0, 2, 4, 6, 8 µl 5 sequenced well, and each well's volume was completed to 50 µl. Also, 1 and 6 µl control samples were prepared with sarcosine standard and completed to 50 µl after pipetting in 2 different wells. After the enumeration of 84

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patients' urine samples between 1 and 84, samples were pipetted to 50 µl wells in order. For each well, 50 µl reaction mix was added, and the wells plate was mixed with confounder. After incubation for 1 hour in 37 °C, EX/Em=544/590 nm fluorometric and 540 nm colorimetric was read. Concentration unit was determined as nmol/µl or millimolar.

Study was performed with 84 male patients aged between 45-79 with a mean of 60.49±6.81 years between 15.12.2013-15.03.2014 dates in the Urology Clinic of Taksim Training and Research Hospital.

Statistical Analysis

The Number Cruncher Statistical System Statistical Software (Utah, USA) program was used for statistical analysis. While evaluating the study data, quantitative variables were shown with mean, standard deviation, median, minimum and maximum values, and qualitative variables were shown with descriptive statistical methods such as frequency and percentage. Shapiro-Wilks test and Box Plot charts were used to evaluate the conformity of the data to the normal distribution. Mann-Whitney U test for the evaluation of non-normally distributed variables according to two independent groups; Wilcoxon Signed-Rank test was used in the evaluation of dependent groups according to their follow-up. Fisher's Exact test was used for the comparison of the qualitative data. Spearman's correlation analysis was used for the evaluation of the relationships between the pre-biopsy PSA parameters and PSA parameters on the day of the biopsy in colorimetric and fluorometric sarcosine measurements. The significance was evaluated at the levels of $p<0.05$.

RESULTS

Pathology results were benign in 71.4% (n=60), while they were malignant in 28.6% (n=24) of the patients. Gleason score was observed to be 6 in 79.2% (n=19), while it was 7 in 20.8% (n=5) of the malignant patients. Prostatitis was present in 63.1% (n=53) while it was not observed in 36.9% (n=31) of the patients.

Pre-biopsy PSA values of the patients ranged between 2.57 ng/mL and 9.89 ng/mL and mean was 5.85±1.97 ng/mL; PSA values on the day of the biopsy ranged between 0.84 ng/mL and 13.31 ng/mL and mean was 5.73±2.23 ng/mL. Percentage change in PSA values ranged between -90.7 ng/mL and 50.57 ng/mL and mean was -0.86±21.94 ng/mL.

While colorimetric sarcosine measurements ranged between 0 and 1.21, and the mean value was 0.44±0.24; fluorometric sarcosine measurements ranged between 0 and 1.3, and the mean was 0.36±0.27.

Prostate volume ranged between 15 ccs and 160 ccs, and the mean was 49.01±27.80 ccs.

No statistically significant difference was determined between pre-biopsy PSA measurements and PSA measurements on the day of the biopsy according to pathology results ($p>0.05$).

In benign group, change in PSA measurements on the day of the biopsy in the direction of reduction compared to pre-biopsy PSA measurements wasn't statistically significant ($p=0.123$; $p>0.05$), in malignant group, average increase in PSA measurements on the day of the biopsy compared to pre-biopsy PSA measurements wasn't statistically significant ($p=0.338$; $p>0.05$) (Table 2).

A statistically significant difference was determined between percentage change in pre-biopsy and on the day of the biopsy PSA measurements according to pathology results ($p=0.050$; $p<0.05$). While the mean percentage change in the benign patients was determined to be -3.44±22.43, it was 5.58±19.63 in malignant patients (Figure 1).

No statistically significant difference was determined between colorimetric and fluorometric sarcosine measurements of the patients according to pathology result ($p>0.05$) (Table 3).

A statistically significant difference was determined between prostate volume of the patients according to pathology result ($p<0.05$) and prostate volume in the malignant group was less than the benign group.

A statistically significant difference was determined between the prevalence rates of prostatitis in the patients ($p<0.01$). Prevalence

Table 1. Sarcosine kit content

Sarcosine tamp	25 mL
Sarcosine prob (DMSO, anhidrosis)	0.2 mL
Sarcosine enzyme mix (lyophilised)	1 vial
Sarcosine standard (10 µmol, lyophilized)	1 vial

Table 2. Assessment of PSA measurements according to pathology result

	Pathology		[a]p
	Benign (n=60)	Malignant (n=24)	
	Median (min-max)	Median (min-max)	
Pre-biopsy PSA	5.42 (2.7-9.9)	6.06 (2.6-9.5)	0.443
PSA on the day of the biopsy	5.537 (0.8-13.3)	6.09 (3.2-10.5)	0.080
[b]p	0.123	0.338	
Pre-biopsy - PSA on the day of the biopsy percentage change (%)	-4.19 (-90.7/47.34)	1.05 (-29.5/50.6)	0.050*

[a]Mann-Whitney U test, [b]Wilcoxon Signed-Rank test, *$p<0.05$, PSA: prostate specific antigen, min: minimum, max: maximum

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of prostatitis in the malignant group was significantly less than the patients of the benign group.

No statistically significant correlation was determined between pre-biopsy PSA values and colorimetric and fluorometric sarcosine measurements and between PSA values on the day of the biopsy and colorimetric and fluorometric sarcosine measurements of the benign cases (p>0.05). No statistically significant correlation was determined in the malignant group, either (p>0.05).

No statistically significant correlation was determined between percentage change in PSA values and colorimetric sarcosine measurements of the benign patients (p>0.05). A statistically significant positive correlation was found between percentage change in PSA values and fluorometric sarcosine measurements (fluorometric sarcosine value increased with the increase of percentage change in PSA values) at a level of 31.8% (r=0.318; p=0.013; p<0.05).

A statistically significant negative correlation was found between percentage change in PSA values and colorimetric sarcosine measurements (as the percentage change in PSA values increased, colorimetric sarcosine value decreased) at a level of 41.5% in malignant patients (r=-0.415; p=0.044; p<0.05). It was also found the same in fluorometric sarcosine measurements at a level of 41.8% in malignant patients (r=-0.418; p=0.042; p<0.05) (Table 4).

There was no statistically significant difference between pre-biopsy PSA measurements and PSA measurements on the day of the biopsy (p>0.05) and between percentage change in PSA values of the malignant patients according to Gleason scores (p>0.05).

There was no statistically significant difference between colorimetric and fluorometric sarcosine measurements (p>0.05) and between prostate volumes of the malignant patients according to Gleason scores (p>0.05).

There was also no statistically significant difference between the prevalence rates of prostatitis in malignant patients according to Gleason scores (p>0.05) (Table 5).

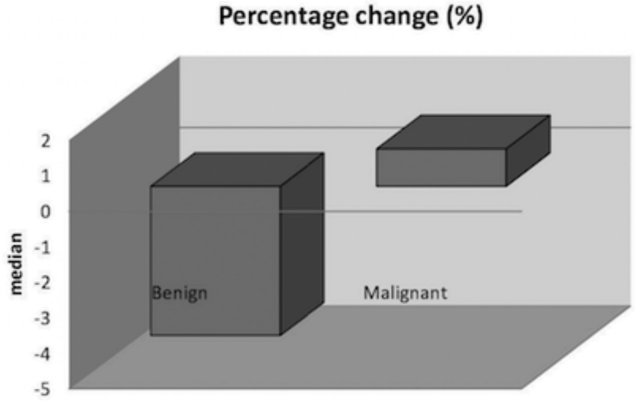

Figure 1. Percentage change (%) in pre-biopsy PSA measurements and PSA measurements on the day of the biopsy according to pathology results

PSA: prostate specific antigen

DISCUSSION

After the examinations of USA 2013 prostate cancer (PC) incidence-mortality data, it was seen that PC had the highest incidence (238.590) and second highest mortality (29.720) in all male cancer types (9). Due to those high ratios, early diagnosis of PC is vital. PSA is an organ-specific reagent. However, it has no specificity for disease or its degree. There are two main problems with PSA. The first problem is the great ratio of negative prostate biopsy results because PSA isn't specific for PC.

The second problem is that low level (0-20 ng/mL) PSA can't predict PC (3). Thus, it's thought that PSA is inadequate in PC screening and the researchers are looking for alternative reagents (10). Nowadays, the most used reagents in non-invasive disease are PCA-3 and annexin (3,4,11) in urine. New markers, such as urine alfa methyl alkyl CoA, which increases in adenocancer and high-grade intraepithelial neoplasia, are being discussed to be used in prediction of cancer progression (5,12-14). Another reagent is the ratio of fPSA/tPSA. It's reported that this ratio is especially effective in differentiating patients with PC and benign situations. Kallikrein 2, urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor, IL-6/IL-6 receptor, pigment epithelium-derived factor, fibronectin 1, chromogranin A, ceruloplasmin are remarked as high potential bioreagents (15,16). In addition, sarcosine; related to methionine and one-carbon metabolism, has been searched recently. Glycine N-methyltransferase (GNMT) is the main component that affects the sarcosine syntheses (17-19). GNMT syntheses is controlled with the same-named gene. It has been recently identified that this gene is on the sixth chromosome's short arm's 12th position (19). The increase of GNMT production causes glycine to transform sarcosine and increase its excretion in urine. Stabler et al. (20)

Table 3. Assessment of colorimetric sarcosine measurements and fluorometric sarcosine measurements according to pathology result

	Pathology		[a]p
	Benign (n=60)	Malignant (n=24)	
	Median (min-max)	Median (min-max)	
Colorimetric sarcosine	0.41 (0-1.2)	0.44 (0.01-0.9)	0.365
Fluorometric sarcosine	0.30 (0-1.3)	0.40 (0-0.9)	0.513

[a]Mann-Whitney U test, min: minimum, max: maximum

have proven that the increased GNMT increases homocysteine and sarcosine formation with increasing s-adenosyl methionine usage. This fact caused sarcosine to be thought as a bioreagent in the non-invasive cancer field.

In 2009, Sreekumar et al. (21), examined metabolomic characters of PC and suggested that urine sarcosine levels could be used as reagent for prediction of PC progression. After this study, number of studies about this subject increased and many studies with different methods were performed. Some of those studies supported sarcosine as a reagent in cancer determination; however, some of them didn't.

Urine sarcosine levels were evaluated with colorimetric and fluorometric methods in our study, which aimed to determine the efficiency of sarcosine in predicting PC, especially in patients with low-level PSA. There was no statistically significant difference detected between the benign and malignant patients in terms of urine sarcosine levels ($p>0.05$). Our finding matches Jentzmik et al.'s (6) and Struys et al.'s (7) studies in literature, however, it doesn't match with Bianchi et al.'s (22) and Cernei et al.'s (5) studies.

In Jentzmik et al.'s (6) study in 2010, the urine sarcosine levels were evaluated with gas chromatography and spectrometry in 106 patients with PC and 33 controls without cancer. It was seen that sarcosine/creatinine ratio was 13% less in patients with PC. As a result, it has no additional extender to PSA in benign-malignant differentiation and it's more inadequate than fPSA (6).

Struys et al. (7) reported that there was no significant difference between patients with increased serum PSA level, patients with metastatic PC and the control group (patients were chosen from patients whose vitamin B12 levels were examined). They even identified that serum sarcosine levels were not helpful with serum PSA level increase; therefore, it wasn't helpful in prediction of cancer progression.

In 2011, Bianchi et al. (22) evaluated urine sarcosine levels of a total of 56 participants consisting of healthy controls, patients with benign prostate hypertrophy (BPH) and patients with prostate gland localized cancer with fully automated solid-phase microextraction-fast gas chromatography and mass spectrometry. They found that the sarcosine/creatinine ratio in stated participants were 103, 137, and 267 µg/g, respectively. The highest sensitivity was 79%, and specificity was 87% with cut-off sarcosine value of 179 µg sarcosine (g creatinine)$^{-1}$ and in case of usage of this cut-off value, sarcosine had an important relationship with the cancer presence ($p<0.0001$). The correlation between patients with clinical localized cancer and patients with no evidence of tumour was presented with receiver operating characteristic analysis (22).

Table 4. Assessment of colorimetric sarcosine measurements and fluorometric sarcosine measurements in the cases according to pathology result

	Benign (n=60)		Malignant (n=24)	
	r	p	r	p
Pre-biopsy PSA - colorimetric sarcosine	0.071	0.591	-0.013	0.953
Pre-biopsy PSA - fluorometric sarcosine	0.018	0.894	0.149	0.487
PSA on the day of the biopsy - colorimetric sarcosine	0.118	0.369	-0.176	0.410
PSA on the day of the biopsy - fluorometric sarcosine	0.127	0.335	-0.025	0.906
Percentage change in PSA (%) - colorimetric sarcosine	0.211	0.189	-0.415	0.044*
Percentage change in PSA (%) - fluorometric sarcosine	0.318	0.013*	-0.418	0.042*

r=Spearman's correlation coefficient, *p<0.05, PSA: prostate specific antigen

Table 5. Assessment of PSA, sarcosine, prostate volume and prostatitis parameters in the cases with malignant pathology result according to content Gleason score

		Gleason score 6	Gleason score 7	
		Median (min-max)	Median (min-max)	p
Pre-biopsy PSA		6.12 (3.35-9.50)	5.87 (2.57-8.68)	[a]0.696
PSA on the day of the biopsy		6.16 (3.21-10.51)	5.65 (3.67-10.30)	[a]0.804
Percentage change in PSA (%)		0.13 (-29.51/50.57)	13.51 (-3.75/42.8)	[a]0.166
Colorimetric sarcosine		0.42 (0.01-0.85)	0.57 (0.26-0.69)	[a]0.374
Fluorometric sarcosine		0.30 (0-0.9)	0.40 (0.2-0.7)	[a]0.389
Prostate volume		32.40 (18-140)	40.0 (20-75)	[a]0.749
		n (%)	n (%)	
Prostatitis	Present	8 (42.1%)	0 (0.0%)	[b]0.130
	Absent	11 (57.9%)	5 (100.0%)	

[a]Mann-Whitney U test, [b]Fisher Exact test, min: minimum, max: maximum, PSA: prostate specific antigen

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Urine sarcosine values of patients with PC -were evaluated with ion-exchange chromatography developed by Cernei et al. (5), and it was seen that those patients had significantly higher sarcosine levels than treated patients. It was shown that urine sarcosine levels of healthy people could be ignored. In our study, which had a starting point accordingly, it was identified that patients who were reported as having BPH and healthy people had sarcosine in their urines. There was no statistically significant difference found in malignant patients, and our study didn't reveal the fact that sarcosine levels in healthy people could be ignored.

Koutros et al. (8), examined sarcosine levels of 1,122 patients with PC (813 non-aggressive and 309 aggressive) and 1,112 controls with liquid chromatography-mass spectrometry. They found that as the sarcosine level increased, PC risk increased (p=0.03). As a result, Koutros et al. (8) reported that high serum sarcosine levels accompanied increased PC risk and that sarcosine could be used as bioreagent.

Our findings support most of the studies in literature. For example, Koutros et al. (8) classified PC according to tumor aggressiveness in 4 groups (Q1-Q4) and evaluated the relationship between sarcosine levels and the aggressiveness of the disease. They reported strong relationship in non-aggressive patients [for Q4-Q1 odds ratio =1.44, 95% confidence interval (CI): 1.11, 1.88; P-trend 0.006]. However they didn't report a significant relationship in the aggressive patients (for Q4-Q1 odds ratio =1.03, 95% CI: 0.73, 1.47; P-trend 0.89).

Cao et al. (12) identified that urine sarcosine and sarcosine/creatinine ratio were incompatible with Gleason score and T phase. Similarly, Jentzmik et al. (6) found that sarcosine levels weren't related to tumor phase or Gleason score (<7 vs. ≥7) (19). In our study, there was no statistically significant difference between colorimetric and fluorometric sarcosine measurements of the malignant patients according to Gleason scores as well (p>0.05).

Struys et al. (7), reported that sarcosine levels weren't correlated with tumor progression. Wu et al. (4) showed that sarcosine/creatinine ratio wasn't sufficient for cancer diagnosis and wasn't determinant for histological degree and identifying the tumour behavior. According to those studies and our study, sarcosine levels and tumor aggressiveness are not related and disease aggression cannot be evaluated with sarcosine levels.

Apart from all these data, our study found statistically significant negative correlation in malignant group and positive correlation in benign group with percentage change in PSA values and fluorometric sarcosine measurements.

Study Limitations

The main limitation was the low amount of sample size. However, the study was designed to be prospective, all groups' features were analyzed without a control group. Sarcosine kit was obtained from an abroad country and only 90 kit contents could be received. Sample storage had to be in -40 °C and the limiting storage time was maximum 3 months. Despite all these challenging difficulties and lack of technical issues, we believe that we have designed a good study to make an addition to the present literature.

CONCLUSION

In our study, which we used not only fluorometric technic but also colorimetric technic, sarcosine levels were found inadequate in predicting PC, differentiating benign-malignant patients, and predicting the aggressiveness of the disease. However, the correlation between percentage change in PSA values and fluorometric sarcosine measurements might be used in grey zone PSA patients. Combining this correlation with newly popular Multiparametric Prostate MR results may lead us avoid unnecessary biopsies in especially patients with low level PSA and PI-RADS 2-3.

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A New Questionnaire for Aesthetic Gynecology: A Self-appraisal Questionnaire for Female Genital Cosmetic Procedure Demand (Q-FGCP)

Estetik Jinekoloji için Yeni Bir Anket: Kadın Genital Kozmetik Prosedür Talebine Yönelik Kendini Değerlendirme Anketi (Q-FGCP)

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ABSTRACT

Objective: Today, demand for genital cosmetic procedures is increasing among women. However, despite the rapid increase in popularity of these procedures, research questionnaires to determine whether there is a real need for genital aesthetic procedures for patients are still limited. The objective of this study is to develop a self-assessment questionnaire for female genital cosmetic procedures, to use this questionnaire for genital appearance satisfaction within the general population and to evaluate the correlation between total item score and demand.

Methods: The questionnaire was conducted on 100 women who were thought to reflect the general population who presented to the Gynecology and Obstetrics clinics of our tertiary hospital. The questionnaire, which consisted of 11 items, was prepared to screen the women who needed cosmetic genital procedures. The first 10 items included factor analysis regarding aesthetic appearance of the genital organs (items 1-8) and its impact on sexual pleasure (items 9 and 10). Item 11 questioned how much participants needed a cosmetic gynecologic procedure.

Results: A strong need for genital aesthetic procedures was observed in 41% of the participants. When demographic variables were analyzed in terms of the desire for cosmetic procedures; only the first 10 questions were statistically significantly related to the total score. It was shown that a total score >19 was an important factor for women desiring to undergo genital cosmetic procedures (odds ratio: 3.9, confidence interval: 1.7-9.2, p=0.011).

Conclusion: This scoring system will be helpful in determining whether there is a real need for genital aesthetic procedures in the general population, and whether patients with an actual need for genital aesthetic procedures can be directed with this scoring since perception of women with regards to vaginal cosmetic procedures can differ from that of professionals.

Keywords: Aesthetic gynecology, aesthetic vaginal plastic surgery, questionnaire, genital cosmetics

ÖZ

Amaç: Günümüzde kadınlar arasında genital kozmetik işlemlere olan talep artmaktadır. Ancak, bu işlemlerin popüleritesindeki hızlı artışa rağmen, hastalar için genital estetik işlemlere ihtiyaç olup olmadığını belirlemeye yönelik araştırma anketleri hala sınırlıdır. Bu çalışmanın amacı, kadın genital kozmetik prosedürünü arzulamak için bir öz değerlendirme anketi geliştirmek, bu anketi genel popülasyonda genital görünüm memnuniyeti için kullanmak ve toplam madde puanı ile talep arasındaki ilişkiyi değerlendirmektir.

Yöntemler: Anket, hastanemizin kadın hastalıkları ve doğum polikliniklerine başvuran genel popülasyonu yansıttığı düşünülen 100 kadına uygulandı. On bir maddeden oluşan anket, genital estetik işlem ihtiyacı olan kadınları taramak amacıyla hazırlanmıştır. İlk 10 madde, genital organların estetik

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görünümü (1-8. maddeler) ve cinsel hazza etkisi (9. ve 10. maddeler) ile ilgili analizini içermektedir. On birinci madde, katılımcıların kozmetik jinekolojik prosedüre ne kadar ihtiyaç duyduğunu sorguladı.

Bulgular: Katılımcıların %41'inde genital estetik işlemlere güçlü bir ihtiyaç olduğu gözlemlendi. Estetik işlem isteği açısından demografik değişkenler incelendiğinde; sadece ilk 10 soru toplam puanla ilgili istatistiksel olarak anlamlıydı. Genital kozmetik işlem yaptırmak isteyen kadınlarda toplam puan >19 olmasının önemli bir faktör olduğu gösterildi (olasılık oranı: 3,9, güven aralığı: 1,7-9,2, p=0,011).

Sonuç: Bu skorlama sistemi, genel popülasyonda genital estetik işlem ihtiyacı olup olmadığının belirlenmesinde yardımcı olacaktır ve kadınların normal algısı farklı olabileceğinden, genital estetik işlem ihtiyacı olan hastalar bu skorlama ile yönlendirilebilir.

Anahtar kelimeler: Estetik jinekoloji, vajinal estetik ameliyat, anket, genital kozmetik

INTRODUCTION

Recently, female plastic/cosmetic genital procedures have increasingly become popular among women (1). There are a series of genital procedures such as vaginoplasty, labiaplasty, clitoral hood reduction, labia majora augmentation/reduction hymenoplasty and G-spot amplification (2). Among these procedures, the number of labiaplasty operations rose to 12,756 in 2018 from 8,341 in 2014 by an increase of 53% only over four years (3,4). However, despite this rapid dramatic increase in demand for these procedures, research questionnaires to determine whether there is an actual need for such aesthetic procedures are still limited. The primary goals of female genital aesthetics/vaginal cosmetic procedures are to improve patient satisfaction and quality of life (QoL) (5).

The satisfaction with the genital appearance of women is associated with overall genital appearance, sexual esteem, and sexual satisfaction (6). Women who are satisfied with their body image report to have confidence with their sexual life, more sexual activity, and orgasm more often (7). A positive body image has been strongly correlated with sexual functioning and satisfaction, while a negative body image has been associated with a need for physical attraction (7). A persons' high respect to her/his body refers to satisfaction of this person from personal and interpersonal relations, and it can help in estimating sexual satisfaction (8). It is known that the perceived body image levels widely affect clinical scenarios, but this association is often overlooked or is not noticed by clinicians. Women increasingly think that the appearance of their genitalia is crucial, and more and more women are interested in their genital appearance possibly by the impact of media (9). It has been argued that women with low sexual satisfaction can benefit from existing treatment methods that target certain regions of their body (8).

The need for genital aesthetic procedures by the patient is determined by specially designed and approved questionnaires. These questionnaires provide the measurement of the need for a clinical intervention to determine health status and the image regarding QoL from patients' perspective (10). Such questionnaire applications will possibly improve evidence-based implementation, will be helpful for potential improvements in the development of surgical methods and will facilitate the decision-making process for both patients and surgeons and make it more effective (11,12).

The objective of this study was to develop a self-assessment questionnaire for those desiring female genital cosmetic procedures (Q-FGCP), to use this questionnaire for satisfaction with genital appearance within the general population and to evaluate the correlation between total item score and demand.

METHODS

The questionnaire was conducted on 100 women who were thought to reflect the general population who presented to the Gynecology and Obstetrics clinics of our tertiary hospital. Informed consents were obtained before applying the questionnaires. An illustration of female genital organs with anatomic markings was given to the subjects who responded to the questionnaire. The questionnaire, which consisted of 11 items, was prepared to screen the women needing cosmetic genital procedures (Appendix). The first 10 items included factor analysis regarding aesthetic appearance of the genital organs (items 1-8) and the impact of aesthetics on sexual pleasure (items 9 and 10). Each question was scored between 0 and 3 points for the first 10 items. Zero point was defined as adequate satisfaction related to genital organ appearance and sexual satisfaction. One point was evaluated as normal related to genital organ appearance and sexual satisfaction whereas, 2 and 3 points were graded as dissatisfaction related to genital organ appearance and sexual satisfaction.

Item 11 questioned how much participants needed a cosmetic gynecologic procedure. Women were asked to evaluate the degree of their desire between 0 and 3 points. Zero point was accepted as no desire, while 3 points was graded as a very strong desire. The scores of 2 and 3 were assessed as a strong need for female cosmetic procedures. One point was assessed as a mild need.

The study was approved by the Zeynep Kamil Women and Children's Diseases Training and Research Hospital Ethics Committee (decision no: 33, date: 10.02.2017) in accordance with the requirements of the Declaration of Helsinki.

Statistical Analysis

Statistical analysis of the data obtained from the study was performed using SPSS version 25.0 (IBM, SPSS, Chicago, IL, USA) statistical package software. Descriptive data were expressed as frequency and percentage. The optimal cut-off value for desire score for vaginal aesthetic procedure was determined using

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receiving operating characteristics (ROC) analysis by calculating the areas under the ROC curve. Risk coefficient for categorical variable (such as a total desire score for aesthetic procedure ≤19 and >19 points) was evaluated using logistic regression analysis and expressed as "odds ratio (OR)". The correlation between continuous variables was tested using the Spearman's correlation analysis. The variable used in the correlation analysis was the score for desire for vaginal aesthetic procedure that was a continuous variable. The results were evaluated at 95% confidence interval (CI). A p-value below 0.05 was considered statistically significant.

RESULTS

Ages of the participants varied between 19 and 53 years. Educational level of the participants was high school degree or lower. While 55 (55%) of the participants had normal vaginal delivery, 12 (12%) had cesarean sections and 33 (33%) were nulliparous. Score analysis of the item 11 is shown in (Table 1). The correlation analysis was calculated between the total 10-item score and the score of the item 11. A strong need for genital/vaginal aesthetics procedures was observed in 41% of the participants. When demographic variables were analyzed in terms of the desire for cosmetic procedures, a statistically significant correlation was found only with the total score of the first 10 questions (p=0.007) (Table 2). No statistically significant correlation was found with age, weight, height, body mass index, duration of marriage, gravida, parity and abortus. ROC analysis revealed that a total score >19 was an important factor for women desiring to undergo genital cosmetic procedures (OR: 3.9, CI: 1.7-9.2, p=0.011) (Table 3). This questionnaire seems to be and will be a valuable screening tool to determine the need for cosmetic gynecology procedures among women presenting to gynecology outpatient clinics (Figure 1).

Table 1. Number of patients with the score analysis of the 11th item

11th item score	Number of patients	
0	51	51.0%
1	8	8.0%
2	37	37.0%
3	4	4.0%
Need for genital cosmetic procedures	**Number of patients**	
Little or no need	59	59.0%
Strong need	41	41.0%

Table 2. The relationship between the desire for aesthetic procedures and demographic variability

Correlation analysis	r	p-value
Total score	0.267	0.007
Age	-0.061	0.548
Height	0.010	0.919
Weight	0.078	0.440
BMI	0.047	0.645
Duration of marriage	0.067	0.557
Gravidity	0.014	0.891
Parity	0.012	0.904
Abortus	-0.040	0.693

BMI: body mass index

DISCUSSION

Regarding genital morphology, it is possible for individuals who are not even interested in the appearance of sexual organs to create a "normal" genital concept. The term "normality" and establishment of the "normal" can play a role in the determination of how much satisfaction women derive from their own physical image. If the sources to which women refer include bias or if these sources are contrary to certain morphological features and "standards", the possibility of dissatisfaction with the appearance of sexual organs is higher among women, and this causes an increase in demand for genital aesthetic procedures. With the Q-FGCP questionnaire, we aimed to create awareness of genital appearance and demonstrated the real need of individuals for surgery. We found with Q-FGCP that 41% of the participants had a strong desire for genital aesthetic procedures.

Recently, Yurteri-Kaplan et al. (13) evaluated the perception of women regarding their vulvas and whether this perception affected their desire for genital aesthetic procedures. Majority of the 354 participants argued that the appearance of their vulvas was important. Both 44-year-old women and those aged >44 years showed anatomy sources to their physicians for obtaining information about vulvar and labial appearance. However, significant portion of younger women used pornography as a source of information (13). Therefore, increased usability of

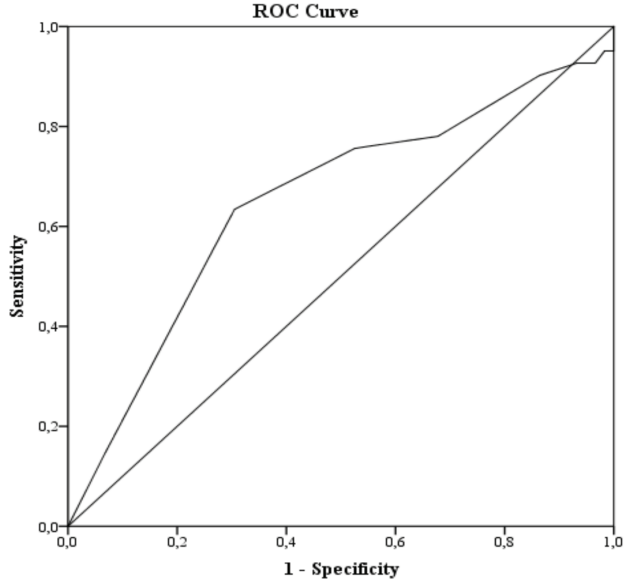

Figure 1. Vaginal aesthetic requirement
ROC: receiving operating characteristics

Table 3. Eleventh item score and vaginal aesthetic requirements

11th item score (vaginal aesthetic requirement)		No need (0+1)	Strong desire (2+3)	AUC (SE)	Odds ratio (%95 CI)	p-value
Total score	≤19	41 (69.5)**	15 (36.6)	0.650 (0.058)	3.9 (1.7-9.2)	0.011
	>19	18 (30.5)	26 (63.4)*			

**specificity, *sensitivity, SE: standard error, AUC: area under the curve, CI: confidence interval

pornographic material in media and the Internet influence the attitudes of women regarding the appearance of the genitalia and sensation of personal satisfaction. As a result of the mentioned study, Yurteri-Kaplan et al. (13) concluded that the age of a woman had no effect on the perception of a normal vulva, that majority of women perceived their vulvas as normal and were satisfied with their appearance, but older women were more interested in cosmetic vulvar surgery. We believe that the results of that study support our theory and thus, a weak-to-moderate negative correlation should be expected between physical appearance of women and demand for genital aesthetic procedures.

In another study from Netherlands (4), when demands of the women desiring cosmetic surgery were examined, the reason was the request to regain the appearance of the prior younger vulvar. During menopause, genital prolapsus and estrogen insufficiency can alter the appearance of the vulva. Therefore, the restoration of previous anatomy and appearance can be a source of demand for cosmetic vulvar surgery instead of a perception of a distorted vulvar.

Most women in the Western culture perceive a normal vulva with the following features: symmetrical labia majora and labia minora with a clitoral hood (5). According to the cosmetic data from the United States of America, there may be regional differences in the perception of the vulva, and demand for these procedures can be higher among the regions (3). As the results of ethnic origin, regional, social needs and demands vary, this situation also applies to our country. Given these results, further studies are necessary to generalize these results to a wider population (14). The motivation of women for requesting cosmetic vulvar surgery should be investigated when they are admitted for these procedures. Since healthcare workers play a critical role in providing patients with medical education, based on the unrepresentative concept of women variation in morphology, women reporting concerns about their physical image should be informed through images or tools explaining the normal anatomy. Physicians should be aware that perceptions can be influenced by a distorted perception of what is normal or a demand for regaining the previous anatomy because of postnatal genital prolapsus or estrogen insufficiency. As physicians, we must train our patients about the variations in vulvar anatomy and potential risks of such procedures.

Recently, the rate of women requesting elective surgery is increasing, and these women seem to be significantly influenced by the media (15). Schick et al. (16) reported a shifting in the ideals of genital appearance over 50 years and concluded that this perception promoted an important physical image dissatisfaction among women. In a study by Laan et al. (17), effects and self-awareness of women graduated from college were investigated by showing them images of a natural vulva. The authors observed that exposure to the natural vulva images affected genital self-image positively (17). Hummel et al. (14) reported that cognitive-behavioral therapy was effective in sexual dysfunction and physical image-related procedures were effective among victims of breast cancer. Q-FGCP has the potential for using the questionnaire in such approaches before performing elective female cosmetic procedures. Physical body image, dyspareunia and sexual function can be affected by infertility, pregnancy, gestational diabetes mellitus, and endometriosis, which are among the most common causes of presentation to gynecology outpatient clinics (18,19). It would be an interesting experience to observe the changes in self-image and sexual functioning level during and after the treatment of these disorders in obstetrics and gynecology clinics.

Study Limitations

This questionnaire should have been applied to more people since there may be geographical, traditional and expected differences. Hence, that would have created a truly random sample of people answering our questions for the survey/questionnaire.

CONCLUSION

There is no uniform definition for "abnormal" labial size and genital appearance. Most sexual medicine specialists think that physical self-image is an important determinant in the demand for genital aesthetic procedures among women. Many specialists advise that these women should be referred to a consultation with a psychiatrist or a psychologist before being admitted for genital aesthetic procedures. A scoring system questionnaire can be used to determine whether individuals in general population need genital/vaginal aestetic procedures. Since perception of "normal" appearance can differ among women, only patients with an actual need for these procedures can be directed to aesthetic procedures.

Ethics Committee Approval: The study was approved by the Zeynep Kamil Women and Children's Diseases Training and Research Hospital Ethics Committee (decision no: 33, date: 10.02.2017) in accordance with the requirements of the Declaration of Helsinki.

Informed Consent: Informed consents were obtained before applying the questionnaires.

Peer-review: Externally and internally peer-reviewed.

Author Contributions: Surgical and Medical Practices - D.H., B.H.; Concept - S.Ş., M.E.; Design - C.K.; Data Collection and/or Processing

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- B.H., C.Ö.; Analysis and/or Interpretation - C.K., S.Ş.; Literature Search - C.Ö., M.E.; Writing - D.H.

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Appendix. Questionnaire for Female Genital Cosmetic Procedures (Q-FGCP)

QUESTIONNAIRE FOR FEMALE GENITAL COSMETIC PROCEDURES (Q-FGCP)

NAME/SURNAME: ..... DATE: .....

AGE: ..... SINGLE/MARRIED (DURATION): .....

FILE NO: .....

HEIGHT/WEIGHT: .....

GRAVIDA/PARITY/ABORTUS: .....

MODE OF DELIVERY (IF ANY): .....

PREVIOUS OPERATION(S): .....

COMORBIDITY: .....

EDUCATION/PROFESSION:

1- Do you like the appearance of your sexual organ?
1) Very much
2) Normal
3) Little
4) Very little

2- Do you think that the size of your labia majora is adequate?
1) Very adequate
2) Normal
3) Large
4) Very large

3- Is the appearance of your both labia majora symmetrical?
1) Very symmetrical
2) Normal
3) Little symmetrical
4) Not symmetrical

4- Do you think that the size of your labia majora is adequate?
0) Very adequate
1) Normal
2) Large
3) Very large

5- Is the appearance of both labia minora symmetrical?
0) Very symmetrical
1) Normal
2) Little symmetrical
3) Not symmetrical

6- Is the size of the hairy area and fatty portion above the labia majora adequate for you?
1) Very adequate
2) Normal
3) Large
4) Very large

7- Is the size of the folding above the clitoris adequate for you?
1) Very adequate
2) Normal
3) Large
4) Very large

8- Do you like the darkness of your sexual area?
1) Very much
2) Normal
3) Not much
4) Don't like

9- Are you satisfied with the width of your vagina?
1) Very much
2) Normal
3) Wide
4) Too wide

10- Does adequate wetting occur during intercourse?
1) Very well
2) Normal
3) Not sufficient
4) Never sufficient

11- Are you considering rejuvenation and restoration procedures for your genitalia?
1) No
2) May be
3) Yes
4) Definitely Yes

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Investigation of *in vitro* Biofilm Formation and Its Correlation with Antibiotic Resistance Pattern Among Clinical Isolates of *Staphylococcus aureus*: A Cross-sectional Study in Northern Cyprus

Staphylococcus aureus Klinik İzolatları Arasında *in vitro* Biyofilm Oluşumunun ve Antibiyotik Direnç Paterniyle Korelasyonunun Araştırılması: Kuzey Kıbrıs'ta Kesitsel Bir Çalışma

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ABSTRACT

Objective: *Staphylococcus aureus* (*S. aureus*), including methicillin resistant *S. aureus* (MRSA), can produce biofilm leading to increased morbidity and mortality in hospital infections. Antibiotic resistance is an inherent feature of bacterial biofilms, and the formation of biofilms is more widespread in MRSA. This study aimed to reveal the phenotypic biofilm-forming abilities of *S. aureus* isolates and to investigate the relationship of antibiotic resistance of biofilm-forming *S. aureus* with biofilm formation.

Methods: A cross-sectional descriptive study was carried out in the microbiology laboratory at the Near East University Hospital in the Turkish Republic of Northern Cyprus. A total of 67 non-duplicative samples (wound/pus, sputum, aspirate, blood and urine) for the study were collected between January 2020 and April 2021 from samples of inpatients and outpatients from various hospital departments. VITEK 2 system was used for bacterial identification and antibiotic susceptibility testing, biofilm formation was evaluated using Congo red agar (CRA).

Results: It was observed that 56 (84.3%) of 67 *S. aureus* isolates cultured on CRA produced biofilm, while the remaining 11 (15.7%) were not biofilm producers. A statistically significant relationship was found between methicillin resistance and biofilm formation in *S. aureus* isolates. Accordingly, a significantly higher biofilm formation was observed in MRSAs compared to those with negative methicillin resistance (92.1% vs. 72.4%, p=0.034). A high proportion of isolates of *S. aureus* showed susceptibility towards tigecycline (100%) and gentamycin (100%).

Conclusion: The findings of this study indicated that methicillin-resistant strains produced more biofilms and exhibited a high degree of resistance to most antibiotics.

Keywords: *Staphylococcus aureus*, MRSA, biofilm, Congo red agar, Northern Cyprus

ÖZ

Amaç: *Staphylococcus aureus* (*S. aureus*), metisiline dirençli *S. aureus* (MRSA) da dahil olmak üzere hastane enfeksiyonlarında morbidite ve mortalite artışına yol açan biyofilm üretme özelliği göstermektedir. Antibiyotik direnci, bakteriyel biyofilmlerin doğal bir özelliği olmakla birlikte biyofilm oluşumu MRSA'da yaygın bir şekilde görülmektedir. Bu çalışmada, *S. aureus* izolatlarının fenotipik biyofilm oluşturma yetenekleri ve biyofilm oluşumu ile antibiyotik direncinin ilişkisini araştırmak amaçlanmıştır.

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Yöntemler: Çalışma, Ocak 2020-Nisan 2021 tarihleri arasında Kuzey Kıbrıs Türk Cumhuriyeti'nde Yakın Doğu Üniversitesi Hastanesi Mikrobiyoloji Laboratuvarı'nda gerçekleştirildi. Hastanenin farklı bölümlerinden, yatan ve ayaktan olmak üzere toplam 67 hastaya ait örnekler (yara/irin, balgam, aspirat, kan ve idrar) incelendi. Bakteri identifikasyonu ve antibiyotik duyarlılık testi için VITEK 2 sistemi kullanıldı ve biyofilm oluşumunu tespit etmek için ise Kongo kırmızısı agar (KKA) kullanılarak değerlendirme yapıldı.

Bulgular: KKA'ya ekimi yapılan toplam 67 *S. aureus* izolatının 56'sının (%84,3) ürettiği, geri kalan 11'inin (%15,7) ise biyofilm üreticisi olmadığı görülmüştür. *S. aureus* izolatlarında metisilin direnci ile biyofilm oluşumu arasında istatistiksel olarak anlamlı bir ilişki tespit edilmiştir. Buna göre, MRSA'larda metisilin direnci negatif olanlara göre anlamlı düzeyde daha yüksek biyofilm oluşumu gözlendi (%92,1 vs. %72,4, p=0,034). *S. aureus* izolatları yüksek oranda tigesiklin (%100) ve gentamisine (%100) karşı duyarlılık göstermiştir.

Sonuç: Bu çalışmanın bulguları, MRSA suşlarının daha fazla biyofilm ürettiğini ve çoğu antibiyotiğe karşı yüksek oranda direnç sergilediğini göstermektedir.

Anahtar kelimeler: *Staphylococcus aureus*, MRSA, biyofilm, Kongo kırmızısı agar, Kuzey Kıbrıs

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a gram-positive commensal opportunistic pathogen which poses a threat to public health. It is responsible for bacteremia acquired in hospitals with a mortality rate of 20-30%. Other infections caused by *S. aureus* are bloodstream infections, surgical site infections, skin, and soft tissue infections, infectious endocarditis, osteomyelitis, device-related infections and pneumonia (1).

S. aureus can colonize and spread by attaching to the host's extracellular matrix components and serum proteins. In the pathogenesis of *Staphylococcal* infection, it has been observed that biofilm production has a very active role in protecting the colony from environmental factors, antibacterial therapy, and immune reactions of the host (2). Biofilms are complex assemblages of bacteria embedded in an extracellular matrix of exopolysaccharides, proteins, and macromolecules like DNA. They can grow on both living and non-living surfaces. Studies using molecular methods and scanning electron micrographs have shown that biofilms colonize on wounds. They shield the microorganisms from host immunity and prevents antibiotics from reaching the site of infection, causing wound healing to be hindered (3).

In developed countries, methicillin resistant *S. aureus* (MRSA) is now endemic in nearly all medical centers (4). The *mecA* or *mecC* genes are found on the *Staphylococcal* chromosomal cassette and encode penicillin-binding protein 2A (PBP2A), an enzyme that crosslinks the peptidoglycans in the bacterial cell wall, which confers methicillin resistance. Beta lactam antibiotics are ineffective against these enzymes, which results in resistance. Vancomycin has been used as a first-choice antibiotic to treat MRSA infections for years. Outbreaks of multidrug-resistant, medium and high-level vancomycin-resistant *S. aureus* (VRSA) have occurred over the last two decades, posing an important public health risk (5). *S. aureus* can be easily transmitted between individuals in the healthcare and in the community settings, owing to its commensal presence with immunocompetent individuals. Furthermore, it is a growing matter of concern due to its relation to hospital-acquired infections and antibiotic resistance (6). MRSA is a serious and widespread problem due to its capacity to colonize and cause disease in humans and animals (7). Therefore, the aim of our study was to investigate the phenotypic biofilm forming abilities of *S. aureus* isolates and the relationship of antibiotic resistance of biofilm forming *S. aureus* with biofilm formation.

METHODS

Design of Study

A cross-sectional descriptive study was carried out in the microbiology laboratory at the Near East University Hospital in the Turkish Republic of Northern Cyprus. A total of 67 non-repeated samples for the study were collected between January 2020-April 2021 from hospitalized patients from various hospital departments.

Samples Collection

Collected samples were cultured on blood agar (Merck, KgaA, Germany) and Eosin Methylene Blue agar (Becton Dickinson, Sparks, MD 211 52, USA) and incubated at 35 °C for 24-48 hours to obtain pure colonies. Only colonies that grew on blood agar media were loaded into the VITEK 2 (bioMérieux SA, F-69280 Marcy l'Etoile, France) system for bacterial identification and antibiotic susceptibility patterns; then, when the VITEK 2 device identified *S. aureus*, the bacterial colonies were transferred and stored in bacteria storage tubes (OR-BAK, Ankara, Turkey) at -30 °C until used.

Samples Isolation and Culturing

To revive the stored samples, *S. aureus* strains were inoculated on blood agar for growth and incubated for 24-48 hours at 35 °C to get pure colonies, then Congo red agar (CRA) was prepared and pure colonies from blood agar were inoculated on CRA for biofilm detection and subsequently incubated for 24-48 hours at 35 °C, colonies that were black were considered biofilm positive whereas colonies that showed red were considered biofilm negative. Both blood agar and CRA were prepared as per the manufacturer's directions.

Antibiotic Susceptibility Testing

For bacterial identification and antibiotic susceptibility, VITEK 2 system was employed. All *S. aureus* isolates were tested for their sensitivity against 16 commonly used antibiotics which were as follows: benzylpenicillin, cefoxitin, gentamicin, ciprofloxacin,

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levofloxacin, clindamycin, linezolid, daptomycin, teicoplanin, vancomycin, tetracycline, tigecycline, fosfomycin, fusidic acid, mupirocin and cotrimoxazole. Vancomycin resistant strains were confirmed by the E-test method using Vancomycin MIC Test Strip (Liofilchem s.r.l., Italy).

Detection of Biofilm Production

For the formation of biofilm in *S. aureus* clinical isolates, CRA method was utilized. In CRA method, *S. aureus* was inoculated in CRA comprising Blood Base 2 media (40 gr/L supplemented with 10 gr/L glucose and Congo red (0.4 gr/L). It was incubated at 37 °C for 24-48 hours. The biofilm produced was observed and interpreted; a positive result indicated black color colonies (Figure 1) with a dry crystalline consistency and negative result indicated red color colonies (Figure 2).

To ensure quality control of test organisms, three bacterial strains were used as controls for the experiment: *S. aureus* ATCC29213 was used as the positive control for biofilms, while *S. aureus* ATCC6538 and *S. epidermidis* ATCC11047 were used as negative biofilm controls, respectively. They were then incubated on CRA plates to determine whether they produced black colonies. For growth and biofilm formation, all control species were cultured on both blood agar and CRA. The isolates were then incubated at 37 °C for 24-48 hours.

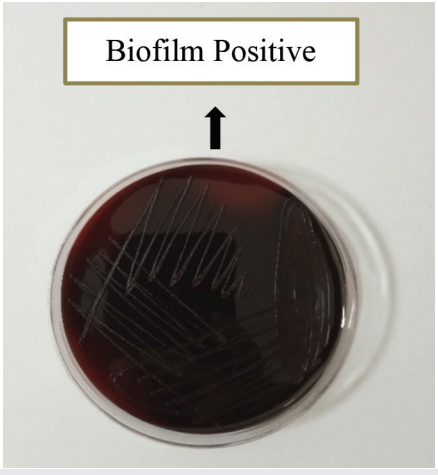

Figure 1. Black colonies positive biofilm

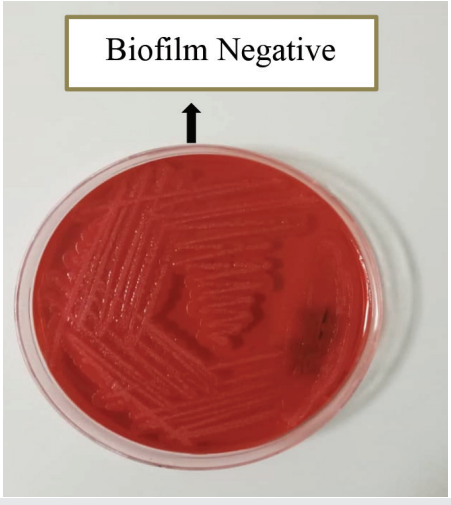

Figure 2. Red colonies negative biofilm

Statistical Analysis

All data acquired were statistically analyzed with a computer-based SPSS 22 software package. Frequency and cross-tabs analysis were used to test the totals. To discover an association between two variables, a Pearson chi-square test was utilized with a significance level of $p<0.05$.

Ethical Approval

This study was approved by the Scientific Research Ethics Committee of Near East University on 25.02.2021 (2021/88-1194). Patient consent was not required because the samples sent to the routine laboratory were examined. The names of the patients were covered, and the privacy of data was maintained.

RESULTS

A total of 67 samples for the study were collected between January 2020 and April 2021 from hospitalized patients from various hospital departments and subjected to microbiological analysis to isolate *S. aureus* strains. The mean age of the patients with MRSA isolated was 63.32±26.10 (between 3-97 years), while the average age of patients isolated with methicillin-sensitive *S. aureus* (MSSA) was 44.24±28.50 (between 1-92 years). According to the data obtained, the frequency of MRSA infection increased significantly as the age got older (p=0.006) as shown in Table 1. Among a total of 67 *S. aureus* isolates, 38 (56.7%) were identified to be MRSA by using VITEK 2 antibiotic susceptibility testing system with cefoxitin performed and the remaining 29 (43.3%) were identified to be MSSA.

Out of 38 MRSAs, 29 (76.3%) of them were recovered from inpatients and 9 (23.7%) from outpatients. The association between MRSA occurrences in inpatients was statistically significant (p=0.018), which demonstrated the fact that the possibility of finding MRSA in admitted patients was high as compared to the outpatients as shown in Table 2.

Among 67 *S. aureus* isolates, 29 were MSSA and 38 were MRSA. Of those 35 were male and 32 were female patients. However, no significant relationship between gender and growth of MRSA was identified (p=0.675). Among 67 *S. aureus* strains recovered, 52.2% were MRSA, 43.3% were MSSA, while 4.5% were VRSA.

A total of 67 *S. aureus* isolates undergoing CRA method demonstrated 56 (84.3%) as biofilm producer and the rest

Table 1. Distribution of MRSA due to age

	No of patients	Mean age of patients	Standard deviation	p-value
MSSA	29	44.24	28.50	0.006
MRSA	38	63.32	26.10	

MSSA: methicillin-sensitive *S. aureus*, MRSA: methicillin resistant *S. aureus*

11 (15.7%) as non-biofilm producer. A statistically significant relationship was found between methicillin resistance and biofilm formation in *S. aureus* isolates. Accordingly, significantly higher biofilm formation was observed in MRSA (92.1%) compared to MSSA (72.4%) isolates (p=0.034) as shown in Table 3.

All *S. aureus* isolates were tested for their sensitivity against 16 commonly used antibiotics. Resistance rates of the MRSA isolates were significantly higher towards benzylpenicillin 33 (97.1%), clindamycin 27 (75%) and tetracycline 17 (47.2%) compared to MSSA isolates. Of the MSSA isolates 22 (81.5%) were resistant to benzylpenicillin, 10 (34.5%) to clindamycin, and 5 (17.2%) to tetracycline. Lower rate of resistance was observed in 1 (3.7%) MSSA against linezolid, however MRSA showed no resistance against linezolid, making it the most effective antibiotic for severe MRSA infections and it could be used as empiric therapy. On the other hand, both MRSA and MSSA showed less resistance against ciprofloxacin, levofloxacin, daptomycin, mupirocin and trimethoprim/sulfamethoxazole. Furthermore, the MRSA isolates showed a statistically significant resistance pattern against the following antibiotics: clindamycin and tetracycline compared to MSSA (p=0.001 and 0.011, respectively). Almost all isolates were sensitive to tigecycline and gentamycin. Interestingly, MRSA isolates were even resistant to vancomycin and teicoplanin [3 (8.3%) and 2 (5.7%), respectively]. On the other hand, no MSSA was found resistant to vancomycin, but it was resistant to teicoplanin 1 (3.6%) as shown in Table 4.

DISCUSSION

Bacteria in biofilms are of considerable concern as they represent up to 65% of human infections and they have high resistance (10-1000 times) to normal antibiotics (2). Nosocomial infections are a severe and persistent issue in the hospital settings. MRSA is a significant human pathogen which causes various diseases in humans, ranging from skin infections to severe infections like pneumonia, soft tissues, bones, heart valves, and even fatal septicemia (8). In this study, the CRA method was used to detect biofilm production of *S. aureus*. A total of 67 *S. aureus* isolates were incorporated, together with two control strains of *S. aureus* and *S. epidermidis*, as positive and negative biofilm controls, respectively.

Table 2. Distribution of MRSA and MSSA in outpatients and inpatients

Patient's type	Number of MRSA (%)	Number of MSSA (%)	Total number (%)	p-value
Inpatients	29 (76.3)	14 (48.3)	43 (64.2)	0.018
Outpatients	9 (23.7)	15 (51.7)	24 (35.8)	
Total	38 (100)	29 (100)	67 (100)	

MSSA: methicillin-sensitive *S. aureus*, MRSA: methicillin resistant *S. aureus*

Table 3. Correlation between biofilm production and methicillin-resistance

Biofilm	MRSA (%)	MSSA (%)	p-value
Producer	35 (92.1)	21 (72.4)	0.034
Non-producer	3 (7.9)	8 (27.6)	
Total	38 (100)	29 (100)	

MSSA: methicillin-sensitive *S. aureus*, MRSA: methicillin resistant *S. aureus*

This study discovered that patient age was a risk factor for MRSA infection in admitted patients. The mean age of patients with MRSA infection was 63.32±26.10 (range: 3-97 years), whereas the average age of patients with MSSA infection was 44.24±28.50 (between 1-92 years). According to the data obtained, the prevalence of MRSA infection increased significantly with age (p=0.006), which correlated with the findings of a previous study by Kshetry et al. (9), who found that 29 strains of MRSA were isolated from adults and 18 strains were isolated from pediatric patients, with the difference being statistically significant. In our current study, the prevalence of MRSA was found to be high (n=38, 56.7%) compared to MSSA (n=29, 43.3%), with a similar rate reported by Belbase et al. (10). The numbers and rates of and MSSA were 36 (47.4%) and 17 (22.4%), respectively in the study by Piechota et al. (8). However, a lower prevalence of MRSA was reported as 26.12% by Pandey et al. (11). Of the 38 (56.7%) MRSA strains, 3 (4.5%) were resistant to vancomycin which was comparable to the results of Jahanshahi et al. (12). In our study, substantial proportion of MRSA isolates were obtained from hospitalized patients (n=29, 76.3%). Colonized health care workers in hospitals are the primary source of MRSA infection in hospitalized patients, resulting in increased infection rates. However, the isolation rate of MRSA among outpatients was low, (n=9, 23.7%). Additionally, admitted patients who became colonized during their hospital stay might act as secondary sources of community-acquired MRSA infections. The higher rate of MRSA infection in admitted patients was statistically significant (p=0.018), which was consistent with the findings of Belbase et al. (10), 54.5% and 41.9% in inpatients and in outpatients, respectively. This difference could be explained by a prolonged hospital stay, instrumentation, and other invasive devices, as well as the fact that *S. aureus* was mostly associated with nosocomial infections.

Numerous studies have been conducted on producing biofilms by *Staphylococcus* species using various methods (13,14). It was revealed in this study that the technique used could detect the formation of biofilms between isolated strains. The current study evaluated the production of biofilms/ESPs by 67 *S. aureus* strains on CRA. Out of 67 cultures inoculated on CRA, 56 (84.3%) were identified as *S. aureus* producing biofilm, which was comparable to the results of Sharma et al. (15), (2021), which identified 53 (80%) as biofilm producing *S. aureus*. However, Haghi Ghahremanloi Olia et al. (16) reported a higher rate of biofilm production (n=57, 95%), which could be explained by the imprecision with which this method identified moderate biofilm-producing strains (17).

Because biofilms are protective, bacteria growing in them are intrinsically resistant to a wide variety of antibiotics. Positive biofilm producers were detected in 92.1% of MRSA samples and 72.4% of MSSA samples. A statistically significant relationship between methicillin resistance and biofilm formation in *S. aureus*

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Table 4. Resistance pattern of *S. aureus* from different clinical specimens (n, %)

Antibiotics	MSSA	MRSA	p-value	Biofilm-producer	Non-biofilm producer	p-value
Benzylpenicillin	22/27 (81.5)	33/34 (97.1)	0.055	46/51 (90.2)	9/10 (90.0)	0.676
Gentamicin	0/29 (0)	0/35 (0)	-	0/54 (0)	0/10 (0)	-
Ciprofloxacin	1/29 (3.4)	7/36 (19.4)	0.054	5/52 (9.6)	0/10 (0)	0.402
Levofloxacin	1/29 (3.4)	5/38 (13.2)	0.174	4/54 (7.4)	0/10 (0)	0.498
Clindamycin	10/29 (34.5)	27/36 (75.0)	0.001	30/54 (55.6)	7/11 (63.6)	0.441
Linezolid	1/27 (3.7)	0/35 (0)	0.435	1/50 (2.0)	0/11 (0)	0.823
Daptomycin	0/28 (0)	2/35 (5.7)	0.305	2/53 (3.8)	0/10 (0)	0.706
Teicoplanin	1/28 (3.6)	2/35 (5.7)	0.584	3/52 (5.8)	0/11 (0)	0.557
Vancomycin	0/28 (0)	3/36 (8.3)	0.171	3/54 (5.6)	0/10 (0)	0.595
Tetracycline	5/29 (17.2)	17/36 (47.2)	0.011	20/54 (37.0)	2/11 (18.2)	0.199
Tigecycline	0/29 (0)	0/35 (0)	-	0/54 (0)	0/10 (0)	-
Fosfomycin	0/29 (0)	3/34 (8.8)	0.151	3/53 (5.7)	0/10 (0)	0.590
Fusidic acid	0/27 (0)	3/34 (8.8)	0.166	3/52 (5.8)	0/9 (0)	0.614
Mupirocin	1/27 (3.7)	0/32 (0)	0.458	1/50 (2.0)	0/9 (0)	0.847
SXT	3/29 (10.3)	2/38 (5.3)	0.372	5/56 (8.9)	0/11 (0)	0.396

SXT: trimethoprim/sulfamethoxazole, MSSA: methicillin-sensitive *S. aureus*, MRSA: methicillin resistant *S. aureus*

isolates (p=0.034) was observed, consistent with the results of Khasawneh et al. (18), indicated that 90.9% of MRSA and 71.4% of MSSA isolates were resistant to methicillin. According to a study conducted by Grinholc et al. (19), only 45-47% of MRSA strains and 66-69% of MSSA strains could form biofilms *in vitro*. Certain strains have been reported to produce no biofilm despite the presence of a locus. Biofilm formation is widely regarded as a significant factor in the virulence of antibiotic-resistant bacteria, particularly MRSA. Further phenotypic and genotypic characterization of the *ica* locus genes is required to better understand the mechanism of biofilm production in *Staphylococcal* infections (20).

The development of MRSA among *S. aureus* strains leads to problems in the treatment of these infections. Monitoring *S. aureus'* antimicrobial susceptibility patterns is of prime significance to understand new emerging resistance trends and to treat infections in hospitals and in community (21). This study found that commonly used antibiotics were more resistant to MRSA than to MSSA; the highest resistance rates were observed against benzylpenicillin (n=33, 97.1%), clindamycin (n=27, 75%), and tetracycline (n=17, 47.2%). In this study, a high proportion of isolates (97.1%) were penicillin-resistant. This was expected, as only a minority of *S. aureus* strains did not produce beta-lactamases. In a study carried out by Ansari et al. (21), a comparable rate of resistance to penicillin was observed (94.7%).

The MRSA is commonly treated with clindamycin. Other types of antibiotics, like macrolides, can also lead to macrolide-resistant strains of *S. aureus*. Resistance to macrolides, on the other hand, can occur due to mutation of the 23S rRNA encoded by the *erm* gene, known as MLSB resistance, and is also referred to as clindamycin resistance or MLSB resistance (due to efflux mechanism encoded by the *msrA* gene). Failure can occur if the treatment is applied to a strain of bacteria that contains an *erm* gene, which can induce resistance (21). In our study, we identified 27 (75%) MRSA resistant strains and 10 (34.5%) MSSA resistant strains against clindamycin which were in line with the findings of Horváth et al. (22), indicating that clindamycin resistance was present in 79.1% of patients.

Study Limitations

This study was done only phenotypically. Therefore, molecular analyses of genes responsible for biofilm formation and antibiotic resistance are needed. Also, this study was cross-sectional with small size of 67 samples from a single center and therefore, did not represent an overall prevalence of biofilm forming MRSA in hospitals in Northern Cyprus. Multicenter studies with large number of samples collected from patients are required to estimate the overall prevalence of biofilm forming MRSA in hospitals across the country. In addition, the fact that teicoplanin-resistant strains were nor confirmed by a different method (such as the E-test) was another limitation of our study.

CONCLUSION

According to the findings of this study, *S. aureus* formed biofilms and this finding was clinically significant as biofilm formation was associated with the pathogenicity of organisms that caused device-related infections and exhibited high resistance to antibiotics. The CRA method used in the study to detect biofilm was reliable and the prevalence rate of MRSA isolated from hospitalized patients with *S. aureus* was high.

In the hospital setting, the wound/pus was the primary source of *S. aureus* and MRSA. Tigecycline and gentamycin (100%) were the prior drugs of choice for the treatment of *S. aureus*

infections, including MRSA, followed by linezolid, mupirocin, and daptomycin. MRSA strains exhibited multidrug resistance and were unusually resistant to vancomycin, the drug of choice, indicating that MRSA was a vibrant organism. As a result, this threat can be mitigated through the implementation of sound infection control policies, regular surveillance of the antibiotic profile of *Staphylococcus* isolates to establish antibiotic policies, and the reasonable use of antimicrobial agents. Additionally, as this study only qualitatively presents biofilm in isolates, additional research is recommended that further research be conducted on the molecular mechanisms involved. There is a need for detailed information on the molecular mechanisms underlying biofilm formation and its relationship to other microbial processes such as virulence and antibiotic resistance.

Ethics Committee Approval: This study was approved by the Scientific Research Ethics Committee of Near East University on 25.02.2021 (2021/88-1194).

Informed Consent: Patient consent was not required because the samples sent to the routine laboratory were examined.

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Comparison of Broth Microdilution Method with BD Phoenix, Micro Scan and E-test for Carbapenem-resistant *Enterobacterales*: Colistin Susceptibility Testing

Karbapenem Dirençli *Enterobacterales* için Sıvı Mikrodilüsyon Yöntemi ile BD Phoenix, Micro Scan ve E-test Yöntemlerinin Karşılaştırılması: Kolistin Duyarlılık Testi

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ABSTRACT

Objective: In the past years, due to the increasing carbapenem resistant *Enterobacterales* (CRE) infection rates, colistin use has been on the rise. Multi-drug resistant Gram-negative bacteria and colistin resistance are increasing simultaneously; therefore, an accurate method for antimicrobial susceptibility testing of colistin is crucial. Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing recommend the standard broth microdilution (BMD) method for colistin minimum inhibitory concentration testing. In this study, we aimed to examine the performance of BD Phoenix, MicroScan, and E-tests on CRE isolates on the determination of colistin susceptibility. The existing commercial tests were compared to the reference BMD method.

Methods: One hundred and twenty non-duplicate clinical *Enterobacterales* isolates such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloace* (*E. cloacae*) were collected between August 2017 to June 2018. The BD Phoenix, MicroScan systems, and E-tests were used to test colistin susceptibility. Commercial methods were compared with the reference method BMD.

Results: Colistin susceptibility was evaluated in 120 Gram-negative clinical isolates, including 108 *K. pneumoniae*, 10 *E. coli*, 2 *E. cloacae*, during the study period. Among the isolates, 66 (55%) were susceptible, and 54 (45%) were resistant to colistin, according to BMD. BD Phoenix, MicroScan, and E-test had 90.90%, 95.45%, and 96.96% sensitivity, respectively, when colistin was tested.

Conclusion: In routine clinical practice, the worldwide reference method can hardly be implemented, and commercially available systems are used for the interpretation of colistin susceptibility. Colistin use is increasing for the treatment of multiresistant Gram-negative infections, further and more extensive studies are needed for precise susceptibility testing methods for this compound. We recommend that laboratories use the BMD method at least in selected patient groups in the face of increasing antimicrobial resistance.

Keywords: Colistin susceptibility, carbapenem-resistant *Enterobacterales*, broth microdilution

ÖZ

Amaç: Son yıllarda artan karbapenem dirençli *Enterobacterales* (CRE) enfeksiyon oranlarına bağlı olarak kolistin kullanımı artmaktadır. Çoklu ilaca dirençli Gram-negatif bakteriler ve kolistin direnci aynı anda arttığı için, kolistinin antimikrobiyal duyarlılık testi için doğru bir yöntemin seçimi çok önemlidir. Klinik Laboratuvar Standartları Enstitüsü ve Avrupa Antimikrobiyal Duyarlılık Testleri Komitesi, kolistin minimum inhibitör konsantrasyon testi için standart sıvı mikrodilüsyon (BMD) yönteminin kullanılmasını önermektedir. Bu çalışmada, CRE izolatları üzerinde BD Phoenix, MicroScan ve E-testlerinin kolistin duyarlılığının belirlenmesindeki performansını incelemeyi amaçladık. Mevcut ticari testler, referans BMD yöntemiyle karşılaştırıldı.

Yöntemler: Ağustos 2017 ile Haziran 2018 arasında *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloace* (*E. cloacae*) gibi 120 klinik *Enterobacterales* izolatı toplandı. Kolistin duyarlılığı için BD Phoenix, MicroScan ve E-test kullanıldı. Referans yöntem BMD ile ticari yöntemlerin karşılaştırması yapıldı.

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Bulgular: Çalışma süresi boyunca 108 *K. pneumoniae*, 10 *E. coli*, 2 *E. cloacae* dahil olmak üzere 120 Gram-negatif klinik izolatın kolistin duyarlılığı değerlendirildi. BMD'ye göre izolatlardan 66'sı (%55) kolistine duyarlı, 54'ü (%45) kolistine dirençli idi. BD Phoenix, MicroScan ve E-test ile kolistin duyarlılığı test edildiğinde sırasıyla %90,90, %95,45 ve %96,96 idi.

Sonuç: Rutin klinik uygulamada, dünya çapında referans yöntemin uygulanması neredeyse imkansızdır ve kolistin duyarlılığının yorumlanması için ticari olarak mevcut sistemler kullanılmaktadır. Çoklu dirençli Gram-negatif enfeksiyonların tedavisi için kolistin kullanımı artmakta ve bu bileşiğe yönelik hassas duyarlılık test yöntemleri için daha fazla ve daha kapsamlı çalışmalar yapılmaktadır. Artan antimikrobiyal direnç karşısında laboratuvarların en azından seçilmiş hasta gruplarında BMD yöntemini kullanmasını öneriyoruz.

Anahtar kelimeler: Kolistin duyarlılığı, karbapenem dirençli *Enterobacterales*, sıvı mikrodilüsyonu

INTRODUCTION

Multi-drug resistant organisms are the source of a growing burden with challenging solutions globally. Recently, carbapenem-resistant *Enterobacterales* (CRE) has become a problem worldwide. The treatment of CRE infections is challenging, and the available treatment options are limited (1). In the past years, due to the increasing CRE infection rates, polymyxin use has been on the rise (2,3).

Antimicrobial treatment should be managed carefully by considering the benefits, potential toxicities; therefore, susceptibility testings play an important part in antimicrobial treatment guidance. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) issued the breakpoints of colistin for *Enterobacterales* and reported susceptible and resistant breakpoint of <2 mg/liter and >2 mg/liter, respectively (4). The available methods are limited regarding the performance, reproducibility, and accuracy for the susceptibility testing of colistin (5,6). In 2016, the ISO-20776 standard broth microdilution (BMD) method was recommended for colistin minimum inhibitory concentration (MIC) testing by Clinical and Laboratory Standards Institute (CLSI) and EUCAST (7). Other test techniques such as gradient diffusion, agar dilution, and disk diffusion are not suggested today. Colistin shows a poor diffusion on the agar; therefore, the disk diffusion method has high interpretation error levels, which decreases the reliability (8,9). Reference BMD use is not practical to perform for susceptibility testing due to the individual laboratory burden; also, the production and use of BMD panels are exhausting. There are commercially produced BMD panels as well, but those panels are expensive for most of the hospitals. Up to this day, the accuracy of automated antimicrobial susceptibility methods is not precise.

In this study, we aimed to examine the performance of BD Phoenix (BD Diagnostic Systems, Sparks, MD), MicroScan (Beckman Coulter, CA, USA) and Colistin E-tests (bioMérieux, Marcyl'Etoile, France) on CRE isolates on the determination of colistin susceptibility. The existing tests were compared to the reference BMD method.

METHODS

Bacterial isolates: One hundred and twenty non-duplicate clinical *Enterobacterales* isolates such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloace* (*E. cloacae*), that were carbapenem-resistant were collected from a tertiary research and education hospital between August 2017 to June 2018 for this study. The evaluation was performed, prospectively, in the Microbiology Laboratory of Bozyaka Training and Research Hospital, İzmir, Turkey. The isolates were stored at -80 °C in brain heart infusion broth medium with 10% glycerol stocks and subcultured twice before the testing. All isolates were inoculated on 5% Sheep Blood agar (BD Diagnostic Systems, Sparks, MD) and EMB agar (BD Diagnostic Systems, Sparks, MD) from the stock medium before assay. It was incubated at 37 °C for 24 hours. *E. coli* ATCC 25922 and clinical isolate with confirmed MCR-1 positivity were used as the control strain for the drug-susceptibility/resistance.

Antimicrobial powder: Sulfate salts of colistin (Carbosynth, Compton, UK) were dissolved in distilled water, and BMD was performed under the CLSI reference method (10). All the colistin susceptibility tests were performed by following commercial methods and BMD.

BMD: The BMD was carried out in duplicate accordingly the CLSI guidelines that used cation- adjusted Mueller- Hinton broth (Difco™ Becton Dickinson, Sparks, MD). Dilutions were prepared with a MIC range of 0.06 mg/liter to 64 mg/liter in 96-well polystyrene microplates (Citotest, Jiangsu, China) (7). The plates had an incubation time of 18 to 24 hours at 37 °C. The isolates were considered resistant when colistin MIC >2, based on the breakpoints of CLSI and EUCAST (11,12).

Identification and colistin susceptibility testing: The isolates were identified by using BD Phoenix (BD Diagnostic Systems, Sparks, MD) and traditional methods. BD Phoenix and MicroScan automated systems and E-tests were used to determine colistin susceptibility. The manufacturer's instructions were followed while semi-automated systems were used for testing of colistin susceptibility. The E-test method was performed with a colistin strip (bioMérieux SA, Marcyl'Etoile, France) using Mueller-Hinton agar (BD Diagnostic Systems, Sparks, MD) medium in accordance with the manufacturers' recommendations. The probable range of MIC readings for each method were as follows: for BMD, <0.06 to >64 mg/liter; for BD Phoenix, ≤1 to >4 mg/liter; for MicroScan, ≤2 to >4 mg/liter; for E-test, <0.016 to >256 mg/liter. Colistin MIC results were interpreted according to the EUCAST breakpoints (susceptible, ≤2 mg/liter; resistant, >2 mg/liter).

Statistical Analysis

Commercial methods were compared with the reference method BMD. Rates of very major errors (VMEs), major errors (MEs),

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essential agreement (EA), and categorical agreement (CA) were established.

The CA and EA (EA: MICs within ±1 dilution of reference MICs) were calculated as claimed by the International Organization for Standardization (ISO) standard 20776-2 (13).

The EA was considered when the E-test MIC was ±1log2 when compared with BMD results.

The EA was not determined for BD Phoenix and MicroScan. The MIC ranges were limited to ≤1, 2, 4, ≥4, and ≤2, 4, >4 mg/liter, respectively. CA was defined as the compatibility of MIC results with commercial kits and BMD. Although the reference method result is resistant, VMEs are defined as the sensitivity of the method result tested. Although the reference method result is sensitive, MEs are defined as the resistance of the method result tested (14).

The acceptable performance was accepted following the criteria of the ISO as >90% for essential or category agreement and VME <1.5%, ME <3.0% (14).

The study was conducted after it was approved by the University of Health Sciences Turkey, İzmir Bozyaka Training and Research Hospital Ethics Committee (decision no: 02, date: 09.10.2019).

RESULTS

Colistin susceptibility was evaluated in 120 Gram-negative clinical isolates, including 108 *K. pneumoniae*, 10 *E. coli*, 2 *E. cloacae*, during the study period. MICs for *E. coli* ATCC 25922 were examined, the isolates were between 0.25 and 1 mg/liter for all testing methods (7). MICs for mcr-1 positive *K. pneumoniae* were between >2 and 4 mg/liter for all testing methods. The dispersions of colistin MICs determined by BMD and other testing methods for the isolates are presented in Table 1. Among the isolates, 66 (55%) were susceptible, and 54 (45%) were resistant to colistin, according to BMD (Table 2). BD Phoenix, MicroScan, and E-test had 90.90%, 95.45%, and 96.96% sensitivity, respectively, when colistin was tested. The comparison of performance characteristics of different methods with BMD is presented in Table 3. The BD Phoenix system, MicroScan and E-test failed to detect 7, 7, and 13 colistin-resistant *K. pneumoniae* isolates, respectively. The commercial methods and E-test revealed poor performance in species other than *E. coli* and *E. cloacae*.

There was >85% CA between BD Phoenix, MicroScan, E-test, and BMD for all the 120 isolates. Although BD Phoenix, MicroScan, and E-test resulted in close to >85%, in general VME rate was high (10%, 7.5%, and 12.5%, respectively). E-test showed 71.17% EA.

E-tests with colistin showed higher VME rates (12.5%). BD Phoenix had six (5%) MEs, MicroScan had three (2.5%) MEs, and E-test had two (1.7%) MEs for colistin. None of the commercial testing methods for colistin satisfied the CLSI-committed performance standards for commercial AST systems (VME <1.5%, ME <3.0%, CA >90%, EA >90%) (14). Only Micro Scan met the CA and ME performance standards recommended by CLSI for colistin.

DISCUSSION

Multi-resistant *Enterobacterales* can cause severe infections, and colistin is an agent used in the treatment. A false susceptible and false resistant results in this last resort agent should be considered equally serious. Colistin is often among the limited treatment options. Therefore, reliable colistin susceptibility testing should be performed before the use of colistin in clinical practice.

Multi-drug resistant Gram-negative bacteria and colistin resistance are increasing simultaneously; therefore, an accurate method for AST of colistin is crucial. The reference methodology is MIC determination with BMD according to the ISO standard 20776-1 for AST (15).

According to EUCAST experience, it was shown that the correct categorization was difficult, especially in MICs in the range of 2-8 mg/L (https://www.eucast.org/ast_of_bacteria/warnings/). For this reason, according to our study results, MIC should be confirmed with BMD, especially for colistin determined by semi-automated systems between 2-8 mg/liter. The EUCAST uses the BMD method as the reference method according to the latest recommendations of the joint CLSI and EUCAST subcommittee on the polymyxin susceptibility testing and breakpoints (16). The performance of commercial AST systems for colistin is as follows: VME <1.5%, ME <3.0%, EA >90%, and CA >90%, according to CLSI -recommended performance standards (14).

Colistin susceptibility testing studies are limited to Micro Scan. Lee et al. (17) compared the Micro Scan system with agar dilution as a colistin sensitivity test. They found the CA value of 87.3% for Micro Scan. In our study, Micro Scan susceptibility testing for colistin, the rates of VME, ME, and CA were 7.5%, 2.5%, and 90%, respectively.

The BD Phoenix, Micro Scan, and E-test have VMEs of <1.5% rate, which is the recommended value by CLSI (18). In this study,

Table 1. Colistin MICs distribution determined by BMD and other testing methods for all isolates

MIC detected by BMD (mg/L)	BD Phoenix (n)	Microscan (n)	E-test (n)
≤2 (n=66)	60 (90.9%)	63 (95.45%)	64 (97%)
2-8 (n=13)	8 (61.54%)	7 (53.85%)	3 (23.08%)
16 (n=41)	37 (90.24%)	38 (92.69%)	36 (87.80%)

MIC: minimum inhibitory concentration, BMD: broth microdilution

Table 2. Colistin MIC results of isolates determined by BMD, BD Phoenix, Microscan automated systems and gradient test

	S	R
BMD	66 (55%)	54 (45%)
BD Phoenix	73 (60.8%)	47 (39.2%)
Microscan	73 (60.8%)	47 (39.2%)
E-test	79 (65.8%)	41 (34.2%)

MIC: minimum inhibitory concentration, BMD: broth microdilution, S: susceptible, R: resistant

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Table 3. The comparison of the overall performance characteristics of the different methods with BMD

	CA	VME	ME	Sensitivity	Specificity	Positive predictive value	Negative predictive value
BD Phoenix	101 (85%)	13 (10%)	6 (5%)	90.9%	75.9%	82.2%	87.2%
Microscan	107 (90%)	10 (7.5%)	3 (2.5%)	95.5%	81.5%	86.3%	93.6%
E-test	103 (85.8%)	15 (12.5%)	2 (1.7%)	97%	72.2%	81%	95.1%

BMD: broth microdilution, CA: categorical agreement, VME: very major errors, ME: major error

the percentage of VME exceeded the recommendation of CLSI; however, this might be caused by the limited number of isolates. The MEs for all methods (except E-test colistin testing) also exceeded the CLSI recommendation of >3.0% (14).

The use of semi-automated systems for diagnostic purposes has become quite common even in microbiology laboratories of developing countries. It is quite difficult to ensure quality at BMD in small-scale laboratories. In addition, the scarcity of trained technical personnel also requires the use of semi-automated systems (19). For example, it has been reported that VitekVR 2 can be used as a reliable colistin susceptibility test method in studies (20). In addition, another recent study reported that colistin susceptibility testing might not be reliable with a VME rate of 36% (21). Bartoletti et al. (22) found that VME (42%) was common in the colistin susceptibility test performed on semi-automated systems. According to this result, VME was associated with inappropriate antibiotic use and worse outcomes. In another study evaluating six commercial products for colistin susceptibility testing in *Enterobacterales*, the performances of the semi-automated systems Vitek 2 and BD Phoenix were found to be unacceptable (due to the large number of false susceptible results) (23). Colistin heteroresistance is defined as colistin-resistant subpopulations. These subpopulations arise from the colistin-susceptible population under colistin pressure. It can be proved by the presence of skip wells in the BMD (9). A general limitation for semi-automated systems is that they use panels/cards that contain a certain number of colistin concentration, most of the tests has only one or two dilutions up and down from breakpoint for resistance. In our study, the possible range of MIC readings for automated systems was as follows: for BD Phoenix, ≤1 to >4 mg/liter; and for MicroScan, ≤2 to>4 mg/liter. Semi-automated systems commonly exhibit false-sensitivity results, possibly due to the presence of colistin hetero-resistant subpopulations (24). Limited colistin concentrations used in semi-automated systems may not be able to detect colistin heteroresistance due to the presence of skip wells observed in BMD.

Several studies stated that there were methodological difficulties in colistin MIC (25,26). In some studies, different susceptibility testing methods were reviewed (27,28). Recently, Micro Scan Microbiology Systems, Beckman Coulter Inc., conducted an experiment that revealed that the addition of polysorbate-80 caused higher colistin MICs higher (29). BMD without polysorbate-80 supplementation is the current reference method (7).

The E-test allows evaluation of the wider colistin MIC range. However, poor diffusion of the large colistin molecule in the agar causes errors in the interpretation of the results. Colistin E-tests have varying error rates, and the E-test is not an adequate testing method (7,17,30,31). After an E-test, the highest reported rate of VMEs of colistin was 41.5% (17). In our study, the rates of VME, ME, and CA for E-tests were 12.5%, 1.7%, and 85.8%, respectively. EA was particularly poor for E-tests (71.17%) in our study, which could be due to the poor diffusion of colistin molecules causing inhibition of a narrow zone close to the MIC. Colistin disk diffusion testing is also unreliable due topoor diffusion of colistin molecules (18,31,32).

Study Limitations

Our study had some limitations. First of all, the number of species other than *K. pneumoniae* used to determine the compatibility of colistin susceptibility tests was insufficient. In addition, the inability to include Gram-negative non-fermentative rods in the study might be another limitation.

CONCLUSION

This study evaluated automated systems and E-test for colistin MIC determination. According to our results, the reference BMD method should be performed for colistin MIC determination. Colistin MIC should be confirmed with BMD, particularly by using automated systems between 2-8 mg/liter.

The data on the use of colistin in clinical treatment is limited. The subjects related to colistin susceptibility testing methods remain unclear. BMD requires experienced staff, and manual preparation of antibiotic solutions, therefore, is time-consuming and hard to perform in routine laboratories. Nevertheless, BMD is the only test that is recommended by EUCAST and CLSI until further studies are carried out.

In routine clinical practice, the worldwide reference method can hardly be implemented, and commercially available systems are used for the interpretation of colistin susceptibility. Colistin use is increasing for the treatment of multiresistant Gram-negative infections, further and more extensive studies are needed for precise susceptibility testing methods for this compound. We recommend that laboratories use the BMD method at least in selected patient groups in the face of increasing antimicrobial resistance.

Ethics Committee Approval: The study was conducted after it was approved by the University of Health Sciences Turkey, İzmir Bozyaka Training and Research Hospital Ethics Committee (decision no: 02, date: 09.10.2019).

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The Effect of Diet on ECP, IL-4 and IL-31 in Patients with Persistent Allergic Rhinitis

Persistan Alerjik Rinitli Hastalarda Diyetin ECP, IL-4 ve IL-31 Üzerine Etkisi

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ABSTRACT

Objective: The aim of this study is to evaluate the effects of dietary changes on late phase of allergic reaction in patients with allergic rhinitis (AR) by measuring eosinophilic cationic protein (ECP), interleukin (IL)-4 and IL-31 levels.

Methods: Sixty participants (40 patients with AR and 20 healthy control subjects) were included in this study. Forty patients with confirmed diagnosis of AR were randomly divided into two groups, all patients were prescribed the same treatment, intranasal azelastine hydrochloride. Patients in group 1 were asked to change their diet from Western to Mediterranean diet by eliminating processed meat, processed sugar, and polyunsaturated fatty acids. Blood samples of every subject were driven at the beginning and three months later to study ECP, IL-4 and IL- 31 levels. By using ELISA method IL-4 and IL-31 levels were determined and compared between healthy volunteers and patients.

Results: There was a significant difference in terms of ECP levels in group 2. The posttreatment ECP levels were significantly higher than pretreatment levels in group 2 ($p=0.025$ and $p<0.05$). There was no significant difference in terms of other parameters.

Conclusion: In our study, we showed that the laboratory parameters associated with AR were positively affected by the Mediterranean diet.

Keywords: Allergic rhinitis, diet, eosinophil cationic protein, interleukin-4, interleukin-31

ÖZ

Amaç: Bu çalışmanın amacı, alerjik rinitli (AR) hastalarda diyet değişikliklerinin alerjik reaksiyonun geç fazındaki etkilerini eozinofilik katyonik protein (ECP), interlökin (IL)-4 ve IL-31 düzeylerini ölçerek değerlendirmektir.

Yöntemler: Bu çalışmaya 60 katılımcı (AR'li 40 hasta ve kontrol grubuna 20 sağlıklı birey) dahil edildi. AR tanısı doğrulanmış 40 hasta rastgele iki gruba ayrıldı, tüm hastalara aynı tedavi, intranazal azelastin hidroklorür, verildi. Grup 1 hastalarına diyetlerinden işlenmiş et, işlenmiş şeker ve çoklu doymamış yağ asitlerini ortadan kaldırarak Akdeniz tipi beslenmeye uygun beslenmeleri söylendi. Her denekten ECP, IL-4 ve IL-31 düzeylerinin ölçülmesi için başlangıçta ve üç ay sonra kan örneği alındı. Elde edilen veriler istatistiksel olarak incelendi.

Bulgular: Grup 2'de ECP düzeylerine göre anlamlı bir fark saptandı. Grup 2'de tedavi sonrası ECP düzeyleri tedavi öncesi değerlere göre anlamlı derecede yüksekti ($p=0,025$ ve $p<0,05$). Diğer parametreler açısından anlamlı bir fark yoktu ($p>0,05$).

Sonuç: Çalışmamızda AR ile ilişkili laboratuvar parametrelerinin Akdeniz diyetinden olumlu yönde etkilendiğini gösterdik.

Anahtar kelimeler: Alerjik rinit, diyet, eosinofil katyonik protein, interlökin-4, interlökin-31

INTRODUCTION

Allergic rhinitis (AR) is a common nasal inflammatory disease characterized by runny nose, itching and nasal congestion (1). AR is categorized as intermittent or persistent according to the frequency and duration of symptoms (2). AR, which is accepted as the most common chronic disease in children by the World Health Organization, affects 10-30% of children in developed countries and its prevalence has increased worldwide, especially in industrialized countries (3-5). It has been hypothesized that a change in lifestyle, especially dietary habits, is considered as an important factor of AR development (4).

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AR is chronic inflammatory disease of nasal airway. Imbalance in T helper 1 (Th1)/T helper 2 (Th2) response is responsible for this allergic reaction (6). Eosinophils and released mediators promote Th2 maturation and shift in response contributes to AR (7). Eosinophils and mediators localized in their granules such as eosinophilic cationic protein (ECP) are effectors in allergic reactions such as AR (8,9). ECP has been measured in body fluids, including serum, and nasal secretions of patients with allergic and other inflammatory diseases (10).

One of the key cytokines in Th2 maturation and shifting is IL-4. IL-4's receptor shares same receptor subunit with IL-13 receptor which induces IgE isotype switching, T-cell population shifts to Th2 cell (11,12). IL-4 also promotes eosinophils and Th2 migration to the inflammatory site (13). In addition to these effects, it has been reported that IL-31 is produced by many cell groups in the presence of IL-4, and this triggers Th2-mediated inflammation (14). It is thought that IL-31 is produced by many cell groups in the presence of IL-4, and this triggers Th2-mediated inflammation (14).

The IL-31 is another effector cytokine of Th2. It plays important role in the pathogenesis of atopic and allergic diseases. IL-31 is released from activated Th2 cells (15). Antigen-induced IL-31 production in the AR process has a special and independent role in the pathophysiology of AR, unlike other Th2 cytokines such as IL-5 and IL-13 (16).

Environmental exposures, climate changes and lifestyle are important risk factors for AR. Many hypotheses are considered for the development of allergies. One of these hypotheses is the triggering effect of allergic inflammation of the Western diet (WD), which is rich in antioxidants and rich in polyunsaturated fatty acids (17). It is hypothesized that increased consumption of high protein and fatty foods and processed dairy products and less consumption of fresh fruits, vegetables and whole grains in the WD contribute to AR (18). Mediterranean diet (MD) is characterized by high intake of fruit, vegetables, fish and olive oil (19). It is characterized with balanced ratio of n-6/n-3 essential fatty acids, fiber, antioxidants and monounsaturated fatty acids and anti-oxidants such as fresh fruits and vegetables (20,21). It is hypothesized to have a protective effect on chronic inflammatory airway diseases such as asthma and AR (22-24).

In this study, the effects of removing trans fatty acids, milk, and dairy products from the diets of patients diagnosed as having persistent AR by prick test, that is, converting a WD to a MD, on serum IL-4, IL-31 and ECP levels were evaluated.

METHODS

This study was conducted prospectively on patients and volunteers who were admitted to Istanbul University-Cerrahpaşa, Cerrahpaşa Medical Faculty Ear Nose Throat Department's Allergy Clinic with the approval of Istanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (no: 83045809/18849, date: 12.07.2013).

Forty patients with AR were included in this study. Informed consent form was obtained from each subject or subject's custodian for under aged subjects. All patients were questioned for allergic symptoms, endoscopic nasal examinations were performed, and skin prick tests were planned. The skin prick test (Multi Test®, Lincoln Diagnostics Ltd, USA) was performed according to European Academy of Allergology and Clinical Immunology guidelines to support the diagnosis of allergy and to determine the allergen or allergens in the etiology of the disease.

The exclusion criteria were as follows: lack of mental capacity, history of anaphylaxis, being diagnosed as having vasomotor rhinitis or non-AR, having any other positive allergic reaction on prick test, presence of a chronic disease, use of any medication, alcohol dependence and smoking, and refusing to participate in the study. Considering these criteria, 40 patients, with allergic symptoms and positive prick test only for house dust mite Dermatophagoides farinea and/or Dermatophagoides pyteronysinus, were included in the study. The control group was composed of 20 healthy individuals aged 18 to 49 years with negative prick test.

Patients were randomly divided into two groups. Patients in group 1 were prescribed azelastine hydrochloride two times daily and given a list that contained banned and allowed foods and a check list to mark the progress each day. Dairy products, processed sugar and trans fatty acid consumptions were restricted. Group 2 was only prescribed azelastine hydrochloride twice a day and it was stated that they could eat whatever they wanted. The control group was named as group 3.

A full ENT examination was performed every month for each patient and the diet check lists of the patients in group 1 were controlled. Five cc of venous blood was collected from all patients at the beginning of the study (Day 0) and at third month (3rd Month) for measuring ECP, IL-4 and IL-31 levels, and restored in a -70 °C refrigerator. Control group's blood samples were only collected at the beginning to measure IL-4 and IL-31 levels. ECP was not allergy specific and therefore it was not studied in the control group (9,10).

ECP

Blood samples from each patient were collected in anti-coagulant free tube by the same nurse, tubes were slowly turned upside down for 5 times, restored 1-2 hours at the room temperature, then studied in the laboratory.

ELISA Detection Method

Human IL-4 ELISA Kit Diaclone® (France) and Human IL-31 ELISA Kit Diaclone® (France) were used to determine the concentration of IL-4 and IL-31 levels in serum samples. Standard and samples were read with 450 nm reading filter and linear regression analysis was used to calculate results.

Statistical Analysis

Statistical analysis was performed with SPSS Version 21.0 (SPSS Inc., USA) program. It was evaluated whether the groups showed

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normal distribution by Kolmogorov-Smirnov test. Homogeneity of variances was evaluated by Levene test. The values of the parameters in groups at the beginning and the end of the research were analyzed with Wilcoxon signed-ranks test. The comparison between the groups was performed with the Kruskal-Wallis test. Paired comparison of groups was performed with Mann-Whitney U test. Bonferroni correction was used to counteract the multiple comparisons problem. The significance level was accepted as $p<0.05$. The significance level for Mann-Whitney U test with Bonferroni correction was accepted as $p<0.025$.

RESULTS

Twenty-three of 40 patients (57.5%) were female, and 17 of 40 patients (42.5%) were male. Group 1 had 9 (45%) female 11 (55%) male patients, mean age was 18.95±9.16, and range was 6-36 years. Group 2 had 14 (70%) female, 6 (30%) male patients, mean age was 22.58±9.19, and range was 8-36 years.

The ECP levels of group 1 and 2 and their comparisons were given in Table 1. In the evaluation made in terms of the ECP levels, a statistically significant difference was found between the beginning of the research and the end of the 3rd month in group 2 (p=0.025). In other comparisons made in terms of ECP values, no statistically significant difference was found in the groups (p>0.05) (Table 1).

IL-4 levels of groups were given in Table 2. A statistically significant difference was found between the groups in terms of the IL-4 values at the beginning of the research (p=0.001 and p=0.001, respectively). In paired comparison of the groups, IL-4 levels in group 1 and 2 were found to be statistically significantly higher than the control group at the beginning (p=0.001 and p=0.001, respectively). However no statistically significant difference was found in the comparison between patient groups (p=0.704). And also, a statistically significant difference was found between the patient and control groups in terms of the IL-4 levels at the end of the research (p=0.001 and p=0.001, respectively). In paired comparison of the groups, IL-4 levels in both group 1 and 2 were found to be statistically significantly higher than the control group at the end of the research (p=0.001 and p=0.001, respectively). However, no statistically significant difference was found in the comparison between patient groups (p=0.913). There was no statistically significant difference between the patient groups in the comparison made in terms of the initial and final IL-4 levels (p=0.925 and p=0.432, respectively) (Table 2, 3).

The IL-31 levels of groups were given in Table 2. A statistically significant difference was found between the patient and control groups in terms of the IL-31 levels at the beginning of the research (p=0.001). In paired comparison of the groups, IL-31 levels in both group 1, 2 were found to be statistically significantly higher than the control group at the beginning (p=0.001 and p=0.001, respectively). However, no statistically significant difference was found in the comparison between patient groups (p=0.542). And a statistically significant difference was found between the patient and control groups in terms of the IL-31 levels at the end of the research (p=0.125). In paired comparison of the groups, IL-31 levels in both group 1 and 2 were found to be statistically significantly higher than the control group at the end (p=0.001 and p=0.001, respectively). However no statistically significant difference was found in the comparison between patient groups (p=0.125). There was no statistically significant difference in both groups in the comparison made in terms of the initial and final IL-31 levels (p=0.709 and p=0.341, respectively) (Table 3, 4).

DISCUSSION

AR is one of the most common allergic diseases (1). In addition to genetic predisposition, many environmental factors have an effect on this disease (17). One of these factors is eating habits (17). In this study, we applied a MD in addition to local

Table 1. Comparison of ECP levels between groups and between months

ECP	Before treatment day 0 Median (min-max)	After treatment 3rd month Median (min-max)	p*
Group 1 (n=20) (µg/L)	48.75 (5.48-108)	59.95 (13.3-152)	0.179
Group 2 (n=20) (µg/L)	32.6 (4.56-90.8)	49.65 (18-201)	0.025
p**	0.138	0.433	

ECP: eosinophilic cationic protein, min: minimum, max: maximum, *Wilcoxon signed-ranks test p<0.05, **Mann-Whitney U test with Bonferroni Correction p<0.025

Table 2. Comparison of groups according to IL-4 levels

IL-4	Day 0 Median (min-max)	3rd month median (min-max)	p**
Group 1 (n=20) (pg/mL)	0.41 (0.04-2.04)	0.38 (0.17-2.03)	0.925
Group 2 (n=20) (pg/mL)	0.34 (0.14-0.73)	0.38 (0.18-1.46)	0.432
Group 3 (n=20) (pg/mL)	0.2 (0.18-0.31)		
p*	0.001	0.001	

*Kruskal-Wallis test p<0.05, **Wilcoxon signed-ranks test p<0.05, min: minimum, max: maximum, IL: interleukin

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Table 3. Statistical evaluation of interleukin levels

	Compared groups	IL-4		IL-31	
		Day 0	3rd month	Day 0	3rd month
p*	1-2	0.704	0.913	0.542	0.125
	1-3	0.001	0.001	0.001	0.001
	2-3	0.001	0.001	0.001	0.001

*Mann-Whitney U test with Bonferroni correction p<0.025

Table 4. Comparison of groups according to IL-31 levels

IL-31	Day 0 Median (min-max)	3rd month Median (min-max)	p**
Group 1 (n=20) (pg/mL)	21.2 (0.19-138)	21.4 (0.19-81.4)	0.709
Group 2 (n=20) (pg/mL)	18.74 (0.19-85)	18.74 (0.19-100)	0.341
Group 3 (n=20) (pg/mL)	3.1 (0.2-7.5)		
p*	0.001	0.001	

*Kruskal-Wallis test p<0.05, **Wilcoxon signed-ranks test, IL: interleukin, min: minimum, max: maximum

treatment in patients with AR. As a result, there was no significant increase in ECP level, which was an indicator of systemic inflammation, at the end of the study in the group fed with MD, while a significant increase was observed in the free-fed group (p=0.025). In addition, IL-4 level, which had an important role in the inflammation mechanism due to AR, decreased in the MD group, while an increase was observed in the free-fed group. However, this change was not statistically significant (p=0.925). There was no significant difference in terms of IL-4 levels between the two patient groups after treatment (p=0.913). Although IL-31 level, which was involved in the pathogenesis of AR and was associated with disease severity, decreased in both groups, and this decrease was not significant (p=0.709 and p=0.341, respectively). There was no significant difference in terms of IL-31 levels between the two patient groups after treatment (p=0.125).

Turkey is geographically located in the easternmost part of the MD belt. However, in parallel with the socio-cultural life in Turkey, of which integration with the West is increasing, it has begun to shift to WD. We planned this study to examine the possible effects of this change on patients with AR. Since nutrition was a physiological event with systemic effects, we chose local treatment to minimize the systemic effects of AR with the treatment we would give, and for the same reason, we used azelastine hydrochloride, an antihistamine, locally, instead of corticosteroids. ECP is the major protein in AR and its serum levels correlate with AR (10,25). IL-4 and IL-31 are involved in the pathophysiology of AR (16,26,27). IL-31 levels also correlate with the severity of the AR clinic (28,29). By determining the levels of these markers, we aimed to determine both the effect of nutrition on the inflammatory process associated with AR and the possible effect on the clinical severity of AR.

Eosinophils are cells that are at the center of allergic reactions. ECP is secreted from the granules in these cells in the last phase of allergic reactions, and the ECP level is higher in patients with AR than in non-allergic patients and shows a positive correlation with AR (8-10,25). In our study, ECP levels increased during the study period in both patient groups, probably due to the fact that a systemic anti-allergic treatment was not used. However, while this increase was statistically significant in the free-fed group (p=0.025), no significant increase was observed in the Mediterranean-type fed patients. This result may show us that the MD reduces inflammation associated with AR.

The IL-4 is involved in the development and maintenance of allergic inflammation seen in airway mucosa of patients with allergic respiratory disorders, such as AR (30,31). IL-4 plays a critical role in the pathogenesis of AR, especially in the late phase of the disease (32). While the level of this marker, which was directly related to inflammation in AR, increased in the free-fed group in our study, it decreased in the Mediterranean-type fed patient group. This result can be interpreted that MD reduces allergic inflammation.

The IL-31 levels increase in AR and its levels correlate with the clinical severity of the disease (33). No significant difference was found in the statistical analyzes of IL-31, which was the marker most closely associated with the AR clinic among the laboratory parameters we examined in our study. This can be explained by the small number of patients and the short study period.

Study Limitations

There were some limitations of this study, in which we examined the relationship between MD and laboratory parameters of AR. The first of these was that we determined a short study period of 3 months to evaluate the parameters of AR, which was a chronic disease. The shortness of this period was in order to ensure the compliance of the patients with the diet. Our most important limitation was the small number of patients included in our study. This was because our budget was limited for the kits we used for laboratory evaluation.

CONCLUSION

AR is one of the most common chronic allergic diseases. This disease, which is affected by many environmental factors, may also be affected by the type of nutrition. As a result of our study, we determined that the laboratory parameters associated with AR were positively affected by the MD. Based on the results of our study, we may say that WD may negatively affect AR, while MD may reduce inflammation of AR and severity of AR.

Ethics Committee Approval: This study was approved by the Istanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (no: 83045809/18849, date: 12.07.2013).

Informed Consent: Informed consent form was obtained from each subject or subject's custodian for under aged subjects.

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Frequencies, Variations, and Significance of Rouviere's Sulcus in the Context of Difficult Laparoscopic Cholecystectomy

Zor Laparoskopik Kolesistektomi Bağlamında Rouviere Sulkusunun Sıklığı, Varyasyonları ve Önemi

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ABSTRACT

Objective: Researchers described several anatomical landmarks, a method of identification of the cystic structures referred as "critical view of safety", and operative techniques to avoid vascular and biliary injuries due to the laparoscopic cholecystectomy (LC). In this present study, we aimed to determine the frequencies, variations, and significance of Rouviere's sulcus (RS) in the context of difficult laparoscopic cholecystectomy (DLC).

Methods: The video records of 102 patients with gallbladder disease who underwent LC were reviewed. DLCs were determined according to Tokyo Guidelines. RS type and dimensions, if present, were noted. The features of RS were shown using descriptive statistics and collected data were analyzed using SPSS version 25.0.

Results: Out of 102 patients RS was present in 80 (78.4%) patients. The most frequent type of the sulcus was horizontal, less frequently oblique, and rarely vertical. The most frequent sulcus type was type 1A, less frequently type 1B and type 2, and rarely type 3. The average dimension was 12.5 mm in length and 6.9 mm in width. A vessel or a biliary structure was commonly seen in type 1A sulci. We identified almost half of the cholecystectomy operations as DLCs and the RS was present in two-third of these DLCs while the RS was present in majority of standard LC. LC was performed in majority of the patients except for four patients in whom open surgery was preferred due to intense inflammation. No mortalities or bile duct injuries were identified.

Conclusion: The frequency and type of RS in this sample from Turkey were found to be like the reports in literature. When available (present and visualized), RS contributes a lot to the safety measures of LC. When it comes to DLCs, the availability and convenience of RS might be limited or misleading for surgeons.

Keywords: Rouviere's sulcus, laparoscopic cholecystectomy, liver, anatomy

ÖZ

Amaç: Araştırmacılar, laparoskopik kolesistektomide (LK) birkaç anatomik dönüm noktası, "critical view of safety" olarak adlandırılan kistik yapıların tanımlanması için bir yöntem ve vasküler ve biliyer yaralanmalardan kaçınmak için ameliyat teknikleri tanımladılar. Bu çalışmada, zor laparoskopik kolesistektomi (ZLK) ameliyatları bağlamında Rouviere sulkusunun (RS) sıklığını, varyasyonlarını ve önemini belirlemeyi amaçladık.

Yöntemler: Safra kesesi hastalığı olan ve LK yapılan 102 hastanın video kayıtları incelendi. ZLK'ler Tokyo Rehberi'ne göre belirlendi. RS tipi ve varsa boyutları not edildi. RS'nin özellikleri tanımlayıcı istatistikler kullanılarak belirtildi ve toplanan veriler SPSS sürüm 25.0 kullanılarak analiz edildi.

Bulgular: Yüz iki hastanın 80'inde (%78,4) RS mevcuttu. Sulkusun en sık görülen tipi yatay tip idi, daha az sıklıkla oblik tip ve nadiren dikey tip idi. En sık görülen sulkus tipi tip 1A, daha az sıklıkla tip 1B ve tip 2 ve nadiren tip 3 idi. Ortalama boyut, 12,5 mm uzunluğunda ve 6,9 mm genişliğindeydi. Tip 1A sulkuslarda yaygın olarak bir damar veya safra yapısı görüldü. Kolesistektomi operasyonlarının neredeyse yarısını ZLK'ler olarak belirledik ve bu ZLK uygulanan hastaların üçte ikisinde RS mevcutken, standart LK'lerin çoğunda RS mevcuttu. Yoğun enflamasyon nedeniyle açık cerrahinin tercih edildiği dört hasta dışında hastaların çoğuna LK uygulandı. Hiçbir ölüm veya safra kanalı yaralanması tespit edilmedi.

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Sonuç: Türkiye'den bu örnekte; RS sıklığı ve tipi literatürdeki raporlara benzer bulundu. RS mevcut olduğunda (mevcut ve görselleştirilmiş), LK'nin güvenlik önlemlerine çok katkıda bulunur. ZLK'ler söz konusu olduğunda, RS'nin mevcudiyeti ve uygunluğu cerrahlar için sınırlı veya yanıltıcı olabilir.

Anahtar kelimeler: Rouviere sulkusu, laparoskopik kolesistektomi, karaciğer, anatomi

INTRODUCTION

Gallstones are frequently seen clinical entities causing significant morbidity and mortality in the United States and when associated with symptomatic gall bladder disease, they require surgical removal (1). Similarly, cholecystectomy is the most performed surgical procedure after caesarean section in Turkey. It was reported that more than 120,000 cholecystectomies were performed by surgeons working in state hospitals per 2017 due to the State Hospitals Report of Turkey (2). After the introduction of laparoscopic cholecystectomy (LC) by Erich Mühe of Germany on September 12, 1985 under direct scope vision, it has been accepted rapidly and become the gold standard surgical procedure for treatment of gallbladder stones due to its advantages such as need for small incision, short hospital stay, cost-effectiveness, and others (3). Despite its advantages, biliary duct injury rates increased significantly at the beginning and showed a fluctuating course during the past three decades. Vasculobiliary injuries, one of the most feared iatrogenic complications, have been encountered in 0.3-0.6% of LCs. Although the mortality rates declined from 90-100% to 2-4%, thanks to the evolution of diagnosis and treatment of these injuries, morbidity is still as high as 40-50% (4,5). Misidentification of the bile duct anatomy is the prominent reason for these iatrogenic injuries to the bile ducts or hepatic arteries.

Some safety measures and anatomical landmarks have been proposed to avoid these iatrogenic injuries (6). The most well-known and accepted identification technique is the critical view of safety (CVS). The CVS has three components: dissecting fat and fibrous tissues at the triangle of Calot, separating the lowest part of the gallbladder from the cystic plate, which is the flat fibrous surface to which the non-peritonealized side of the gallbladder is attached, and lastly defining two structures, and only two, entering the gallbladder (7). Once these three criteria have been met, the CVS has been secured. In addition, the imaginary line between Rouviere's sulcus (RS) and umbilical fissure at the base of segment IVb of the liver to the hepatic ligament was defined as R4U line, and dissecting ventrally to this was suggested to be safe to achieve CVS while avoiding bile duct injuries during LC operations (6,8,9).

The RS is a transverse sulcus on the inferior surface of the right lobe of the liver, running to the right of the hepatic hilum. It was first described by Henrie Rouviere in 1924 and named as "sillon du processus caudé". Gans named it "incisura dextra" in 1955, Couinaud modified it as "le sillion du processus caudé de Rouviere" in 1957, and it was used as "incisura dextra of Gans" in 1991. Subsequently, the researchers preferred the term "RS" (8). RS is seen best when the gallbladder neck is retracted towards the umbilical fissure during LC (10). This anatomical landmark, laparoscopically easily identified structure was thought to be unaffected from inflammation of the gallbladder and biliary tract. On the other hand, in difficult cholecystectomy surgeries, the identification and feasibility of this sulcus are not established well. Inflammation around the hilum of the liver and gallbladder can interfere with safe dissection (11). The anatomical researchers reported the presence of RS in 11-82% of the livers, either as open or fused type, perhaps due to geographical differences (12,13).

In this present study, we aimed to determine the frequency of RS in Turkish population and assess its surgical relevance and impact in difficult cholecystectomy operations (LCs).

METHODS

Study Subjects

This retrospective descriptive study was conducted in coordination with the University of Health Sciences Turkey Gaziosmanpaşa Training and Research Hospital. We obtained the Ethics Committee approval from the University of Health Sciences Hamidiye Scientific Research Ethics Committee (decision no: 12/9, date: 02.04.2021).

In accordance with ethical provisions, LC video records of 102 patients who were operated in between August 2019 and December 2019 were reviewed. The patients who were older than 18 years and presented to the hospital with symptomatic benign gallbladder disease secondary to gallstones were included. Patients with previous upper abdominal surgeries (laparoscopic or open) and suspected malignant diseases were excluded. The consent form was not required in this study because it was a retrospective study.

Procedure

The four-trocar LC method was preferred in all patients. After dissecting the hepatocystic triangle (also known as Calot's triangle), two structures entering the gallbladder were identified, clipped, and cut. Intraoperative findings related to inflammation of the gallbladder, like fibrotic adhesions, edematous, necrotic, or easily bleeding at dissection were identified according to the video records. As outlined and recommended in Tokyo Guidelines (14); we attempted to assess intraoperative findings as objective indicators of surgical difficulty and when we did not obtain a CVS, we converted to open cholecystectomy or other bail-out procedures, and it was evaluated as difficult cholecystectomy (14). The RS was identified when seen at any moment of the entire video record and classified according to the Péré et al.'s (8) classification (Table 1). Directions of RS were classified as horizontal, vertical, or oblique according to Cantlie's

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line (15). The dimensions of RS were measured intra-operatively by a ruler or a feeding tube. If the length was not measured intra-operatively, the measurement was performed on the video screen by comparing it with a known sized instrument such as a dissector. Also, we noted any other sulcus-like structures during the evaluation of the records. We conducted a cadaveric dissection. After opening the abdominal cavity, the falciform ligament was cut off. The liver was released from the visceral surface, keeping the gallbladder in the statute. The section at the falciform ligament was advanced to the coronal ligament and the coronal ligament with the right and left triangular ligaments were cut off. The free border of the lesser omentum and hepatic portion of the inferior vena cava were cut off. The diaphragmatic surface through which inferior vena cava passed was cut off, and the liver was released (Figure 1). After dissection of the hepatic tissue around the RS, we exposed the right portal vein of the liver sulcus (Figure 2).

Statistical Analysis

According to the power analysis result using G power 3.1.9.7 version, we determined that a total of 108 images should be obtained with 80% effect size and 95% power ratio. The features of RS were displayed using descriptive statistics and collected data were analyzed using SPSS version 25.0 (IBM SPSS Statistics for Windows. Armonk, NY: IBM Corp.). The analysis of RS presence was performed using chi-squared test. The statistically significant value was set as $p<0.05$.

Table 1. Types of Rouviere's sulcus due to morphology of the sulcus

Type 1A	A deep sulcus, open at the hilar side
Type 1B	A deep sulcus, closed at the hilar side
Type 2	Slit-like sulcus
Type 3	Scar-like sulcus appears as a white line of fusion

Figure 1. At the visceral surface of the liver Rouviere's (type 1A) sulcus exposed

RESULTS

Out of the 102 patients included in the study, 70 (68.6%) were female and 32 (31.4%) were male patients. The mean ages were 43.71 and 51.06, respectively. Out of the 102 patients, RS was present in 80 of 102 patients (78.4%).

In terms of the direction of RS, the most frequent type of the sulcus was horizontal, less frequently oblique, and rarely vertical (Table 2). In terms of morphology of the sulcus; the most frequent type was type 1A (a deep sulcus which was continuous medially within the hilum of the liver, Figure 3), less frequently type 1B (a deep sulcus which was fused medially, Figure 4) and type 2 (slit-like, superficial and narrow, Figure 5), and rarely type 3 (as a scar, since it appeared as a fused line, Figure 6) (Table 2).

In terms of dimension of the sulcus, the average dimension was 12.5 mm in length and 6.9 mm in width. We measured the width only in deep ones. A vessel or a biliary structure was frequently seen in type 1A sulci. We also noted additional sulci at the inferior surface of the right liver in 25 of 102 (24.5%) video records. They were in various dimensions and directions, but two of them were very close to RS and thought to be duplicate RS at first sight (Figure 7, 8).

Out of the 102 operations, 48 (47.1%) were classified as difficult laparoscopic cholecystectomies (DLCs) according to the Tokyo Guidelines (14). We identified the RS in 29 of 48 (60.4%) of DLC operations while identified the RS in 51 of 54 (94.4%) standard LCs ($p<0.01$) (Table 3).

The LC was performed in 98 of 102 (96.1%) patients. The surgeons converted to open surgery in 4 patients (3.9%) because of intense inflammation around the Calot's triangle which prevented the achievement of CVS. One patient appeared to have Mirizzi's syndrome type 3, and a T-tube was placed after cholecystectomy. There were no mortalities or bile duct injuries.

DISCUSSION

In this present study, the RS was present in most patients and was nicely visible during laparoscopy, being visible in one form

Figure 2. The right portal vein of the liver within the sulcus

or another such as a sulcus, or a variation. It was visible in 78.4% of the patients. In terms of the direction of RS, the most frequent type was horizontal, less frequently oblique, and rarely vertical. In terms of morphology of the sulcus, the most frequent type was type 1A (a deep sulcus which was continuous medially within the hilum of the liver), less frequently type 1B (a deep sulcus which was fused medially) and type 2 (slit-like, superficial and narrow), and rarely type 3 (as a scar, since it appeared as a fused line). In

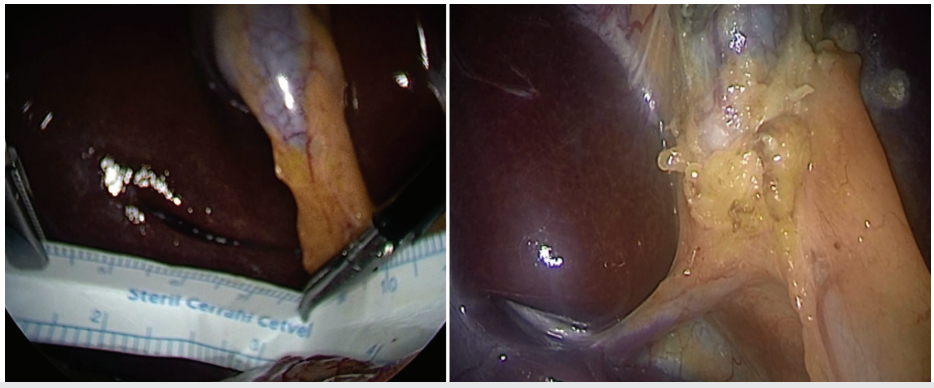

Figure 3. Type 1A

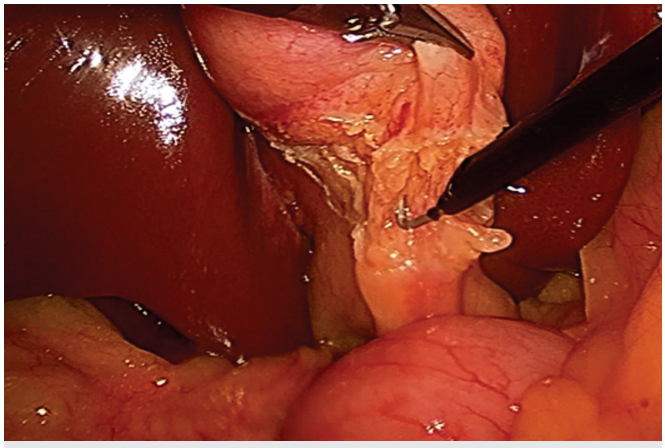

Figure 4. Type 1B

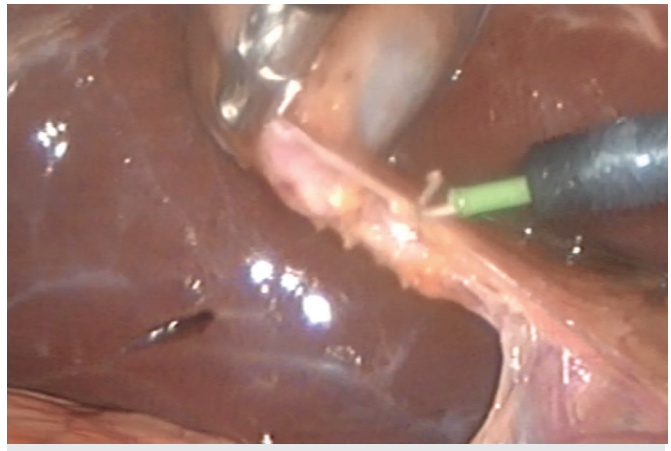

Figure 5. Type 2

Table 2. The morphology and direction of the sulcus of Rouviere's sulcus

Rouviere's sulcus type	Horizontal	Oblique	Vertical	Total	%
Type 1A	39	11	1	51	63.75
Type 1B	4	9	1	14	17.50
Type 2	2	4	1	7	08.75
Type 3	4	4	-	8	10.00
Total	49	28	3	80	-
%	61.25	35.00	3.75		100

Table 3. Statistical comparison per Rouviere's sulcus availability

	Rouviere's sulcus			p
	Available	Absent	Total	
Normal LC	51 (94.4%)	3 (5.6%)	54 (100%)	0.001
DLC	29 (60.40%)	19 (39.6%)	48 (100%)	<0.01
Total	80 (78.40%)	22 (21.60%)	102 (100%)	-

Chi-square test; χ^2=17.394; df=1
LC: laparoscopic cholecystectomy, DLC: difficult laparoscopic cholecystectomy, df: degrees of freedom

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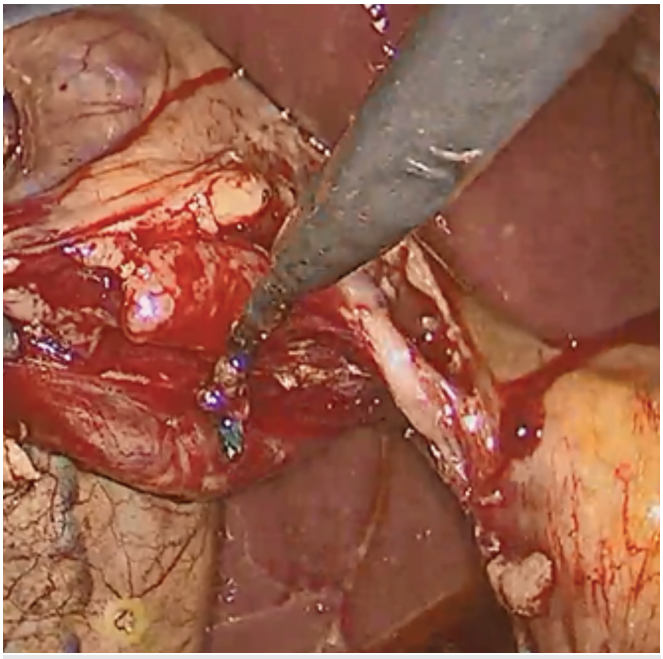

Figure 6. Type 3

terms of dimension of the sulcus, the average dimension was 12.5 mm in length and 6.9 mm in width. A vessel or a biliary structure was commonly seen in type 1A sulci. We also observed additional sulci at the inferior surface of the right liver in one fourth of the patients with various dimensions and directions. We identified almost half of the cholecystectomy operations as DLCs and the RS was present in two-third of these DLCs while the RS was present in majority of standard LCs. Finally, LC was performed in majority of the patients except for four patients in whom open surgery was preferred due to intense inflammation around the Calot's triangle which prevented the achievement of CVS. No mortalities or bile duct injuries were identified (15).

The frequency of RS (78.4%) in this present study is similar to the frequencies reported in the literature, ranging from 68 to 90.6% (16). According to a recent meta-analysis, overall pooled prevalence of RS was 83% (95% confidence interval: 78-87), which was similar to our results. In addition, no significant differences were found in terms of prevalence between cadaveric studies and laparoscopic studies (17). When we compared difficult and normal cholecystectomies, we found a significant difference in terms of frequencies. Since laparoscopic studies differ

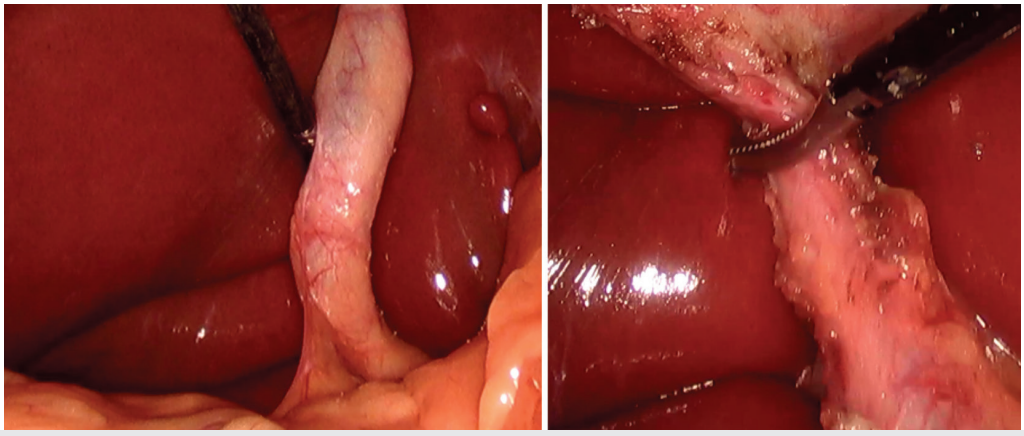

Figure 7. Double RS 1
RS: Rouviere's sulcus

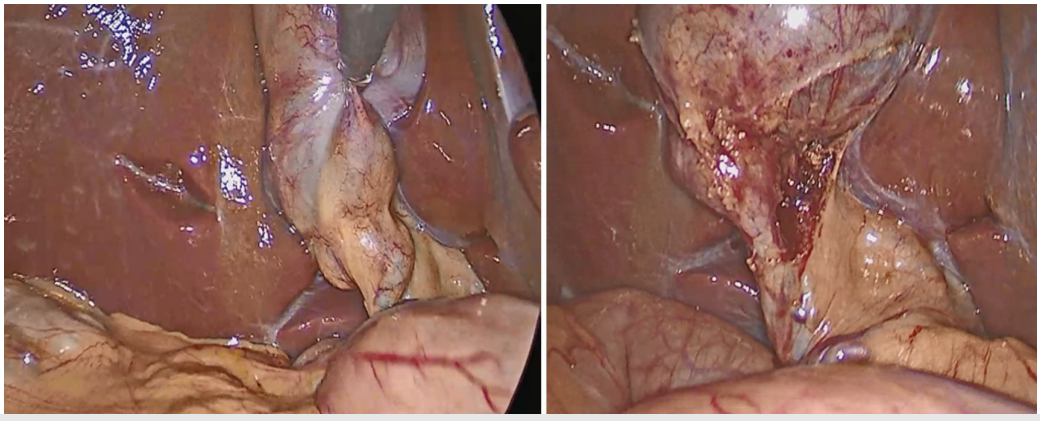

Figure 8. Double RS 2
RS: Rouviere's sulcus

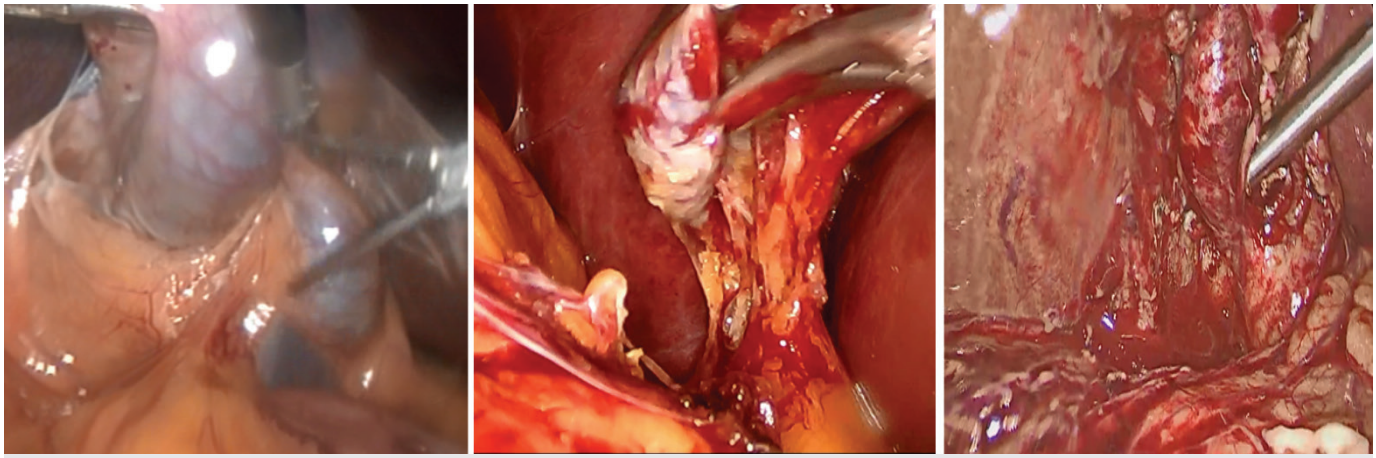

Figure 9. RS was not available due to chronic adhesions or was absent in normal and inflammatory patients
RS: Rouviere's sulcus

from cadaveric ones, the first no harm rule must be obeyed. Dissection around the inflamed area might cause injury to adjacent tissue including the common bile duct, portal vein, hepatic artery, duodenum, or colon. So, additional dissection was not performed to identify RS (18). This may cause inability to see RS due to inflammation around. Moreover, other fissures can be misidentified as RS and can be misleading surgeons during difficult cholecystectomies.

The clinical significance of the types of RS is still unclear (17). It has not been associated with a clinical condition so far. Emphasis is placed on the ability to guide the surgeon to the dissection site, and when there is an RS, it guides well. The CVS is not a dissection method but an identification tool to prevent bile duct injuries. If a surgeon cannot perform safe dissection and achieve CVS, bail-out strategies should be used freely for patient safety. As the inventor of CVS Strasberg and Brunt (7) once said, "The simple method to handle the difficult gallbladder is do not handle!". Those patients should be evaluated carefully preoperatively, and operation should be performed after the inflammation is treated (17). Fibrotic adhesions may not regress, even if an appropriate antibiotic therapy and a sufficient waiting period in some patients have been commenced. Although the surgeries in our study were elective surgeries, 48 of them were difficult cholecystectomies according to the Tokyo Guidelines (14). We recognized RS in 29 (60.4%) of the 48 DLCs. In these patients, RS cannot be seen due to adhesions and cannot guide the surgeon (Figure 9). Therefore, surgeons should use other safety measures during the operations like perioperative cholangiography, subtotal cholecystectomy, or simply drainage.

Study Limitations

The present study has certain limitations. First, our study sample was composed of video recordings of LCs. Second, our sample size was small to generalize our findings. However, despite these limitations, one of the strengths of the study was that it provided data for significance of RS in DLCs. On the other hand, due to relatively small number of our patients, it cannot be claimed that not detecting the RS during the operation will increase the complications. Then again, visualizing RS and not dissecting below that line certainly give surgeons a sigh of relief.

CONCLUSION

In summary, the present study contributes to an understanding of the significance of RS in DLC operations. The frequency and type of RS in this sample from Turkey were found to be similar to the reports in literature. When available (present and visualized), RS contributes a lot to the safety measures of LC. On the other hand, when it comes to DLC, the availability and convenience of RS might be limited or misleading for surgeons.

Besides precise knowledge of anatomy, awareness of variations and misidentification set-ups are the key elements for patient safety during surgery. It is especially true for laparoscopic surgeries that with two-dimensional images and perspective misidentification might end up with a surgical disaster. Since the LC is among the most performed surgeries worldwide, surgeons need to be aware of anatomical landmarks, safety measurements, and bail-out strategies.

Ethics Committee Approval: We obtained the ethics committee approval from the University of Health Sciences Hamidiye Scientific Research Ethics Committee (decision no: 12/9, date: 02.04.2021).

Informed Consent: The consent form was not required in this study because it was a retrospective study.

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The Relationship Between Ideal Lumbar Pedicle Screw Position and Superior Facet in Adolescent Idiopathic Scoliosis

Adölesan İdiyopatik Skolyozlu Hastalarda İdeal Lomber Pedikül Vidalama Uygulamasının Superior Faset Eklem ile İlişkisi

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ABSTRACT

Objective: The purpose of this study was to investigate the angular relationship between the anatomic inclination of the superior facet and the desired safe straight-forward (SF) transpedicular screw trajectory in the surgical treatment of patients with adolescent idiopathic scoliosis (AIS).

Methods: The study was conducted as a retrospective evaluation. One hundred and ten lumbar vertebrae were analyzed from the preoperative computed tomography scans of 22 patients with AIS scheduled for surgery. Each lumbar vertebral segment was prepared using reformat images obtained with three-dimensional (3D) volume-rendered images. The axial angles between the relative trajectory of the implant, which was planned to be placed according to the ideal SF transpedicular pedicle screw technique, and the inclination of the superior facet joint were measured.

Results: Two hundred and twenty pedicle-facet angles of 110 vertebrae were measured on image slices showing 3D volume rendering of pedicle and facet joint together. The ideal SF orientation angle at each vertebral level was more laterally oriented than the facet tilt. Fifteen (68%) of the patients had Lenke type 1 and 7 (32%) had Lenke type 5. When the patients were divided into two groups according to Lenke type (type 1 and 5), there was no statistically significant difference in terms of the angular values of the lumbar spine ($p>0.05$).

Conclusion: When implanting a transpedicular screw to correct a scoliotic deformity, a trajectory that is no more medially inclined than the inclination of the facet joint will reduce adverse events such as medial breeching. This information can be used as supporting information when placing pedicle screws in addition to other anatomical landmarks.

Keywords: Pedicle screw, facet joint, medial breeching

ÖZ

Amaç: Bu çalışmanın amacı, adölesan idiyopatik skolyozlu (AIS) hastaların cerrahi tedavisinde superior fasetin anatomik eğimi ile istenen güvenli straight-forward (SF) transpediküler vida yörüngesi arasındaki açısal ilişkiyi araştırmaktır.

Yöntemler: Çalışma retrospektif bir değerlendirme olarak yapılmıştır. Ameliyat planlaması yapılan AIS tanılı 22 hastanın 110 lomber vertebrası bilgisayarlı tomografi taramalarından analiz edildi. Her lomber vertebral segment, üç boyutlu (3B) hacimle işlenmiş görüntülerle elde edilen yeniden formatlanmış görüntüler kullanılarak hazırlandı. İdeal SF transpediküler pedikül vida tekniğine göre yerleştirilmesi planlanan implantın göreceli yörüngesi ile superior faset eklemin eğimi arasındaki aksiyal açılar ölçüldü.

Bulgular: Yüz on vertebranın 220 pedikül-faset arası açısı, 3D olarak pedikül ve faset eklemi birlikte gösteren görüntü kesitleri üzerinden ölçüldü. Her vertebra seviyesinde ideal SF yönelim açısı, faset eğiminden daha lateral yönelimli idi ($p<0,001$). Olguların 15'i (%68) Lenke tip 1, yedisi (%32) Lenke tip 5 idi. Hastalar Lenke tipine göre (tip 1 ve 5) iki gruba ayrıldığında bel omurlarının açısal değerleri arasında istatistiksel olarak anlamlı fark görülmedi ($p>0,05$).

Sonuç: Skolyotik deformitenin düzeltilmesi için transpediküler vida yerleştirirken, faset eklem eğiminden daha mediale doğru eğimli olmayan bir vida yönelimi, medial kanal ihlali gibi istenmeyen olayları azaltacaktır. Bu bilgi, pedikül vidası uygulamalarında diğer anatomik işaretlere ek olarak destekleyici bir bilgi olarak kullanılabilir.

Anahtar kelimeler: Pedikül vidası, faset eklemi, medial ihlal

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INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a three-dimensional (3D) deformity with coronal, sagittal, and axial components (1). The current gold standard treatment method in the surgical treatment of AIS is instrumented fusion of the posterior segments with transpedicular screws (2). The incidence of misplacement of pedicle screws in the lumbar spine is reported to be 5% to 41% (3). To avoid complications in scoliosis surgery, it is crucial to avoid misplacement of transpedicular screws (4). The screws should be firmly seated in the medullary canal of the bony pedicle to also provide adequate correction of the rotational deformity (5).

Multiplanar images can be thickened into slabs according to the anatomical area of interest using projection techniques such as volume rendering. Volume rendering is a technique that combines 3D perspective with versatile and interactive rendering of the entire volume of reconstructed data (6). Radiological images obtained by volume rendering are closest to the image that the surgeon sees with the naked eye in the surgical field (7).

The purpose of the present study was to investigate the relationship between the anatomic inclination of the superior facet and the desired safe transpedicular screw trajectory in the surgical treatment of AIS. To this end, the angular relationship between the superior facet joint and the pedicle screw trajectory was evaluated using postoperative computed tomography scans and volume rendering projection techniques to obtain a reliable orientation angle in the axial plane.

METHODS

Two hundred and twenty pedicle facet regions of lumbar vertebral segments from 22 patients with AIS surgically treated in our department of orthopedics and traumatology between July 2020 and March 2022 were included in the evaluation based on preoperative tomography images. Demographic data (age, sex, Lenke subtypes) were recorded. Surgical treatment of AIS was accepted as an inclusion criterion, and patients with a diagnosis of known etiology or those with inadequate or poor imaging for measurements were excluded from the study. It was confirmed that no intracanal pathology was present on magnetic imaging studies. The study protocol was approved by the Ethics Committee of Başakşehir Çam and Sakura City Hospital (decision no: 2022.04.102). Informed consent was not needed given the nature of the study.

Radiological Evaluation

Each of the 3D lumbar vertebral images created using the volume rendering technique was sliced using the scalpel function of the program [RadiAnt DICOM Viewer (Software). Version 2021.1. Jun 27, 2021]. In this way, a bird's-eye view of the tip of the superior facet joint and the pedicle of each vertebra was obtained on the same screenshot. The screenshot was then opened in the Surgimap© measurement system (Nemaris Inc. USA) (https://www.surgimap.com/). For the lumbar vertebrae from L1 to L5, it was marked on the tomography that the junction of the middle and lateral thirds of the intersection of the midline of the transverse process and the corresponding superior articular process was the entry point of the pedicle screw. The trajectory of the pedicle screw was determined by combining this point with the most central point of the pedicle. The angle between the superior articular facet and the trajectory of the ideal screw passing through the pedicle was determined using the straight-forward (SF) technique (Figure 1, 2) (8). This measurement was performed for both the right and left sides. Radiographic measurements (measurements in the Surgimap app) were measured again 3 weeks later by the same observer.

Statistical Analysis

Data analysis was performed using the SPSS 24.0 program. For the descriptive characteristics, analyzes of number, percentage, mean, and standard deviation were performed. While the Mann-Whitney U test was used for paired groups, Kruskal-Wallis analyzes were performed for more than two groups. Spearman correlation test was performed for relational analysis. $P<0.05$ was considered significant.

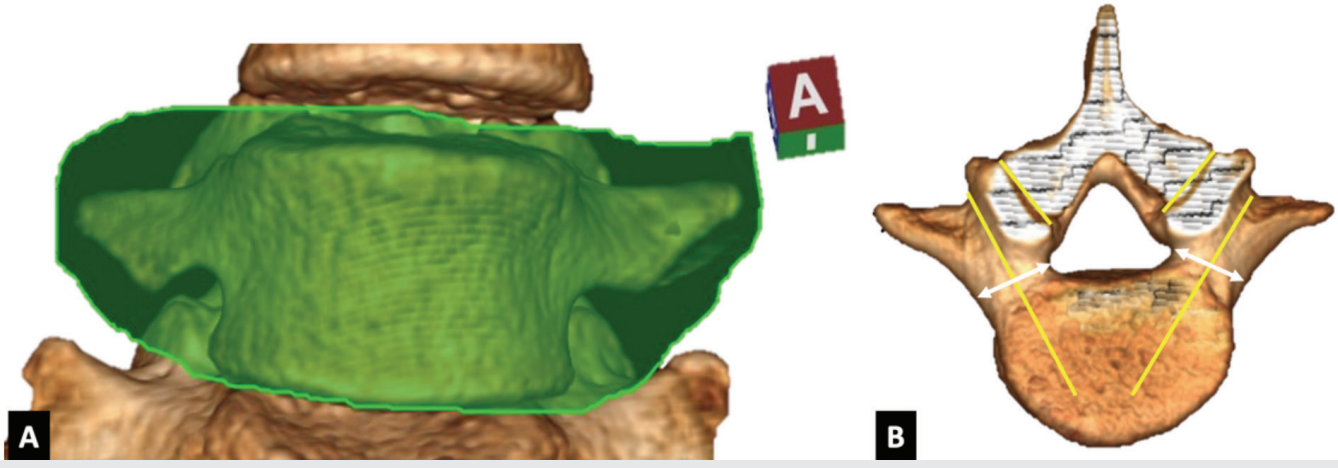

Figure 1. Cutting a three-dimensional volume-rendering image with the scalpel feature at the L5 vertebral level (A). Identification of the planned ideal transpedicular pedicle trajectory and the superior facet inclination with the top view of the obtained single vertebral level (B)

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RESULTS

The mean age of the 22 patients included in the study was 14.5±1.33 and two of them were male. In 3 of the patients, L5-S1 transitional vertebrae were observed as an additional anatomical variation. The angles between the axial superior facet inclination and the targeted SF pedicle trajectory of the measured vertebrae are shown in Table 1.

According to the values for skewness and kurtosis, except for the left L5 lumbar vertebra, a normal distribution was found for the angular measurements of all segments, as indicated in the methodology, since they were all in the nominal range (-1.5)-(+1.5), parametric tests were used for the statistical analyzes. The consistency of the correlation coefficient between two separate calculation time intervals was above 0.8, indicating good agreement. According to Lenke classification, 68% of patients belonged to type 1 and 32% to type 5, and 32% had the level of lumbar apex at L2 (Table 2).

Considering the distributions of mean and standard deviation of angle measurements, the highest angles were observed at the L5 Right (18.47±8.62) and L5 Left (18.4±10.34) spine levels (Table 3).

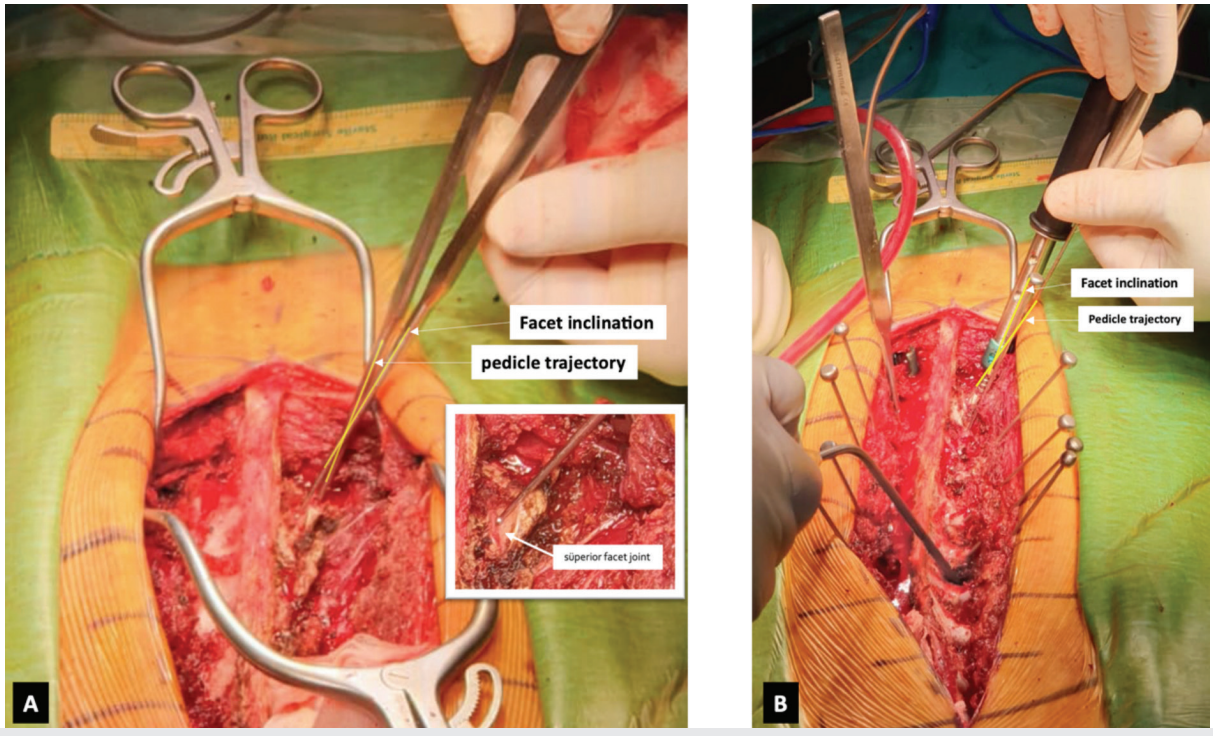

Figure 2. In one of the patients included in the study, the angle between the pedicle and the inclination of the superior facet joint is observed during surgery. Angle between two pedicle guides in A. In B, the relationship to the facet joint during pedicle screw application is seen. Note how the imaginary lines drawn in yellow cross each other. The axis of the ideal pedicle screw is more laterally directed to the inclination of the facet joint and does not go further medially than the inclination of the facet joint

Table 1. Bilateral facet-SF pedicle trajectory angular measurements for each lumbar vertebral level

Level	Mean	Minimum	Maximum	SD	Skewness	Kurtosis
L5 right	16.5	10	45.7	10.3	0.92	1.86
L5 left	15.9	12	42.6	8.9	1.2	2.92
L4 right	11.4	2.3	21.1	5.4	0.19	-0.64
L4 left	11.4	10	25.3	6.6	0.43	-0.18
L3 right	8.5	0.3	19.9	5.6	0.28	-1.03
L3 left	8.1	0.2	19.5	5.3	0.28	-0.86
L2 right	9.1	0.4	18.5	4.8	0.13	-0.34
L2 left	7.7	0.5	15.5	5.0	0.08	-1.2
L1 right	10.8	0.6	23.7	5.0	0.17	1.4
L1 left	10.4	0.5	24.8	5.4	0.45	1.3

SD: standard deviation, SF: straight-forward, L: lumbar vertebra

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No statistically significant difference was found between the age groups of the patients and the mean value of the angle measurement (p>0.05) (Table 4).

No statistically significant difference was found between the sex and mean angular measurements of the patients (p>0.05) (Table 5).

Of the patients, 15 (68%) had Lenke type 1 and seven (32%) had Lenke type 5. No statistically significant difference was found when patients' Lenke types were compared with the mean of the angle measurement (p>0.05) (Table 6).

The lumbar modifier type was type A in 4 of the patients and type C in the remaining 18 patients. The lumbar apex was at the L1 level in 5 (23%) of 22 patients, at the L2 level in 7 (32%), at the L3 level in 6 (27%), and at the L4 level in 4 (18%) patients. No statistically significant difference was found when comparing the Lenke Lumbar Modifiers and the patients' mean angle measurements (p>0.05) (Table 7).

No statistically significant difference was found when comparing measurements of Lumbar Apex and mean angle (p>0.05) (Table 8).

According to the Spearman correlation test, a positive and statistically significant relationship was found in the relational analyzes of the following angle measurements: Between L1 L and L1 R (strong relationship), L2 L (moderate), L2 R (moderate), and L3 R (moderate); between L1 R and L2 L (moderate), L2 R (moderate), and L3 R (moderate); between L2 L and L2 R (strong), L5 L (moderate), L5 R (moderate), between L2 R and L5 R (moderate); between L3 L and L3 R (strong), L4 L (moderate), L4 R (moderate), L5 L (moderate), and L5 R (moderate); between L3 R and L4 L (moderate), L4 R (moderate), L5 L (moderate), and L5 R (moderate); between L4 L and L4 R (strong), L5 L (strong), and L5 R (strong); between L4 R and L5 L (moderate), L5 R; and finally between L5 L and L5 R (strong) (p<0.05) (Table 9).

Table 2. Lenke type and lumbar apex distributions

	n (22)	%
Lenke type		
1	15	68.2
5	7	31.8
Lumbar apex		
L1	5	22.7
L2	7	31.8
L3	6	27.3
L4	4	18.2

L: lumbar vertebra

Table 3. Distribution of mean and standard deviation values of angle measurements

Facet-screw angle	Mean + SD		Mean ± SD
L1 R	11.1±4.61	L1 L	10.78±5.05
L2 R	9.62±4.46	L2 L	8.82±3.95
L3 R	9.75±5.15	L3 L	9.41±5.07
L4 R	12.43±5.6	L4 L	12.46±5.84
L5 R	18.47±8.62	L5 L	18.4±10.34

R: the angle between the superior articular facet and the trajectory line of the ideal screw at the right side, L: same angle measurement fort he left side, SD: standard deviation

It was found that the angles measured at each lumbar vertebral level varied within a wide scale, regardless of the patients' demographic characteristics and Lenke type classification (p<0.05), even at the same vertebral level in different patients. In all patients, the right and left angle measurements for each vertebra were found to be similar and did not show statistical significance (p>0.05). Statistically significant differences were observed in the angle measurements between the same vertebral levels in different patients (p<0.05).

In all patients, the calculated angle between the facet inclination and the imaginary trajectory of the pedicle screw had a positive numerical value, meaning that the trajectory of the pedicle was more laterally directed than the inclination of the facet cartilage (100%).

DISCUSSION

The current study reports that osteotomy of the inferior facet before pedicle screw placement helps surgeons avoid injury to nerve tissue in the canal and prevent possible medial breaching by guiding the pedicle screw at a lower inclination (more lateral) than the axial inclination of the exposed superior facet cartilage.

The open surgical technique for AIS uses a midline skin incision and subperiosteal dissection to expose the spinous processes, laminas, facet joints, and transverse processes. Inferior facet osteotomies are performed before or after screw placement in the fusion area to help identify pedicle screw entry points, promote arthrodesis, and allow easier correction of the deformity except at the superior and inferior levels (1,9). Mattei et al. (9) recommended partial facetectomy of the inferolateral third of the inferior articular process of the upper spine. With this osteotomy, the boundaries of the facet joints can be clearly determined, and the entry point for the pedicle screw can be properly designed by removing the hypertrophic and misleading tissue; it can be ensured that the screw head can sit on a smooth surface. The current study shows that it is probably a dangerous idea to guide a transpedicular screw trajectory more medially than the inclination of the superior facet joint in the axial plane. This information may be a valuable contribution to the literature.

Amaral et al. (10) studied the safe angle with the facets that could prevent facet joint violation during screw insertion regardless of the screw insertion technique in degenerative spines. The authors analyzed imaging of patients operated for degenerative lumbar spine pathologies and defined the angle between pedicle screw and facet joint inclination as Δ-angle. Accordingly, the rate of facet injury in patients with a Δ-angle of

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Table 4. Comparisons of age groups and mean angle measurements

	Age group	n (22)	Mean ± SD	U	p
L5 R	<15	10	15.93±6.97	42	0.235
	≥15	12	20.6±9.56		
L5 L	<15	10	18.35±12.72	48.5	0.448
	≥15	12	18.43±8.46		
L4 R	<15	10	11.02±6.15	46.5	0.373
	≥15	12	13.61±5.07		
L4 L	<15	10	11.7±6.65	52	0.598
	≥15	12	13.1±5.27		
L3 R	<15	10	9.19±4.9	53.5	0.668
	≥15	12	10.21±5.51		
L3 L	<15	10	9.17±5.72	56.5	0.817
	≥15	12	9.6±4.72		
L2 R	<15	10	8.64±2.95	48	0.429
	≥15	12	10.44±5.41		
L2 L	<15	10	8.1±3.08	50.5	0.531
	≥15	12	9.42±4.6		
L1 R	<15	10	10.9±4.42	59	0.947
	≥15	12	11.25±4.96		
L1 L	<15	10	11.56±4.03	45	0.323
	≥15	12	10.13±5.86		

SD: standard deviation, U: The Mann-Whitney U value, L: lumbar vertebra, R: right

Table 5. Comparisons of gender and mean angle measurements

	Gender	n	Mean ± SD	U	p
L5 R	Female	18	19.1±9.37	29	0.551
	Male	4	15.65±3.2		
L5 L	Female	18	19.33±11.2	26	0.394
	Male	4	14.15±2.85		
L4 R	Female	18	11.97±5.58	26	0.394
	Male	4	14.52±6.04		
L4 L	Female	18	12.13±5.53	34.5	0.898
	Male	4	13.92±7.83		
L3 R	Female	18	10.7±5.12	14.5	0.067
	Male	4	5.5±2.74		
L3 L	Female	18	10.21±5.04	16	0.089
	Male	4	5.8±3.87		
L2 R	Female	18	9.91±4.17	31	0.67
	Male	4	8.32±6.2		
L2 L	Female	18	8.92±3.7	32	0.733
	Male	4	8.35±5.63		
L1 R	Female	18	11.57±4.55	27	0.443
	Male	4	8.9±4.9		
L1 L	Female	18	11.47±4.93	22	0.233
	Male	4	7.67±5.04		

SD: standard deviation, U: The Mann-Whitney U value, L: lumbar vertebra, R: right

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Table 6. Comparisons of lenke types and mean angle measurements

	Lenke type	n (22)	Mean ± SD	U	p
L5 R	1	15	16.94±5.97	45	0.597
	5	7	21.77±12.6		
L5 L	1	15	18.08±10.28	52	0.972
	5	7	19.07±11.25		
L4 R	1	15	12.3±6.07	51.5	0.944
	5	7	12.74±4.87		
L4 L	1	15	12.1±6.06	47.5	0.724
	5	7	13.25±5.7		
L3 R	1	15	9.78±5.6	51.5	0.944
	5	7	9.67±4.42		
L3 L	1	15	9.13±5.63	45.5	0.622
	5	7	10±3.93		
L2 R	1	15	9.24±4.76	44	0.549
	5	7	10.42±3.97		
L2 L	1	15	8.68±4.13	49	0.805
	5	7	9.11±3.84		
L1 R	1	15	11.11±5.54	51.5	0.944
	5	7	11.04±1.73		
L1 L	1	15	10.92±5.74	51	0.916
	5	7	10.48±3.5		

SD: standard deviation, U: The Mann-Whitney U value, L: lumbar vertebra, R: right

Table 7. Comparison of lenke lumbar modifier and mean angle measurements

	Lenke lumbar modifier	n (22)	Mean ± SD	U	p
L5 R	A	4	15.07±5.5	26	0.395
	C	18	19.23±9.12		
L5 L	A	4	19.7±19.07	26	0.394
	C	18	18.1±8.21		
L4 R	A	4	10.725	26.5	0.418
	C	18	12.81±5.42		
L4 L	A	4	9.55±6.64	23	0.268
	C	18	13.11±5.64		
L3 R	A	4	6.46.46	16	0.089
	C	18	10.5±4.7		
L3 L	A	4	6.77±8.53	20	0.173
	C	18	10±4.11		
L2 R	A	4	7.97±5.5	31	0.67
	C	18	9.98±4.3		
L2 L	A	4	8.42±5.51	30	0.609
	C	18	8.91±3.73		
L1 R	A	4	8.75±5.76	27.5	0.469
	C	18	11.61±4.34		
L1 L	A	4	9.35±6.46	34	0.865
	C	18	11.1±4.85		

SD: standard deviation, U: The Mann-Whitney U value, L: lumbar vertebra, R: right

less than 5 degrees was found to be 65%. They found that it was 11% in patients with a Δ-angle between 5-15 degrees and 3% in patients with a Δ-angle higher than 15 degrees. Because an open surgical approach was used in our study and we assumed that there would be no force against the superior facet joint, scenarios that could lead to screw misplacement in the canal were investigated. As the relative angular value decreased, the extent of facet injury in our study appeared to be correlated with the risk of medial breaching.

Table 8. Comparisons of lumbar apex and mean angle measurements

	Lomber apex	n (22)	Mean ± SD	X^2	p
L5 R	L1	5	26.02±12.54	3.789	0.285
	L2	7	16.91±5.03		
	L3	6	16.28±7.92		
	L4	4	15.07±5.5		
L5 L	L1	5	22.86±11.21	2.493	0.477
	L2	7	16.28±5.19		
	L3	6	16.26±8.06		
	L4	4	19.7±19.07		
L4 R	L1	5	14.34±4.95	0.925	0.819
	L2	7	11.97±4.72		
	L3	6	12.53±7.09		
	L4	4	10.72±6.97		
L4 L	L1	5	14.78±6.04	1.983	0.576
	L2	7	12.21±3.68		
	L3	6	12.76±7.67		
	L4	4	9.55±6.64		
L3 R	L1	5	10.94±4.72	5.004	0.172
	L2	7	8.21±3.95		
	L3	6	12.78±4.98		
	L4	4	6.4±6.46		
L3 L	L1	5	10.92±4.35	3.41	0.333
	L2	7	8.28±3.86		
	L3	6	11.21±4.21		
	L4	4	6.77±8.54		
L2 R	L1	5	10.54±4.85	0.281	0.964
	L2	7	10.27±5.08		
	L3	6	9.2±3.44		
	L4	4	7.97±5.5		
L2 L	L1	5	9.26±4.64	1.732	0.63
	L2	7	9.74±4.06		
	L3	6	7.65±2.7		
	L4	4	8.42±5.51		
L1 R	L1	5	10.54±1.75	1.376	0.711
	L2	7	12.17±5.45		
	L3	6	11.85±4.94		
	L4	4	8.75±5.76		
L1 L	L1	5	9.4±3.47	0.706	0.872
	L2	7	12.31±6.1		
	L3	6	11.1±4.6		
	L4	4	9.35±6.46		

X^2: chi-square, , SD: standard deviation, L: lumbar vertebra, R: right

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Table 9. Relational analysis of angle measurements

		L1 L	L1 R	L2 L	L2 R	L3 L	L3 R	L4 L	L4 R	L5 L	L5 R
L1 L	r	1.000	-	-	-	-	-	-	-	-	-
	p	-	-	-	-	-	-	-	-	-	-
L1 R	r	0.878**	1.000	-	-	-	-	-	-	-	-
	p	0.000	-	-	-	-	-	-	-	-	-
L2 L	r	0.511*	0.499*	1.000	-	-	-	-	-	-	-
	p	0.015	0.018		-	-	-	-	-	-	-
L2 R	r	0.609**	0.625**	0.909**	1.000	-	-	-	-	-	-
	p	0.003	0.002	0.000	-	-	-	-	-	-	-
L3 L	r	0.394	0.380	0.398	0.379	1.000	-	-	-	-	-
	p	0.069	0.081	0.066	0.082	-	-	-	-	-	-
L3 R	r	0.448*	0.494*	0.367	0.411	0.946**	1.000	-	-	-	-
	p	0.037	0.019	0.093	0.058	0.000	-	-	-	-	-
L4 L	r	0.185	0.227	0.333	0.343	0.565**	0.553**	1.000	-	-	-
	p	0.409	0.310	0.130	0.118	0.006	0.008	-	-	-	-
L4 R	r	0.169	0.230	0.189	0.224	0.500*	0.524*	0.897**	1.000	-	-
	p	0.451	0.304	0.399	0.317	0.018	0.012	0.000	-	-	-
L5 L	r	0.265	0.361	0.459*	0.373	0.525*	0.567**	0.728**	0.592**	1.000	-
	p	0.232	0.099	0.031	0.087	0.012	0.006	0.000	0.004	-	-
L5 R	r	0.325	0.419	0.518*	0.487*	0.517*	0.565**	0.711**	0.646**	0.931**	1.000
	p	0.140	0.052	0.014	0.021	0.014	0.006	0.000	0.001	0.000	-

*Significant at the p<0.05 level, **Significant at the p<0.001 level. L: lumbar vertebra, R: right

There are numerous studies in the literature on superior facet injury after pedicle screw placement (11-13). The aim of these studies was to increase surgical safety by revealing the risk factors influencing screw placement in the correct position. In this regard, Cong et al. (12) found that the use of an intraoperative navigation system was an important factor in preventing pedicle malposition. Because of the high cost of navigation systems and the fact that they cannot be used in every spine surgery clinic, special attention should be paid to the factors such as the use of fluoroscopy, printing of 3D spine models, neuromonitoring to increase reliability, and sensitive attention to anatomic markers during freehand techniques (14-16).

Facet tropism describes the presence of asymmetric angles on both sides of the facet joints and is common in various pathologies of the lumbar spine (17). In our study, we found that there was no statistical difference in the angle between the facet and pedicle trajectories on the right and left sides at each spinal level, except for the L5 vertebra in the 3 patients with sacralization. This finding suggests that there is no facet tropism in the scoliotic spine, unless there is some other anatomic variation. Can et al. (18) also found a possible link between facet tropism and sacralization, as observed in our study.

Study Limitations

One of the strengths of the study was the 3D evaluation of tomographic sections via reformat studies, so that the study came closest to real anatomy (6). Because most studies in the literature use tomographic images without reformatting them to a focused true plane, it is possible that the anatomy will be evaluated in an incomplete plane due to the natural lordosis of the lumbar spine. The weakness of our study was that the patients were not evaluated for postoperative pedicle screw applications. We have two explanations for this. First, screw implantation was not performed at all levels of the lumbar spine (especially at L4 and L5) in patients with AIS, and second, we did not yet implement this method, which we examined in our study, sufficiently in our routine practice to test its effectiveness.

According to our results, the conclusion that "screw insertion with more lateral alignment than facet joint inclination is reliable" applies only to the transpedicular SF screw technique method. In this technique, the midpoint of the anterior cortex of the vertebral body of the screw tip is targeted through the junction of the entry point and the midpoint of the pedicle. In cases where the anatomical methodology is different, such as the cortical bone trajectory technique, this conclusion will not be valid (19).

In the statistical analysis, we could not find a practical margin of safety for any level as a general rule for the placement of pedicle screws. Each patient should be evaluated on his/her own merits in this regard. However, a screw inserted at an angle no more medial than the facet joint, has a higher probability of being in the correct trajectory. Careful review of radiographic images should be

performed in each individual patient prior to surgical intervention when considering the angular inclination of the facet.

CONCLUSION

When implanting a transpedicular screw to correct a scoliotic deformity, a trajectory that is no more medially inclined than the inclination of the facet joint in the axial plane will reduce undesirable events such as medial breaching. This information can be used as supporting information in addition to other anatomical landmarks in pedicle screw applications.

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of Başakşehir Çam and Sakura City Hospital (decision no: 2022.04.102).

Informed Consent: Informed consent was not needed given the nature of the study.

Peer-review: Externally and internally peer-reviewed.

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The Effects of Acute and Chronic Metformin Treatment on Penicillin Induced Epileptiform Activity in Rats

Sıçanlarda Penisilinle Oluşturulan Epileptiform Aktivite Üzerine Akut ve Kronik Metformin Tedavisinin Etkileri

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ABSTRACT

Objective: The aim of this study is to investigate acute and chronic administered metformin on epileptiform activity induced by penicillin and antioxidant activity in rats.

Methods: Eighty-four adult male Wistar albino rats were used in this study. The rats were divided into two large groups as acute and chronic groups, and later on each group was divided into different subgroups as control, sham, penicillin, metformin 100 mg/kg (Met_100), 200 mg/kg (Met_200) and only metformin 200 mg/kg (OMet_200) intraperitoneally. The substances were applied to the chronic groups for 21 days, while acute groups received them just before the initiation of epileptiform activity. In the present study, onset of first epileptiform activity, spike wave frequency and amplitude, and superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) parameters were evaluated.

Results: No epileptiform activity was observed in the control, sham, and OMet_200 groups. When metformin doses of 100 mg/kg, 200 mg/kg were compared with the penicillin group in both acute and chronic groups, the onset of first epileptiform activity was prolonged, spike wave frequency and spike wave amplitude decreased significantly. SOD, CAT and GPx levels were found to be significantly different in the acute and chronic metformin groups compared to the penicillin group.

Conclusion: In conclusion, this study shows that metformin can decrease epileptic seizures and increase the level of antioxidant enzymes and it can be used in the treatment of epilepsy in the future.

Keywords: Antioxidant, electrocorticography, epilepsy, epileptiform activity, metformin

ÖZ

Amaç: Bu çalışmanın amacı, akut ve kronik olarak uygulanan metforminin, sıçanlarda penisilin ile indüklenen epileptiform aktivite ve antioksidan aktivite üzerine etkisinin araştırılmasıdır.

Yöntemler: Bu çalışmada 84 adet yetişkin erkek Wistar albino sıçan kullanıldı. Sıçanlar akut ve kronik gruplar olmak üzere iki büyük gruba ayrıldıktan sonra her bir grup kontrol, sham, penisilin, metformin 100 mg/kg (Met_100) intraperitoneal (i.p), 200 mg/kg (Met_200) (i.p) ve sadece metformin 200 mg/kg (SMet_200) (i.p) dozlarda farklı alt gruba ayrıldı. Kronik gruplara maddeler 21 gün, akut gruplara ise sadece epileptiform aktivite öncesi uygulandı. Bu çalışmada ilk epileptiform aktivitenin başlangıcı, spike dalga frekansı ve amplitüdü ile süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx) ve katalaz (CAT) parametreleri değerlendirildi.

Bulgular: Kontrol, sham ve sadece metformin gruplarında hiçbir epileptiform aktivite görülmemiştir. Metforminin 100 mg/kg, 200 mg/kg'lik dozları hem akut hem de kronik gruplarda penisilin grubu ile kıyaslandığında, ilk epileptiform aktivite başlangıç latensi uzamış, diken dalga sayısı ve diken dalga genliği anlamlı olarak azalmıştır. Akut ve kronik metformin gruplarında SOD, CAT ve GPx seviyeleri penisilin grubuna göre anlamlı bulunmuştur.

Sonuç: Sonuç olarak bu çalışma metforminin, epileptik nöbetleri hafifletebileceği ve antioksidan enzimleri artırabileceği ve gelecekte epilepsi tedavisinde kullanılabileceğini göstermektedir.

Anahtar kelimeler: Antioksidan, elektrokortikografi, epilepsi, epileptiform aktivite, metformin

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1. INTRODUCTION

Epilepsy is a neurological brain disease that affects approximately 50 million people of all ages worldwide. It is characterized by recurrent seizures, transient occurrence of abnormal, excessive, and/or synchronous neuronal activity in the brain, and sometimes accompanied by loss of consciousness and poor control of bowel and bladder function. Of the people with epilepsy 80% live in low and middle income countries (1,2).

In general, epileptic seizures indicate the presence of an imbalance between inhibitory and excitatory neurotransmission. However, the mechanism of epileptogenesis is still unclear (3).

Until now, anti-epileptic drugs could not been developed to completely heal epilepsy. Therefore, the current therapeutic approach is symptomatic therapy to improve patients' lifetime and quality of life (4,5). The epileptogenesis process involves a variety of molecular and cellular changes that can be used as potential targets for the treatment and prevention of epilepsy, but most of the currently available drugs only work by suppressing the seizure without affecting the underlying pathological conditions (6).

Metformin is the most commonly prescribed oral antidiabetic drug in the world and has been used in the treatment of type 2 diabetes for many years. In addition, this drug is used in the treatment of prediabetes, polycystic ovary syndrome and insulin resistance. The positive effects of metformin on the cardiovascular system and its anti-cancer activity are positive aspects of the drug. Diabetes associations recommend the use of metformin at all levels of the national and global type 2 diabetes treatment algorithms (7).

Metformin is an inexpensive drug that is well tolerated, effective and easy to access. Besides its known antihyperglycemic effects, it has been shown to have positive effects on the cardiovascular system in patients with insulin resistance and diabetes. Increasing the studies on metformin, which is one of the oldest drug known, leads to an increase in its use in medicine (8).

It has been shown that metformin can decrease the symptoms of epileptic seizures as well as modifying the molecular and cellular changes, including oxidative stress, neuroinflammation, apoptosis, and neuronal loss. In experimental studies, the neuroprotective effects of metformin were investigated by using pentylenetetrazole (PTZ), pilocarpine, and kainic acid models that produced epileptiform activity. In the study of Mehrabi et al. (9), it was shown that metformin decreased brain-derived neurotrophic factor (BDNF) and TrkB expression, increased AMPK expression, and decreased mTOR protein expression in an animal model of temporal lobe epilepsy (TLE) created by pilocarpine administration. In the study conducted by Yang et al. (10), it was found that administration of metformin decreased mortality, increased AMPK levels and facilitated seizure termination compared to the control group in the kainic acid and PTZ epilepsy models in mice.

In this study, different from the studies which were mentioned above, the experimental epilepsy model was created with penicillin. In the present study, the onset of first epileptiform activity, spike wave frequency and spike wave amplitude were examined electrophysiologically to determine the chronic and acute effects of metformin on epileptiform activity induced by penicillin. In addition, in order to determine the antioxidant properties of metformin, it was aimed to investigate the enzyme values of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in blood samples taken from rats.

2. METHODS

2.1. Animals Used in the Experiment

The ethical approval was obtained from Duzce University Animal Experiments Local Ethics Committee with the decision number 2019/6/1 (date: 02.07.2019), and the animals to be used in the experiment were obtained from Duzce University Experimental Animals Application and Research Center. Wistar albino male rats weighing 290±30 and 12-14 weeks old were used for the experiment. The animals were kept at 23 °C room temperature, 60±5% humidity and 12:12 light-dark cycle, and free to access food and water.

2.2. Experimental Groups

The animals were divided into two experimental groups as acute (n=42) and chronic (n=42) groups. Then, each of the acute and chronic groups was divided into 6 subgroups (Table 1, 2). The substances specified in Table 1 were administered to the rats in the acute group and the substances specified in Table 2 were administered to the rats in the chronic group intraperitoneally (i.p). Electrocorticography (ECoG) recordings were obtained on

Table 1. Acute metformin administered groups

Group no	Groups	Substances supplied	Amount given	Delivery method	Number of animals
1	Sham group	-	-	-	7
2	Control group	Saline	1 mL/kg	i.p	7
3	Penicillin group	Penicillin	500 IU/2 µl	i.c	7
4	AOMet_200	Metformin	200 mg/kg	i.p	7
5	AMet_100 group	Metformin	100 mg/kg	i.p	7
6	AMet_200 group	Metformin	200 mg/kg	i.p	7

i.c: intracortically, i.p: intraperitoneally

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the same day in the acute groups, and 22nd day in the chronic groups. Metformin used in the study (BioVision incorporated 155 S. Milpitas Boulevard, Milpitas, CA 95035 USA) was dissolved in saline. Metformin was administered at the doses of 100 mg/kg and 200 mg/kg i.p. to the rats. As an anesthetic, urethane (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) was used at a dose of 1.25 g/kg i.p. To induce epilepsy, 500 IU/2µl penicillin (I.E. Ulagay Ilac Sanayi Turk A.S. Istanbul, Turkey) was injected intracortically (i.c.) to the rats. The substances used in the study were prepared daily.

2.3. Electrophysiological Study Procedure

2.3.1. Surgical Procedure

In all groups, rats were fixed to the stereotaxic frame (Harvard Instruments, South Natick, MA, USA) under the 1.25 g/kg urethane anesthesia, and bone on left cerebral cortex was precisely removed with tour motor (FST Rechargeable Microdrill, KF Technology, Rome, Italy).

2.3.2. Stimulation of the Epileptiform Activity

Epileptiform activity was induced by intracortical administration of 500 IU/2 µl penicillin with a Hamilton microinjector (701N, Hamilton Co. Reno, NV, USA) to 2 mm lateral, 1 mm in front of the bregma line and 1.2 mm cortex depth in the skull of rats.

2.3.3. Electrophysiological Recordings

For electrophysiological recordings, two Ag-AgCl ball electrodes were placed in the visible somatomotor cortex area by opening the lateral part of the bregma line on the left hemisphere. A reference electrode was placed to the right ear of the rats. The first electrode to take recordings was placed 1 mm in front of the bragma line and 2 mm lateral to the sagittal suture, the second was placed 5 mm posterior to the bregma line and 2 mm lateral to the sagittal suture. After the electrodes were placed, ECoG was recorded by the PowerLab/8SP (ADInstruments Pty Ltd. Castle Hill, NSW, Australia) data acquisition recording system.

Five minutes of basal activity was recorded before injecting the substances for both acute and chronic groups. In acute groups, metformin was given i.p after basal activity recording, and ECoG was recorded for another 30 minutes (Figure 1A). After 30 minutes of ECoG recording, intracortical penicillin was injected i.c. at the 35th minute. Then, another 120-minute recording was made. In the only metformin group without penicillin, after 5 minutes of basal activity recording, 200 mg/kg metformin was injected, and another 150-minute ECoG recording was made. Thus, a total of 155 minutes of ECoG recording was obtained from each animal (Figure 1A).

In the chronic metformin group, substances were given i.p at the same time intervals for 21 days. ECoG recording was made on the 22nd day in chronic groups. After removing the bone on the left somatomotor area as described in the surgical procedure, two recording electrodes were placed in this area. Five minutes of basal activity was recorded after the electrodes were placed. After basal activity recording, penicillin was injected i.c. and another 120-minute recording was made. A total of 125 minutes of ECoG recording was made in each animal (Figure 1B).

After 5 minutes of basal activity recording to both acute and chronic groups, penicillin was administered i.c and ECoG recording was made for another 120 minutes. These recordings were digitized at a sampling rate of 1024 Hz. The onset time of first epileptiform activity, general epileptiform activity, spike wave frequency and spike wave amplitude were evaluated. The data analyses were made with the PowerLab Chart v.8 software program. For each animal, the average spike wave frequency and amplitudes of the 5-minute time intervals of the ECoG recordings were used as data.

2.4. Determination of Antioxidant Activity

In order to determine the CAT, SOD and GPx levels in blood serum samples taken after ECoG from rats, samples were centrifuged at 4000 rpm for 15 minutes (Heraeus Labofuge 400, Thermo Scientific, Waltham, Massachusetts, USA). Serums taken after centrifugation were taken into eppendorf tubes and stored at -80 °C until the test day. SOD, CAT, GPx (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, China) were used as ELISA kits in our study. Spectrophotometer (Bio-Rad model 680 microplate reader, Bio-Rad Labrotories, Inc, USA) at 450 nm light wavelength was used to perform plate reading, and optical densities were recorded.

2.5. Statistical Analysis

Analysis of epileptiform activity records were made in periods of five minutes. The differences between the groups in terms of the onset of the first epileptiform activity, the spike wave frequency and the spike wave amplitude in each period were examined with the One-Way ANOVA test. Homogeneous subgroups multiple

Table 2. Chronic metformin administered groups

Group no	Groups	Substances supplied	Amount given	Delivery method	Number of animals
1	Sham group	-	-	-	7
2	Control group	Saline	1 mL/kg	i.p	7
3	Penicillin group	Penicillin	500 IU/2 µl	i.c	7
4	COMet_200 group	Metformin	200 mg/kg	i.p	7
5	CMet_100 group	Metformin	100 mg/kg	i.p	7
6	CMet_200 group	Metformin	200 mg/kg	i.p	7

i.c: intracortically, i.p: intraperitoneally

comparison method was used to determine different groups. In the comparison of groups in terms of CAT, SOD, GPx values, the ANOVA test was used, and homogeneous subgroups multiple comparison method was used to determine the different groups. IBM SPSS program was used in the analyses of the data. P<0.05 was considered as statistically significant.

3. RESULTS

3.1. The Effects of Only Metformin Administration in Rats

It was found that the administration of different doses of metformin (100 and 200 mg/kg) had no effect on basal activity. Similarly, no epileptic activity was found in the control and sham groups.

3.2. Epileptiform Activity Recording in Rats

In the penicillin groups, epileptic discharges appeared with spike wave formations 3-5 minutes after penicillin injection.

3.2.1. The Effect of Metformin on the Latency of First Epileptiform Activity

The effect of metformin on the onset of first epileptiform activity induced by penicillin was compared with the penicillin group. Onset of first epileptiform activity was significantly prolonged in CMet_100, CMet_200, AMet_100, AMet_200 groups compared to penicillin group (P values respectively p=0.050, p=0.009, p=0.040, p=0.042) (*p<0.05 is significantly higher than penicillin group) (Figure 2, Table 3).

3.2.2. The Effect of Metformin on Spike Wave Frequency

Epileptiform activity as spike waves started within 3-5 minutes in penicillin groups. When the spike wave frequencies were compared in the first period (0-5 min) no significant difference was found (p>0.05) between acute, chronic and penicillin groups.

In the CMet_100 group, the spike wave frequency was significantly less than the penicillin group until the 5th period (20-25 min) (p<0.05). After the 5th period (20-25 minutes), the spike wave frequency of the CMet_100 group showed a tendency to decrease in parallel with the penicillin group without a significant difference until the end of the recording (p>0.05). The spike wave frequency in the CMet_100 group was statistically higher than AMet_100 and AMet_200 groups in the 6th-7th and 8th period (25-40 minutes) (p<0.05). In the 9th period (41-45 minutes), the spike wave frequency in the CMet_100 group was statistically and significantly higher than in the AMet_100 group (p<0.05). When CMet_100 and the other groups were compared, there was no significant difference between CMet_100 and CMet_200, AMet_100, and AMet_200 groups in terms of spike wave frequency starting from the 10th period (45-50 minutes) until the end of the 24th period (115-120 minutes) (p>0.05) (Figure 3). The spike wave frequency in the CMet_200 group compared to the penicillin group until the end of the 6th period except the 5th period was statistically higher (p<0.05). In the 8th period, the spike wave frequency in the CMet_200 group was statistically and significantly higher than the

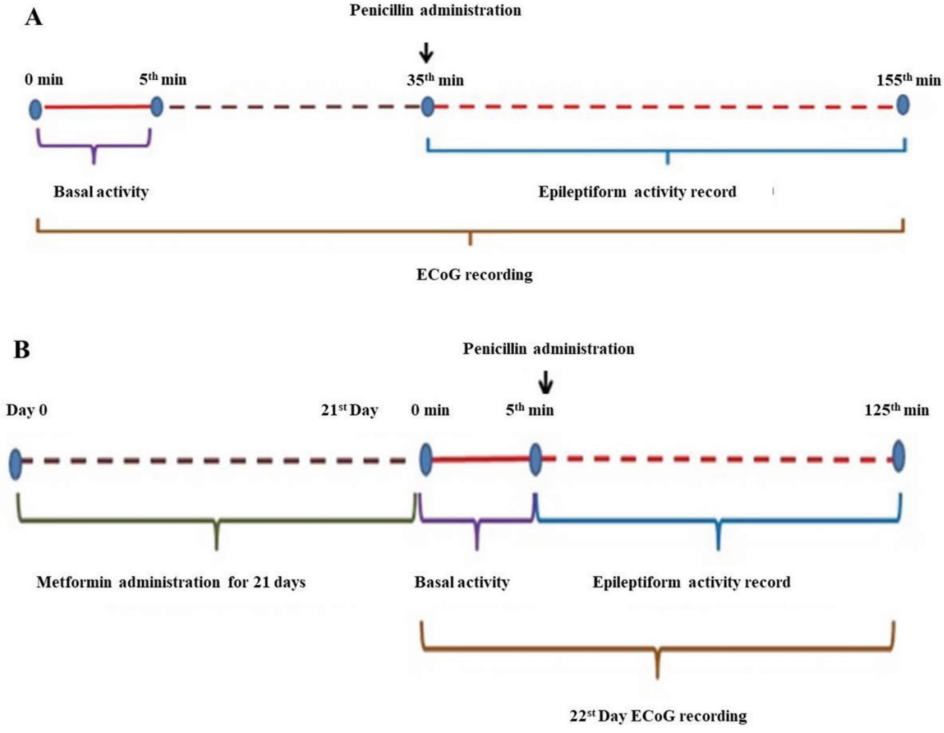

Figure 1. A) Experiment procedure and ECoG recording periods applied to acute groups. B) Experiment procedure and ECoG recording times applied to chronic groups
ECoG: electrocorticography

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AMet_100 and AMet_200 groups (p=0.004 and 0.005, respectively). In the 9th period, the spike wave frequency in the CMet_200 group was statistically and significantly higher than the AMet_100 group (p=0.050). When the spike wave frequency in CMet_200 group was compared with the other groups starting from the 10th period (55-60 minutes), there was no significant difference until the end of the 24th Period (115-120 minutes) (p>0.05) (Figure 3). When the spike wave frequency in the AMet_100 group was compared with the penicillin group up to the 10th period (except the 5th period), the spike wave frequency in the penicillin group was found statistically higher (p<0.05). The spike wave frequency in the AMet_100 group was statistically lower than the CMet_100 group in the 6th and 7th periods and the CMet_200 group in the 8th and 9th periods (p<0.05). When AMet_100 and other groups were compared, there was no significant difference between AMet_100 and CMet_100, CMet_200, and AMet_200 groups in terms of the spike wave frequency starting from the 10th period (45-50 minutes) to the end of the 24th period (115-120 minutes), (p>0.05), (Figure 3).

When the spike wave frequency in the AMet_200 group was compared with the penicillin group until the 9th period (except for the 5th period), the spike wave frequency in the penicillin group was statistically higher (except for the 5th period) (p<0.05).

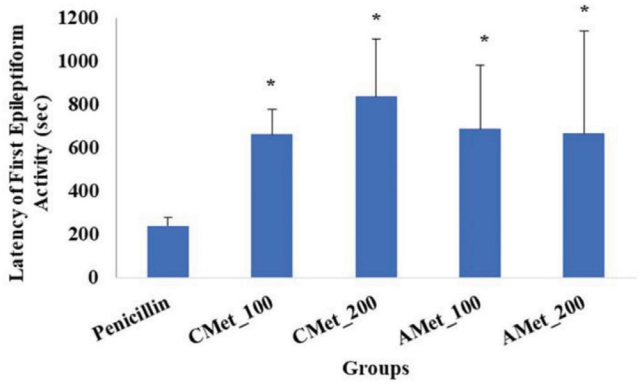

Figure 2. The effect of acute and chronic metformin administration on epileptiform activity onset time compared to penicillin group (mean values marked with * are statistically significant compared to penicillin group p<0.05)

Table 3. The effect of metformin on the latency of first epileptiform activity

Groups	n	Mean ± SD (second)	Min (ng/mL)	Max (ng/mL)	p
Penicillin	7	240±22	196.00	265.00	
AMet_100	7	688±111*	240.00	1114.00	
AMet_200	7	666±158*	0.00	1500.00	0.001
CMet_100	7	663±52*	544.00	793.00	
CMet_200	7	837±109*	494.00	1200.00	

The effect of acute and chronic metformin on the latency of first epileptiform activity compared to penicillin group (*Compared to penicillin group p<0.05). SD: standard deviation, min: minimum, max: maximum

The spike wave frequency of AMet_200 group was statistically and significantly lower than the spike wave frequency of CMet_100 group in the 6th and 7th periods, and the spike wave frequency of CMet_200 group in the 8th period (p<0.05). When the AMet_200 and other groups were compared, there was no significant difference between the AMet_200 and CMet_100 mg/kg, CMet_200, and AMet_100 groups starting from the 10th Period (45-50 minutes) to the end of the 24th Period (115-120 minutes) in terms of the spike wave frequency (p>0.05) (Figure 3, Table 4).

3.2.3. The Effect of Metformin on Spike Wave Amplitude

The effect of metformin on spike wave amplitude was compared with penicillin groups and between CMet_100, CMet_200, AMet_100, AMet_200 groups. Significance value was accepted as p<0.05. AOMet_200 group and COMet_200 group were not compared, since seizures did not occur.

In the 1st and 2nd periods (0-5 min and 6-10 min), the spike wave amplitudes in the penicillin group were significantly higher than the CMet_100, CMet_200, AMet_100, AMet_200 groups (p=0.000; p=0.000, respectively. p=0.000; p=0.000). In the 3rd and 7th periods, the spike wave amplitudes in the penicillin group were significantly higher than the CMet_200, AMet_100, AMet_200 groups (p<0.05). Between the 5th and 7th periods, the spike wave amplitudes in the CMet_100 group were significantly higher than the CMet_200, AMet_100 and AMet_200 groups (p<0.05). In the 8th period, the spike wave amplitude in the penicillin group was significantly higher than the amplitude of the AMet_100 group (p=0.026). The spike wave amplitude in CMet_100 group was significantly higher than in AMet_100 group (p=0.004). Spike wave amplitude in AMet_100 group was significantly higher than CMet_100 group (p=0.006). In the 9th period, the spike wave amplitude in the penicillin group was higher than the spike wave amplitude in the AMet_100 group (p=0.017). Spike wave amplitude in CMet_100 group was higher in AMet_100 group (p=0.025). The spike wave amplitude in the penicillin group was higher than the spike wave amplitude in the AMet_100 group (p=0.042). The spike wave amplitude in the CMet_100 group was higher than the AMet_100 group (p=0.005). In the 11th period, the spike wave amplitude in CMet_100 group was higher than the spike wave amplitude in AMet_100 group (p=0.025). In the 12th period, the spike wave amplitude in the CMet_100 group was higher than the AMet_100 group and the AMet_200 group (p=0.008; 0.048). In the 13th period, the spike wave amplitude in the CMet_100 group was higher than the AMet_100 group (p=0.008). In the 15th period, the spike wave amplitude in CMet_100 group was higher than AMet_100 group and AMet_200 group (p=0.042 and p=0.047). Between the 16th and 23rd periods, the spike wave amplitude in the CMet_100 group was higher than the AMet_100 group (p<0.05). In the 24th period, the spike wave amplitude in the CMet_100 group was higher than the AMet_100 group and CMet_200 group (p=0.027) (Figure 4, Table 5).

3.3. Determination of Blood Serum Antioxidant Activity of Metformin

3.3.1. Effect of Metformin Administration on CAT Level

Serum CAT value in COMet_200 group was statistically and significantly higher than penicillin, AMet_100, AMet_200, AOMet_200, CMet_100, CMet_200 groups (p=0.000; p=0.007; p=0.008; p=0.011; p=0.000; and p=0.000, respectively) (Figure 5, Table 6).

3.3.2. Effect of Metformin Administration on GPx Level

Serum GPx value in AOMet_200 group was statistically and significantly higher than CMet_100, CMet_200, COMet_200 and penicillin groups (p=0.049; p=0.007; p=0.005; and p=0.049, respectively) (Figure 6, Table 7).

3.3.3. Effect of Metformin Administration on SOD Dismutase Level

Serum SOD level in AMet_200 group was statistically and significantly higher than the CMet_200 and COMet_200 groups (p=0.009 and p=0.007, respectively). Although serum SOD level in AMet_200 group was higher than penicillin group, it was not statistically significant (p>0.05), (Figure 7, Table 8)

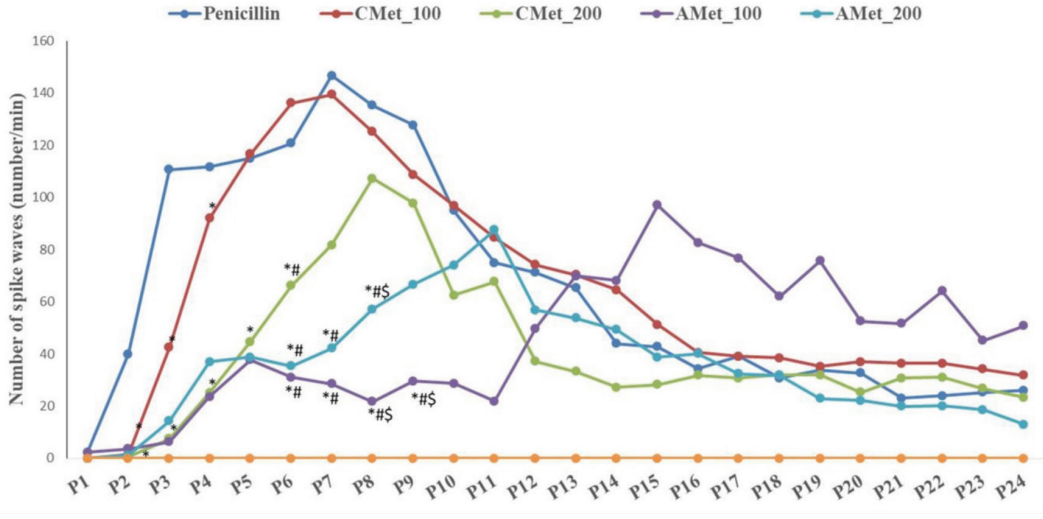

Figure 3. Number of spike waves in penicillin group and acute and chronic groups [mean values marked with * are statistically significant compared to penicillin group (p<0.05); mean values marked with # are statistically significant compared to CMet_100 group (p<0.05); and mean values marked with $ are statistically significant compared to the CMet_200 group (p<0.05)]

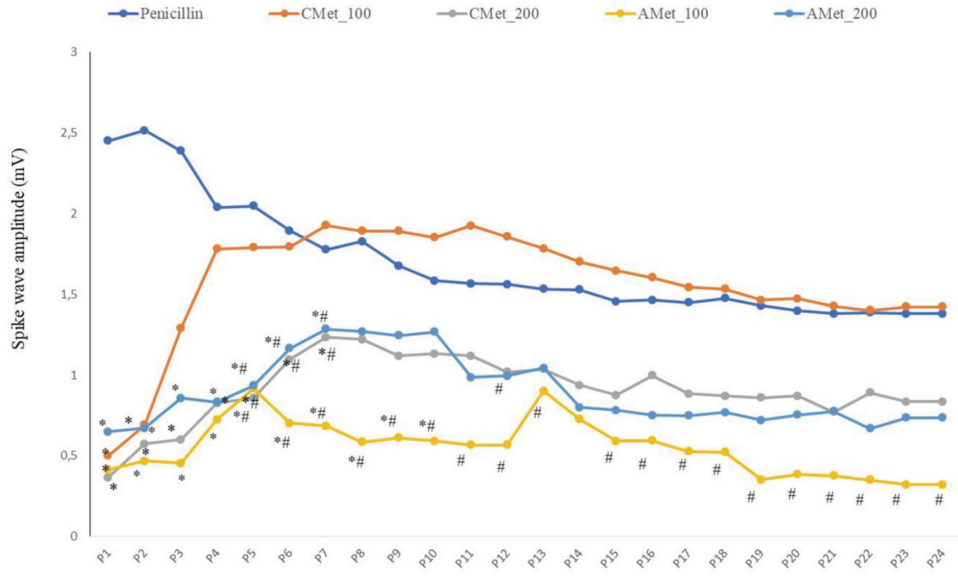

Figure 4. Spike wave amplitude [mean values marked with * are statistically significant compared to penicillin group (p<0.05); mean values marked with # are statistically significant compared to CMet_100 group (p<0.05)]

Table 4. The effects of penicillin, AMet_100 mg/kg, AMet_200 mg/kg, CMet_100 mg/kg, CMet_200 doses of metformin on frequency of penicillin-induced epileptiform activity

Time	Penicillin			AMet_100			AMet_200			CMet_100			CMet_200			p
	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	
0-5	2.33±1.4529	0.00	5.00	2.28±2.28	0.00	16.00	0±0	0.00	0.00	0±0	0.00	0.00	0±0	0.00	0.00	0.555
6-10	40.00±25.00	15.00	90.00	3.5714±1.82*	0.00	12.00	1.111±0.80*	0.00	7.00	1.40±0.87*	0.00	4.00	0.66±.66*	0.00	4.00	0.001
11-15	250.66±126.1	21.00	456.00	6.28±4.37*	0.00	32.00	14.33±9.27*	0.00	78.00	42.40±15.29*	3.00	76.00	7.50±6.716*	0.00	41.00	0.001
16-20	111.66±105.72	0.00	323.00	23.6±9.1*	2.00	69.00	37±16.2*	0.00	118.00	92±33.0	8.00	161.00	25.2±14.0*	0.00	75.00	0.026
21-25	84.00±55.00	28.00	194.00	37.8±17.0	0.00	113.00	39±20.0	0.00	140.00	117±38	19.00	202.00	45.0±21.4	0.00	120.00	0.068
26-30	121.0±36.0	58.00	182.00	31.1±16.4*#	2.00	126.00	35.4±21.2*#	0.00	169.00	136.2±36.8	33.00	218.00	66.2±28.1*#	0.00	145.00	0.007
31-35	146.7±42.0	78.00	223.00	28.6±17.5*#	1.00	131.00	42.2±22.0*#	0.00	185.00	139.4±25.8	49.00	200.00	81.7±22.1	10.00	150.00	0.001
36-40	135.3±46.4	84.00	228.00	21.7±17.0*#$	1.00	124.00	57.2±19.5*#$	0.00	175.00	125.2±26.1	28.00	185.00	107.2±17.0	51.00	168.00	0.001
41-45	127. 7±63.2	60.00	254.00	29.6±18.2*#	1.00	137.00	66.7±25.8*#	0.00	233.00	108.8±33.5	3.00	189.00	97.8±16.4	48.00	166.00	0.026
46-50	95±36.9	40.00	165.00	28.7±18.9	2.00	141.00	74±26.5	0.00	230.00	96.8±33.7	2.00	174.00	62.5±18.1	21.00	142.00	0.092
51-55	75±26.0	23.00	103.00	21.9±6.5	1.00	40.00	87.6±36.4	0.00	292.00	84.6±30.3	1.00	153.00	67.7±32.4	3.00	208.00	0.216
56-60	71.3±26.7	19.00	107.00	49.7±29.4	5.00	211.00	56.9±25.1	.00	206.00	74.2±30.5	2.00	160.00	37.2±15.4	2.00	99.00	0.445
61-65	65.3±24.2	18.00	98.00	69.8±49.3	2.00	360.00	53.7±24.24.9	.00	213.00	70.4±29.9	2.00	155.00	33.3±15.6	0.00	82.00	0.656
66-70	44±23.1	10.00	88.00	68.1±48.1	6.00	353.00	49.3±22.7	0.00	196.00	64.6±28.5	0.00	156.00	27.2±13.8	0.00	70.00	0.671
71-75	42.7±22.7	18.00	88.00	97±74.7	2.00	543.00	38.7±22.1	0.00	194.00	51.2±27.4	0.00	153.00	8.3±15.01	0.00	87.00	0.731
76-80	34.3±20.4	12.00	75.00	82.7±69.8	2.00	500.00	40.1±21.7	.00	193.00	40.6±27.3	.00	145.00	31.8±17.05	0.00	98.00	0.831
81-85	39±24	15.00	87.00	76.7±65.8	2.00	471.00	32.4±20.3	.00	186.00	39±25	0.00	132.00	30.8±17.2	0.00	106.00	0.844
86-90	30.7±23.7	7.00	78.00	62.1±56.0	0.00	398.00	31.8±17.5	0.00	159.00	38.4±27.1	0.00	139.00	32±18.0	0.00	113.00	0.887
91-95	33.7±25.7	6.00	85.00	75.7±55.0	0.00	386.00	22.9±15.0	0.00	138.00	35.2±25.2	0.00	129.00	32±18.3	0.00	111.00	0.705
96-100	32.7±23.4	4.00	79.00	52.6±42.3	0.00	300.00	22.1±16.9	0.00	155.00	37±26.7	0.00	137.00	25.3±13.3	0.00	69.00	0.844
101-105	23±19.6	1.00	62.00	51.7±40.3	.00	286.00	19.9±15.09	.00	138.00	36.4±25.2	0.00	128.00	30.8±16.4	0.00	93.00	0.816
106-110	24±22.5	0.00	69.00	64.1±42.4	0.00	271.00	20.1±16.1	0.00	147.00	36.4±24.9	0.00	126.00	31±17.0	0.00	96.00	0.682
111-115	25.3±20.9	1.00	67.00	45.1±34.4	0.00	243.00	18.6±15.4	.00	141.00	34.2±22.2	0.00	109.00	26.8±14.2	0.00	78.00	0.826
116-120	26±21.1	0.00	68.00	50.7±37.4	0.00	262.00	13±10.7	0.00	98.00	31.8±20.9	0.00	104.00	23.3 ±12.1	0.00	67.00	0.707

All values are number/minute. p<0.05 is considered statistically significant (*Compared to penicillin group, #Compared to CMet_100 mg/kg group, $Compared to CMet_200 mg/kg group)
SD: standard deviation, min: minimum, max: maximum

Table 5. The effects of penicillin, AMet_100 mg/kg, AMet_200 mg/kg, CMet_100 mg/kg, CMet_200 doses of metformin on amplitude of penicillin-induced epileptiform activity

Time (minute)	Penicillin			AMet_100			AMet_200			CMet_100			CMet_200			p
	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	
0-5	2.4±0.7	1.58	3.76	0.4±0.1*	0.26	0.75	0.6±0.1*	0.15	1.45	0.5±0.1*	0.31	0.71	0.4±0.1*	0.07	0.68	0.000
6-10	2.5±0.6	1.86	3.69	0.5±0.1*	0.16	1.10	0.7±0.1*	0.16	1.33	0.7±0.1*	0.32	1.06	0.6±0.2*	0.06	1.29	0.000
11-15	2.4±0.6	1.37	3.58	0.5±0.1*	0.11	1.09	0.9±0.2*	0.13	1.98	1.3±0.3	0.33	1.75	0.6±0.1*	0.14	0.89	0.000
16-20	2.2±0.5	1.47	3.27	0.7±0.2*	0.09	1.65	1.2±0.3*	0.12	2.60	1.5±0.3	0.39	2.35	0.7±0.2*	0.17	1.19	0.008
21-25	2.0±0.5	1.34	3.08	0.7±0.2*#	0.11	1.80	0.8±0.2*#	0.13	2.03	1.8±0.4	0.53	2.86	0.8±0.2*#	0.13	1.49	0.004
26-30	2.0±0.5	1.58	2.96	0.9±0.2*#	0.37	1.82	0.9±0.2*#	0.20	2.19	1.8±0.4	0.48	3.25	0.9±0.2*#	0.16	1.53	0.008
31-35	1.9±0.3	1.46	2.64	0.7±0.2*#	0.16	2.12	1.2±0.2*	0.18	2.27	1.8±0.5	0.38	3.16	1.1±0.2	0.25	1.50	0.018
36-40	1.8±0.3	1.35	2.38	0.7±0.2*#	0.17	1.94	1.3±0.08	0.35	2.33	1.9±0.5	0.43	3.49	1.2±0.2	0.32	1.98	0.012
41-45	1.8±0.4	1.24	2.63	0.6±0.2*#	0.13	1.86	1.3±0.3	0.28	2.37	1.9±0.5	0.38	3.55	1.2±0.3	0.34	2.27	0.015
46-50	1.7±0.3	1.26	2.25	0.6±0.2*#	0.09	1.95	1.2±0.2	0.27	2.40	1.9±0.5	0.32	3.71	1.1±0.3	0.34	2.29	0.025
51-55	1.6±0.3	1.06	2.19	0.6±0.2#	0.09	1.91	1.3±0.2	0.27	2.42	1.9±0.6	0.33	4.06	1.1±0.3	0.45	2.43	0.044
56-60	1.6±0.3	1.04	2.20	0.6±0.2#	0.10	1.69	1.0±0.3#	0.16	2.32	1.9±0.7	0.37	4.60	1.1±0.3	0.54	2.37	0.008
61-65	1.6±0.3	1.04	2.13	0.6±0.2#	0.10	1.66	1.0±0.2	0.15	2.26	1.9±0.7	0.46	4.46	1.0±0.3	0.52	2.27	0.008
66-70	1.5±0.3	1.08	2.09	1.0±0.2	0.25	1.68	1.0±0.2	0.17	2.26	1.8±0.7	0.40	4.53	1.0±0.2	0.61	2.10	0.140
71-75	1.5±0.4	0.97	2.20	0.7±0.2#	0.31	1.52	0.8±0.3#	0.18	2.08	1.7±0.7	0.32	4.42	0.9±0.2	0.46	1.97	0.042
76-80	1.5±0.3	0.97	2.01	0.6±0.2#	0.27	1.46	0.8±0.2	0.18	1.99	1.6±0.7	0.32	4.48	0.9±0.2	0.49	1.88	0.026
81-85	1.5±0.4	0.87	2.11	0.6±0.1#	0.20	1.22	0.75±0.2	0.17	1.94	1.6±0.8	0.37	4.50	0.9±0.2	0.47	1.83	0.033
86-90	1.4±0.4	0.85	2.11	0.5±0.1#	0.17	1.14	0.7±0.2	0.20	1.93	1.5±0.7	0.30	4.44	1.0±0.2	0.46	1.64	0.029
91-95	1.5±0.4	0.84	2.13	0.5±0.1#	0.16	0.87	0.8±0.2	0.18	2.08	1.5±0.8	0.18	4.68	0.9±0.2	0.43	1.61	0.042
96-100	1.4±0.4	0.69	2.18	0.4±0.1#	0.17	0.64	0.7±0.2	0.19	2.03	1.5±0.8	0.19	4.53	0.9±0.2	0.44	1.52	0.020
101-105	1.4±0.4	0.70	2.10	0.4±0.1#	0.15	0.99	0.8±0.2	0.16	2.38	1.5±0.8	0.19	4.67	0.9±0.1	0.47	1.50	0.031
106-110	1.4±0.4	0.65	2.07	0.4±0.09#	0.15	0.88	0.8±0.2	0.16	2.28	1.4±0.8	0.22	4.51	0.8±0.2	0.42	1.46	0.031
111-115	1.4±0.4	0.62	2.13	0.3±0.1#	0.13	0.67	0.7±0.2	0.16	2.22	1.4±0.8	0.18	4.47	0.8±0.2	0.43	1.77	0.031
116-120	1.3±0.4	0.69	2.04	0.3±0.2#	0.10	0.75	0.7±0.2	0.17	2.38	1.5±0.8	0.24	4.55	0.8±0.2	0.43	1.95	0.027

All values are number/minute. $p<0.05$ is considered statistically significant. (*Compared to penicillin group, #Compared to CMet_100 mg/kg group)
SD: standard deviation, min: minimum, max: maximum

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4. DISCUSSION

Epilepsy is a neurological disease that is characterized by changes in molecular, cellular and neuronal activity, and affects approximately 1% of the world's population (11). Although the mechanisms of epilepsy are better understood recently, drug resistance and side effects that limit the pharmacological treatment of epilepsy prevent the disease from cure completely. This reflects the need to develop new treatment strategies to end the progression of epilepsy and minimize associated comorbidities (12).

In this study, penicillin was used to induce epileptiform activity. After the intracortical administration of 500 IU penicillin, spike-wave activity was observed within 3-5 minutes. This value was determined as 8-14 minutes in the acute metformin groups and 10-16 minutes in the chronic metformin groups. It was observed that this activity reached the maximum frequency and amplitude in about 35th minute in penicillin groups. Cortical pyramidal cells play an active role in the development of epileptiform

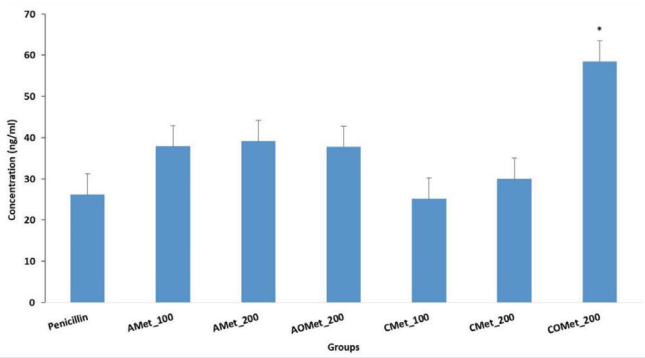

Figure 5. Showing the effects of acute and chronic metformin applied groups on serum catalase value in column graph (mean values marked with * are statistically significant from penicillin, AMet_100, AMet_200, AOMet_200, CMet_100, CMet_200 groups, p<0.05)

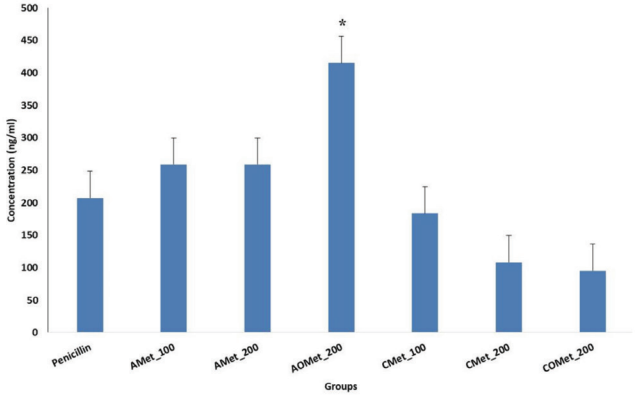

Figure 6. Showing the effect of acute and chronic metformin applied groups on serum GPx value (mean values marked with * are statistically and significantly higher than penicillin, CMet_100, CMet_200, COMet_200 groups, p<0.05)
GPx: glutathione peroxidase

Table 6. The effect of acute and chronic metformin applied on serum CAT level

Parameter	Groups	n	Mean ± SD (ng/mL)	Min (ng/mL)	Max (ng/mL)	p
CAT	Penicillin	7	26.2±3.8	14.00	37.00	0.001
	AMet_100	7	37.9±6.1	21.00	67.00	
	AMet_200	7	39.1±2.3	29.00	50.00	
	AOMet_200	7	37.8±2.0	32.00	43.00	
	CMet_100	7	25.1±4.1	15.00	45.00	
	CMet_200	7	30±4.1	17.00	49.00	
	COMet_200	7	58.5±10*	44.00	88.00	

p<0.05 is considered statistically significant, *compare to Penicillin, AMet_100, AMet_200, AOMet_200, CMet_100 and CMet_200 groups. SD: standard deviation, min: minimum, max: maximum, CAT: catalase

Table 7. The effect of acute and chronic metformin applied on serum GPx level

Parameter	Groups	n	Mean ± SD (ng/mL)	Min (ng/mL)	Max (ng/mL)	p
GPx	Penicillin	7	207±70.3	109.00	411.00	0.001
	AMet_100	7	258.7±46.5	83.00	409.00	
	AMet_200	7	258.6±55.6	4.00	441.00	
	AOMet_200	7	415±39.7*	336.00	461.00	
	CMet_100	7	183.3±130.3	39.00	443.00	
	CMet_200	7	108±34.3	13.00	165.00	
	COMet_200	7	94.8±35.1	14.00	178.00	

p<0.05 is considered statistically significant, *compare to Penicillin, CMet_100, CMet_200, COMet_200 groups. SD: standard deviation, min: minimum, max: maximum, GPx: glutathione peroxidase

activity induced by penicillin. Studies have shown that penicillin administration causes both focal and generalized seizures (13). The penicillin model is frequently used to answer questions about the mechanism of epilepsy (14).

Penicillin performs its activity through the GABAA receptor and blocks the inhibition of the GABAA receptor. There are many studies which claim that penicillin blocks the GABA receptor and induces epileptiform activity in rats. Arslan et al. (15) triggered epileptiform activity with penicillin (2.5 µl, 500 units, ic) in rats. Zhou et al. (16) also induced epileptiform activity with intraperitoneal injection of penicillin (10 million units/kg). In this study, we also obtained similar results which were compatible with previous studies and triggered epileptiform activity by administering penicillin. It was observed that only acute and chronic administration of metformin did not cause any epileptiform activity in rats. These data show that metformin does not have any epileptic effect in animals.

It has been shown in many experimental epilepsy studies that merformin, which is not used as an anti-epileptic drug, has anti-epileptic properties such as delaying the onset of seizures, decreasing the frequency and duration of seizures, as well as it causes behavioral and cognitive improvement, and it has antioxidant and neuroprotective effects (9,10,17,18).

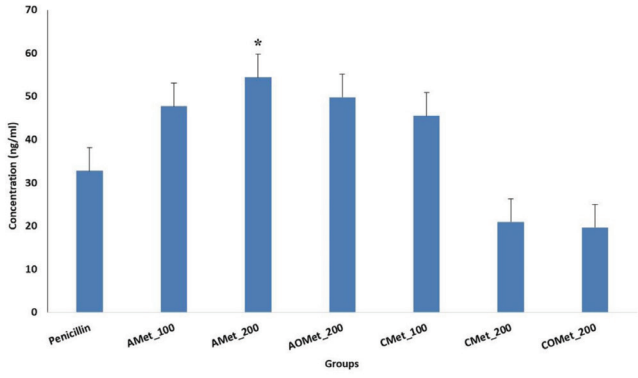

Figure 7. Showing the effect of acute and chronic metformin applied groups on serum SOD value in column chart (mean values marked with * are statistically and significantly higher than CMet_200, COMet_200 groups, p<0.05)
SOD: superoxide dismutase

Nevertheless, the mechanism underlying the anti-epileptic effect of metformin is not fully understood, but the antiepileptic effect of metformin is associated with its potential to improve brain oxidative damage, activate AMPK, inhibit mTOR and reduce apoptotic neuronal cell death, α'-synuclein, BDNF, and TrkB (19).

The AMPK activation may attenuate the generation of seizures by delaying the onset of epilepsy, reducing neuronal loss in the hippocampus, and preventing cognitive impairments in both acute and chronic epilepsy models. In the case of metformin, its antiepileptic effects could be attributed to an amelioration of oxidative brain damage, activation of the AMPK pathway, inhibition of the mTOR signaling, downregulation of BDNF, and TrkB levels or improvement of proteostasis (6,19).

Metformin has beneficial effects on several neurological disorders that could be attributed to both AMPK-dependent and AMPK-independent mechanisms of action. As a whole, metformin improves mitochondrial function [thereby reducing reactive oxygen species (ROS) production and oxidative stress], reduces the inflammatory response, reduces glucose production, and improves proteostasis (enhances the degradation of toxic aggregates) (20).

In our study, epileptogenesis was not examined molecularly, but it was shown electrophysiologically that it delayed seizures. We think that the anti-epileptic effect of metformin is through the pathways listed above.

Kainic acid and pilocarpine, which are used to induce TLE, status epilepticus, and other types of epilepsy in animals, have been the most widely used agents to provide a better understanding of the epileptogenesis process (21). In a study, abnormal neurogenesis is significantly increased in KA-induced status epilepticus, and metformin pretreatment has been shown to reduce this abnormal neurogenesis. In the same study, they reported that metformin significantly reduced spontaneous seizure activity and the number of epileptic spikes one month after KA administration, and that metformin was an antidiabetic drug with the potential to be used as a safe and well-tolerated antiepileptic drug (22).

Both acute and chronic epilepsy models induced by PTZ, which is an another agent to induce epileptiform activity, are frequently used in the studies (18,23). Results from these studies showed that

Table 8. The effect of acute and chronic metformin applied on serum SOD level

Parameter	Groups	n	Mean ± SD (ng/mL)	Min (ng/mL)	Max (ng/mL)	p
SOD	Penicillin	7	32.7±10.9	9.00	75.00	0.040
	AMet_100	7	47.7±8.9	22.00	77.00	
	AMet_200	7	54.4±7.1*	33.00	97.00	
	AOMet_200	7	49.8±18.5	8.00	114.00	
	CMet_100	7	45.5±5.1	25.00	61.00	
	CMet_200	7	21.0±8.1	0.78	47.00	
	COMet_200	7	19.7±12.7	6.00	45.00	

p<0.05 is considered statistically significant, *compare to CMet_200, COMet_200 groups. SD: standard deviation, min: minimum, max: maximum, SOD: superoxide dismutase

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metformin had a significant antiepileptic potential in experimental epilepsy models with PTZ, kainic acid and pilocarpine, by delaying seizures, reducing the frequency and duration of seizures, and reducing oxidative stress (9,10,18,23,24).

Experimental epilepsy model induced by penicillin was used in the present study. It was noticed in previous studies that metformin was not used in the penicillin model.

In addition, in our study, unlike other literature, the time periods in the acute and chronic models were different, and epilepsy was evaluated electrophysiologically and with antioxidant enzyme activity. In some literature, it has been reported that epileptiform activity may be mediated by behavioral scores (racine scoring) and the potential to activate AMPK and inhibit mTOR at the molecular level, reduced apoptotic neuronal cell death, downregulation of α'-synuclein, BDNF, and TrkB (19,25).

It was shown electrophysiologically in the present study that metformin in different doses (CMet_100, CMet_200, AMet_100, AMet_200) in both acute and chronic groups delayed the onset of seizures and decreased the frequency and amplitude of spike waves. The spike wave frequency in the CMet_200 group was significantly less than in the penicillin group until the end of the 6th period. The spike wave frequency in the AMet_100 group was significantly less than in the penicillin group up to the 10th period. The spike wave frequency in the AMet_200 group was significantly less than in the penicillin group until the 9th period. Epileptiform activity was significantly less in the CMet_200 and AMet_200 dose groups than the penicillin group, up to the 8th period, with spike wave amplitude. Spike wave amplitude in AMet_100 group wass significantly less than penicillin group until the end of the 10th period.

It is known that the risk of epilepsy is higher in diabetic patients (26). Therefore, metformin may be a potential pharmacological agent, especially for patients with diabetes and epilepsy. Seizures cause cognitive impairment in patients with epilepsy in the long term. In experimental epilepsy studies, metformin therapy improved epilepsy-related behavioral disorders (19). All these data demonstrate the potential role of metformin in preventing symptoms associated with epilepsy. Metformin is known to prevent cellular changes that cause epilepsy, such as neuronal cell loss, gliosis, and apoptosis (27,28). Metformin is also thought to inhibit oxidative stress, which plays a major role in the initiation and progression of epilepsy (29).

The role of oxidative stress in the initiation and progression of epileptic seizures has been widely recognized. Oxidative damage caused by neuronal hyper-excitability and excessive free radical production trigger the onset and progression of epilepsy (30). Metformin is known to exhibit antioxidant activity by reducing free radicals, including lipid peroxidation and enhanced glycation end products (18,23). Substantial evidence shows that metformin exerts antioxidant effects. Some of these can be attributed to the inhibition of mitochondrial complex I, which reduces production of ROS by the oxidative phosphorylation system respiratory chain (31). In addition, metformin has other functions related to the activation of the AMPK pathway: (1) reduction of ROS levels by upregulating the expression of antioxidant enzymes, such as thioredoxin, through the AMPK-FOXO3 pathway; (2) modulation of the expression of sirtuin 3 deacetylase, of which activity promotes antioxidant effects in the cell; (3) downregulation of NADPH oxidase, one of the main producers of cellular ROS; and (4) enhancement of mitochondrial biogenesis by enhancing the function of PGC1 alpha transcription factor (32). In addition, it has an antioxidant effect by increasing the levels of SOD, CAT and (GSH) glutathione, which are antioxidant enzymes (18,23). It is estimated that the anti-epileptic effect of metformin is partially due to its antioxidant properties (18). In a study, postnatal metformin administration to rats exposed to valproic acid in the uterus increased CAT activity by 1.87-fold in prefrontal cortex (PFC), by 1.55-fold SOD activity in the hippocampus, by 2.08-fold GSH in the hippocampus, by 1.68-fold in the hippocampus, and by 2.63-fold in PFC (33). Another study demonstrated that metformin inhibited apoptosis by decreasing caspase 3 and 9 expression in epileptic mice (34).

In the present study, blood serum samples were studied to determine antioxidant activity. Serum CAT value in COMet_200 group was statistically and significantly higher than penicillin, AMet_100, AMet_200, AOMet_200, CMet_100, CMet_200 groups. Serum GPx value in the AOMet_200 group was statistically and significantly higher than the CMet_100, CMet_200, COMet_200 and penicillin groups. Serum SOD level in the AMet_200 group was statistically and significantly higher than CMet_200 and COMet_200 group. Although serum SOD level in the AMet_200 group was higher than the penicillin group, it was not statistically significant. When we evaluate our blood serum antioxidant enzyme values and tissue antioxidant enzyme levels shown in the literature, we can say that metformin increases the antioxidant enzyme activities and partially delays the development of epilepsy.

4.1. Study Limitations

Molecular parameters were not evaluated due to budget constraints.

5. CONCLUSION

In our study, it was concluded that metformin prolonged the onset of seizure latency, decreased spike wave frequency and amplitude, and increased the level of antioxidant enzymes in the epilepsy model induced by penicillin. The good safety profile of metformin suggests that the drug can be used in the treatment of epilepsy, alleviating epileptic seizures, decreasing the level of free radicals, and strengthening the antioxidant system. However, more experimental, preclinical, and clinical studies are needed to determine the long-term efficacy and safety of metformin in epilepsy.

Ethics Committee Approval: The ethical approval was obtained from Düzce University Animal Experiments Local Ethics Committee with the decision number 2019/6/1 (date: 02.07.2019), and the animals to be used

in the experiment were obtained from Düzce University Experimental Animals Application and Research Center.

Informed Consent: Animal experiment study.

Peer-review: Externally and internally peer-reviewed.

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Evaluation and Long-term Monitoring of Patients with MODY, and Description of Novel Mutations

MODY Olgularının Değerlendirilmesi ve Uzun Dönem İzlem Sonuçları, Yeni Mutasyonların Tanımlanması

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ABSTRACT

Objective: Maturity-onset diabetes of the youth (MODY) is a genetically and clinically heterogeneous group of diseases which is often misdiagnosed as type 1 diabetes or type 2 diabetes. The aim of this study is to identify the occurence of mutations in subjects classified clinically as having MODY, and to determine phenotypic features and their long-term monitering consequences.

Methods: Eighteen probands were selected based on the clinical criteria of MODY. Firstly, in patients with mild stable fasting hyperglycemia who did not progress, Sanger sequencing of GCK gene was performed as GCK-MODY was the most common cause of persistent and incidental hyperglycemia in the pediatric population. Patients without a GCK gene mutation or without mild fasting hyperglycemia were analysed by using targeted next-generation sequence for seven known monogenic genes of diabetes (ABCC8, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11) to identify the molecular pathology.

Results: We identified 11 GCK, 2 HNF1A, 2 KCNJ11 mutations in 18 probands. Eleven of them (73%) were previously reported and 4 of them (27%) were assessed as novel mutations. In two patients who were treated with insulin before the molecular analysis, insulin was switched to sulfonylurea and glibenclamide, after determination of pathogenic variants in HNF1A and KCNJ11, respectively. Retinopathy or nephropathy was not detected among the patients.

Conclusion: The MODY has a large spectrum of clinical presentations. We detected 4 novel mutations among our cohort. Although GCK-MODY was the most frequent type of our study population, identification of rare MODY types and follow-up of these patients would help us better understand monogenic diabetes.

Keywords: Diabetes, MODY, next-generation sequencing

ÖZ

Amaç: Gençlerin erişkin tipi diyabeti (Maturity onset diabetes of young - MODY), genellikle tip 1 diyabet veya tip 2 diyabet olarak yanlış teşhis edilebilen, genetik ve klinik olarak heterojen bir hastalık grubudur. Bu çalışmanın amacı, klinik olarak MODY olarak sınıflandırılan olgularda mutasyonlarını, fenotipik özelliklerini tespit etmek ve bunların uzun vadeli izlem sonuçlarını göstermektir.

Yöntemler: MODY klinik kriterlerine göre 18 olgu seçildi. İlk olarak, pediatrik popülasyonda kalıcı, tesadüfi hipergliseminin en yaygın nedeni GCKMODY olduğundan, ilerleyici olmayan, hafif stabil açlık hiperglisemisi olan hastalarda, GCK geninin Sanger dizilemesi yapıldı. GCK gen mutasyonu olmayan veya hafif açlık hiperglisemisi olmayan hastalar, moleküler patolojiyi belirlemek için bilinen yedi monogenik diyabet geni (ABCC8, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11) için hedeflenen yeni nesil diziyle analiz edildi.

Bulgular: On sekiz probandda 11 GCK, 2 HNF1A, 2 KNCJ11 mutasyonu belirledik. Bunların 11'i (%73) daha önce tespit edilmiş ve 4'ü (%27) yeni mutasyonlar olarak değerlendirilmiştir. Moleküler analizden önce insülin ile tedavi edilen iki hastada, sırasıyla HNF1A ve KCNJ11'de patojenik varyantların belirlenmesinden sonra tedaviler sülfonilüre ve glibenklamid olarak değiştirildi. Hastalar arasında retinopati veya nefropati saptanmadı.

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Sonuç: MODY, geniş bir klinik sunum yelpazesine sahiptir. Kohortumuzda 4 yeni mutasyon tespit ettik. GCK-MODY çalışma popülasyonumuzda en sık görülen tip olmasına rağmen, nadir MODY tiplerinin belirlenmesi ve bu hastaların takibi monogenik diyabeti daha iyi anlamamıza yardımcı olacaktır.

Anahtar kelimeler: Diyabet, MODY, yeni nesil dizileme

INTRODUCTION

Maturity onset diabetes of the youth (MODY), which is estimated to account for 1-2% of all patients with diabetes, is a monogenic form of diabetes that is inherited in an autosomal dominant manner. It encompasses a genetically and clinically heterogeneous group of diseases affecting pancreatic β-cells and resulting in impaired insulin secretion (1,2). At least 14 MODY subtypes with distinct genetic etiologies have been identified to date (GCK, HNF1A, HNF4A, HNF1B, PDX1, NEUROD1, KLF11, CEL, PAX4, INS, BLK, KCNJ11, ABCC8, APPL1) (3).

The MODY may be misdiagnosed as type 1 diabetes (T1DM) or type 2 diabetes (T2DM) and there can be a significant overlap in clinical features (4). The importance of diagnosing MODY includes the application of optimal treatment. It will be possible to switch to sulfonylurea therapy in patients with some MODY types such as GCK-MODY previously misdiagnosed as T1DM and in whom insulin therapy has been started. The diagnosis of MODY can also positively affect glycemic control, prognosis and quality of life. Moreover, it would be possible to determine the family members under risk because the disease is inherited in an autosomal dominant manner (5). Targeted next-generation sequencing (tNGS) is reported to be utilized in diagnosis of monogenic diabetes with up to 100% sensitivity (6).

The aim of this study is to identify the occurence of both reported and novel mutations in subjects classified clinically as having MODY by using tNGS, and to determine phenotypic variability, clinical and metabolic features and their long-term monitering consequences.

METHODS

Patients who fulfilled at least two criteria mentioned below were included in the study: 1-a family history of diabetes in one parent, 2-negative antibodies for glutamic acid decarboxylase or islet cells, 3-low (<0.5 IU/kg/day) insulin requirements 3 years after the diagnosis and with C-peptide level ≥0.6 ng/mL, 4-persistent but nonprogressive fasting hyperglycemia, and 5-clinical presentation resembling T2DM with normal fasting C-peptide levels but without obesity, acanthosis nigricans, or insulin resistance. Eighteen children with a clinical diagnosis of MODY aged between 1 and 18 years, who were followed up at three different pediatric endocrinology clinics between January 2015 and December 2019 were included. Sanger sequencing of GCK was performed in 16 patients with a fasting glucose level between 99 and 145 mg/dL, and with HbA1c level ≤7.5%. Other patients who did not have mild fasting hyperglicemia or without GCK mutation were analysed by using tNGS for seven known monogenic diabetes genes. The genetic testing stratergy is illustrated in Figure 1.

Data about presenting complaints, age at diagnosis, duration of diabetes, medical history of the proband and family, diagnosis if any before MODY diagnosis, treatment plans, duration of follow-up, baseline anthropometric measurements, and physical examination findings were obtained from the hospital records. Laboratory results including fasting blood glucose (FBG), urine glucose-ketone, venous blood gases, serum insulin, C-peptide and/or insulin, HbA1c levels in admission and diabetes autoantibodies were recorded. Standard deviation (SD) scores of weight, height and body mass index (BMI) of patients were calculated by using reference values for Turkish children (7). According to age and

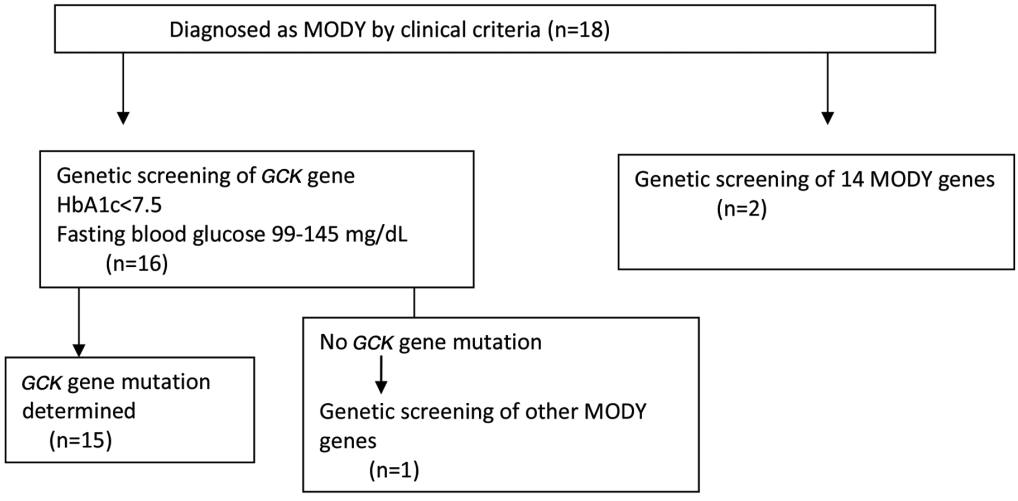

Figure 1. Genetic testing algorithm

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gender, patients with BMI between the 85th and 95th percentiles was considered as overweight, and patients with BMI ≥95th percentile was considered as obese. Diabetic ketoacidosis was biochemically defined as venous pH <7.3 or serum bicarbonate concentration <15 mmol/L, and serum glucose concentration >200 mg/dL together with ketonemia, glucosuria, and ketonuria (8). All patients were evaluated by the ophthalmologist with direct ophthalmoscopy for diabetic retinopathy 2 years after diagnosis. Urinary albumin/creatinine ratio (ACR) was analyzed. According to the 2018 International Society for Pediatric and Adolescent Diabetes guidelines, patients with ACR value above 30 mg/g (spot urine) were evaluated for diabetic nephropathy (9).

Genetic Analysis

After obtaining written informed consent, blood samples were obtained from the patients and available parents. Automatic DNA isolation was performed in accordance with the standard protocols of the QIAAmp DNA Mini (Qiagen) kit from EDTA-anticoagulated peripheral blood samples.

Sanger sequencing: Sanger sequencing of all coding exons of GCK was performed using BigDye terminator chemistry 3.1 on the 3130 Genetic Analyzer (Applied Biosystems). Primer sequences and PCR conditions are available on request.

Targeted NGS: Within the scope of the test, the sequencing was done on Miseq (Illumina) Next Generation Sequencing platform using SOPHIA Clinical Exome Solution using Illumina V2 chemicals. The targeted gene panel consisted of 7 (*ABCC8, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11*) genes associated with MODY. Sequence analysis covered coding regions of each gene, including all coding exons, +/- 10 base pairs of adjacent intronic sequences and each nucleotide was read at a depth of at least 50X. Any variants that fall outside these regions and exonic variants with a minor allele frequency of less than 10% were considered as false positives and not analyzed. With this analysis, copy number variations were not examined. The DNA sequences were aligned to the NCBI Build36 (hg18) version of the human genome. Variant calling and data analysis were performed by Sophia-DDM-V5.2 bioinformatics analysis program.

Interpretation of Mutations

The interpretation of the variants were performed according to the 2015 ACMG standards and guidelines (10). Since there were not enough genome and exome databases for the Turkish population, 1000 genome projects, dbSNP, ExAC, GnomAD data were used as control population. The effects of the variants on protein function were investigated by using prediction programs such as SIFT, Polyphen, and MutationTaster. Human Gene Mutation Database (HGMD) and ClinVar databases were used to investigate mutations which were previously associated with MODY.

Statistical Analysis

Statistical analyses in our study were performed using the SPSS (Version 22.0, SPSS Inc., Chicago, IL, USA) package program. The normality distribution of the retrospective data was evaluated with the Kolmogorov-Smirnov test. Descriptive statistics for continuous variables were presented as mean ± SD in normally distributed data, median (minimum-maximum) in non-normally distributed data, and categorical data as numbers and percentages (%).

The study was approved by Taksim Training and Research Hospital Clinical Research Ethics Committee (decision no: 83, date: 12.06.2019) and was performed in accordance with the Helsinki Declaration.

RESULTS

Clinical Features of the Patients

A total of 18 patients, 12 females (66.6%), 6 males (33.3%), diagnosed as having MODY by using clinical criteria were included in the study. The mean age of the patients at diagnosis was 13.9±5.4 years (range 5-17). The mean BMI was 18.2±1.9 kg/m^2 (range 15.5-26.5), and all patients had normal weight, except for patient 8 (GCK-MODY) who was overweight (26.5 kg/m^2).

The presenting complaints of two patients (11%) were polydipsia, polyuria, whereas the remaining 16 (89%) patients had incidental hyperglycemia. Mean duration of follow-up was 40.1±10.6 months (range 9-156 months). None of the patients were diagnosed as having ketoacidosis at the initial evaluation or during the follow-up. Mean FBG, HbA1c, C-peptide and insulin levels at presentation were 138.7±15.9 mg/dL, 6.7±0.4%, 1.5±0.8 ng/mL, and 7.3±1.2 IU/mL, respectively. Glucosuria was detected in P17 (HNF1A-MODY) and P18 (KCNJ11-MODY). Treatment consisting of medical nutrition therapy alone was given to 15 (83.3%) patients, whereas insulin was used in 1 (5.5%) patient, sulfonylurea in 2 (11.1%) patients. None of the patients had retinopathy or nephropathy during follow-up. Clinical characteristics of the patients are summarized in Table 1.

Moleculer Genetic Test Results

We identified 15 patients with GCK variants (5 pathogenic and 10 likely pathogenic), 2 patients with HNF1A variants (1 pathogenic, 1 variant), and 1 patient with 2 differerent KCNJ11 mutations (variant/pathogenic). Two patients who had GCK and HNF1A mutations also had novel ABCC8 mutations classified as variant of uncertain significiance (VUS) (Table 2). The frequency of GCK-MODY, HNF1A-MODY and KCNJ11-MODY was 83.3% (15/18), 11.1% (2/18) and 5.5% (1/18), respectively in our cohort. All variants were heterozygous. There were 4 novel GCK gene mutations (c.74T>C, c.106_108delAGAinsTGG, c.534delG, c.583G>A). Genetic characteristics are summarized in Table 2. Segregation analysis could be performed only in P15 and P17.

Characteristics of Patients with GCK Mutation

In patients with a GCK mutation (10 females and 5 males), mean age was 11±4.4 years (range 4.8-17 years) and mean duration of follow-up was 3.8 years (1-13 years). Mean FBG, HbA1c, C-peptide and insulin levels were 120.3±13 mg/dL, 6.5±0.2%, 1.4±0.7 ng/mL, and 7.5±1.2 IU/mL, respectively. Patients 12 and 13 were siblings.

Table 1. Clinical characteristics of the patients

Patient no.	Age at diagnosis, year	Symptoms at diagnosis	Parents with diabetes	Affected relatives	Fasting blood glucose (mg/dL)	HbA1c at admission (%)	Urine glucose	Previous diagnosis	Treatment
P1	12.6	Coincidental		Positive	123	6.6	Negative		Medical nutrition therapy
P2	10	Coincidental	Mother	Positive	116	6.7	Negative		Medical nutrition therapy
P3	4.8	Coincidental		Positive	114	6.4	Negative		Medical nutrition therapy
P4	6	Coincidental	Father	Positive	115	6.2	Negative		Medical nutrition therapy
P5	15	Coincidental	Mother	Positive	117	6.3	Negative		Medical nutrition therapy
P6	5.1	Coincidental	Father	Positive	142	6.1	Negative		Medical nutrition therapy
P7	15	Polyuria, polidpsia	Father	Positive	120	6.1	Negative		Medical nutrition therapy
P8	17.2	Coincidental	Father	Positive	115	6	Negative		Medical nutrition therapy
P9	8.4	Coincidental	GD in mother	Positive	126	6.2	Negative		Insulin (0.1 U/kg/day)
P10	6.6	Coincidental	GD in mother	Positive	116	6	Negative		Medical nutrition therapy
P11	17	Coincidental		Positive	132	6.9	Negative		Medical nutrition therapy
P12	8.4	Coincidental	GD in mother	Positive	122	6.1	Negative	T2DM	Medical nutrition therapy
P13	7.1	Coincidental	GD in mother	Positive	118	6.1	Negative	T2DM	Medical nutrition therapy
P14	15.8	Polyuria, polidpsia		Positive	113	6.4	Negative		Medical nutrition therapy
P15	12.6	Coincidental	Mother	Positive	116	6.6	Negative	T1DM	Medical nutrition therapy
P16	11	Coincidental	GD in mother	Positive	107	6.2	Negative		Medical nutrition therapy
P17	17.3	Coincidental	Father	Positive	4635	11.7	Positive	T1DM	Sulfonylure
P18	4.5	Coincidental	GD in mother	Positive	178	9.7	Positive	T1DM	Glibenclamide

GD: Gestational diabetes, T1DM: type 1 diabetes mellitus, T2DM: type 2 diabetes mellitus

Table 2. Genetic characreristics of the patients

Patient no.	Gene	Status	c.DNA	Mutation type	Protein effect	Mutation taster	Polyphen	SIFT prediction/score	HGMD
P1	*GCK*	Novel	c.74T>C	Missense	p.Leu26Pro	Disease causing	Probably damaging	Damaging /0	NA
P2	*GCK*	Known	c.506A>G	Missense	p.Lys169Arg	Disease causing	Probably damaging	Damaging /0	CM141531 (DM)
P3	*GCK*	Known	c.676G>A	Missense	p.Val226Met	Disease causing	Probably damaging	Damaging /0	CM970636 (DM)
P4	*GCK*	Novel	c.106_108delAGAinsTGG	Indel	p.Arg36Trp	Disease causing	Probably damaging	Damaging	NA
P5	*GCK*	Known	c.506A>G	Missense	p.Lys170Arg	Disease causing	Probably damaging	Damaging /0	CM141531(DM)
P6	*GCK*	Novel	c.534delG	Frameshift	p.Asn180ThrfsTer25	Disease causing	Probably damaging	Damaging	NA
P7	*GCK*	Known	c.661G>A	Missense	p.Glu221Lys	Disease causing	Probably damaging	Tolerated/0.116	CM970635 (DM)
P8	*GCK*	Known	c.1178T>C	Missense	p Met394Thr	Disease causing	Possibly damaging	Damaging /0.002	CM096945 (DM)
P9	*GCK*	Known	c.1178T>C	Missense	p Met394Thr	Disease causing	Possibly damaging	Damaging /0.002	
P10	*GCK*	Known	c.676G>A	Missense	p.Val226Met	Disease causing	Probably damaging	Damaging /0	CM970636 (DM)
P11	*GCK*	Known	c.898G>A	Missense	p.Glu300Lys	Disease causing	Probably damaging	Damaging /0.001	CM930305 (DM)
P12	*GCK*	Novel	c.583G>A	Missense	Asp195Asn	Disease causing	Probably damaging	Damaging /0.025	NA
P13	*GCK*	Novel	c.583G>A	Missense	Asp195Asn	Disease causing	Probably damaging	Damaging /0.025	NA
P14	*GCK*	Known	c.898G>A	Missense	p.Glu300Lys	Disease causing	Probably damaging	Damaging /0.001	CM930305 (DM)
P15	*GCK/ ABCC8*	Known/ Novel	c.544G>A/ c.2888A>G	Missense/ Missense	p.Val182Met p.Asp963Gly	Disease causing/ Disease causing	Probably damaging/benign	Tolerated/ Tolerated	CM930298 (DM) /-
P16	*HNF1A*	Known	c.716C>T	Missense	p.Ala239Val	Disease causing	Probably damaging	Damaging	CM023588 (DM)
P17	*HNF1A/ ABCC8*	Known/ Novel	c.872dupC c.1996C>T	Frameshift /Synonym	p.Gly292Argfs*25 p.Arg657Trp	Disease causing/ Disease causing	Possibly damaging/ Benign	Damaging/ Tolerated	CI962354 (DM)/ NA
P18	*KCNJ11*	Known	c.1054G>C / c.965A>C	Missense/ Missense	p.Asp352His/ p.Glu322Ala	Disease causing/ Disease causing	Possibly damaging/ Probably damaging	Tolerated/ Damaging	CM1111083(DM)/ CM1111082 (DM)

Table 2. Continued

Patient no.	Transcript ID	Zygosty	Clinvar	ACMG variant interpretation criterias	Author comment	PUBMED ID
P1	NM_033507.3	Het.	NA	PM1, PM2, PM5, PP2, PP3	Likely pathogenic	NA
P2	NM_033508.3	Het.	NA	PM1, PM2, PP2, PP3	Likely pathogenic	24411943, 25015100
P3	NM_001354800.1	Het.	Pathogenic	PS1, PM1, PM2, PM5, PP2, PP3	Pathogenic	10525637, 25015100
P4	NM_033507.3	Het.	Pathogenic	PM1, PM2, PP2, PP3, PP5	Pathogenic	NA
P5	NM_033508.3	Het.	NA	PM1, PM2, PP2, PP3	Likely pathogenic	24411943, 25015100
P6	NM_033507.3	Het.	NA	PVS1, PM1, PM2, PP3	Pathogenic	NA
P7	NM_001354800.1	Het.	Pathogenic/ Likely pathogenic	PM1, PM2, PP2, PP3, PP5	Pathogenic	29056535, 28170077
P8	NM_033507.3	Het.	NA	PM1, PM2, PP2, PP3	Likely pathogenic	19790256
P9	NM_033507.3	Het.	NA	PM1, PM22, PP2, PP3	Likely pathogenic	NA
P10	NM_001354800.1	Het.	Pathogenic	PS1, PM1, PM2, PM5, PP2, PP3	Pathogenic	10525637, 25015100
P11	NM_001354800.1	Het.	NA	PM1, PM2, PM5, PP2, PP3, PP5	Likely pathogenic	9078243, 10525657
P12	NM_033507.3	Het.	NA	PM1, PM2, PP2, PP3	Likely pathogenic	NA
P13	NM_033507.3	Het.	NA	PM1, PM2, PP2, PP3	Likely pathogenic	NA
P14	NM_001354800.1	Het.	NA	PM1, PM2, PM5, PP2, PP3, PP5	Likely pathogenic	9078243, 10525657
P15	NM_001354800.1 / NM_000352.6	Het./ Het.	Pathogenic/NA	PM1, PM2, PM5, PP2, BP4/ PP2	Likely pathogenic/ VUS	10525657/ 3019164
P16	NM_000545.8	Het.	VUS	PP2,PP3, BS1	VUS	25525159, 2839597
P17	NM_000545.8/ NM_000352.6	Het./ Het.	Pathogenic/NA	PVS1, PM1, PP3, PP5/PM2, BP7	Pathogenic/ VUS	29417725, 30814848/ NA
P18	NM_000525.3/ NM_000525.3	Het./ Het.	NA/NA	PM2, PP2, PP3/PM2, PM5, PP2, PP3 PP5	VUS/ Pathogenic	22308870,291831/ 22308870, 29183106

NA: not available, Het: heterozygous, VUS: variant of uncertain sihnificiance, SIFT: scale-invariant feature transform, HGMD: Human Gene Mutation Database, ACMG: American College of Medical Genetics and Genomics, DM: disease causing mutation

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All six patients (P2, P3, P4, P10, P12, P13) had been diagnosed before puberty. While P7 and P14 had been diagnosed due to polyuria and polydipsia, others had been diagnosed due to incidental hyperglicemia. None of the patients had ketonemia, ketoacidosis at the time of diagnosis or during follow-up. Hyperglycemia was not detected in the parents of P1, P3, P11 and P14, however they had affected relatives. Mothers of two siblings (P12-P13), P9 and P10 had gestational diabetes. All of the patients were consuming low carbohydrate diet. P9 needed low dose of insulin (0.1 U/kg). Before the moleculer diagnosis of MODY, P15 was followed up for 9 months diagnosed as having T1DM, and P12 and P13 (two siblings) were diagnosed as having T2DM for 5 years. After the genetic diagnosis, insulin and antidiabetic drug were discontinued.

Eleven different heterozygous mutations were identified in 15 patients. P3 and P10, P8 and P9, P12 and 13, P11 and P14 had same variants. There were 9 (82%) missense mutations, 1 (9%) frameshift mutation and 1 (9%) indel variants. Seven of the variants had already been reported (c.506A>G, c.676G>A, c.506A>G, c.661G>A, c.1178T>C, c.898G>A, c.544G>A). The remaining four variants (c.74T>C, c.106_108delAGAinsTGG, c.534delG, c.583G>A) were not listed in HGMD, ClinVar or PUBMED and were classified as novel mutations. In P15, a heterozygous missense ABCC8(c.2888A>G) VUS variant was also identified along with the likely pathogenic variant in GCK. Mother of P15 was heterozygous for the variant in ABCC8 and the father was heterozygous for the variant in GCK.

Characteristics of Patients with HNF1A Mutation

The mean age at diagnosis was 12.2±3.6 years (8.6-17 years). All patients were diagnosed during adolescence. None of them was obese. The mean HbA1c level at diagnosis was 8.5±2.5% (6-11%). They had been diagnosed due to incidental hyperglycemia. One of patients had positive family history of diabetes and one of the mothers was diagnosed during pregnancy. None of the patients had ketosis at the time of diagnosis or during follow-up. Before the diagnosis of MODY, P17 was followed up for 1 year with a diagnosis of T1DM. After the genetic diagnosis, insulin therapy was switched to sulfonylurea treatment. P16 was monitored without any treatment. Two different mutations in *HNF1A* gene were identifed in 2 patients. In P16, a previously reported missense VUS variant (c.716C>T) was detected. P17 had a known a frameshift variant in HNF1A (c.872dupC) and a synonymous variant in ABCC8 (c.1996C>T) classified as pathogenic and VUS, respectively. Father of P17 was also heterozygous for *HNF1A* (c.872dupC) variant.

Characteristics of Patient with *KCNJ11* Gene

A KCNJ11 mutation was detected in a boy. Incidental hyperglycemia was detected at the age of 14. Before the diagnosis of MODY, he was followed up for 3 years with the diagnosis of T1DM. A two-generation positive family history was noted. The FBG, HbA1c, C-peptide and insulin levels were 178 mg/dL, 9.2%, 3.22 ng/mL, and 7.8 IU/mL, respectively at diagnosis. Urine glucose was positive. He did not have ketosis or ketoacidosis. Two known missense mutations were detected in the *KCNJ11* gene (c.1054G>C and c.965A>C) classified as VUS and pathogenic, respectively. After the genetic diagnosis, insulin treatment was switched to glibenclamide. He has been followed up for 4 years, so far, he has no retinopathy or nephropathy.

DISCUSSION

In this study, 18 patients were selected with the clinical criteria of MODY. We assessed mutation distribution, novel mutations, clinical characteristics, and prognosis. Today, tNGS is routinely performed in all newly diagnosed patients. Early diagnosis and correct diagnostic approach are essenstial for appropriate treatment and for patients who have a milder phenotype. GCK mutations were the most frequent in our cohort. We detected GCK mutations in 15 of the 18 patients with MODY (83.3%). The frequency of GCK-MODY varies according to different healthcare systems. In countries which perform routine blood glucose screening such as Italy, France, and Spain, GCK mutations are the most common cause of MODY. However, in the United Kingdom, where the studies mainly included symptomatic patients, HNF1A mutations were more common than GCK mutations (11). Similar to our study, variants were most frequently found in the *GCK* gene in several studies in Turkey (12-15). Heterozygous loss-of-function mutations in the *GCK* gene cause GCK-MODY. Mutations in GCK usually presents with mild hyperglycemia. Almost 15% of all patients with incidental hyperglycemia may be caused by GCK mutations (16). In our study, except 2 patients, all the other patients with a GCK mutation were diagnosed due to incidental hyperglycemia. In the remained 2 patients (P7 and P14), polyuria and polydipsia were the presenting symptoms. These osmotic symptoms are rarely observed in patients with GCK-MODY (17). Patients with GCK-MODY usually have a lower HbA1c level and good metabolic control. In support of this view, among patients with GCK-MODY in our cohort, HbA1c levels were between 6.1-6.9%. Normal levels of blood glucose were achieved with only medical nutrition therapy in 14 of 15 patients. The remained one patient needed low dose insulin (0.1 U/kg) therapy because of non-compliance to diet and HbA1c level rising up to 7.1%. We discontinued oral antidiabetic drugs in 2 siblings (P12 and P13) who were misdiagnosed as having T2DM before the molecular diagnosis. Therapy was continued with only medical nutrition therapy in such patients. The similar incidence of microvascular complications compared with controls without diabetes was reported in patients with GCK-MODY (18). Based on this, none of our patients with GCK-MODY in our cohort had any microvascular complications. In one patient (P15), a VUS variant in the *ABCC8* gene was detected in addition to the GCK variant. However, we did not consider that the ABCC8 variant to have a modifying effect, since this patient's clinical status was not different from patients with GCK mutations.

In HNF1A-MODY, response of insulin secretion to blood glucose levels is severely reduced. However, our patients (P16 and P17)

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with HNF1A mutations had been diagnosed with incidental hyperglicemia. This situation may be related to the pathogenicity of mutations. We detected 2 different HNF1A mutations in 2 patients. Variant in P16 was classified as VUS and this patient had mild hyperglycemia, and so far, did not need treatment. P17 was misdiagnosed as having T1DM and blood glucose levels were high even with the insulin treatment before the molecular diagnosis. In addition to the pathogenic variant in HNF1A, there was an additional synonym VUS variant in ABCC8 in this patient and we considered that this variant had no contribution to the phenotype.

Heterozygous mutations in KCNJ11 which encode the 2 subunits (Kir6.2 and SUR1, respectively) of the pancreatic β-cell ATP sensitive potassium (K ATP) channels have been shown to cause a wide spectrum of phenotypes, ranging from neonatal diabetes to MODY (19). Moreover, heterozygous KCNJ11 mutations were identified in a Chinese family with early onset T2DM (20). In our study, two KCNJ11 variants were detected only in P18. Among heterozygous KCNJ11 mutations, c.965A>C (p.Glu322Ala) variant was classified as pathogenic and c.1054G>C (p.Asp352His) variant was classified as VUS. In previous studies, c.965A>C (p.Glu322Ala) variant was identified in two patients with transient neonatal diabetes and it was reported that sulfanylurea was effective in the treatment of these patients (21,22). P18 was misdiagnosed as T1DM and treatment was switched to glibenclamide after the molecular diagnosis. Sulfanylurea was also effective in our patient. He was using glibenclamide for four years and HbA1c level decreased after the treatment change.

Study Limitations

There are some limitations in this study. Segregation analysis could not be performed in all parents of the patients. So, we could not detect if the mutation was *de novo* or not. We also could not perform functional anayses of the new mutations.

CONCLUSION

Since a wide spectrum of phenotypes of MODY has been shown, identification of new mutations and experiences of the clinics are very important. This study provides molecular-clinical features of MODY in three different centers.

Ethics Committee Approval: The study was approved by Taksim Training and Research Hospital Clinical Research Ethics Committee (decision no: 83, date: 12.06.2019) and was performed in accordance with the Helsinki Declaration.

Informed Consent: After obtaining written informed consent, blood samples were obtained from the patients and available parents.

Peer-review: Externally and internally peer-reviewed.

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