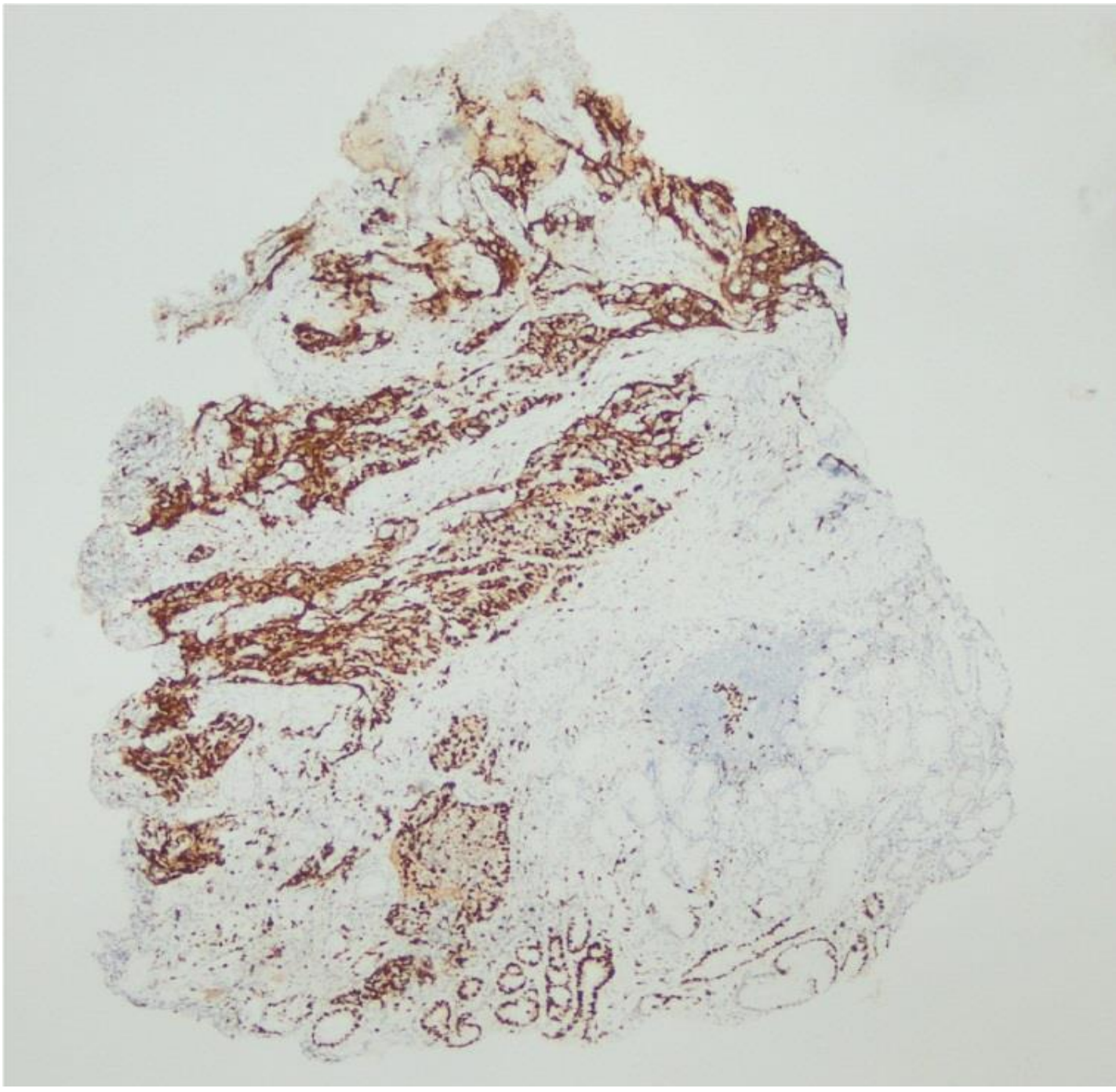
MSD April. 2021, Vol 8, No 4

MSD

Medical Science & Discovery



International Journal of Medical Science and Discovery
Open Access Scientific Journal
www.medscidiscovery.com,
Lycia Press London UK ISSN: 2148-6832

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ISSN: 2148-6832 (Print) E-ISSN: 2148-6832 (Online)

Category: Multi Disciplinary Health Science Journal

Abbreviated key title: Med. Sci. Discov.

Frequency: Monthly

Review System: Double Blind Peer Review

Circulation: Globally, Online, Printed

Article Processing Charge (APC): Free

Licensing: CC-BY-NC 4.0 International License Environmental

Editor-in-Chief: Assoc. Prof. Dr. Dr. Ahmad Rajabzadeh, Anatomical Department of lorestan, University of Medical Sciences, Tabriz, Iran

Established: 30.04.2014

Web address: www.medscidiscovery.com

E-mail : editor [at] medscidiscovery.com

Phone : +44 020 3289 9294

Design and preparation of PDFs, Language editing, Web site design, Graphical design Services of international Journal of Medical Science and Discovery has been contracted with Lycia Press LONDON, UK (as Publisher), by the MSD Board of Directors

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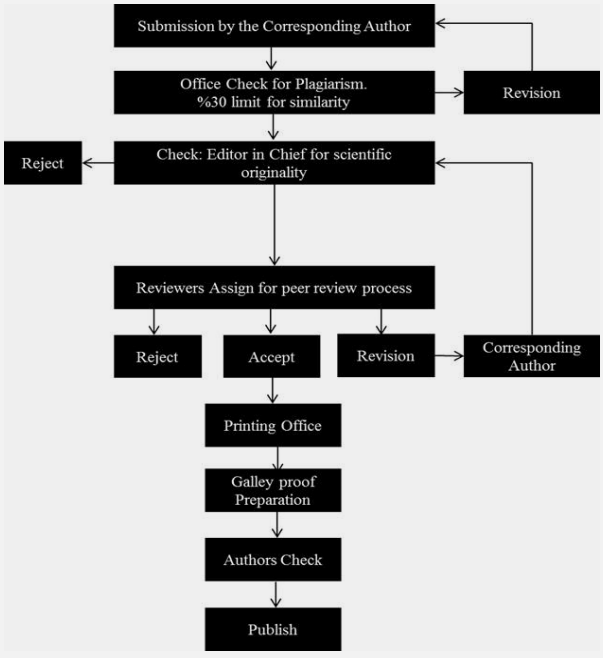

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Medical Science and Discovery
ISSN: 2148-6832

Academic health services and health needs of college students around the era of the Covid-19 pandemic

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ABSTRACT

Objective: The university medical services vary around the world (even within each university), but there are only a few publications on the utilization of these services by the students. The available on-campus services of public health care might include general health care, women's centers, mental health care, disability services, wellness resource centers, career counseling, and alcohol and other drug education programs.

Evidence Acquisition: This paper reviews the current literature on the overtime and current (due to Covid-19 pandemic) public health needs of college students based on studies that report the commonest specific diagnostic reasons for using the on-campus health care services.

Results: Special reference is done on mental health problems among students generally and the students of health professions fields (a specific category themselves). Besides, other issues of interest are the substance-related problems among students and their perceptions about mental health problems and on- campus help- seeking services.

Conclusions: It is unanimous that we need further educational and promotional campaigns to enhance the students; help-seeking behaviors, reduce stigmatizing behaviors and create more preventive public health services on campus, but also out-campus due to the Covid-19 pandemic.

Keywords: Health, Service, Covid, 19, College, Student, pandemic, COVID-19

INTRODUCTION

Context: This paper reviews the current literature on the overtime and current public health needs of college students and the use of current on-campus health care services. It is crucial for the academic authorities to know whether the available health care services cover the students; needs and if they don't new educational and promotional campaigns should be scheduled to enhance the students; help-seeking behaviors, reduce stigmatizing behaviors and create more preventive public health services on campus, but also out-campus due to the Covid-19 pandemic

Evidence Acquisition: The university medical services vary around the world (even within each university) and are widespread on college campuses, but there are only a few publications on the utilization of these services by the students. The authors searched the current literature published in Medline, setting appropriate keywords and retrieved articles both from online magazines, structured journals and scientific books. The included articles referred to students and college universities around the world and the supplied health care services, both those of primary care and prevention. Priority was given to scientific publications of the last 10 years which encountered a large sample of students. Although many on-campus providers and administrators retain electronically medical data of the students and their on- campus needs, these information are rarely published in the literature and cannot be used by the public health professionals and academic authorities to reorient the provided services just upon their students; demands (1).

Review Article

Received 05-03-2020

Accepted 02-04-2021

Available Online: 04-04-2021

Published 30-04-2021

Currently, clinical data from campus medical facilities are limited to single schools, whereas data are very rare concerning populations of students who seek medical care in university college health services, in spite of the thousands of studies on habits, thoughts of on-campus students (**2-5**). The American College Health Association (ACHA), records the existence of around 1,500 health services on college campuses addressed to their students, although there are no data on the utilization of these services. According to the literature, the different on-campus services of public health care might include general health care, women's centers, mental health care, disability services, wellness resource centers, career counseling, and alcohol and other drug education programs (**1**). This paper reviews the current literature on the overtime and current public health needs of college students and aims to stress the need for preventive public health services on campus, thus out-campus due to the Covid-19 pandemic.

RESULTS

On-campus medical needs

In 2014 the College Health Surveillance Network (CHSN) published the results of a large study enrolling thousands of students from 23 universities and reported the demand of university health services. This study concluded that 60% of college students' visits concerned primary care, 13% mental health, 9% vaccination, and 31% other miscellaneous services. The specific diagnostic categories claimed by the students were sorted by descending prevalence and were sorted as preventive, respiratory, skin, hair, and nails; infectious non–sexually transmitted infection, and mental health. This large study used the ICD-9 codes to classify the students visit by symptom or by the preventive or not character of this medical visit (**6, 7**). They used the models of ADG19 and MDC20 and adjusted the most frequently occurring codes into 118 diagnostic groups categorized by anatomic symptoms or signs, descriptive diagnoses, or specific etiology (e.g., acute upper respiratory tract infection, anxiety, infectious mononucleosis) and finally merged these diagnostic groups only 20 diagnostic categories (e.g., respiratory, mental health, infectious non–sexually transmitted infection (STI), respectively).

In this study the annual utilization rates were significantly higher at private schools than at public ones for all visit types, with females and age categories "<18" and "22–29" years having the highest rates for primary care and mental health visits. Moreover, females tend to use preventive services more than males, especially for reasons of contraceptive advice. Also, younger students; visits usually concern with respiratory and non–sexually transmitted infections. In addition, after a rough statistic evaluation, the previous study concluded that 16% of college students proceeded annually the preventive health services of the campus. Besides, the overall utilization of services by the 3 principal minority groups of the included students (African American, Asian, and Hispanic) was similar, but higher than the whites and Native Americans (**6**), with the Asians to have the lowest rate on demand of the mental health services probably due to psychological perceptions (**3-4**).

Also, the most used services were the Prevention-related ones (e.g. contraceptive management, physical examinations for athletics, travel, and work and screenings for STIs, lipid abnormalities, and hypertension, followed by the monitoring of the respiratory system and skin disorders. Dermatologic diseases followed and included contact dermatitis, acne, and skin infections. The next category of specific illnesses (sorted by descending prevalence) was the non–sexually transmitted infections (e.g., influenza, infectious mononucleosis, streptococcal pharyngitis, mumps, chicken pox, conjunctivitis, hepatitis B, herpes zoster, pertussis, and latent tuberculosis), with the mental health disorders coming next. Musculoskeletal disorders, injuries, and abdominal/gastrointestinal symptoms, followed by the disorders of eye, ear and mouth and female reproductive diagnoses filled out the 10 most popular diagnostic categories addressed to the college health services. Remarkably, the sexually transmitted illnesses appeared to the bottom of the ranking category, including human papillomavirus (HPV), genital herpes, chlamydia, gonorrhea, immunodeficiency virus (HIV), and other/unspecified STIs infections (**6**).

On the other hand, the mental health disorders were among the top 5 diagnostic categories and had the highest number of visits per patient (4.93). This specific category group included anxiety and depression (44% and 34%, respectively), psychosocial stressors (19%), adjustment disorders (17%), drug abuse (13%), attention-deficit/hyperactivity disorder (ADHD) (12%) and alcohol-related disorders (4%). From all the mental health disorders those which reported the highest rate of visits were the eating and personality disorders. These findings stress the need of through-24h available behavioral health care service on-campus for the college students (**6**).

The previous analyzed data provided by CHSN had been widely known recently and may help the health services campus worldwide to plan and organize adequate preventive and behavioral health care services for their college students. Factors such as sex, age, race should be taken in mind proportionally to the rates of each sub-population into the specific academic community. The epidemiologic data available from CHSN provide a better understanding of the clinical health care needs of subpopulations of students and may help colleges plan appropriate services (**6**).

Mental health problems among students

Students and stress: A cross-sectional study including students of seven public Krakow universities, investigated the possible relationship between insomnia, stress, stresscoping strategies and selected social and medical factors in this population. They used the Perceived Stress Scale 10, an abbreviated version of the Coping Orientation to Problems Experienced inventory (mini-COPE inventory) and the Athens Insomnia Scale, and found that one tenth of Krakow students perceive a high level of stress especially those with chronic diseases (p=0.006) and the cigarette smokers (p=0.004). Besides, one-fifth of the respondents suffered from insomnia whose positive correlation with the level of stress was very strong (p=0.00; r=0.44) (**8**).

Students of health professions fields and stress: Several studies agree that students in the health professions fields (e.g. students in pharmacy and medical degree programs) are of high risk to deal with multiple stressors factors, like academic

overload, the pressure to succeed, and competition (9-10). The majority of these publications suggest that medical students overall, compared with individuals in the general population of similar age, experience significantly more stress and lower mental health quality of life, with burnout affecting up to 50% of US medical students (11). Besides, 19% -44% of medical students experience anxiety and 27% of them feel depression, although estimates vary widely across studies (18% to 58%) (12-14).

On the other hand, other research data report depression (51%) and anxiety (29%) among pharmacy students, yet lower than those found among medical students (58% and 48% respectively). Although, research studies report conflicting results for the prevalence of anxiety between pharmacy and medical students, it is unanimous that both groups meet few stress (12, 15). Further, a recent study on pharmacy residents found a 40% self-reported depression during their residency similar to the rate of medical students and the general college student population (9, 16).

The previously described psychological distress, including anxiety and depression, degrades students' happiness and well-being and is a definitely risk factor for suicidality (17). Over 25% of medical students have ever considered suicide, with 11% having seriously contemplated suicide within the last year and almost one fifth of them have already attempted suicide (13), whereas there is little respective evidence among pharmacy students. Although the prevalence of suicidal ideation reported among all undergraduate and graduate academic fields is low (4%-11%), scientists of public health emphasize the need of early identification of suicidal ideation, symptoms of hopelessness and depressive symptoms in these students (18-20).

Another issue is the substance-related problems among this group of students which ranges from 17% to 58% during their clinical training (12, 21). Substance use is prevalent among the medical students, with 17%-34% of them reporting alcohol abuse or binge drinking, and about one third are using illicit substances (21-22). Pharmacy students report similar rates, with 25% of them exhibiting excessive drinking, 21% indicating marijuana use, and 41% reporting use of substances without a prescription (23).

All published studies report a high rate of alcohol intake in the past two weeks (77.4%) among students of the health professions fields of both sexes, whereas cigarette smoking rate in the past 30 days tends to be low (3.5%). On the contrary, marijuana use in the previous 30 days differs among medicine (14%) and pharmacy (4.8%) students (p5.03), and cocaine use respectively (3.6% vs 0.2%), while past research data showed lower rates (22).

Perceptions about mental health problems among students: Another cross-sectional analysis was conducted including students from 23 institutions in the United States, aiming to compare the prevalence of mental health problems, help-seeking attitudes, and perceptions among US pharmacy and medical students. This study reported similar rates of depression (18%) between the two groups. Although the prevalence of anxiety in pharmacy students (21% vs 11%) was higher, in the previously mentioned study this group of students used less frequently the student counseling services (only 11% vs 49%) due to fear of the stigma and very often they ignored where to seek help on campus if needed (18, 24).

The approach or not of the on-campus help services for mental health depends on several perceptions of the college students, taking into account the perceived risk, stigma, insurance, living on- or off-campus, and knowledge of how to use services. Generally, it is reported that despite the awareness of the available services, only a few students (13%) finally use them when they face mental health problem and this depends directly to the disorder, gender, and race/ethnicity. The mental health disease itself affects the possibility of utilization of the respective services, e.g. anxiety (5–35%), depression (3–39%), alcohol-related concerns (3–39%). Females tend to utilize more services than males for all health reasons, whereas non-white race college students (Black, Asian, or Hispanic) usually seek fewer services and receive less treatment versus white students. Those finding prove that it is crucial to increase awareness on mental health diseases and encourage students facing those problems to address to the appropriate health services by ensuring the confidence and the avoidance of stigma (1, 25).

Medical students may be less likely to seek help from campus-based services due to concerns around confidentiality, especially when seeking help for stigmatized conditions, including mental health problems (26). Among pharmacy residents, a perceived need for mental health services exists, with 26% indicating they believe they would benefit from mental health resources (16). However, less is known about the help-seeking attitudes and behaviors of pharmacy students. Studies including students of all health professions fields found that they prefer rather to speak about their problems with friends (43%), significant others (37.7%), family members (36.9%), and/ or roommates (16.8%) than address to other professionals for mental or emotional health support. However, one third of this population avoid speaking to anyone and just one tenth finally ask for academic personnel help. (27, 28) Although, medical students often face distress, burnout, anxiety, depression and suicidal ideation (8-9, 18), they rarely seek mental health services (10-13), thinking about lack of confidentiality, waste of time, cost, perceived stigma, potential repercussions, and unwanted interventions (21, 29). Furthermore, antidepressants are the most commonly prescribed category of drugs in most studies (10.8%), with the pharmacy students to account the most prescriptions from a primary health care physician versus the medical students (6).

Very often the medical students already have mental health diseases even prior to medical school (36%), or report a decline in their mental health during medical school training (47%) (27, 30). Actually, few reports converge to this finding (8, 31) attributing this mental decline of medical students to excessive workload, pressure to succeed, fatigue, ethical conflicts, accumulating debt, and exposure to death and human suffering (13, 32-33). The disclosure of this mental disease on a Medical Board license application is usually avoided by the college medical students, due to fear of stigmatization, of repercussions, and perception of the irrelevance of this information (30, 32).

Finally, it is unanimous that we need further educational and promotional campaigns to enhance the students; help-seeking

behaviors and reduce stigmatizing attitudes. Academic authorities should focus on information and promotion about the available health services and strengthen their discretion and confidentiality. It is encouraging that the American Association of Colleges of Pharmacy Student Affairs Standing Committee recently charged institutions to focus on student wellness by promoting self-awareness and well-being. Nowadays, the interest of academic authorities and ministries of Education around the world is focused on stressing the awareness about mental health issues, suicide prevention and stigma reduction into the college campuses (**30, 34**).

Impact of COVID-19 in students

The COVID-19 pandemic has influenced the academic community in various ways. Education procedures are mainly done via internet platforms, social relationships and on-campus athletic and cultural activities have been postponed and minimal physical activity is limited in-house. Several studies have assessed mental health issues and reported many reasons acting as stress factors between various groups of population. In the Covid- 19 era, many people face stress, anxiety or even depressive symptoms due to fear of death, frustration, boredom, inadequate supplies, inadequate information, financial loss, and stigma (**35**). So far most research studies are focused on the impact of this pandemic on health workers, patients, children, and the general population (**36, 37**). Currently, only a few preliminary studies coming from China, detect a slight trend on psychological or mental health worsening of college students due to quarantine and reduction of physical activity and social isolation (**38-41**). Another study conducted in the university system of Texas reported increased stress and anxiety (71%) within the students; population due to the COVID-19 outbreak. The most commonly mentioned stress factors were fear and worry about their own health and of their loved ones (91%), difficulty in concentrating (89%), intermittent sleep (86%), decreased social relationships due to isolation (86%), and worries for academic performance (82%). All these degrade the mental health of the campus students and make urgent the generation of out-campus preventive initiatives and services which will be primarily scheduled to support the mental health of the college students. (**34, 42, 43**).

CONCLUSIONS

The university medical services vary around the world (even within each university), but there are only a few publications on the utilization of these services by the students. The available on-campus services of public health care might include general health care, women's centers, mental health care, disability services, wellness resource centers, career counseling, and alcohol and other drug education programs. Academic authorities and Ministries of Education should focus on serving the overtime and current (due to Covid-19 pandemic) public health needs of college students, their mental health problems (generally and among students of health professions fields) and the substance-related problems among students. Finally, it is unanimous that we need further educational and promotional campaigns to enhance the students; help-seeking attitudes, reduce stigmatizing behaviors and create more preventive public health services on campus, thus out-campus due to the Covid-19 pandemic.

Author contributions: IK, ES, EK, AL; Literature search and study design, **IK;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

Point of Care Ultrasound during the medical teaching rounds in the COVID-19 Era; The Hospitalist Perspective

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ABSTRACT

Objective: In Internal Medicine, POCUS is gaining significant favorability. An increasing number of clinicians are interested in being trained for POCUS. The newer portable ultrasounds are small and can be transported easily during rounds. Their design is now for a more intuitive use. Training of Internists now involves assessing patients utilizing POCUS technology in residency. Here at Danbury Hospital, we have formal POCUS training. Attending internists are now attempting to incorporate POCUS training as a part of continuing medical education. POCUS in the hospitalist or general practitioner world has not been completely defined. Generally, the patient seen in the medical ward is not ill as the patients seen in intensive care units (ICU), Emergency Department (ED), and other high acute-care settings. However, from time to time, internists need to treat high acuity patients on the medical floors before transferring them to a higher level of care or when they are required to cover an open ICU or Progressive Care Unit (step-down unit). The role of POCUS while managing stable patients may differ significantly compared to the role in more acute patients. A well-defined spectrum for the use of POCUS does not currently exist. However, there are efforts in this regard.

POCUS is an emerging and exciting diagnostic modality in the medical ward. We believe that the pandemic has given it a new meaning for the hospitalist and general practitioner, and we expect that its use and significance will only grow in the few years ahead.

Keywords: Point-of-care-ultrasound, Ultrasound, Lung, COVID-19, POCUS

INTRODUCTION

Point of Care Ultrasound (POCUS) is widely used in many disciplines such as Cardiology, Emergency Medicine, Anesthesia, Critical Care, and others.

POCUS is used commonly for hemodynamic, functional, and anatomical assessment as well in procedural medicine.

In critically ill patients has proven useful in evaluating dyspnea and shock [1, 2]. POCUS is not limited to critical medical care and plays a role in other settings [3].

POCUS is a tool that requires technical training, anatomical knowledge, and a deep physiologic understanding of the processes that imaging documents. It is essential to understand the limitations of POCUS since this is not a comprehensive examination.

It requires a portable ultrasound machine that is appropriate to perform the type of examination required.

POCUS is different from other comprehensive ultrasound studies. It is used to answer a question that the clinician has while visiting a patient and can often yield useful diagnostic data in real-time to aid in clinical decision making. Some examples include but are not limited to evaluating the ejection fraction of the heart or the presence of abnormal fluid in cavities such as pleural effusions and ascites [3].

Short Communication

Received 12-03-2020

Accepted 29-03-2021

Available Online: 02-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.510

In Internal Medicine, POCUS is gaining significant favorability. An increasing number of clinicians are interested in being trained for POCUS. The newer portable ultrasounds are small and can be transported easily during rounds. Their design is now for a more intuitive use [4].

Training of Internists now involves assessing patients utilizing POCUS technology in residency. Here at Danbury Hospital, we have formal POCUS training. Attending internists are now attempting to incorporate POCUS training as a part of continuing medical education.

POCUS in the hospitalist or general practitioner world has not been completely defined [4, 5]. Generally, the patient seen in the medical ward is not ill as the patients seen in intensive care units (ICU), Emergency Department (ED), and other high acute-care settings. However, from time to time, internists need to treat high acuity patients on the medical floors before transferring them to a higher level of care or when they are required to cover an open ICU or Progressive Care Unit (step-down unit). The role of POCUS while managing stable patients may differ significantly compared to the role in more acute patients. A well-defined spectrum for the use of POCUS does not currently exist. However, there are efforts in this regard [6].

We believe that training Internists and Hospitalists is critical, helping better physicians with better clinical outcomes.

Training internists in POCUS seems feasible since studies have found that medical students can better identify heart conditions with ultrasound than cardiologists using a stethoscope [7]. Trained nurses can also distinguish the patient with pulmonary edema in the outpatient setting when adequately trained in lung ultrasound [8]

We use POCUS in a myriad of procedures.

The use of POCUS by the general practitioner seems necessary.

In the acute setting as POCUS lung ultrasound (LUS) may lead to the identification of the cause of dyspnea of a patient and start treatment as necessary [1]; in contrast, in the Medical Ward, an evaluation of a more stable patient by an internist, may need a more detailed and thorough examination of the lungs to advance diagnosis and treatment. Proper identification of scanned areas and proper positioning of the patient while being scanned is essential to obtain a more anatomical idea of the patient's affliction; this should always be paired with the lungs' auscultation.

For LUS, we have found that use of 8 frontal zones [9] (figure 1) and at least four posterior zones (as per our definition with a two-point examination for each hemithorax: The low point in the mid-scapular line usually near the 10th intercostal space with the patient sitting; the superior point in the space between the spine and the midpoint of the scapular body) (figure 2) is useful to evaluate for most lung disease. We also advise that every lung ultrasound is paired with thorough auscultation of the chest and evaluation of other imaging if available. The images should be documented and adequately assigned to specific scanning zones. They should be well described in the medical records of the patients. We recommend the use of four-second clips for all point-of-care ultrasounds for recording. As such, all images and interpretations should be subjected to systematic quality analysis by peers to identify opportunities for further training and improvement. We use POCUS to evaluate the heart and systemic veins better to assess patients' volume status and hemodynamics better. This is especially important when heart failure, thrombosis, sepsis, or acute kidney injury is suspected [10].

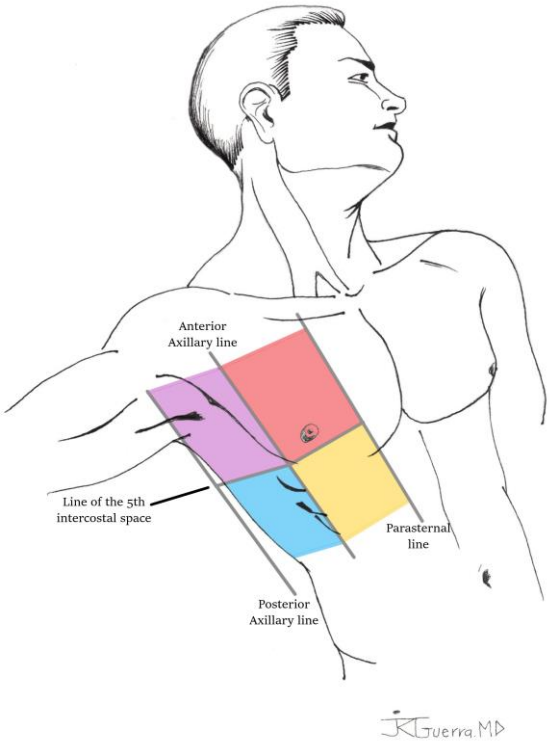

Figure 1: The 8 frontal zones for LUS

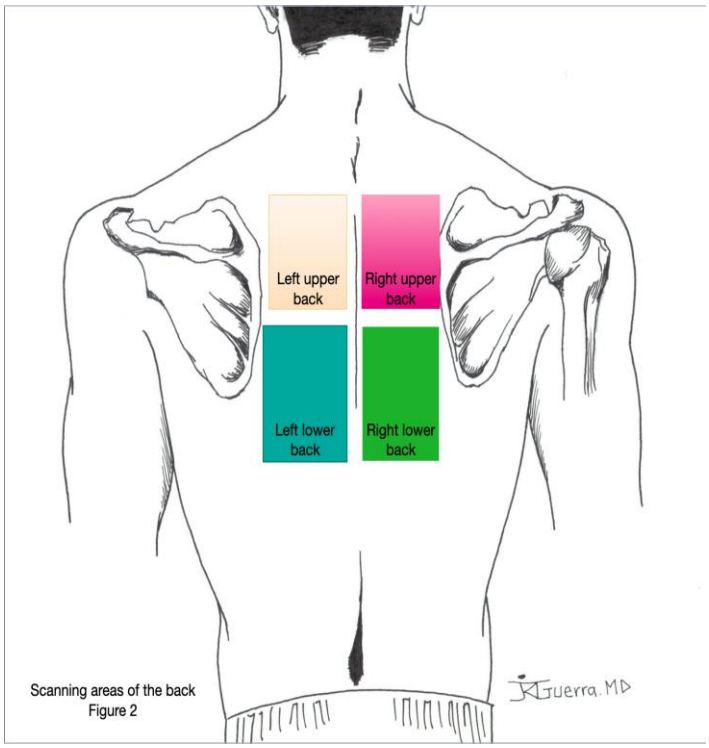

Figure 2: four posterior zones for LUS

Some of the limitations for POCUS use are lack of equipment, deficiency of consistent training, absence of motivation, lack of sufficient time during rounds, questions about documentation, and liability [4, 11]. Also may be limited by the perception that there is no medical decision associated with the procedure and revenue. Hospitalists, especially teaching hospitalists, may face large lists of patients making time to teach during rounds scarce and difficult.

POCUS appears to improve clinical results:

An observational study implemented at a tertiary level ICU aimed to investigate whether incorporating POCUS during the morning round on a routine basis improved clinical outcomes in critically ill patients with sepsis. This, by measuring mortality, duration of mechanical ventilation, vasopressor utilization, volume status. The intervention group had shorter durations of mechanical ventilation (MV) (4.5 ± 1.2 vs. 5.7 ± 1.0 days; p = 0.034) and more negative fluid balance (−143 vs. 48 ml/24 h; p = 0.003) on day 3. In association with the multivariable model, routine incorporation of POCUS lowered the risk of prolonged (>7 days) ICU stay (OR: 0.39, 95% CI: 0.29–0.88; p = 0.029) [14].

A systematic review and a proportion meta-analysis focused on the frequency of abnormalities found on LUS in Covid-19 patients revealed that almost all SARS-CoV-2-infected patients have abnormal LUS. The most common abnormality is interstitial involvement depicted as B-pattern. These findings emphasize the potential role of POCUS in the triage, diagnosis, and follow-up of COVID-19 patients [13].

The rise of POCUS in rounding in the patient with COVID 19:

Since March of 2020, Danbury Hospital saw an exponential increase of admissions secondary to COVID-19, in turn making Danbury Hospital one of the de-facto COVID hospitals of the Nuvance Health system in Connecticut and New York. During the first wave of the pandemic and now with the second wave, we have seen a surge of patients in severe respiratory failure where classic clinician tools, such as the stethoscope, have shown to be of little help (unpublished data), and this has complicated the evaluation of the disease and volume status.

We used the POCUS approach to these problems. We began to perform lung ultrasound (LUS) and heart POCUS to help evaluate the lungs and heart in patients with COVID-19, which to our experience, lacks enough auscultatory and physical examination findings (unpublished data).

POCUS helps evaluate volume status, which seems to be paramount in these patients suffering from various COV-19 pathologies[14].

Like many other studies, we have found that the infection of SARS-COV-2 has a characteristic appearance in the LUS, and the prognosis of these patients and the clinical status, can be affected severely by the volume status and presence of other complications such as venous and pulmonary thromboembolism.

Because of that, we started to perform LUS in our patients, scanning eight frontal zones looking for the common artifacts in COVID in the lung, and performing a heart ultrasound and systemic veins to evaluate volume status. When patients can cooperate, we perform back ultrasounds. We paired the LUS with the assessment of the heart function and right ventricle, and in patients with acute kidney injury, we have been able to quickly evaluate the kidneys and ureters of the patients avoiding transporting these patients to the ultrasound and CT scan suits.

We have stored all POCUS and proposed some of our findings for publication (results not published).

Results from cardiac views were always compared with formal echocardiograms when available.

Using POCUS gave us light in the general ward to manage those patients to add to the primary diagnosis and its complications.

We modified our morning rounds to take advantage of POCUS during this pandemic with good results even when patients' census was high.

Implementing POCUS in COVID Teaching Rounds:

During the first peak of COVID 19, the team scanned all patients with COVID 19 and compared the images to what was already published. We evaluated all the images with a pulmonologist, and we have submitted our findings to a peer-reviewed journal (unpublished data). During the second peak of COVID 19, we used the experience gained during the first peak. All patients who presented with worsening shortness of breath were flagged for LUS and cardiac ultrasound to evaluate volume status. All patients with stable findings would be evaluated by ultrasound on a case-by-case basis.

Some patients with improvement will be evaluated by ultrasound in order to document changes.

Methodology and Considerations during POCUS utilized Morning Rounds:

1. We will present and discuss all cases before entering the patients' room.
2. The case presentations will be concise and based on the specifics of the patient.
3. The sicker patients will be evaluated and scanned.
4. From these patients, those who will need further workup or particular intervention will be discussed with subspecialties and the lung ultrasound results and point of care cardiac ultrasound if possible.
5. The less urgent ultrasounds will be performed in a second clinical round after the first evaluation, especially those improving.
6. All ultrasounds performed by residents or students were performed under direct supervision.
7. All ultrasound findings will be documented in the paper chart, and the images properly identified and saved for future evaluation.

8. We found out that using a small ultrasound with a small footprint, in our case, a Mindray TE7 with a phased array probe (2-4Mh in lung setting), was appropriate for the relatively small-sized rooms. The use of this probe made it easy to change the settings to evaluate other organs rapidly.
9. We covered the ultrasound with the available PPE and letting only the probe and the screen be exposed to avoid contamination. The equipment was easy to clean with the available peroxide solution and antiviral solution in the hospital.
10. Planning was crucial since the use of ultrasound by trainees may exceed the 5 minutes proposed in other articles [15]. Before ultrasound rounds with the residents and students, we supervised the proper use of PPE.
11. To avoid cross-contamination, we scanned patients with multi-resistant bacteria at the end of the round.
12. All ultrasound results and images were discussed compared with other imaging and diagnosis with the residents.
13. All decisions based on the findings of the ultrasound were discussed during and after rounds. These discussions include the imaging description, reasoning on why to obtain imaging, quality of the imaging, and the physiology behind the ultrasound. On every patient admitted with the diagnosis of COVID-19, an LUS was performed to establish a baseline.
14. All findings were documented in the medical record and associated with a medical decision when appropriate.

We believe that the POCUS use gave us light in the general ward to manage those patients to add to the primary diagnosis and its complications.

Last thoughts

It appears that the use of ultrasound during rounds improves satisfaction in medical students and residents. It also appears to improve patients satisfaction [16]. It also appears that may improve clinical outcomes.

Despite (perhaps because of) the pandemic, POCUS has increasingly used on the general medical wards. We believe the need for POCUS will continue to grow, and it is equally important to implement consistent and quality training. It is also vital to supply adequate equipment and focus on the financial costs of utilizing POCUS.

It is necessary to support and evaluate if the use of POCUS in the medical Wards improves outcomes, and for that, studies will need to be done.

CONCLUSION

We recommend that medical schools, as well as internal medicine residencies, implement the study of POCUS. We also recommend that the hospitals make available portable ultrasounds that are intuitive to use, producing images that are of good quality for decision-making and

storing it for quality improvement. We must offer continuous continuing medical education opportunities for the attending, residents, and students to improve and maintain ultrasound use quality. It is also essential to educate how to describe and document POCUS use in charts and how this can help in revenue.

POCUS is an emerging and exciting diagnostic modality in the medical ward. We believe that the pandemic has given it a new meaning for the hospitalist and general practitioner, and we expect that its use and significance will only grow in the few years ahead.

Aknowledgements: Thanks to Dr. Rodolfo Guerra (MD) for illustrations Figure 1 and 2

Author contributions: CES, WA; Literature search and study design, **WA;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

An investigation of the effects of melatonin administration on biochemical parameters in rats with experimental cartilage damage

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ABSTRACT

Objective: This study was intended to show the effects of melatonin (MEL) in the treatment of cartilage damage in a rat model as a novel field of application.

Materials and Methods: Male Sprague Dawley rats aged 3-4 months were assigned into four groups of six rats each. Group I represented the sham group. In groups 2, 3, and 4, the right knee medial meniscus was surgically destabilized. MEL was administered to groups 3 and 4 twice a week at dosages of 0.4 µg/ml and 4 µg/ml, respectively. The application continued for four weeks. Histological examinations, imaging studies [computed tomography and magnetic resonance imaging], and biochemical tests [cartilage and bone turnover markers (COMP and CTX-I)] were performed.

Results: The application of MEL initiated regeneration in the damaged areas. However, cartilage repair was not observed in areas with experimental cartilage damage without MEL application. MEL-treated rats had higher T2 scores compared to Group 1 in the median femoral condyle at the 12th week ($p<0.05$). Serum COMP and CTX-I levels at 12 weeks were significantly higher in Group 2 compared to Group 1 ($p<0.05$). Serum COMP and CTX-I levels at 12 weeks were lower in groups 3 and 4, but were also significantly higher than in Group 1 ($p<0.05$).

Conclusion: We recommend MEL therapy for diseases related to cartilage damage. MEL seems to exert its therapeutic effect on cartilage damage through its antioxidant properties.

Keywords: Melatonin, rats, cartilage damage, COMP and CTX-I

Research Article

Received 07-03-2020

Accepted 17-04-2021

Available Online: 19-04-2021

Published 30-04-2021

INTRODUCTION

Melatonin (MEL), released from the pineal area, is the hormone that regulates the human sleep–wake cycle (1, 2). It was first discovered in 1958. MEL is a prescription-only medication, but is not approved for any medical use by the US Food and Drug Administration (FDA). However, in the European Union and Australia, it may be prescribed for sleeping problems or insomnia. It was approved for medical use in 2007 (3, 4, 5). MEL is used in the treatment of insomnia in children and adolescents aged 2–18 with autism spectrum disorder (ASD), and especially in Smith–Magenis syndrome (6).

MEL is also beneficial to cartilage tissue. The protective role of MEL was found to be as significant as that of dexamethasone by Yang et al. in a study in which mouse knee joints were treated with MEL, dexamethasone, EX527, and siRNA targeted for SIRT6 (7).

According to World Health Organization (WHO) data, 25% of individuals over the age of 65 complain of joint pain and dysfunction caused by the progressive and irreversible loss of joint cartilage (osteoarthritis and calcification) (8). Despite the prevalence of cartilage damage, modalities for preventing and/or curative treatment are limited (9).

http://dx.doi.org/10.36472/msd.v8i4.507

New therapeutic options for osteoarthritis have therefore become an important research topic in recent years. MEL is a natural agent with well-known antioxidant and immunomodulatory effects.

Cartilage tissues surgically damaged through intra-articular intervention have been treated with MEL, and its effects observed, in rat models in previous studies. Such rat models can be used to evaluate the therapeutic effects of MEL for all chondral injuries. The purpose of this study was to show the effects of MEL in the treatment of cartilage damage in a rat model as a novel field of application.

MATERIAL and METHODS

This animal study was designed using a rat model of cartilage damage to show the effects of MEL on cartilage structures. The experiments commenced following approval from the Kafkas University Animal Experiments Local Ethical Committee (Turkey) (no. 2019/007 dated 21/06/2019). Twenty-four male Sprague Dawley rats aged 3-4 months and weighing 250-300 g were assigned into four groups of six animals each.

The animals were housed in a controlled room temperature (22±2°C) and in a 12-hour dark, 12-h light cycle. All experiments were performed in line with International Association for Pain Studies rules (1983). The animals were permitted ad libitum access to chow and drinking water. Eighteen-gauge soft gavage tubes have generally been used (16-20 sizes) for MEL treatment in similar studies.

Rats were randomly divided into four groups of six animals each. The right knee medial meniscus was surgically destabilized in three of these groups. MEL applications commenced after waiting four weeks for cartilage damage to develop. MEL was administered to two groups subjected to surgical destabilization through addition to drinking water twice a week at two different doses, 0.4 µg/mL and 4 µg/mL. Application continued for four weeks, at the end of which time all rats were sacrificed, and blood and tissue samples were collected.

Group I (n=6) (sham group): The articular cavity was opened in the right knee joint, but the suturing procedure was performed without transecting the medial meniscotibial ligament.

Group II (n=6) (control group): Rats in this group were subjected to medial meniscus destabilization with transection of the ligament of the medial meniscotibial band.

Group III (n=6): Rats in this group underwent medial meniscus destabilization with transection of the ligament of the medial meniscotibial band, followed by MEL application at 0.4 µg/mL twice a week for four weeks.

Group IV (n=6): This group underwent medial meniscus destabilization with transection of the ligament of medial meniscotibial band, followed by MEL application at 4 µg/mL twice a week for four weeks.

Histological Examinations: Tissue samples collected from the knee joints for histological evaluation were fixed in 10% formaldehyde solution. Tissues were then blocked in paraffin after routine processing, and 5-micrometer (µm) thick sections were taken from the paraffin blocks. For histological examinations, the sections were stained with Hematoxylin-Eosin (H&E) and evaluated under a light microscope (Olympus Bx43). Histopathological images of the slides were then taken using a digital camera (Olympus DP21).

Imaging Studies: Animals were sacrificed at week 12. The knee joints were separated from soft tissues for histological examination. Magnetic resonance imaging (MRI) was performed 12 weeks following surgery. MRI (T1 and T2) scans were used to evaluate changes in the articular cartilage area.

Biochemical Tests: The bone and cartilage turnover markers employed were cartilage oligomeric matrix protein (COMP) and carboxy-terminal collagen I (CTX-1). These were measured initially and at 3, 6, and 12 weeks. Cartilage damage was assessed by means of biochemical measurement of the serum levels of these markers using a Biotek 50 TS plate washer and ELX808 plate reader ELISA device (BioTek Instruments Inc., Winooski, VT, USA).

RESULTS

Histological Results: Histopathological tissue examination revealed no difference between the control and sham (no damage groups) groups. However, in the experimental groups, the integrity of the knee joint cartilage was impaired in some areas due to external injury. Regeneration was observed to have commenced in the damaged areas of the knee joint as a result of MEL application in the groups with experimental cartilage damage. Partial cartilage formation was observed with fibrous connective tissue following regeneration. However, repair was negligible in rats with experimental cartilage damage without MEL application, and in some regions, no repair was observed at all (Figures 1, 2, 3, and 4). All histological examinations in this study involved cartilage tissues. The histological grading of the groups is shown in Table 1.

Imaging Studies: T1 scores at 12 weeks were higher in the lateral tibial (LT), medial tibia (MT), and lateral femoral condyle (LFC) compartments ($p<0.05$) compared to the control group. T1 scores were lower in the groups treated with MEL compared to Group 2 rats in MT, LT, LFC, and the median femoral condyle (MFC) at the 12th week ($p<0.01$). T2 scores in the cartilage were higher in Group 1 compared to Group 1. The scores were higher on the 12th week in MFC, LFC, MT, and LT ($p<0.01$) compared to the control group. T2 scores of rats treated with MEL were lower than those in Group 2 at 12 weeks in MT ($p<0.001$), MFC ($p<0.01$), and LFC ($p<0.05$). T2 scores of rats treated with MEL were higher compared to Group 1 in MFC at the 12th week ($p<0.05$) (Figures 5, 6, 7). The groups' MRI scores are shown in Table 2.

Serum Biomarkers: No change was observed in serum COMP and CTX-I levels in Group 1 compared to the other groups ($p>0.001$). Group 2 serum COMP and CTX-I levels at week 12 were higher than those in Group 1 ($p<0.05$). Serum COMP and CTX-I levels were lower in groups 3 and 4 at 12 weeks, but were higher than in Group 1 at 12 weeks ($p<0.05$). The groups' serum COMP and CTX-I levels are shown in tables 3 and 4.

http://dx.doi.org/10.36472/msd.v8i4.507

Table 1. Histological Grading of Groups.

	Group 1	Group 2	Group 3	Group 4
12th week	1.65	1.76	2.22	2.58

Table 2. Magnetic Resonance Imaging (MRI) Grading of Groups.

	Group 1	Group 2	Group 3	Group 4
Initation	32.37	33.65	33.41	33.45
12th week	15.65	15.87	26.78	28.88

Table 3. Serum COMP levels in groups

	Group 1	Group 2	Group 3	Group 4
Initation	2.65	2.98	2.78	2.75
3 rd week	2.27	5.75	2.64	2.60
6 th week	1.78	4.04	2.22	2.12
12 th week	1.65	3.17	1.87	1.78

Table 4. Serum CTX-I levels in groups

	Group 1	Group 2	Group 3	Group 4
Initation	32.37	33.65	33.41	33.45
3 rd week	25.27	31.75	28.64	27.60
6 th week	19.78	25.04	18.22	16.12
12 th week	15.65	23.17	15.87	14.78

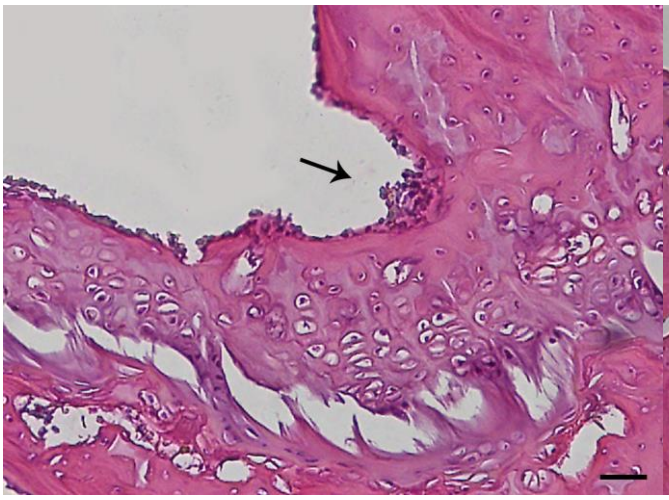

Figure 1. The histologic image of Group 1 (sham group)

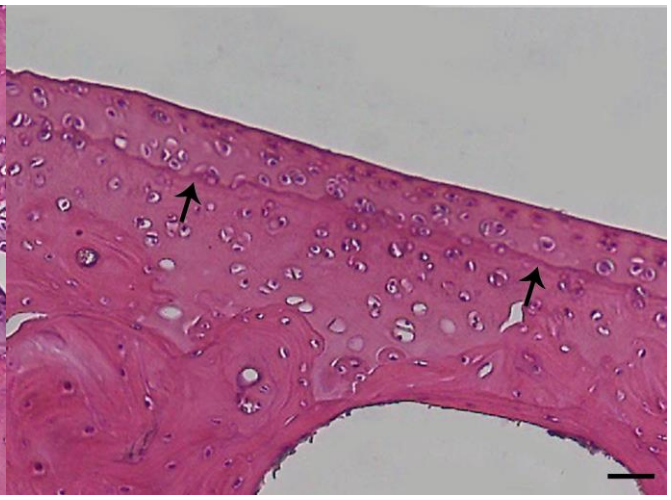

Figure 2. The histologic image of Group 2 (right knee medial meniscus surgically destabilized)

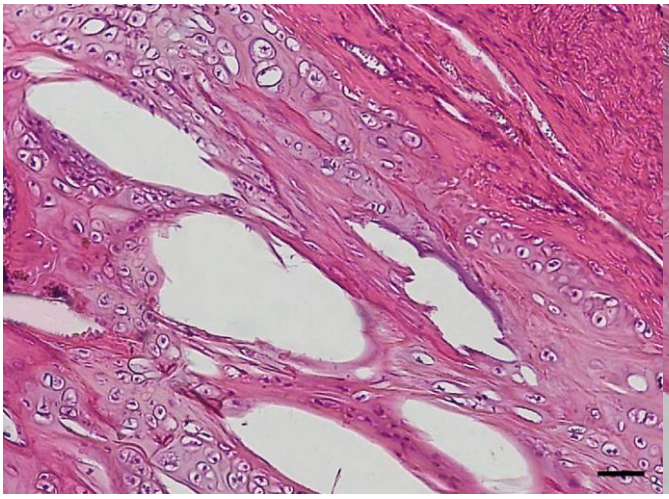

Figure 3. The histologic image of Group 3 (right knee medial meniscus surgically destabilized, MEL administration, a dose of 0.4 µg/mL, for 4 weeks)

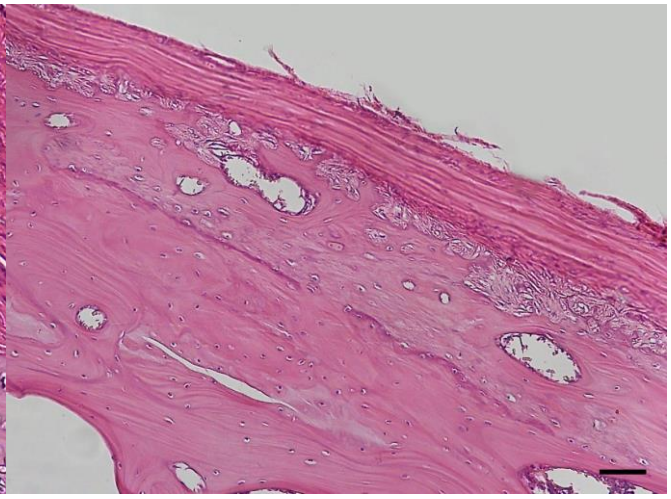

Figure 4. The histologic image of Group 4 (right knee medial meniscus surgically destabilized, MEL administration, a dose of 4 µg/mL, for 4 weeks)

http://dx.doi.org/10.36472/msd.v8i4.507

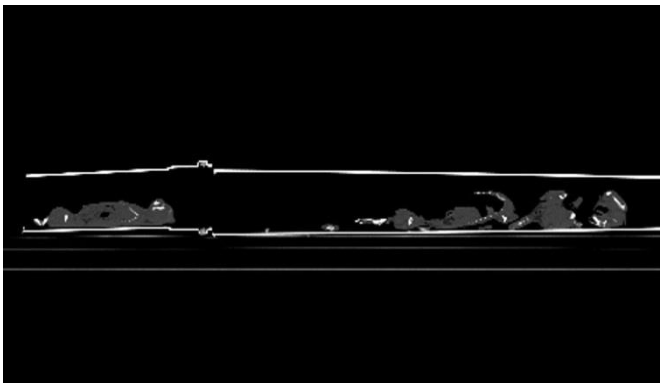

Figure 5. Computed Tomography images of rats

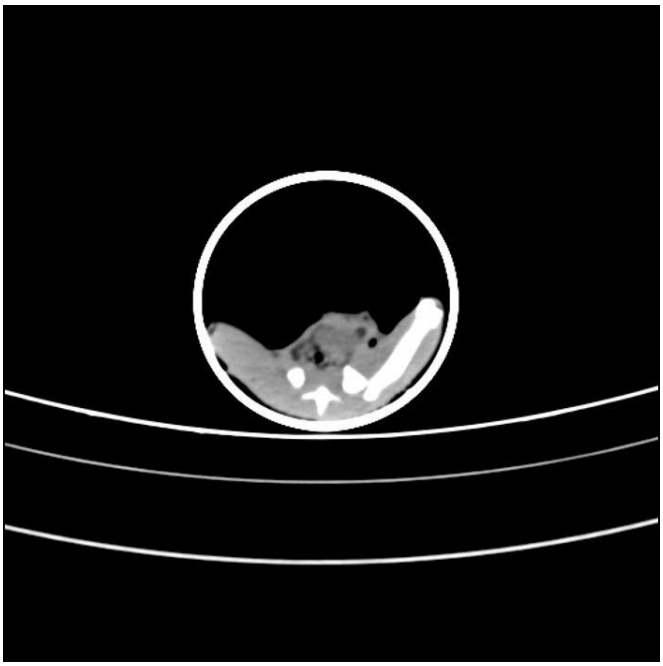

Figure 6. Computed Tomography axial images of rats

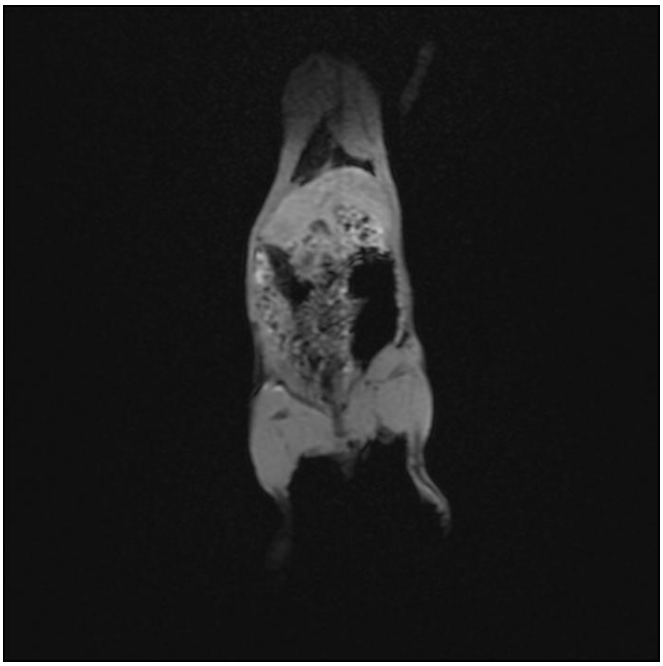

Figure 7. Magnetic Resonance Imaging scans of rats

DISCUSSION

No histological difference was observed between the rats in the control and sham (no damage) groups. In the experimental groups, the integrity of the joint cartilage was impaired in some areas due to external damage to the joint. In the groups with experimental cartilage damage, the application of MEL initiated regeneration in the damaged areas. Following regeneration, partial cartilage formation with fibrous connective tissue was observed. However, no cartilage repair was found in damaged areas with experimental cartilage damage without MEL application. T1 scores at 12 weeks were higher in MT, LT, and LFC compared to the control group ($p<0.05$). MEL treatment resulted in lower T1 scores at 12 weeks compared to Group 2 in MT, LT, LFC, and MFC ($p<0.01$). T2 score elevation was observed in cartilage in groups 3 and 4. At week 12, the scores were higher in MT, LT, MFC, and LFC compared to the control group ($p<0.01$). MEL treatment resulted in lower T2 scores compared to Group 2 in MFC ($p<0.01$), LFC ($p<0.05$), and MT ($p<0.001$) at 12 weeks. MEL-treated rats exhibited higher T2 scores than those in Group 1 in the MFC at 12 weeks ($p<0.05$). No significant difference was determined in serum COMP and CTX-I levels in Group 1 compared to the other groups ($p>0.001$). Serum COMP and CTX-I levels were significantly higher at 12 weeks in Group 2 compared to Group 1 ($p<0.05$). Serum COMP and CTX-I levels were lower in groups 3 and 4 at 12 weeks, but were higher than in Group 1 ($p<0.05$).

A previous study reported that MEL reversed the adverse effects of dexamethasone. Inhibition of SIRT1 was also observed to block the protection provided by MEL. The most important results of that study were that the chondroprotective effects of MEL increase with NAD-dependent SIRT1 activity, and that MEL reduces dexamethasone-induced cartilage damage (7). The removal of the pineal gland accelerates intervertebral disc degeneration (IDD). Li et al. demonstrated the presence of MT1 and MT2 (melatonin membrane receptors) in the intervertebral disk tissues and the cells of the nucleus pulposus (NP). MEL inhibits the cellular proliferation of NP in a dose-dependent manner and regulates its cell function. It is also an important factor in the prevention of IDD (**10**). MEL exhibits cytoprotective effects through a pathway dependent on numerous molecules and receptors, including NF-κB. The disturbance of circadian timekeeping has been linked to inflammatory arthritis. MEL stimulates the cartilage destruction/regeneration process through direct/indirect modulation of the main circadian clock genes. These genes are BMAL, CRY, and/or DEC2. BMAL, CRY, and/or DEC2. The effects of MEL on major arthritis disease as rheumatoid arthritis and osteoarthritis demonstrated by Jahanban-Esfahlan R et al. (**11**).

MEL is a powerful antioxidant capable of scavenging a variety of reactive oxygen species. MEL levels in vivo are relatively low, and it is difficult to explain this antioxidant activity in terms of scavenging activity. It is probably capable of altering the antioxidant activity of the cell in an indirect manner. High local concentrations of MEL in the brain produce significant protective effects against head trauma in rats (**12**).

CONCLUSION

MEL application provided regeneration in the damaged areas of the knee joint following experimental cartilage damage. The fibrous connective tissue was maintained following this regeneration process. Cartilage repair was almost non-existent in rats without MEL application. We recommend the use of MEL therapy for cartilage diseases.

Acknowledgement: None

Author contributions: KY, VT, FB, SK, VY, ED; Study design, Data collection, Literature search, Experimental studies, Data analyzes **KY;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and a specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

The prevalence of Helicobacter pylori and its effect on the prognosis of patients with COVID-19

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ABSTRACT

Objective: SARS-CoV-2 RNA positivity in stool in COVID-19 infection has been reported at rates varying between 6-83%. The purpose of this study was to determine the prevalence of H.pylori and investigate whether it determines the disease prognosis in COVID-19 patients.

Methods: This study was conducted on 117 confirmed COVID-19 patients who were hospitalized due to symptomatic pneumonia and tested for stool H.pylori antigen. Stool H. pylori test outcomes, demographic parameters, laboratory findings, and prognostic predictors of disease were recorded. The effect of the presence of H.pylori in patients with COVID-19 was analyzed.

Results: The mean age of 117 included patients was 49.68 ± 14.62 years, 78 (66.7%) had COVID PCR positivity and 32 (27.35%) had H.pylori positivity. There was no statistical difference in demographic data, prognosis, and laboratory parameters between those with and without H.pylori.

Conclusion: H.pylori positivity was detected as 27.35% in patients with COVID-19 infection. However, we could not find the positive or negative effect of H.pylori on the prognosis of COVID-19 disease. In conclusion, according to the results of this study, H. pylori positivity or negativity neither determined the severity of the COVID-19 disease nor the poor prognostic indicators of the disease.

Keywords: H. pylori, COVID-19, prognosis

Research Article

Received 11-03-2020

Accepted 14-04-2021

Available Online:15-04-2021

Published 30-04-2021

INTRODUCTION

New coronavirus (COVID-19, SARS-CoV-2) has become a pandemic that has spread all over the world, starting in China in 2019, causing severe acute respiratory failure (1). New information is being added every day about the characteristics and treatment of COVID-19 disease. However, our knowledge of COVID-19 infection and its treatment is still limited.

SARS-CoV-2 RNA positivity in stool in COVID-19 infection has been reported at rates varying between 6-83% (2-5). Detection of viral genetic material in the stool does not mean that viable infectious virions are present in the stool, but a long-term positive gastrointestinal specimen is interpreted as the virus can replicate actively in the patient's gastrointestinal system (6). ACE-2 receptors used by SARS-CoV-2 to enter the cell are highly expressed in the small intestine and the binding affinity of ACE affects the infectivity of the virus. A number of viruses, such as coronavirus, rotavirus, and noroviruses, can invade absorbent enterocytes through the ACE-2 receptors on absorbent enterocytes in the ileum and colon, causing gastroenteritis.

http://dx.doi.org/10.36472/msd.v8i4.509

There have been studies showing that intestinal epithelial cells expressing ACE-2 may be at increased risk of attack by SARS-CoV-2 and that ACE2 is highly expressed in the small intestine, especially in proximal and distal enterocytes. Therefore, the digestive system can be invaded by SARS-CoV-2 and used as an entrance of infection. It has been shown that approximately 12% of patients with SARS-CoV-2 infection have gastrointestinal symptoms including diarrhea, nausea and vomiting (7). In the meta-analysis examining the studies investigating whether the gastrointestinal system symptoms are associated with mortality in COVID-19 disease; no significant difference was shown between groups with and without gastrointestinal symptoms (8).

H.pylori is one of the most common infections affecting the human race with a high prevalence in developing countries (9). With respect to the distribution map of COVID-19 cases and mortality rates in different countries, mortality rates per million population are low in Russia, Portugal (https://www.worldometers.info/coronavirus/) whereas higher in these regions (Russia 78.5%, Portugal 86.4%). Therefore, it is assumed that H. pylori may play a role in preventing serious infections in COVID-19 infection (9,10).

Conflicting results have been reported between non-COVID viral infections and H. pylori positivity in the literature (11-13).

In our literature searches, we did not find any studies investigating COVID-19 and H. pylori infection together. To our knowledge, we have investigated for the first time the determination of the prevalence of H. pylori in patients with confirmed COVID-19 disease and whether it determines the prognosis of COVID-19 disease.

MATERIAL and METHODS

Study population and sample collection

A total of 117 patients, whose diagnosis of COVID-19 pneumonia was confirmed by nasopharyngeal (NP) PCR-RT swab and thorax computed tomography between 15 March 2020 and July 2020, were included in the study. Stool H. pylori antigen test was investigated in all patients. Patients with a history of H. pylori eradication therapy, a history of gastric malignancy and NP PCR-RT negative patients were excluded from the study. PCR results of the cases, H. pylori test results, demographic parameters, other laboratory parameters and prognosis indicators were recorded. The effect of the presence of H. pylori in patients followed-up for COVID-19 infection was statistically analyzed.

H. pylori antigen test in faeces

Stool samples from patients with confirmed diagnosis of COVID-19 disease were taken into special stool containers and transferred to the laboratory. H. pylori antigen test [H.pylori Antigen Rapid Test Device (feces), China] was studied in stool samples by immunochromatographic method. After all the reagents and stool samples reached room temperature, the applicator stick coming out of the kit was dipped into three different areas of each stool sample and approximately 50mg sample was collected. After placing the applicator stick in the diluent tube and closing its cap, it was shaked. The results were interpreted after 10 minutes of dropping 3 drops of the immunochromatographic cassette from the liquid sample in the tube to the sample section of the test. The formation of a colored line, even faint, was considered positive in patients with a control line showing the validity of the test. The whole procedure has been done in line with the manufacturer's recommendations.

It has been reported that the use of H. pylori antigen test in stool is reliable as a non-invasive test (14-16).

Nucleic acid isolation and SARS-CoV 2 RT-PCR

Combined nasopharynx and oropharynx swab samples were collected by dacron swab and placed in Viral transport medium immediately, and delivered to the laboratory by keeping them at 2-8 °C. The samples were delivered to the laboratory in accordance with the rules of cold chain with the triple transport system, complying the infection prevention and control procedures.

After taking the samples in microbiology laboratory, samples were taken to a negative pressure chamber with 3rd level bio-safety. Bio-Speedy® Viral Nucleic Acid Isolation Kit (Bioeksen, Turkey) was used for total nucleic acid isolation from the specimens. The isolation procedure was carried out according to the recommendations of the manufacturer.

Bio-Speedy® COVID-19 RT-qPCR Detection Kit (Bioeksen, Turkey) was used for the RT-PCR assays. The PCR amplification and evaluation of the results were carried out according to the recommendations of the manufacturer.

Statistical Analysis

Data analysis was performed by using IBM SPSS Statistics version 21.0 software (IBM Corporation, Armonk, NY, USA). Whether the distributions of continuous variables were normally or not being determined by visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov and Shapiro Wilk tests). Descriptive analyses were presented using means and standard deviations for normally distributed variables; using medians and and 1st-3rd quarter for the non-normally distributed variables. Categorical variables are specified as numbers and percentages. Independent-Samples T test, Mann-Whitney U test, Chi Square test and Fisher's Exact test were used to analyze the data. The significance level for all of the statistical tests was set at $p<0.05$.

RESULTS

The mean age of 117 patients with COVID-19 pneumonia was 49.68 ± 14.62 years and 48.7% were male. H. pylori was detected in 32 (27.35%) of the patients. In terms of COVID-19 PCR-RT positivity, there was no significant difference between the H.pylori positive group (Group 1) and negative group (Group 2) ($p = 0.769$).

Demographic characteristics of both groups were similar (Table 1). During the follow-up period, no patient died in Group-1, while 4 (4.7%) patients in group-2 died, but there was no significant difference between the two groups ($p = 0.574$). Concerning prognostic markers levels such as white blood count (WBC), neutrophil, lymphocyte, thrombocyte, D-Dimer, ferritin, albumin, lactate dehydrogenase (LDH), procalcitonin and CRP were similar in both groups ($P> 0.05$) (Table 2).

http://dx.doi.org/10.36472/msd.v8i4.509

Table 1: Distribution of demographic and clinical characteristics according to H. pylori status in patients with COVID-19

Parameters	All patient (n=117)	Stool H. pylori + (n=32) (%27.35)	Stool H. pylori – (n=85) (%72,65)	p
PCR + n(%)	78 (66.7)	22 (68.8)	56 (65.9)	0.769*
Age (Years) Mean ± SD Median(1st-3rd quarter)	49.68±14.62 47.00 (40.00-60.00)	46.78±13.80 45.50 (37.00-57.00)	50.78±14.85 48.00 (40.50-60.50)	0.189
Sex n (%)				
Male	57 (48.7)	13 (40.6)	47 (55.3)	0.157*
Female	60 (51.3)	19 (59.4)	38 (44.7)	
Having a Chronic illness + n (%)	64 (54.7)	15 (46.9)	49 (57.6)	0.297*
No chronic disease n(%)	53 (45.3)	17 (53.1)	36 (42.4)	
One chronic disease n(%)	45 (38.5)	11 (34.4)	34 (40.0)	
Two chronic disease n(%)	16 (13.7)	4 (12.5)	12 (14.1)	
Three or more chronic disease n(%)	3 (2.6)	-	3 (3.5)	
Diabetes Mellitus n (%)	17 (14.5)	4 (12.5)	13 (15.3)	1.000**
Hypertension n (%)	46 (39.3)	9 (28.1)	37 (43.5)	0.128*
Coronary artery disease n (%)	4 (3.4)	1 (3.1)	3 (3.5)	1.000**
Chronic obstructive Pulmonary disease n(%)	3 (2.6)	-	3 (3.5)	0.561**
Asthma n (%)	7 (6.0)	4 (12.5)	3 (3.5)	0.088**
Blood groups				
A Rh(+)	22 (38.6)	7 (38.9)	15 (38.5)	
B Rh (+)	8 (14.0)	2 (11.1)	6 (15.4)	-
O Rh (+)	20 (35.1)	7 (38.9)	13 (33.3)	
AB Rh (+)	3 (5.3)	-	3 (7.7)	
A Rh (-)	3 (5.3)	2 (11.1)	1 (2.6)	
O Rh (-)	1 (1.8)	-	1 (2.6)	
Oncet of complaintsn (%)				
Cough n (%)	90 (76.9)	23 (71.9)	67 (78.8)	0.427*
Fever n (%)	39 (33.3)	8 (25.0)	31 (36.5)	0.241*
Shortness of breath n (%)	36 (30.8)	9 (28.1)	27 (31.8)	0.704*
Throat ache (%)	31 (26.5)	8 (25.0)	23 (27.1)	0.822*
Diarrhea n (%)	10 (8.5)	5 (15.6)	5 (5.9)	0.134**
Vomiting n (%)	3 (2.6)	3 (9.4)	-	**0.019****
Anosmia n (%)	2 (1.7)	1 (3.1)	1 (1.2)	0.474**
Headache n (%)	2 (1.7)	1 (3.1)	1 (1.2)	0.474**
Support in/Antiviral treatments n (%)				
Hydroxyqloroqine	117 (100)	32 (100)	85 (100)	-
Oseltamivir	117 (100)	32 (100)	85 (100)	-
Favipiravir	19 (16.2)	2 (6.3)	17 (20.0)	0.072*
Azithromycin	110 (94.0)	29 (90.6)	81 (95.3)	0.390**
Antibacterials	25 (21.4)	6 (18.8)	19 (22.4)	0.672*
Radiolojic involvement n (%)				
Bilateral	97 (83.6)	25 (78.1)	72 (84.7)	0.601*
Unilateral	19 (16.4)	6 (18.8)	13 (15.3)	
Mortality n(%)	4 (3.4)	-	4 (4.7)	0.574**

* Chi Square test, ** Fisher's Exact test, IQR =Interquartile range

http://dx.doi.org/10.36472/msd.v8i4.509

Table 2. Distribution of laboratory values according to H. pylori status in Patients with COVID-19

Parameters	All patient	H. pylori +	H. pylori -	p
WBC (10^3*µl) Median(1st-3rd quarter)	6.64 (4.47-8.59)	6.99 (4.71-8.57)	6.32 (4.44-8.68)	0.523*
Lymphocyte (10^3*µl) (Mean±SD)	1.68±0.79	1.71±0.88)	1.67±0.75	0.808**
Neutrophil (10^3*µl) Median(1st-3rd quarter)	3.89 (2.69-5.69)	4.00 (2.80-6.46)	3.89 (2.64-5.69)	0.709*
Platelet (10^3*µl) Median(1st-3rd quarter)	172.00(142.00-225.50)	172.00 (139.25-224.25)	172.00 (145.00-225.50)	0.976*
D-Dimer (ng/mL) Median(1st-3rd quarter)	429.00(283.50-987.75)	378.00 (280.00-714.00)	482.00 (280.00-1047.00)	0.315*
Ferritine (10^3*µl) Median(1st-3rd quarter)	221.00(100.50-543.50)	128.50 (66.70-542.00)	237.00 (125.00-543.50)	0.082*
Albumin (g/L) Median(1st-3rd quarter)	34.25 (32.68-37.65)	35.50 (32.20-37.83)	34.05 (32.68-37.50)	0.551*
LDH (U/L) Median(1st-3rd quarter)	269.00(212.50-354.50)	249.00 (195.50-332.25)	274.00 (219.50-359.00)	0.253*
Procalcitonine (ng/mL) Median(1st-3rd quarter)	0.05 (0.02-0.26)	0.06 (0.02-0.47)	0.05 (0.02-0.20)	0.762*
CRP (mg/L) Median(1st-3rd quarter)	23.30 (4.70-76.90)	20.25 (3.12-78.63)	25.80 (5.31-76.90)	0.446*

* Mann-Whitney U test, ** Independent-Samples T test, IQR =İnterquartile range, WBC: White blood cell. LDH: lactate dehydrogenase. CRP: C reactive protein.

DISCUSSION

To our knowledge, there is no study investigated the frequency of H. pylori and the prognostic significance of H. pylori positivity in patients with COVID-19 disease. In the present study, we determined a 27.35% of H.pylori positivity in patients with COVID-19. Our outcomes are much lower than the rates in the normal and non-COVID viral infectious population. The prevalence of H.pylori, which has lower rates in studies conducted in European countries, varies between 7-87% worldwide (**17**). In a recently published systematic meta-analysis, the mean positive frequency of H.pylori antigen in stool was found to be 49.4% 9. In a study conducted in our country, a prevalence rate of 25.2% was reported in stool H.pylori antigen test studies in the normal population (18).

Studies on the frequency of H. pylori in various non-COVİD-19 viral infections have been identified. For instance; information from epidemiologic works suggest that the frequency of H. pylori infection is clearly lower in HIV-positive compared with the HIV-negative population and that it further declines with the progression of immunodeficiency in HIV-infected patients. In a study of 1095 HIV-positive and 107 HIV-negative patients using the stool antigen test, the prevalence of H. pylori was significantly higher in the HIV-negative group (51.5% vs 88%, respectively, p< 0.05) (11).

In a meta-analysis evaluated 29 studies; all studies, except one study, reported a higher rate of H. pylori infection in HIV negative subjects (**12**). In contrast, another study found a higher prevalence of H. pylori resistant strain in HIV-positive patients than in HIV-negative patients (**13**). In addition, a meta-analysis including 2977 chronic hepatitis B and 1668 control patients, the H. pylori prevalence was found to be higher in patients with chronic hepatitis B positive. Also, the incidence of H. pylori has been shown to be positively correlated with HBV-associated hepatocellular carcinoma (**19**). However, the H. pylori prevalence in 235 asymptomatic HBV carriers, 573 alcohol users and 1637 non-alcoholic individuals was similar as 38.67%, 26.98%, and 35.94%, respectively (**20**).

Recently, the importance of gastrointestinal microbiota as a decisive for the systemic immune response has been recognized, and a number of extraintestinal, immune-related efficacious of H. pylori positivity have been reported (**21-22**).

With respect to the association between the immune system hypoactivation and H. pylori coinfection, it is known that H. pylori decrease markers (HLA DR, CD38, CD4) of the immune activation system by decreasing T-cell activation in HIV-positive and in HIV-negative individuals. This finding might explain the association of H. pylori infection in the intestine with favorable parameters of HIV disease progression (**23**). The immune response to H. pylori infection is predominantly T-cell mediated. It has been shown that the H. pylori vacuolating toxin directly inhibits T-cell activation by interfering with the maturation of antigen present cells and dendritic cells (**24**). It is known that lymphopenia is frequently reported in patients with COVID-19 and is considered to be a determinant of the prognosis of the disease. However, in our study, with respect to lymphopenia, no significant relationship was shown between the H.pylori positive patient group and the negative group. Various poor prognostic markers such as WBC, neutrophil, D-Dimer, ferritin, LDH, procalcitonin, high CRP, thrombocytopenia, and hypoalbuminemia have been demonstrated in COVID-19 infection (**25**). In our study, no significant difference was found in terms of these prognostic markers between groups with and without H. pylori positive. The most important limitation of the present study was that the immune T lymphocyte activation parameters were not detected in H. pylori positive COVID-19 patients.

CONCLUSION

In conclusion, according to the results of this study, H. pylori positivity or negativity neither determined the severity of the COVID-19 disease nor the poor prognostic indicators of the disease. However, larger and controlled studies are needed to confirm these findings.

Acknowledgement: We would like to thank Sakarya University scientific research projects (BAP, project number 2020-6-23-84) for supporting this project.

Author contributions: ABG, SY, HD, DS, EO, KI, DC, HK, CK, EK, AN, AT, MK, OK; Study design, Data collection, Literature search, Data analyzes **SY;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and a specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

Liver Fatty Acid Binding Protein: Is it an early diagnostic and prognostic marker in liver damage?

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ABSTRACT

Objective: The majority of the liver function tests are not specific to the liver. The histological liver damage begins before patients are diagnosed with cirrhosis and continue afterwards. Therefore, there is an increasing demand for early and specific markers that are correlated with liver damage. This study aims to investigate if serum and urinary liver fatty acid-binding protein (L-FABP) levels could be used as an early diagnostic marker of liver cirrhosis.

Material and Methods: This cross-sectional study included 30 patients with compensated liver cirrhosis, 27 patients with decompensated liver cirrhosis, and 30 healthy controls. The patients and healthy controls were tested for serum and urinary L-FABP levels.

Results: The serum and urinary L-FABP levels were higher in patients with cirrhosis than the healthy controls (both $p<0.001$). The cut-off value of serum and urinary L-FABP was computed as 721.78 ng/ml and 621.25 ng/ml, respectively. The sensitivity of serum and urinary L-FABP to detect cirrhosis at this cut-off was 99.8% and 98.9%. The specificity, positive predictive value, and negative predictive value of serum and urinary L-FABP at these cut-off levels were 100%. There was no difference in terms of serum and urinary L-FABP between compensated and decompensated cirrhosis patients. Accordingly, no correlation was determined between serum/urinary L-FABP levels and cirrhosis complications.

Conclusion: L-FABP increases in serum and urine in response to hepatocyte damage that can result in liver fibrosis. We demonstrated that patients with liver cirrhosis had high L-FABP levels. L-FABP may be used as a predictive non-invasive marker of cirrhosis as it can be detected before the clinical symptoms of liver damage.

Keywords: biomarker, cirrhosis, FABP1, L-FABP, liver fatty acid-binding protein

Research Article

Received 18-03-2020

Accepted 12-04-2021

Available Online: 14-04-2021

Published 30-04-2021

INTRODUCTION

Chronic liver disease (CLD) is a global health problem that affects more than 800 million people worldwide (1). The most common etiologies of CLD are hepatitis C virus (HCV), hepatitis B virus (HBV), non-alcoholic fatty liver disease (NAFLD) and alcohol consumption. Regardless of etiological factors, the prevalence of liver cirrhosis in the general population was found to be 0.27% (2). However, considering that a significant proportion of patients are asymptomatic and are only diagnosed with cirrhosis as premortem, it is estimated that the prevalence is higher than it is expected (3). Liver cirrhosis can cause high morbidity and mortality both by its complications as well as hepatocellular carcinoma. Although liver biopsy is still the gold standard method for the diagnosis of cirrhosis, it has some disadvantages because it is an invasive method. Further, false-negative results could be possible in the early stages of cirrhosis. In this regard, researches for inexpensive, safe, specific, and repeatable non-invasive disease markers are proceeding. APRI (AST-Platelet Ratio Index), FIB 4 (Age, AST, platelet count, and ALT), Fibroindex (platelet count, AST, and GGT) are some of the clinically accepted biochemical panels for fibrosis assessment.

In addition to these indirect indicators; hyaluronic acid (HA), tissue inhibitor of metalloproteinase -1 (TIMP -1), amino-terminal of serum procollagen Ⅲ peptide (PIIINP), and a bacterial enzyme as known chondrex (YKL-40) have also been found to reflect extracellular matrix turnover and are directly involved in fibrogenesis. However, all these markers also reflect inflammation and fibrosis in other organs that is why they are not specific to the liver. Besides, they are correlated with advanced fibrosis, but not with early-stage fibrosis (**4**).

Fatty acid-binding proteins (FABPs) are small cytoplasmic proteins that can bind many hydrophobic ligands such as fatty acids and cannot be detected in the serum as they do not possess a secretory signal physiologically. Some FABPs can be used as a diagnostic marker of tissue damage for many organs, including the liver (**5**). Liver FABP (L-FABP) is mainly expressed in the liver and is predominantly considered for liver damage. It comprises 2-5% of the cytosolic proteins in the liver tissue and is involved in the uptake, transport, oxidation, lipid synthesis, storage of fatty acids and the regulation of nuclear receptors (**6**). The L-FABP levels in hepatocytes are altered in response to physiological and pharmacological changes. It increases in parallel to the free oxygen radicals in the cell microenvironment and mediates systemic inflammation by increasing interleukin-6 and interleukin-1α release **(7, 8**). L-FABP can be considered as an early marker for hepatocellular damage while it is released into the circulation quickly due to being small proteins. In the present study, we aimed to investigate whether serum and urinary L-FABP levels could be used as a predictive non-invasive marker of liver cirrhosis and its complications.

MATERIAL and METHODS

Study population and biochemical assessments

This cross-sectional study included 57 patients with a diagnosis of cirrhosis in a hepatology department in a tertiary university hospital between October 2017 – April 2018 and healthy controls. Three groups were defined as follows: compensated cirrhosis (CC) (n=30), decompensated cirrhosis (DC) (n=27), and healthy controls (HC) (n=30). HC consisted of healthy volunteers working in the hospital without comorbid diseases. Patients with radiological (nodular appearance of the liver surface, parenchymal thickening, caudate lobe enlargement, portal vein diameter >13 mm) and endoscopic (varices, portal gastropathy) findings of cirrhosis were considered CC. Patients with ascites, hepatic encephalopathy (HE) or variceal hemorrhage were considered DC and these signs were indicated as a present or not. The exclusion criteria of the study were defined as malignancies, active systemic infections, acute cerebrovascular diseases, chronic kidney disease, psychiatric diseases, and nephrotoxic and sedative medication use. Hepatocellular carcinoma (HCC) was ruled out by radiological evaluation in cirrhotic patients. Demographic variables were recorded for all groups, and Child Turcotte-Pugh (CTP) classification and Model for End-Stage liver disease (MELD) score were calculated for patients with cirrhosis.

All patients and healthy controls were tested for complete blood count parameters, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), albumin, total bilirubin, international normalized ratio (INR), creatinine, serum, and urinary L-FABP levels (ELISA kit; Sun Red, 201-12-2160, China, Shanghai).

Statistical analysis

Statistical data were analyzed using SPSS v.23.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were presented as mean ± standard deviations for continuous variables. Variables were tested for normality using the Kolmogorov-Smirnov test. The Mann-Whitney U test and Kruskal -Wallis H were used to compare nonparametric variables and the chi-squared test was used to compare categorical variables. While investigating the associations between non-normally distributed variables, the correlation coefficients and their significance were calculated using the Spearman test. When a significant cut-off value was observed, the sensitivity, specificity, positive and negative predictive values were presented. A p-value ⩽ of 0.05 was considered statistically significant.

Approval received from the local university ethics committee with the numbers 2017-09/07.

RESULTS

The mean age of the patients and healthy controls included in the study were 67.70 ± 11.32 and 59.67 ± 11.77 years. The mean age of patients with DC was 65.78±11.63 years, and they were older than CC patients and healthy controls. However, there was no statistical difference in age between the groups (p=0.144). The number of men in the study was 48 (55.2%). The male gender ratio in patients with decompensated cirrhosis was 77.8%, and it was found to be significantly higher than other groups (p=0.013). The etiologies of cirrhosis were HBV (n=19), HCV (n=11) and nonalcoholic fatty liver disease (NAFLD) (n=27). The distribution of DC patients according to CTP classification was found as CTP-B (59.3%), CTP-C (40.7%). Hepatic encephalopathy, ascites and variceal hemorrhage were found in 11 (40.7%), 17 (63%) and 7 (25.9%) patients with DC, respectively. Meld Score was calculated as 10.87± 3.80 and 16.89 ±6.41 for CC and DC patients, respectively. The clinical characteristics of the study groups are presented in Table 1.

Mean serum L-FABP were determined as 1273.42± 349.72, 1349.83 ± 321.44, 355.83±32.52 ng/ ml for CC, DC, and healthy controls, respectively (p<0.001). Mean urinary L-FABP were determined as 1450.92± 336.53, 1480.71 ± 178.74, 445.78±24.53 ng/ ml for CC, DC, and healthy controls, respectively (p<0.001). There was no difference between CC and DC cirrhosis in terms of serum and urinary L-FABP levels (p=0.212, p=0.898) (Table 2).

The cut-off value for serum L-FABP was determined as 721.78 ng/ml. For this value, serum L-FABP had a sensitivity of 99.8% and a positive predictive value of 100%. The specificity and the negative predictive value at the stated cut-off value were found as 100%. The cut-off value for urinary L-FABP was determined as 621.25 ng/ml. For this value, urinary L-FABP had a sensitivity of 98.9% and a positive predictive value of 100%.

The specificity and the negative predictive value at the stated cut-off value were found as 100% (Table 3).

Serum and urinary L-FABP levels were statistically shown to have a positive correlation with AST, GGT, INR, total bilirubin, and a negative correlation with haemoglobin, thrombocyte and leukocyte count, and albumin (all p<0.05). Urinary L-FABP but not serum L-FABP positively correlated with creatinine levels. There were no correlations between the MELD score and both serum and urinary L-FABP levels (Table 4).

Serum and urinary L-FABP levels did not differ statistically in terms of cirrhosis etiology. Serum L-FABP was determined as 1490.47±369.09, 1246.36±279.90, and 1208.12±286.71 in patients with cirrhosis with HBV, HCV, NAFLD, respectively (p=0.060). Urinary L-FABP was determined as 1516.99±307.13, 1460.11±291.55, and 1430.48±223.91 in patients with cirrhosis with HBV, HCV, NAFLD, respectively (p=0.681). Besides, there were no correlations between serum/ urinary L- FABP levels and the presence of HE, ascites, and varices (all p>0.05).

Table 1. Clinical characteristics of the study population

	CC (n=30)	DC (n=27)	HC (n=30)	P
Age (years) ±SD	63.73 ± 11.4	65.78 ± 11.63	59.67 ± 11.77	0.144
Sex, male, n (%)	15 (50)	21 (77.8)	12 (40)	0.013
Etiology, n (%)				
HBV	11 (36.7)	8 (29.6)		
HCV	7 (23.3)	4 (14.8)		
NAFLD	12 (40)	15 (55.6)		
CTP grade, n (%)				
A	30(100)			
B		16(59.3)		
C		11(40.7)		
Cirrhosis Complications, n (%)				
Hepatic encephalopathy		11(40.7)		
Ascites		17 (63)		
Variceal hemorrhage		7 (25.9)		
Meld Score ±SD	10.87 ± 3.80	16.89 ± 6.41		

CC, Compensated cirrhosis; CTP, Child Turcott Pugh; DC, Decompensated cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus; HC, Healthy controls; MELD, Model for End-Stage liver disease; NAFLD, non-alcoholic fatty liver disease; SD, standard deviation

Table 2. Laboratory tests of the study population

Parameters (mean±SD)	CC	DC	HC	p*
Wbc (10^3/mm^3)	3791.00±1225.26	3594.44±1275.49	7681.20±1975.96	0.000
Hb (g/dl)	11.96±2.72	10.13±2.28	15.69±1.14	0.000
Plt (10^3/mm^3)m	115.87±45.39	115.19±60.07	266.63±65.59	0.000
AST (U/L)	38.83±31.20	46.67±26.81	20.63±9.80	0.000
ALT (U/L)	29.23±38.68	29.89±19.76	20.90±10.05	0.304
GGT(IU/L)	99.03±140.13	81.15±45.63	36.67±21.12	0.000
T.Bil (mg/dL)	1.13±1.42	3.76±4.44	0.69±0.31	0.001
Albumin (g/L)	3.58±0.64	2.89±0.58	4.15±0.55	0.000
INR	1.26±0.22	1.56±0.42	1.02±0.05	0.000
Cre (mg/dL)	1.06±0.98	1.34±0.89	0.92±0.14	0.633
Urinary L-FABP (ng/ml)	1450.92±326.53	1480.71±178.74	445.78±24.53	0.000
Serum L-FABP (ng/ml)	1273.42±349.72	1349.83±321.44	355.83±32.52	0.000

***: the difference between patients and healthy participants,** AST, aspartate aminotransferase; ALT, alanine aminotransferase; CC, Compensated cirrhosis; Cre, creatinine; DC, Decompensated cirrhosis; GGT, gamma-glutamyl transferase; Hb, Hemoglobin; INR, international normalized ratio; L-FABP, liver fatty acid-binding protein; MELD, Model for End-Stage liver disease Plt, Thrombocyte; SD, standard deviation; T.Bil: Total bilirubin; Wbc: Leukocyte

Table 3. Cut-off values of serum and urinary L-FABP

L-FABP (ng/ml)	Cut -off	Sensitivity	Specificity	PPV	NPV	Accuracy
Urinary	621.25	98.9%	100%	100%	100%	98.9%
Serum	721.78	99.8%	100%	100%	100%	99.8%

L-FABP, liver fatty acid-binding protein; NPV, negative predictive value; PPV: positive predictive value

http://dx.doi.org/10.36472/msd.v8i4.516

Table 4. Correlation of serum and urinary L-FABP levels with other laboratory tests

	L-FABP (ng/ml)			
	Urinary		Serum	
	r	*p*	*r*	*p*
WBC (10^3/mm^3)	-0.608	**<0.001**	-0.612	**<0.001**
Hb (g/dl)	-0.599	**<0.001**	-0.585	**<0.001**
Plt (10^3/mm^3)	-0.627	**<0.001**	-0.581	**<0.001**
AST (U/L)	0.348	**<0.001**	0.349	**<0.001**
ALT (U/L)	0.065	0.548	0.113	0.296
GGT (IU/L)	0.284	**<0.001**	0.366	**<0.001**
T.Bil (mg/dL)	0.344	**<0.001**	0.248	**<0.001**
Albumin (g/L)	-0.433	**<0.001**	-0.466	**<0.001**
INR	0.570	**<0.001**	0.501	**<0.001**
Cre (mg/dl)	0.114	**<0.001**	0.136	**0.208**
Meld Score	0.030	0.822	-0.039	0.772
Urinary L-FABP (ng/ml)	1.000	-	0.754	**<0.001**
Serum L-FABP (ng/ml)	0.754	**<0.001**	1.000	-

AST, aspartate aminotransferase; ALT, alanine aminotransferase; Cre, creatinine; GGT, gamma-glutamyl transferase; Hb, Hemoglobin; INR, international normalized ratio; L-FABP, liver fatty acid-binding protein; Plt, Thrombocyte; T.Bil: Total bilirubin; WBC: Leukocyte

DISCUSSION

L-FABP is an intracellular carrier protein that is synthesized in hepatocytes, and to some degree, in proximal tubule epithelial cells (**9**). Recently, urinary L-FABP is thought to be a useful clinical marker of acute kidney damage in the early period and it could play a role in the prediction of chronic kidney damage and the monitoring of the progression (**10, 11**). Similarly, cellular L-FABP levels are altered due to the changes in cellular lipid metabolism homeostasis in liver diseases such as cirrhosis, hepatitis, iron and copper accumulation, porphyria, and hepatocellular carcinoma (**12**). Besides chronic liver diseases, L-FABP levels were found to be high in acute liver injury that develops secondary to alcohol- or drug-induced toxicity (**13**). In acute hepatocellular damage induced by acetaminophen toxicity, serum L-FABP levels were shown to be negatively correlated with the survey (**14**). Further, L-FABP could be detected in the plasma earlier than alpha glutathione S-transferase (α-GST) and ALT, and that it was more sensitive in detecting hepatocellular damage due to rejection (**15**). Accordingly, its levels were shown to be a novel marker of early parenchymal damage following liver transplantation (**16**). However, there is a limited number of studies in the literature regarding the cut-off levels of L-FABP in acute or chronic liver disease.

In the present study, we found significantly higher serum and urinary L-FABP levels in patients with cirrhosis compared to the healthy control group ($p<0.001$). We determined the cut-off values as 721.78 ng/ml and 621.25 ng/ml for serum and urinary L-FABP, respectively. (Serum AUC= 0.998 and urinary AUC= 0.989). A study that compared patients with acute hepatitis, HE, and CC to healthy controls indicated lower cut-off values for both serum and urinary L-FABP (serum AUC= 0.985 and urinary AUC=1.000) (**17**). We think that these different cut-off levels with similar accuracy may be related to the heterogeneity of the patient groups and the higher number of cirrhosis patients in our study. In some of the heterogeneous validation studies investigating serum biomarkers for detection of cirrhosis; a significant correlation with advanced fibrosis was observed (AUC of HA=0.91, TIMP-1=0.92, PIIINP=0.88, and YKL-40=0.79) (**18-21**).

Although the importance of L-FABP in the pathogenesis of fibrosis is not clear yet, we think that it may be an encouraging marker for the presence of cirrhosis due to its high accuracy rates.

We determined higher serum and urinary L-FABP levels in NAFLD cirrhosis when compared with the healthy controls, although these levels were lower than those in patients with cirrhosis with viral hepatitis. L-FABP bears a critical effect on the ligand-dependent transactivation of peroxisome proliferator-activated receptor α (PPARα) which regulates the transcription of multiple genes involved in the lipid metabolism included in NAFLD pathogenesis (**22, 23**). Since the correlation between FABP levels and fibrosis/inflammation was shown in patients with non-alcoholic steatohepatitis, L-FABP has been introduced to be used as a non-invasive marker (**24**).

We also determined positive correlations between L-FABP (serum/urinary) and AST, GGT, total bilirubin and INR, a negative correlation with albumin. In the present study, creatinine levels were in the normal range and likely higher in patients with CC and DC, respectively. Additionally, there was a weak positive correlation between creatinine and urinary L-FABP but not serum L-FABP. A study conducted on patients who presented to the emergency service for any reason and showed normal serum creatinine levels at presentation showed that urinary L-FABP levels were predictive of acute kidney damage (**25**). Meanwhile, the significantly lower serum L-FABP levels seen in hemodialysis patients with the end-stage renal disease compared to healthy controls are thought to be linked to the decrease in the production of L-FABP in the liver due to severe renal failure (**8**). On the other hand, L-FABP levels were found to have no predictive value for the development of hepatorenal syndrome in decompensated cirrhosis patients (**26**).

In our study, there was no difference between CC and DC in terms of serum and urinary L-FABP. Also, there were no correlations between L-FABP levels and CTP stages, MELD score, and presence of cirrhosis complications such as HE,

ascites, and variceal hemorrhage. However, the present study included only two patients with hepatic coma, thirteen patients with tense ascites, and seven patients with variceal hemorrhage. Accordingly, we cannot completely suggest that L-FABP does not reflect the prognosis of liver cirrhosis due to low patient numbers.

Our study has some limitations. First, all patients had clinical cirrhosis and the liver biopsy was not performed. Second, the patient population is small to evaluate the prognostic importance of L-FABP levels. Third, it is a cross-sectional study that sequential measurements are not warranted.

CONCLUSIONS

In conclusion, L-FABP increases in serum and urine in response to the altered membrane permeability following hepatocyte damage that can result in liver fibrosis. L-FABP may be used as a predictive non-invasive marker of cirrhosis as it can be detected before the clinical symptoms of liver damage. However, L-FABP was not found to be related to the complications of cirrhosis. Further studies must be conducted to determine exact cut-off values and allow L-FABP to be recognized as a specific liver function test in the coming years.

Acknowledgments: We appreciate Selim Çam for his great help in the analysis of the statistics.

Author contributions: PG, EC, HOD, SNY; Literature search and study design, statistical analyzes, **PG, GA, OO, HLD, KO;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding: This work is supported by the Scientific Research Project Fund of Cumhuriyet University under T-761.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

The importance of flowcytometry study with the first aspirate taken during bone marrow aspiration in the diagnosis of multiple myeloma and follow-up of minimal residual disease

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ABSTRACT

Objective: Flow cytometry (FC) is a diagnostic method supporting traditional morphological examination in disease follow-up and the diagnosis of Multiple myeloma (MM). Normal and atypical plasma cells (PCs) can be told apart from each other by means of FC method. The plasma cell rate is the highest in the blood obtained in the first aspirate during bone marrow aspiration in MM.

Material and methods: A total of 60 patients that have been diagnosed with MM between 2018 and 2020, including 30 patients whom flow cytometry was studied with the first aspirate during bone marrow aspiration, and 30 patients whom FC was studied with the second aspirate were included in our study. The characteristics of the patients were analyzed retrospectively from their files.

Results: The median ratio of plasma cells (PCs) detected by FC and bone marrow biopsy was 17,5% and 44%, respectively. While this rate was median 37,5% in patients that flow cytometric study was performed with the first aspirate, the rate was found to be median 7% in patients that FC was performed with the second sample. The PCs rates were statistically significantly higher with the flow cytometric study with the first aspirate than the second one (p=0.000).

Conclusion: Flow cytometric study with the first aspirate during bone marrow aspiration in patients with MM is diagnostically important.

Keywords: Multiple myeloma, flow cytometry, bone marrow aspiration, first aspirate

INTRODUCTION

Multiple myeloma (MM) is a malignant neoplasm presenting with anemia, renal failure, and bone lesions due to the increase in atypical plasma cells in the bone marrow and the accumulation of excessive monoclonal proteins secreted from these cells in serum and urine and causing end organ damage (1). Biochemical parameters, immunofixation studies in serum and urine samples have a great place in the diagnosis of MM (1). Previously, the diagnosis of MM and the decision to start treatment was determined according to the CRAB (hypercalcemia, renal insufficiency, anemia, bone lesions) criteria, together with the bone marrow plasma cell ratio being > 10% (2). The International Myeloma Working Group (IMWG) revised these criteria in 2014 and added new biomarkers to the existing criteria that identify asymptomatic patients at high risk of progression (3). Among these criteria, clonal plasma cells (PC) in the bone marrow 60%, the ratio of free light chains (FLC) 100, and more than 1 focal lesion larger than 5 mm on MRI were accepted as treatment criteria. (3). The definitive diagnosis of MM is made by histopathological examination of the bone marrow biopsy material and microscopic examination of the bone marrow sample taken by bone marrow aspiration together with flow cytometric study (1).

Flowcytometry (FC) is a diagnostic method that supports traditional morphological examination in the diagnosis of MM and disease follow-up (4). With the FC method, normal and atypical plasma cells are distinguished from each other (4-6).

Research Article

Received 22-03-2020

Accepted 04-04-2021

Available Online: 05-04-2021

Published 30-04-2021

It contributes to the evaluation of the possibility of progression to MM, one of the precursor forms that do not require treatment of FC, evaluation of the prognosis, the response to treatment and the minimal residual disease (MRD) (7-9).

Under normal conditions, bone marrow sample taken during bone marrow aspiration for diagnosis of hematological malignancies is aspirated in three stages. In the first stage, 0.5 cc of blood aspirated is spread on the slide and subjected to staining process and used in microscopic examination. Flowcytometric study is performed with 1-2 cc marrow sample taken in the second stage. 2-3 cc marrow sample taken at the last stage is sent to the laboratory for cytogenetic studies. The rate of PCs is highest in the blood taken at the first stage of MM. The proportion of PCs in the bone marrow sample taken in the later stages gradually decreases. This causes less malignant cells to be detected, especially in flowcytometric studies. This results in the detection of fewer PCs both at the time of diagnosis and during post-treatment bone marrow evaluation. Especially nowadays, MRD studies with FC have become more important.

In this study, we aimed to compare the PC rates of the patients who underwent flowcytometric study with the first marrow sample in the bone marrow aspiration performed at initial diagnosis and the PC rates of the patients who underwent flowcytometric study with the second sample and to examine the diagnostic importance.

MATERIAL and METHODS

A total of 60 patients were included in our study, including 30 patients who were diagnosed with MM between 2018-2020, who underwent a flowcytometric study with the first marrow sample during bone marrow aspiration, and 30 patients who underwent flowcytometric study with the second bone marrow sample. The characteristics of the patients were analyzed retrospectively from their files. The sample taken for FC was taken with the first aspirate in the first group and with the second aspirate in the second group during aspiration.

Flowcytometry: Diagnosis with FC in MM and MRD evaluation depends on the detection of immunophenotypic abnormalities in malignant plasma cells rather than clonality evaluation with light chain analysis (10). Normal plasma cells express CD19, CD45, CD138 and bright CD38 and are negative for CD20 and CD56. In MM, PCs generally show abnormal CD56 expression and loss of CD19, and less frequently abnormal CD20, CD28 and CD117 expression (10-12). Panels recommended for disease monitoring in MM, therefore, minimally include the evaluation of CD19 and CD56, because abnormal expression of these antigens should identify neoplastic plasma cells at follow-up in at least 90% of patients with MRD (4, 13).

Analysis was performed on the bone marrow aspirates of patients with a diagnosis of MM or suspected of having MM who were sent for routine diagnostic analysis in our laboratory. Eight-color multiparametric was performed on bone marrow mononuclear cells isolated with the FC Ficoll gradient and stained with antibodies against CD45, CD19, CD38, CD56, CD138 and cytoplasmic kappa and lambda immunoglobulin light chains. Data were collected using BD FACSCanto II devices that collected 150,000 events; FC data were analyzed using BDFacs DIVA Software. Figure 1 shows the FC result of a patient diagnosed with MM.

Statistical Methods: Mean, standard deviation, median, minimum, maximum value frequency and percentage were used for descriptive statistics. The distribution of variables was checked with Kolmogorov-Simirnov test. Kruskal-Wallis and Mann-Whitney U test were used for the comparison of quantitative data. Wilcoxon test were used for the repeated measurement analysis. Chi-Square test was used for the comparison of the qualitative data. SPSS 27.0 was used for statistical analysis.

RESULTS

Twenty-eight (46.7%) of the patients included in the study were female and 32 (53.3%) were male. The median age of the patients was 63 years. Median PC values determined by FC and bone marrow biopsy (BMB) at initial diagnosis, median biochemical results, median involvement with PET / CT, MM subtype, treatment preferences, (and autologous stem cell transplantation (ASCT) if it was performed) and the status of the patients at the last control are summarized in Table 1.

When all patients were evaluated, the median ratios of PCs detected by FC and BMB were 17.5% and 44%, respectively. While this ratio was 37.5% in patients who had FC with the first aspirate, the median was found to be 7% in patients who had FC with the second sample. The PC ratios of the patients who had FC with the first aspirate were statistically significantly higher than the PC ratios detected by FC with the second aspirate ($p = 0.000$). In both groups, the number of PC detected by BMB was higher than that those determined by FC (Figures 1 and 2.), but the PC ratios detected by BMB were similar to the PC ratios obtained by FC with the first aspirate (figure 2). However, the ratios of PC obtained by FC with the second aspirate was significantly lower than the PC ratios detected by BMB ($p <0.05$) (Table 2). In both of the groups in which FC was performed with the first and second aspirate, the ratios of PC detected in BMB were 49.5% and 30%, respectively. There was no statistically significance ($p = 0.261$) (Table 2).

There was no statistically significant difference between patients with remission, refractory disease or with a mortal course in terms of patients' age ($p = 0.490$), gender distribution ($p = 0.087$), PC ratios by FC and BMB ($p = 0.078$ and $p = 0.829$, respectively), biochemical findings and PET / CT involvement. In the exitus group, the first sample PC ratio by FC was significantly lower than the remission and refractory groups ($p = 0.003$). In the group with remission, the first sample PC by FC ratio was significantly lower than the refractory group ($p <0.05$). The lines of treatments in the refractory group were significantly higher than in the exitus and remission group ($p = 0.019$). The proportion of patients who underwent ASCT in the remission group was significantly higher than the exitus and refractory groups ($p = 0.000$). The proportion of patients who underwent ASCT did not differ significantly ($p> 0.05$) between the exitus and refractory groups (Table 3).

http://dx.doi.org/10.36472/msd.v8i4.520

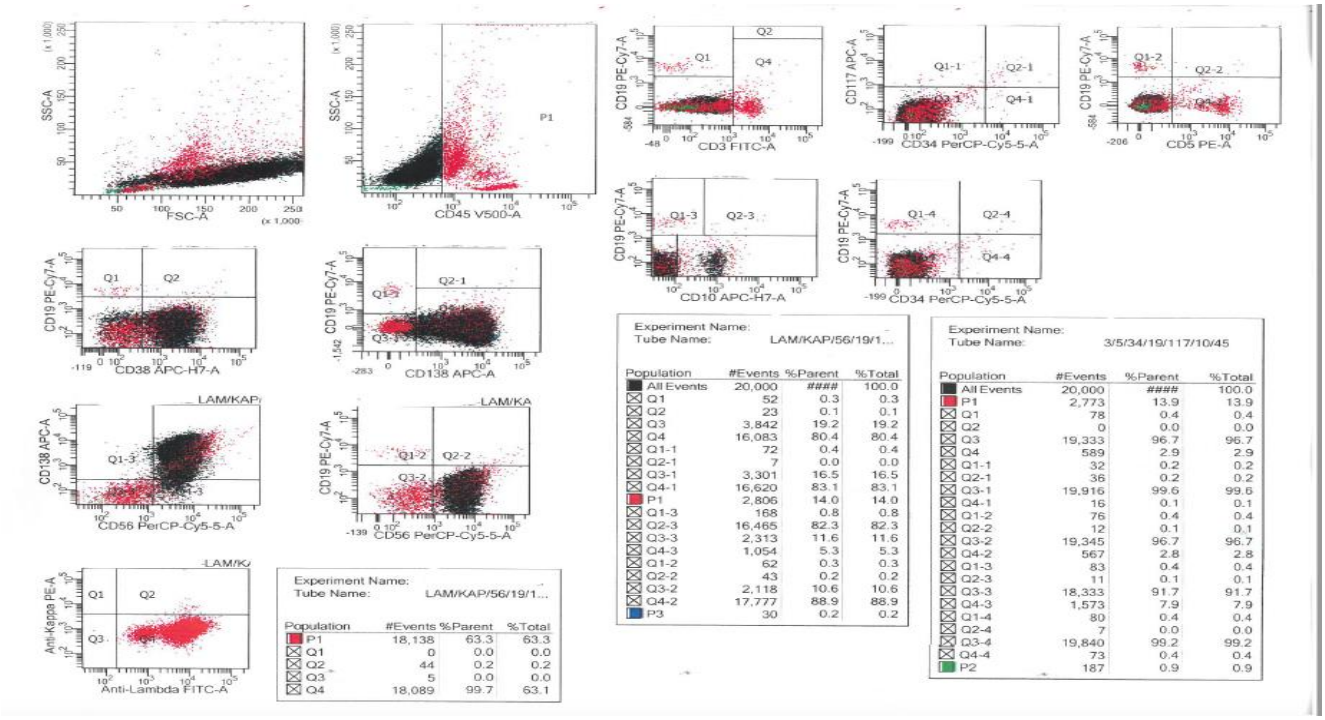

Figure 1: FC result of a MM patient (Analysis on all cells revealed 83.1% abnormal clonal plasma cell population showing CD45-, CD117-, CD38+, CD138+, CD56+, CD19-, Anti-Lambda + immunophentotypic features. These findings are consistent with MM).

Table 1: Demographic characteristics of patients at the time of diagnosis

		Min-Max			Median	Mean±sd/n-%		
Age		40,0	-	85,0	63,0	63,4	±	10,3
Gender	Female					28		46,7%
	Male					32		53,3%
Aspiration	First aspirate					30		50,0%
	Second aspirate					30		50,0%
PC ratio by FC		1,2	-	83,0	17,5	24,8	±	21,2
PC ratio by BMB		10,0	-	90,0	44,0	46,9	±	21,1
Hb		5,0	-	16,0	9,9	9,9	±	2,0
Creatinin		0,5	-	6,8	1,3	2,1	±	1,8
Ca		7,4	-	16,0	9,7	10,1	±	2,0
LDH		100	-	760	188	214	±	110
PET/CT, SUV-MAX		2,0	-	14,5	5,1	6,3	±	3,2
MM Type								
Ig A Kappa						3		5%
Ig A Lambda						3		5%
Ig G Kappa						20		33,3%
Ig G Lambda						7		11,7%
Kappa light chain						19		31,7%
Lambda light chain						8		13,3%
Treatment								
VEL-DEX						1		1,7%
VCD						44		73,3%
VCD/RD						6		10,0%
VCD/VRD						5		8,3%
VCD/VRD-KRD						2		3,3%
VCD/VRD/VRD+DARA						1		1,7%
VEL-DEX/RD						1		1,7%
Treatment line	I					44		73,3%
	II					13		21,7%
	III					3		5,0%
ASCT	(-)					30		50,0%
	(+)					30		50,0%
Remission						38		63,3%
Refractory						10		16,7%
Exitus						12		20,0%

PC: Plasma cell, FC: Flow cytometry, BMB: Bone marrow biopsy, Hb: Hemoglobin, LDH: Lactate dehydrogenase, PET/CT: Positron emission tomography/computorised tomography, Ig: Immunglobulin, VEL-DEX: Bortezomib-Dexamethasone, VCD: Bortezomib-Cyclophosphamide-dexhamethasone, RD: Lenalidomide-Dexhamethasone, VRD: Bortezomib, lenalidomide-dexhamethasone, DARA: daratumumab, ASCT: Autologous stem cell transplantation

http://dx.doi.org/10.36472/msd.v8i4.520

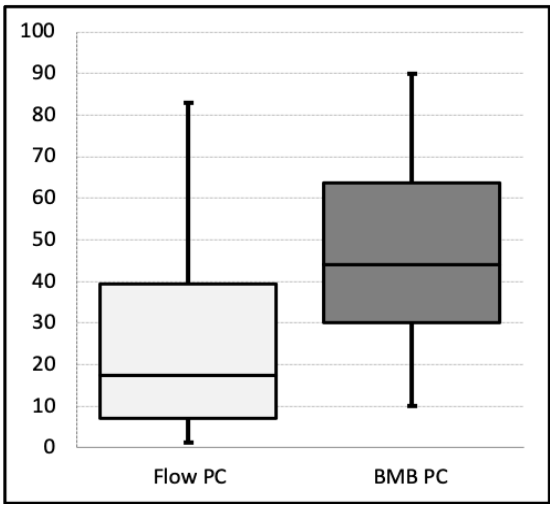

Figure 2: Comparison of plasma cell ratios determined by bone marrow biopsy and flow cytometry in all patients

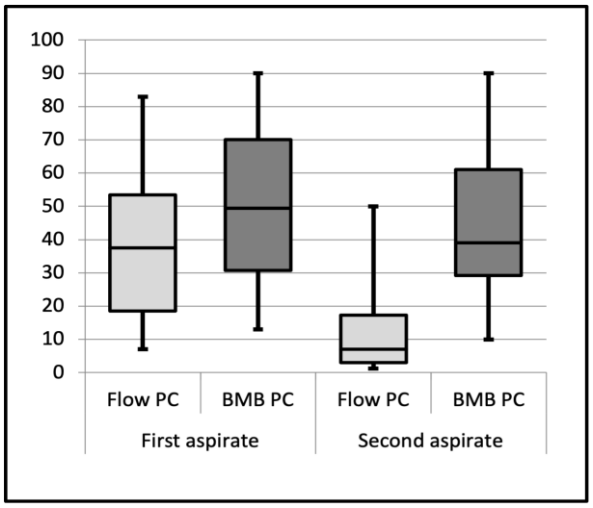

Figure 3: Comparison of plasma cell ratios of the patients whom FC was studied with the first aspirate and the plasma cell ratios of the patients whom FC was studied with the second aspirate

Table 2: Differences of FC study done with first aspirate and second aspirate

	First aspirate		Second aspirate		p	
	Mean±sd	Median	Mean±sd	Median		
PC ratio by FC	37,93 ± 20,25	37,50	11,68 ± 12,07	7,00	***0,000***	m
PC ratio by BMB	50,07 ± 21,59	49,50	43,68 ± 20,43	39,00	0,261	m
Difference	12,13 ± 10,61	10,50	32,00 ± 16,99	30,50	***0,000***	m
Intra Group Difference	***0,000***	w	***0,000***	w		

PC: Plasma cell, FC: Flowcytometry, BMB: Bone marrow biopsy, [m] Mann-whitney u test/ [w] Wilcoxon test

Table 3: FC and BMB and PC ratios in remission, retractory and exitus groups

	Remission		Refractory		Exitus		p	
	Mean±sd/n-%	Median	Mean±sd/n-%	Median	Mean±sd/n-%	Median		
Age	63,68 ± 10,15	63,00	60,67 ± 11,98	60,00	64,33 ± 10,20	68,50	0,490	K
Gender Female	18 47,4%		4 40,0%		6 50,0%		0,887	X^2
Male	20 52,6%		6 60,0%		6 50,0%			
PC by FC	24,54 ± 23,02	15,00	34,30 ± 18,18	33,00	17,73 ± 14,64	14,00	0,078	K
PC by BMB	45,14 ± 23,56	35,50	45,30 ± 18,30	42,00	53,67 ± 13,47	48,00	0,829	K
Hb	9,80 ± 2,17	9,55	10,57 ± 1,44	10,30	9,49 ± 1,82	9,30	0,182	K
Creatinin	1,92 ± 1,71	1,20	2,31 ± 2,08	1,20	2,60 ± 2,09	1,90	0,849	K
Ca	10,14 ± 2,07	9,50	8,99 ± 1,11	9,35	10,66 ± 2,09	10,00	0,199	K
LDH	204,4 ± 96,1	177,0	210,8 ± 63,2	203,5	244,6 ± 171,2	223,5	0,328	K
PET/CT SUVMAX	6,28 ± 3,26	5,30	6,47 ± 2,90	5,85	6,43 ± 3,73	4,95	0,790	K

[K] Kruskal-wallis (Mann-whitney u test) / X^2 Chi-sqaure test, PC: Plasma cell, FC: Flow cytometry, BMB: Bone marrow biopsy, Hb: Hemoglobin, LDH: Lactate dehydrogenase, PET/CT: Positron emissiom tomography/computorised tomography, ASCT: Autologous stem cell transplantation

DISCUSSION

Today, FC is an indispensable method in the diagnosis, treatment and follow-up of plasma cell neoplasms (PCN) together with histopathological and biochemical analyzes. In order to examine PCNs using FC, it is necessary to distinguish the immunophenotype of normal and neoplastic plasma cells (**14**). Numerous studies have been reported on the immunophenotype of PCs and the immunophenotype of polyclonal PCs (**4,7,15-17**). In addition, in order to determine the effectiveness of the treatment, the detection of MRD by FC method has been investigated in several studies (**13,18,19**).

The place of FC in the diagnosis of MM and follow-up of MRD is indisputable. Our aim in this study is not only to reinforce the importance of FC in the diagnosis of MM, but also to determine the contribution of FC with the first marrow sample taken during bone marrow aspiration. To best of our knowledge, our study is first in this field.

Pavia B. et al. revealed that FC and MRD monitoring is one of the most important prognostic factors in elderly MM patients, and cytogenetic risk is complementary and superior to traditional response criteria (**20**).

Cannizzo et al. showed that FC correlates with histopathological studies (14). In our study, PC ratios in patients who had FC with the first aspirate were similar to PC ratios determined by BMB. However, we found that the PC ratio was statistically significantly lower in patients who had FC with the second aspirate compared to BMB. While the median PC ratio was 37.5% in the patients whom FC was studied with the first aspirate, it was found 7% those studied with the second aspirate. In samples obtained by BMB, they were found to be 49.5% and 39% in both groups, respectively.

Nadav et al. showed that FC in MM significantly underestimated the number of PCs compared to counting PCs in aspirate smears. They also provided evidence suggesting that the reason for this underestimation was that FC samples lacked spicules to which MM cells were attached (21).

Terpstra et al. showed that PC was lower in aspirate smears when bone marrow aspirate smears were compared with BMB (22). In our study, the ratios of PCs by FC were found to be lower than BMB in both groups. However, although PC ratio was lower in first aspirate FC results than BMB, it was not statistically significant. The ratio of PC obtained in FC with the second aspirate was statistically lower than the ratio of PC detected by BMB ($p < 0.05$).

Ely S. et al. showed that in MM, their PCs bind strongly to other MM cells and stroma, as they contain large amounts of adhesion factors such as CD138 and CD56 (23).

Probably for this reason, some of the PC's cannot be detected in the aspiration material and less PC is detected in FC compared to BMB. Therefore, it is very important to perform a FC with the first sample during bone marrow aspiration. Because, when the FC is not performed with the first aspirate, the marrow sample becomes diluted at every stage. In addition, the adhesion property of PCs increases in the invitro environment. As a result, the PC ratio is detected lower than expected, which reduces the diagnostic efficiency of FC.

CONCLUSION

Flowcytometric studies are an indispensable diagnostic method in addition to histopathological and biochemical studies in the diagnosis of MM. It has also proven its importance in MRD follow-up. In our study, we aimed to show that performing a FC with the first aspirate sample contributes to the diagnosis. However, prospective studies are needed in larger patient groups.

Author contributions: AE; Literature search and study design, **AE;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

Optimization of head computed tomography scan in a tertiary institution in Edo State, South-South Nigeria

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ABSTRACT

Objective: The study is aimed at optimizing the existing CT protocol for head scans in a Specialist Teaching Hospital in Edo State with a 16-slice Siemens Somatom Emotion scanner. Also, the study determined the vol computed tomography dose index ($CTDI_{vol}$) and Dose Length Product (DLP) from the patient's dose profiles. The results from this study was compared with relevant studies.

Materials and Methods: The scanner was used to acquire head CT of 160 patients retrospectively. Also, a locally designed head phantom was used to simulate individual patients using a similar protocol by changing the tube current (mA) and total scan width (TSW) only from the existing protocol.

Results: Percentage dose reduction (PDR) for the $CTDI_{vol}$ and DLP ranged 42.00-46.80% and 37.13-43.54% respectively. The optimized $CTDI_{vol}$ and DLP were lowest compared to studies in the United Kingdom (UK), Italy, India, Ireland, Sudan, Nigeria, European Commission (EC), United States of America (USA) and Japan. Only the DLP for India was lower than our optimized value.

Conclusion: The need to understudy CT configuration is necessary, this will allow end-users to optimize certain parameters in the CT scanner, which will reduce the patient dose without compromising image quality.

Keywords: Optimization, Computed Tomography (CT), Dose Length Product (DLP), Computed Tomography Dose Index ($CTDI_{vol}$), Peak kilovoltage (kVp), Milliampere-seconds (mAs)

INTRODUCTION

Computerized tomography (CT), has in recent years experienced tremendous technological advances, developing from the first generation in the early 1970s through the seventh generation to multi detector computed tomography (MDCT) (**1, 2**) computed tomography (CT) examinations typically deliver relatively higher radiation dose than other diagnostic imaging machines.

In Europe, diagnostic radiology represents the largest man-made contribution to population dose (**3, 4**), this observation may not be different in developing countries like Nigeria where there is high proliferation of CTs. The radiation dose from CT is relatively higher according to the International Commission on Radiological Protection (ICRP) documents and from research articles (**5, 6**) and CT have the tendencies to increase patient cancer risk (**7, 8**).

Training of personnel on the use of CT scanners may help to improve the quality of care at lower doses and subsequently reduce cancer risk to patients. The risk associated with radiation exposure can be considered as deterministic or stochastic effects. Deterministic risk results from cell death and is quantified in terms of radiation dose to a particular region that has a threshold level beyond which these effects generally occur.

Research Article

Received 23-03-2020

Accepted 09-04-2021

Available Online: 10-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.521

They are rarely seen with diagnostic X-ray based examination but are common in radiotherapy. Radiation risks from stochastic (long term) effects may result to cancer and genetic effects and may occur in the offspring of the irradiated subject. There is no given threshold for this, as any dose received may have the potential to cause lethal damages to the cells (**9, 10**).

Although, several research based work had been done globally and locally in Nigeria to estimate $CTDI_{vol}$ and DLP in a bid to establish reference dose levels (RDL) in CT for different body regions (**11, 12**), there is no evidence(s) that the dose reduction/optimization has been carried out, based on parameter adjustment (kVp, mA, Scan time, pitch) and there are no standardized procedures for CT imaging across the diagnostic hospital in Edo State and in Nigeria at large. This is because each hospital has its own specific protocol, which is largely dependent on the expertise of the radiographer.

Studies have demonstrated that a dose reduction of up to 50% is achievable when mAs and kVp are reduced by half (**13, 14**). Also, the Iterative reconstruction (IT) techniques have demonstrated the potential for improving image quality and reducing radiation dose in CT relative to the filtered back projection (FBP) techniques for conventional CTs (**15-18**).

This work focuses on the adult head CT examination with emphases on reducing milliampere (mA) by 39% and total scan width (TCW) by 75% and keeping kVp, pitch, scan length and other parameters constant. This study is also aimed at comparing the optimized $CTDI_{vol}$ and DLP values with national and international studies.

MATERIAL and METHODS

This study was carried out in the department of radiology in a Specialist Teaching Hospital (STH) in Edo State from the period of June – November, 2020. A 16 slices Somatom Emotion scanner (Siemens) was used (Table 1). A convenient sampling technique was used. This was done retrospectively by accessing the CT workstation to select patients that had head CT. A Digital Imaging and Communications in Medicine (DICOM) MicroDicom software was used to analyze the images obtained. A total of one hundred and sixty real patients (160) between the ages of 18 to 87 years were evaluated retrospectively. A locally designed 16cm head phantom was used with the protocols of the 160 patients to estimate new $CTDI_{vol}$ and DLP values, by reducing the mA by 39% and total collimator width (TCW) by 75% after the radiologist was satisfied with the images. The new protocol was implemented (Table 2). The initial protocol with patients was retrospective while the new protocol with the phantom was prospective. CT parameters that remained constant for both protocols were scan length (SL), kVp, exposure time, and pitch. Parameters that were manipulated in the new protocol were mA, and Collimator width. In general, parameters used included: scan length (SL), collimator width (CW), kVp, mA, exposure time and pitch. Corresponding $CTDI_{vol}$ and DLP were retrieved from the system archiving unit and were recorded. Images were then transferred from the CT monitor to a windows 8 system having a pre-installed microDicom viewer for dose profile evaluation.

Percentage dose reduction for $CTDI_{vol}$ and DLP were expressed mathematically as:

$$\%\ dose\ reduction = \frac{Unoptimized\ CTDI_{vol} - Optimized\ CTDI_{vol}}{Unoptimized\ CTDI_{vol}} \quad (1)$$

$$\%\ dose\ reduction = \frac{Unoptimized\ DLP - Optimized\ DLP}{Unoptimized\ DLP} \quad (2)$$

Statistical analysis: Data analysis was done using SPSS Inc. Released 2018. IBM SPSS Statistics for Windows, Version 22.0. (Chicago, USA). Descriptive statistics was used to determine the mean $CTDI_{vol}$ and DLP. A One-Way ANOVA was used to compare the machine parameters. An independent sample t test was used to compare the mean of the $CTDI_{vol}$ and DLP for unoptimized and optimized. P-value < 0.05 was considered statistically significant.

RESULTS

The average machine parameters according to age of the patients for head CT scans were the scan length (SL), total collimator width (TCW), kVp, mA, pitch and exposure time. Machine parameter that remained constant throughout all scan was the total collimator width (TCW), kVp, mA, and pitch (Table 2).

The same machine parameter was used with a locally designed head phantom to mimic a real patient. The parameters used were the scan length (SL), total collimator width (TCW), kVp, mA, pitch and exposure time. The mA and TCW was reduced by 39 and 75% respectively to achieve our dose optimization process (Table 3)

There was a statistically significant difference between the unoptimized and optimized $CTDI_{vol}$ and DLP respectively ($P < 0.001$). The percentage dose reduction (PDR) for the $CTDI_{vol}$ ranged from 42.00-46.80% and while the percentage dose reduction (PDR) for the DLP ranged from 37.13-43.54% (Table 4).

Comparison of this study with national and international $CTDI_{vol}$ was made. Out of a total of nine comparison made with the unoptimized protocol, four were above while five were below our results. Comparison with the optimized protocol showed that only one country had $CTDI_{vol}$ higher than our study. Relative difference (RF) in $CTDI_{vol}$ between optimized values and the referenced article was in the range of 9-83%. There was no statistically significant difference between unoptimized and optimized $CTDI_{vol}$ ($P = 0.666$) (Table 5).

Comparison of this study with national and international DLP was made. Out of a total of nine comparison made two results (Nigeria and Japan) from other study were above our unoptimized DLP protocol. On the other hand, optimized DLP protocol showed the least when compared to other studies (724mGy.cm) and the relative difference (RF) in DLP between optimized and other studies was 19-60%. There was no statistically significant difference between unoptimized and optimized DLP ($P = 0.606$) (Table 6). Head CT for unoptimized protocol at 180mA and 130kV, had better image contrast compared to the optimized protocol at 110mA and 130kV, with relative image noise (Figure 1 and 2)

http://dx.doi.org/10.36472/msd.v8i4.521

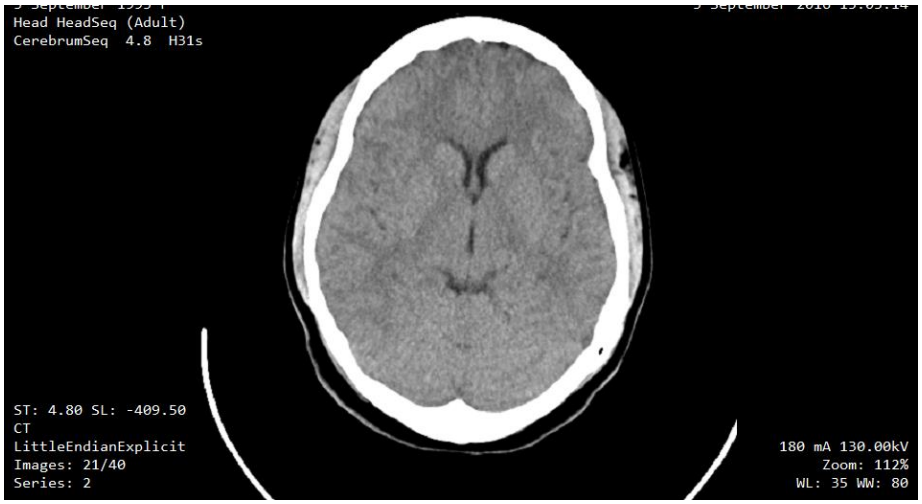

Figure 1. A slice of the unoptimized CT image at 180mA and 130kV

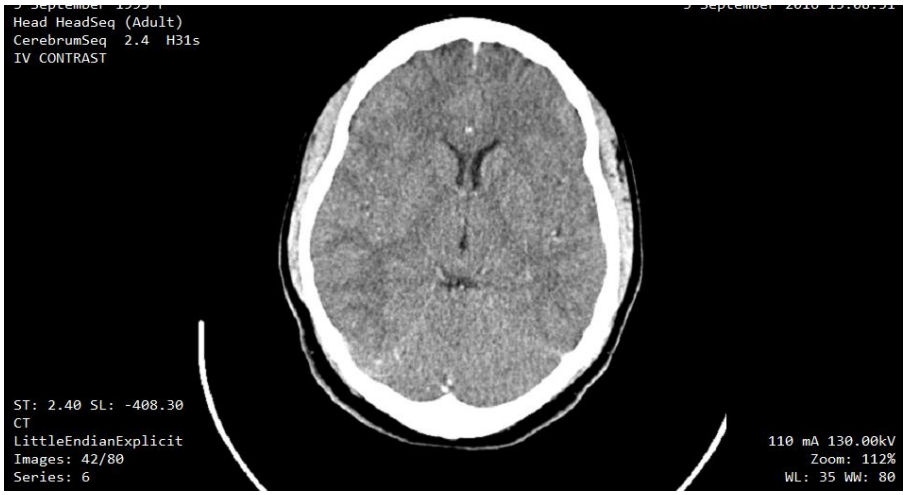

Figure 2. A slice of the optimized CT image at 110mA, 130Kv (with relative image noise)

Table 1. Siemens Somatom Emotion 16-Slice CT machine specification

Generator Maximum output:	50kw
mA range:	20mA-345mA
KV switch:	80KV, 110KV, 130KV
Al equivalent	5.5mmAl
Detector arrangement:	24 rows
Pitch factor:	0.4 to 1.5 (with cone beam correction) 0.4 to 2.0 (without cone beam correction)
Reconstructed slice widths	0.6, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 mm
HU scale	–1,024 to +3,071
Extended HU scale	–10,240 to +30,710
Scan times full scan (360°)	0.6, 1.0, 1.5 s
Slice thickness	0.6–19.2 mm
Scan range:	153cm
Scan speed:	100mm/sec.
FOV variable:	50 cm (70 cm reconstructed FOV available*)
Gantry aperture:	70cm
Gantry tilt:	+/-30°
Spiral acquisition modes	4 x 0.6 mm, 16 x 0.6 mm, 16 x 1.2 mm
Sequence acquisition	modes 4 x 0.6 mm, 12 x 0.6 mm, 16 x 0.6 mm, 2 x 5 mm, 12 x 1.2 mm, 2 x 8 mm, 16 x 1.2 mm

Table 2. Average technical parameters for existing patient protocol

Age range	SL (mm)	TCW (mm)	kVp	mA	Pitch	Exposure time (s)
18-27	187	9.6	130	180	1	33
28-37	189	9.6	130	180	1	27
38-47	196	9.6	130	180	1	30
48-57	214	9.6	130	180	1	33
58-67	187	9.6	130	180	1	32
68-77	190	9.6	130	180	1	30
78-87	192	9.6	130	180	1	28

Table 3. Average technical parameters for the new protocol (phantom)

Age range	SL (mm)	TCW (mm)	kVp	mA	Pitch	Exposure time (s)
18-27	187	2.4	130	110	1	33
28-37	189	2.4	130	100	1	27
38-47	196	2.4	130	110	1	30
48-57	214	2.4	130	100	1	33
58-67	187	2.4	130	110	1	32
68-77	190	2.4	130	110	1	30
78-87	192	2.4	130	110	1	28

* Patients parameters were used with the phantom

Table 4. Percentage dose reduction for optimized $CTDI_{vol}$ and DLP

Age group (yr)	Avg. unoptimized ($CTDI_{vol}$)	Avg. optimized ($CTDI_{vol}$)	PDR	Avg. unoptimized (DLP)	Avg. optimized (DLP)	PDR
18-27	63.50	36.83	42.00	1261	712	43.54
28-37	63.22	34.22	45.87	1196	706	40.64
38-47	62.90	34.70	44.83	1193	723	37.13
48-57	63.54	35.54	44.07	1219	753	38.88
58-67	63.11	34.67	45.06	1231	785	37.45
68-77	63.25	34.63	45.25	1280	732	38.83
78-87	62.50	33.25	46.80	1148	680	39.90

PDR = Percentage dose reduction Avg = Average

Table 5. Comparison of this study's CTDIvol with other studies

Country	$CTDI_{vol}$ (mGy) ǂ	$CTDI_{vol}$ (mGy) ƀ
This study	63	35
UK (24)	58	58
Italy (25)	64	64
India (26)	32	32
Ireland (27)	64	64
Sudan (28)	65.4	65.4
Nigeria (29)	61	61
EC (30)	60	60
USA (31)	57	57
Japan (32)	85	85

ǂ = unoptimized $CTDI_{vol}$, ƀ = optimized $CTDI_{vol}$

Table 6. Comparison of this study DLP with other studies

Country	DLP (mGy.cm) ǂ	DLP (mGy.cm) ƀ
This study	1211	724
UK (24)	890	890
Italy (25)	1086	1086
India (26)	925	925
Ireland (27)	857	857
Sudan (28)	758	758
Nigeria (29)	1310	1310
EC (30)	1000	1000
USA (31)	1011	1011
Japan (32)	1350	1350

ǂ = unoptimized $CTDI_{vol}$, ƀ = optimized $CTDI_{vol}$

DISCUSSION

Parameters like mA have been seen to contribute to patient dose during CT examinations. The product of the tube current and exposure time parameters was statistically significantly different between the unoptimized and optimized values ($P < 0.001$) with percentage dose reduction of 39% on the mA. This have been seen to translate into 45 and 40% dose reduction in $CTDI_{vol}$ and DLP respectively. The study showed that kVp had a significant impact on exposure time, scan length and the optimized mA ($P < 0.001$).

In a study by Frush et al, the principal selectable parameters that contribute to radiation dose are tube current (mA), peak kilovoltage (kVp), pitch, and gantry cycle time (in seconds). The relationship between the tube current and radiation dose was linear. Decreasing tube current by 50% essentially decreased radiation dose by 50% but at increased image noise (**19**). Our study was below the 50% dose reduction achieved in Frush's study because our mA was reduced by 39%. Although, 45% dose reduction was achieved, which was 5% lower than Frush's study.

In a study by Cohnen et al, who investigated CT of the head using reduced current and kilovoltage and the relationship between image quality and dose reduction. It was observed that, in the conventional mode, the highest surface dose was 83.2 mGy (scanner 1: helical mode, 55.6 mGy), and 66.0 mGy (scanner 2: helical mode, 55.9 mGy). By changing kVp and mAs, a dose reduction of up to 75% (scanner 1), and 60% (scanner 2) was achieved. There were no observable differences in image quality between scans obtained with doses from 100% to 60% of standard settings (**20**). This study showed a maximum dose reduction when only the tube current and collimator width were changed. The maximum dose reduction was 47%. Differences obtained may be due to both reductions in mAs and kVp from the above study.

Also, a study by Sodickson et al, who studied strategies for reducing radiation exposure from multidetector computed tomography in the acute care setting.

An average effective mAs of 276 were obtained for the first patient and 272 for the second patient. However, the decrease from 120kVp to 100 kVp resulted in a 42% reduction in $CTDI_{vol}$ from 18.6 mGy to 10.7 mGy. Comparison with our study reveals that a decrease in average effective mAs from 270 to 165 had a maximum dose reduction of 46.8% (63 to 35mGy for the brain) (**21**). The % variation in dose reduction could be associated with a reduction in other machine parameters like scan length, kVp, mAs and pitch.

In addition, other methods for dose reduction have been alighted; one of such methods was in a study by Sulagaesuan et al, who reviewed how to reduce emergency CT radiation doses with simple techniques, using the quality initiative project. The study used automatic tube current modulation (ATCM) method against conventional means. The $CTDI_{vol}$ and DLP for head CT were reduced by 53 and 57% respectively. Although the approach was different from ours where conventional means was used for dose reduction. Our study showed a dose reduction across all age groups, with mean value of 45 and 40% for $CTDI_{vol}$ and DLP respectively.

Dose reduction with ATCM method was better compared to our study (**22**). In another related study by Baskan et al, who investigated the effect of radiation dose reduction on image quality in adult head CT with a noise-suppressing reconstruction system with a 256 slice multi detector computed tomography (MDCT). The study revealed that when the standard dose and low dose groups were compared qualitatively, no significant differences were found in overall quality. By selecting the appropriate level of Iterative reconstruction, 34% dose reduction was achieved without compromising image quality. Dose reduction from our study was better compared to Baskan's study, based on the adjusted parameters. A maximum dose reduction of 47% was obtained from our study (**23**).

Comparison of the optimized value for this study showed that this study $CTDI_{vol}$ was higher than a study that was conducted in India (-9.38%). Similarly, there were difference in $CTDI_{vol}$ and DLP values when optimized values from this study was compared to other studies ($P < 0.001$).

The optimized $CTDI_{vol}$ and DLP were lower compared to studies in the United Kingdom (**24**), Italy (**25**), India (**26**), Ireland (**27**), Sudan (**28**), Nigeria (**29**), European Commission (EC) (**30**), United States of America (USA) (**31**) and Japan (**32**). Only the DLP for a study in India was lower than our optimized value, although the DLP result used a 100 mm long pencil ionization chamber (IC) and polymethylmethacrylate (PMMA) phantom. Studies have shown no statistically significant differences in CTDI and DLP values with either manufacturer's data or phantom measurements with IC or thermoluminescent dosimeters (**33, 34**).

CONCLUSIONS

A study to optimize head CT from a specialist hospital in Edo State Nigeria has been carried out. The dose indicators ($CTDI_{vol}$ and DLP) were reduced by 45 and 40% respectively, with relative noise on the images. The optimized $CTDI_{vol}$ and DLP were lower compared to most studies. The study proved useful and can be implemented for clinical practice. This will help to reduce the patient's exposed dose without compromising image quality.

Author contributions: CUC, ADO, CCN; Literature search and study design, statistical analyzes, Article write up and revision **CCN;** Critical Review

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

Early onset androgenic alopecia: not a cosmetic problem but a sign of life time risk factors. Male phenotypic equivalent of polycystic ovarian syndrome: Is There a Male Phenotype of PCOS

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ABSTRACT

Objective: Polycystic ovarian syndrome (PCOS) was thought to be a gynecologic disorder and then accepted as a general endocrine and metabolic syndrome. The genetic component of PCOS seems to be very important in its etiology. Because of this reason there should be a male PCOS equivalent. Early androgenetic alopecia (EAGA) is a specific pattern of hair loss and it should start before age 30 years and it is claimed to be a male equivalent of PCOS in women.

Materials and Methods: In this study we aimed to investigate the hormonal and metabolic parameters of men with EAGA and compare them with healthy age-matched controls. Thirty men with EAGA and 30 controls were screened for free testosterone, DHEAS, gonadotropins, 17OH progesterone, ACTH, fasting glucose, fasting insulin, homocysteine and metabolic profile. Homeostasis model assessment (HOMA) results were used for the marker of insulin sensitivity. Alopecia classification was made by using the scale of Hamilton with Norwood modification.

Results: Patients with EAGA had higher free testosterone (25,12±3,05 vs 21,3±1,77), DHEAS (634,90±27,09 vs 578±17,82), LH (9,16±0,28 vs 5,13±0,40). The EAGA group had insulin resistance but the control group did not (HOMA results were 3,34±0,47 vs 1,43±0,3). The homocysteine levels of EAGA group were higher than controls (12,37±1,31 vs 9,33±2,12) which is another cardiovascular risk factor. The correlations that we found in our study among HOMA, serum androgen levels, homocysteine and alopecia scores were positive in EAGA patients. We didn't find any correlations among those parameters in control group. Because of these findings men with EAGA can be considered as male synonym to PCOS syndrome. These young men should be followed for the same long time risk profile like PCOS women. Insulin resistance and its results like metabolic syndrome, diabetes and cardiovascular diseases are real risks but there may be even a risk for infertility.

Conclusion: We aimed to investigate whether EAGA can be accepted as the male phenotype of PCOS and if they have elevated risk factors for chronic complications than their age and sex matched controls.

Keywords: insulin resistance, androgenetic alopecia, polycystic ovary syndrome

INTRODUCTION

Polycystic ovarian syndrome (PCOS) was thought to be a gynecologic disorder and then accepted as a general endocrine and metabolic syndrome. It is characterized by irregular menses caused by anovulation, clinical (hirsutism /acne), with or without biochemical findings of high androgenic hormones, small sized (<8mm) multiple cysts in ovaries and some metabolic abnormalities in women who suffers from the syndrome (1). The evidences for the genetic component in PCOS etiology are strong. In the clinic, we see lots of patients with PCOS who clusters in the same families. It seems to be inherited through a polygenic autosomal type of mechanism.

Research Article

Received 24-03-2020

Accepted 08-04-2021

Available Online: 10-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.522

The role of genetic in PCOS etiopathogenesis gives the scientific basis for a male PCOS equivalent. There is a strong possibility for inheriting the same responsible genes for PCOS in male relatives of those women (2).

Early androgenetic alopecia (EAGA) starts before age of 30 even sometimes before age of 20. It is a specific pattern of hair loss that starts from the temporal and occipital parts of the hair. EAGA and accompanied hypertrichosis in men may be the male synonym of PCOS in women. Men with EAGA mostly show similar abnormalities in their sex hormone profiles like PCOS women. Although this phenotype and androgen abnormalities in men are widely accepted, the metabolic abnormalities and long-term risks are still controversial (3).

MATERIAL and METHODS

This study was a prospective case-control hospital-based one and executed in the endocrinology department of Ege University. The ethics committee approved this study as a graduation thesis, and all participants gave written approval for participating in this study. Thirty men aged 18-30 years that has this specific type of hair loss (according to the classification of alopecia scale of Hamilton with Norwood they all had grade 4 or higher alopecia) were accepted as our study patient group. The most important inclusion criteria of our study group are that the patients should have a first degree female relative with the diagnosis of PCOS. Thirty age-matched men without any evidence of androgenetic alopecia were accepted as control.

The exclusion criteria for our study were

i. prior diagnosed endocrine disorder,
ii. cardio-metabolic diseases especially diabetes
iii. usage any medications that effect skin or hormones for hair loss
iv. Men with Body mass index≥35 kg/m2
v. Usage of medications that effect glucose metabolism
vi. Special for the control group, men who have relatives with PCOS were also excluded.

Detailed anamneses and family history were saved in their medical records for each participant. All individuals were assessed by the same physician. The weights of the patients were evaluated with the same Tanita BC-418 early in the morning before breakfast, their hights were also measured. The body mass index (BMI) was calculated using these results.

Blood samples were collected after ten hours overnight fast between 8 AM to 9 AM. Serum lipid profiles (total cholesterol, triglyceride, HDL, LDL) were measured by Olympus AU 2700 automated analyzer. The insulin levels in plasma were assessed using 2 site chemiluminescent immunometric assay by Immunolite 2000.

The glucose oxidase technique was used to evaluate plasma glucose levels (Biobak Laboratory Supplies Trade, Ankara, Turkey). Serum liver function tests [Serum glutamic oxaloasetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) and glutamyl transferase (GGT)], kidney functions (creatinine and urea) and uric acid levels were measured by Olympus AU 2,700 analyzer.

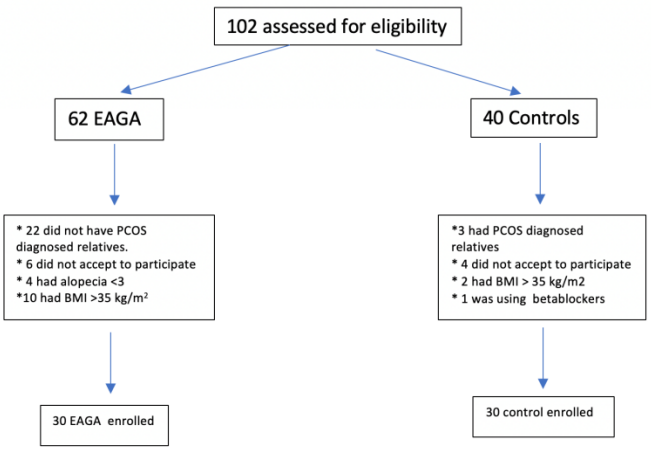
The levels of insulin in plasma were measured by microparticle enzyme immunoassay (Abbott, Wiesbaden-Delkenheim, Germany). Insulin sensitivity was investigated by calculation of [fasting insulin (mU/ml) X fasting glucose (mg/dl)/22.5X18 = HOMA]. Results greater than 1.7 were accepted positive for insulin resistance and >2,7 was a high risk of diabetes mellitus.

Serum homocysteine levels were measured by high-performance liquid chromatography with the Hewlett-Packard 1100 Series System (Waldron, Germany). The serum concentrations of FSH, LH, E2, progesterone, prolactin, and cortisol, were measured by chemiluminescent enzyme immunoassay (ASC 180 (+) Ciba Diagnostics, USA) . The blood sample test results of ACTH, 17-OH progesterone, Free Testosterone, DHEA-S, and thyroid hormones were measured with standard radioimmunoassay.

The Statistical Package for the Social Sciences (version 10.0 for Windows; SPSS, Inc., Chicago, IL) was used for statistical analyses. The normality tests were used to test the characteristic distributions of the data. If the distribution of the variables were normal, students two tailed t-test with or without logarithmic transformation was used to compare all parameters. For the variables which didn't show normal disturbance even after log transformation Man-Whitney U test was performed. Relations between insulin sensitivities, degree of alopecia, metabolic and hormonal parameters were analyzed with simple linear regression analysis, and Pearson (r) correlation coefficients were presented. P values smaller than 0.05 were accepted as statistically significant.

Study design: One hundred two patients were evaluated as the outpatient basis in Ege University endocrinology and dermatology departments. Figure 1 shows the patient enrollment protocol. Sixty-two patients with EAGA were evaluated for the patient group. In this group of patients, 22 did not have first degree PCOS relatives, 6 of them did not accept to participate in our study, one had an alopecia score <4 and 10 of them had body mass index (BMI)>35 kg/m^2. . For the control group we evaluated 30 medical students, assistants and fellows without significant alopecia. Among the control group subjects 3 had PCOS relatives, 4 of them did not want to participate, 2 had BMI>35kg/m^2 and one of them was using betablockers. The remaining 30 participants became our control group.

Figure 1: Study design chart



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RESULTS

The demographic and biochemical features and laboratory results of the studied groups are demonstrated in Table 1. The age in the EAGA group (as mean±SD) was 25,33±2,24 years and it was 24,3±1,72 years in group 2. The difference was not statically significant (P=0,06). The calculated BMI is significantly high in the EAGA group than the control group 2 (27,42±3,3 kg/m^2 vs 24,7±3,13 kg/m^2 respectively P=0,02) The systolic blood pressures were similar in both groups but the diastolic blood pressure was high statistically in EAGA group (8,18±0,41 vs 7,95±0,29 P=0,01). The blood glucose in the fasting state (FBG) was significantly higher in Group-1 than Group-2 (98,93±4,49 mg/dl vs 85,96±4,64 mg/dl respectively P=0,01). There were 6 patients in EAGA group who had impaired fasting glucose (IFG). We performed standard two-hour oral glucose tolerance test with seventy-five grams glucose (OGTT) to those patients and 4 had impaired glucose tolerance and 2 had normal results. None of the control group-participants had IGF. We did not perform an OGTT to the control group as the FBG and HbA1c levels were normal in all control group patients. The HOMA index was higher and it was in insulin-resistant range in all the patients in Group 1. That result was significantly higher than control group (3,34±0,47 vs 1,43±0,3 respectively P<0,01). There were only 7 participants whose HOMA index was higher than 1,7 (risky group) and no one had a HOMA index greater than 2,7. The lipid profile parameters except HDL were significantly higher in EAGA but none of them needed antilipidemic treatment.

HDL levels were similar in the two groups. The mean HbA1c value of EAGA was higher than control group (5,91±0,24 vs 5,29±0,18 respectively P<0,01). In the EAGA group 1 patient had a diabetic HbA1c value but his OGTT was in IGT range. Homocysteine values were higher in AGAE group than control group (12,37±1,31 vs 9,33±2,12 respectively P<0,01).

The hormonal profiles of our study population are demonstrated in Table 2. We found that The EAGA group had significantly higher free testosterone levels than controls. (25,12±3,05 vs 21,3±1,77 respectively P<0,01). When we investigate adrenal androgens DHEAS was significantly higher in the EAGE group (634,90±27,09 vs 578±17,82 p=0,02) but the 17-hydroxiprogesterone values were not significantly different. (2.31±0,18 vs 2,22±0,12 P=0,02).

The ACTH and TSH values were similar in both groups. The prolactin results of EAGA group was significantly lower than controls (12,96±1,58 vs 14,7±2,1 respectively p<0,01) The mean LH results were higher in EAGA group (9,16± 0,28 vs 5,13±0,40 P<0,01) but FSH mean results were similar in both groups (4,20±0,17 vs 4,29±0,16 P=0,8). This resulted in the elevation of LH/FSH ratio in the EAGA group. In our investigation we demonstrated that the mean levels of prolactin in the EAGA group were significantly lower than the control group. But all the results of both groups were in the normal range (12,96±1,58 vs 14,7±2,1 respectively p<0,01)

Table 1: Demographic and biochemical results of the studied groups.

	GROUP-1	GROUP-2	p
AGE (year)	25,33 ± 2,24	24,3 ± 1,72	P=0,06
BMI(kg/m^2)	27,42 ± 3,3	24,7 ± 3,13	P=0,02
SYSTOLIC BLOOD PRESSURE (cm/Hg)	12,12 ± 0.46	11,87 ± 0,55	P>0,05
DIASTOLIC BLOOD PRESSURE (cm/Hg)	8,18 ± 0,41	7,95 ± 0,29	P=0,01
ALOPECİA SCOR	7,93 ± 1,5	1,47 ± 0,5	P<0,01
FASTİNG BLOOD GLUCOSE (mg/dl)	98,93 ± 4,49	85,96 ± 4,64	P<0,01
FASTING INSULIN (mIU/ml)	13,53 ± 1,83	6,8 ± 1,44	P<0,01
HOMA	3,34 ± 0,47	1,43 ± 0,3	P<0,01
T. CHOLESTEROL (mg/dl)	232,96 ± 14,44	193,63 ± 15,817	P<0,01
TG (mg/dl)	245,76 ± 45,92	177,9± 35,48	P<0,01
HDL (mg/dl)	42,16 ± 4,2	41,83 ± 6,2	P=0,81
LDL (mg/dl)	141,40 ± 11,92	118,23 ± 14,32	P<0,05
URIC ASID (mg/dl)	6,1 ± 1,19	5,1 ± 0,55	P=0,83
SGOT (IU/L)	24,42 ± 6,7	23 ± 6,1	P=0,76
SGPT (IU/L)	39,6 ± 9,3	26 ± 9,1	P<0,05
HOMOCYSTEIN (µmol/L)	12,37 ± 1,31	9,33 ± 2,12	P<0,01
HBA1C	5,91 ± 0,24	5,29 ± 0,18	P<0,01

Table 2: The Hormonal Profiles of the Studied Groups

	GROUP-1	GROUP-2	p
F.Testosteron(pg/ml)	25,12 ± 3,05	21,3 ± 1,77	P<0,01
DHEAS (µg/dl)	634,90 ± 27,09	578 ±17,82	P=0,02
17-OHP (ng/ml)	2.31 ± 0,18	2,22 ± 0,12	P=0,11
FSH (mIU/ml)	4,20 ± 0,17	4,29 ± 0,16	P=0,80
LH (mIU/ml)	9,16 ± 0,28	5,13 ± 0,40	P<0,01
ACTH (pg/ml)	26,16 ± 3,90	26,4 ± 3,59	P=0,81
TSH (mU/ml)	2,56 ± 0,51	2,37 ± 0,52	P=0,73
PROLAKTİN (ng/ml)	12,96 ± 1,58	14,7 ± 2,1	P<0,01
AGA SKOR	7,93 ± 1,5	1,47 ± 0,5	P<0,01

http://dx.doi.org/10.36472/msd.v8i4.522

We investigated the correlation between androgenic hormones and insulin sensitivity. The correlation between HOMA and free testosterone levels were positive and strong in EAGA group as shown in Figure-2 (r=0,69 p<0,001) . The correlation between DHEAS and HOMA was also positive and statistically significant but weaker than free testosterone and HOMA correlation shown as shown in Figure 3. (r=0,43 p=0,17)

The levels of 17OHP and HOMA were not significantly correlated in our study (r=0.17 p=0.59). The correlation among homocysteine values and HOMA results were weakly positive but statistically significant (r=0,37 p=0.04). The correlation was also positive between the alopecia score and free testosterone as shown in Figure-4.

Figure 2: The correlation of F. testosterone and HOMA

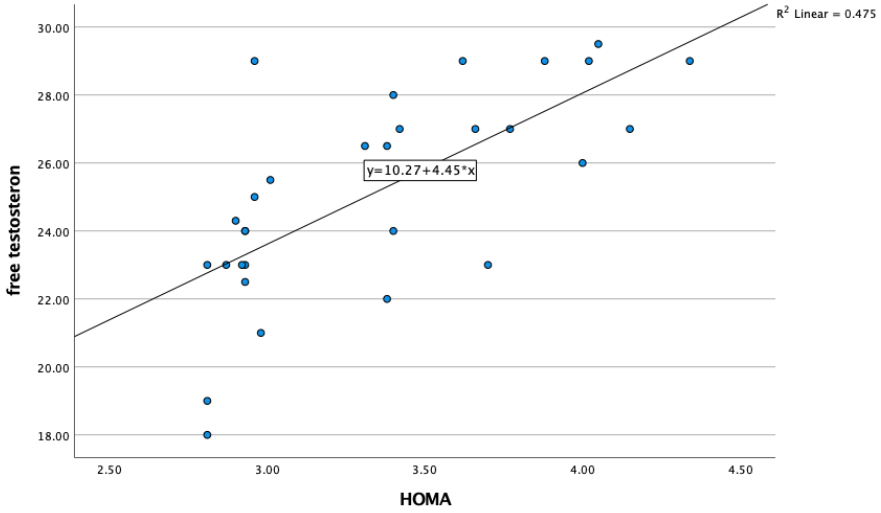


Figure 3: The correlation of Alopecia Score and HOMA

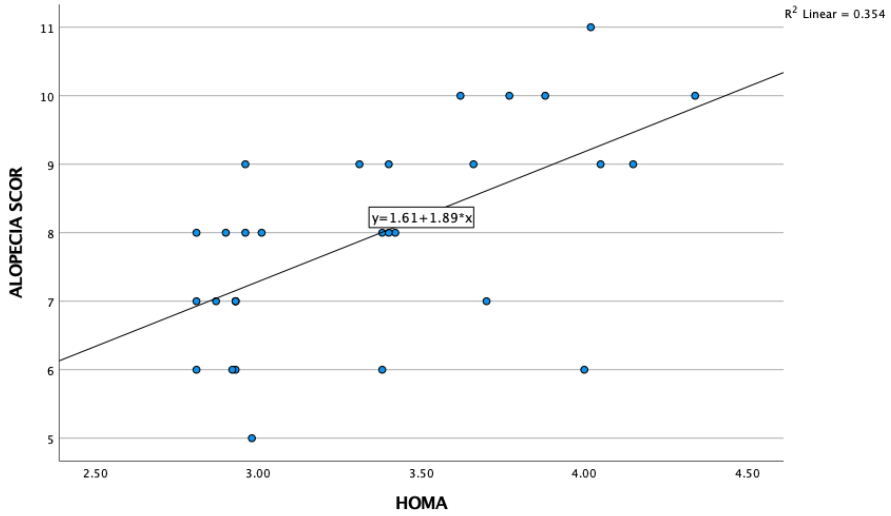
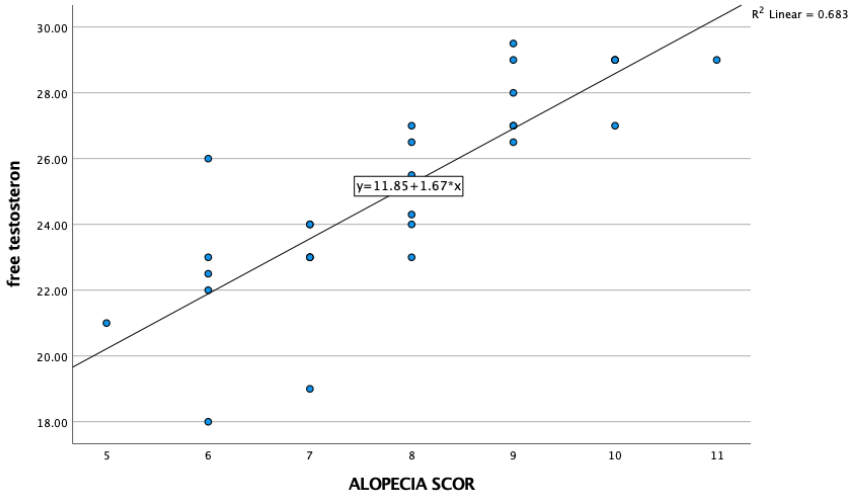


Figure 4: The correlation of Alopecia Score and free testosterone



http://dx.doi.org/10.36472/msd.v8i4.522

DISCUSSION

EAGA is like a dance of androgens and gens. Evaluating the testicular and the adrenal gland hormones in these men is necessary to understand the etiopathogenesis of EAGA and to predict the future risks in this problem. Various studies proved the there are lots of similar findings in the hormonal abnormality styles of EAGA men with those women with PCOS. Now EAGA in men is widely accepted to be the phenotypic equivalent of PCOS (4).

There are a lot of studies showing the association of EAGA with insulin resistance and metabolic syndrome (3). Although it is really difficult to compare the results of the sex hormone parameters in male and female patients, there are studies in the literature that reports similar hormonal changes in men affected by the EAGA as seen in PCOS (5). It was found that this characteristic EAGA type is mostly present in male family members of PCOS women. This supports the hypothesis of the genetic component of this syndrome.

In this study we investigated 30 EAGA men who have first-degree relatives with the diagnosis of PCOS and compared them with age-matched men without EAGA and also without any female relatives with the diagnosis of PCOS.

In our study, the mean free testosterone and DHEAS levels in the EAGA group were significantly elevated than the levels of Group 2. The findings of free testosterone were similar to studies by Sanke et al (6), Narad et al (7) and some others. (8, 9) In contrast with those studies, some investigators found just slightly lower than normal levels of testosterone among their EAGA patients (10, 11). For the high DHEAS levels in EAGA, our findings were similar to the findings of some of the authors (10, 12). But again some authors published normal values of DHEAS (7, 8) in their study group EAGA men. The high DHEAS value is an important finding as DHEAS has some special features such as it has a long half-life and it doesn't have any pulsatility. It is widely accepted as a useful marker of hyperandrogenism in men (5, 13). In this study, we didn't define any correlation between the DHEAS - ACTH levels but there correlation between F. testosterone and DHEAS was significant and positive. This indicates that pituitary-adrenal ax stimulation may not be the main reason of DHEAS elevation (5). But we did not investigate ACTH stimulated DHEAS response. Studies with PCOS women found the increased response to ACTH stimulation even the basal DHEAS values were not correlated to ACTH (13, 14). Additional new studies are needed to estimate the effect of ACTH stimulation on DHEAS levels in EAGA men.

In our study, we found elevated LH/FSH levels than the control group due to the elevation of LH levels in men with EAGA. There are conflicting data in the literature about the high LH/FSH levels and LH elevations in EAGA men (7, 15). We think that elevation in LH is an important finding in EAGA men with PCOS family members as elevated LH/FSH ratio is a significant finding of PCOS. (13) An increase in LH levels results in increased testosterone levels and the finding in our study of significant correlation among those values supports this theory. As far as we know this is the first study that evaluated the correlation among them. In our study, we found decreased levels of prolactin in EAGA group; it is the opposite of the study by Sanke et al (6) as they found increased levels. But in our study all the results of both the EAGA and control group were in the normal range. More research is needed for determining the role of prolactin in this syndrome.

In our study, we found significant increases of fasting blood glucose, fasting insulin, HOMA and HbA1c results in EAGA men than the control group. Insulin resistance was reported frequently in men affected by the EAGA in the literature (16). The brothers of PCOS women are shown to suffer from some metabolic problems such as a higher risk of insulin resistance, elevation in triglyceride levels, and elevation in blood pressure (17, 18). We found insulin resistance and other problems not only in brothers but in all first degree male EAGA relatives of PCOS women. The combination of lower SHBG with higher free testosterone and metabolic problems in the EAGA men were reported in some studies. A case–control study on a Finnish population made of 125 men with EAGA and 104 controls, aged 19–50 years, found patients with early EAGA had elevated BMI with a twofold higher risk of developing insulin resistance when compared to controls (19). In our study, we found higher BMI in the group of EAGA than controls. The interesting finding is that the insulin resistance was significantly higher than the control group even if the BMI effect is eliminated. According to this EAGA may be accepted as one of the predictors for the future diagnosis of hyperglycemia, insulin resistance then type 2 DM in young patients. Framingham Offspring Study has demonstrated that insulin resistance and serum homocysteine level elevations are associated and may partially be the reason for increased risk of CVD (20). The reason for elevated homocysteine is insulin inhibits the hepatic cystathionine β synthetase activity. In our study, we found significantly increased levels of homocysteine in the EAGA group and it was correlated with insulin levels this was similar to the study of AGA and coronary artery heart disease risk (21).

The correlation was not strong but homocysteine levels can be affected by nutrition factors like vitamin B12 and folic acid. It may be the reason for the weak correlation of our results as we did not investigate the nutritional status of our patients. There were few problems in our study; the parameters that we asses alopecia grade were subjective and our study group had a small sample size.

CONCLUSIONS

We found significantly increased LH, free testosterone, DHEAS levels in our EAGA group. The LH/FSH ratio was higher in men with EAGA. These hormonal test results are nearly the same of the profile of women with PCOS. We also found significantly increased levels of fasting blood glucose, fasting insulin and HbA1c levels in EAGA group. These biochemical parameters are also similar to the metabolic abnormality of PCOS women. So we suggest that these men could be accepted as a phenotypic synonym of PCOS women. These young men may have the same increased risks as women with PCOS, including insulin resistance, metabolic syndrome, diabetes, cardiac and vascular diseases and also may be for infertility. These risks need to be confirmed by some multicentered, large studies. And long term follow-up study results of men with EAGA are also needed to confirm these findings.

Author contributions: DDA, CY; Literature search and study design, statistical analyzes, **DDA;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

Relationship between hyperglycemia, insulin resistance and serum apoptosis marker m30- antigen in patients with type 2 diabetes mellitus

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ABSTRACT

Objective: The amount of evidence suggests that the apoptosis marker M30-antigen (an antibody that recognizes the cytokeratin-18 fragment) has an association with hyperglycemia.

Material and Methods: In this study, serum M30 levels of 145 patients diagnosed with prediabetes (n = 28) and type 2 diabetes, which was divided into four groups according to their HbA1c levels, were measured. The health control group (n = 24) was composed of healthy individuals. Serum concentrations of M30 antigen were measured using an enzyme-linked immunosorbent assay system and expressed as mean ± SD. HbA1c concentration was determined by boronate affinity technology according to NGSP standards.

Results: M30 levels were comparable in the healthy control group (64,39±3,9 U/L) and prediabetes group (82,07±13,7). Type 2 diabetes Groups A, B, C, and D had levels of 109,38±16 U/L, 117,46±14,3 U/L, 173,69±48,1 U/L, and 163,40±37,3 U/L, respectively. The analysis of the data has shown that serum levels of M30 in the control and prediabetes groups were significantly lower than Type 2 diabetes Groups C and D (p=0.043). When all groups were taken into consideration, a significant relationship was found between HbA1c and serum M30 levels (r=0.231, p=0.002).

Conclusion: Apparently, the increase in glycemia is followed by a rise in the serum levels of the, suggesting that apoptosis occurs as a secondary effect immediately after hyperglycemia.

Keywords: type 2 diabetes mellitus, prediabetes, hyperglycemia, apoptosis, M30-antigen, insulin resistance

INTRODUCTION

Diabetes Mellitus (DM) is a long-lasting metabolic disease that prevents the body from using carbohydrates, fats, and proteins due to insulin deficiency or defects under the influence of insulin. Furthermore, diabetes is associated with a heavy-economic burden on the healthcare systems since it requires perpetual medical care. Diabetes is an endemic disease worldwide due to the changes in the lifestyles and increasing length of life. It is estimated that more than 700 million people will be affected by this disease by 2045 (1). Since DM appears in earlier ages in the last several decades, a detailed evaluation of the body response to hyperglycemia is required (2). In type 2 diabetes mellitus (T2D), lipotoxicity, increased production of reactive oxygen species (ROT) and insulin resistance cause damage in pancreatic β-cells. Many factors contribute to the development of T2D, including breakdown of insulin secretion, relative insulin deficiency, and IR (3). It has been shown that 25%-50% of necrosis in β-cells occurs through intrinsic and extrinsic apoptotic pathways in T2D (4).

Apoptosis, also called programmed cell death, is a physiological mechanism to maintain body homeostasis. Apoptosis is pathological when the rate of apoptosis is altered. During apoptosis, apoptosis enzymes, such as caspases and cysteine proteinases, cleave many intracellular proteins (4).

Research Article

Received 28-03-2020

Accepted 11-04-2021

Available Online: 13-04-2021

Published 30-04-2021

The cleavage of these proteins releases proteolytic fragments such as cytokeratin-18 fragment (CK-18) into the circulation. CK-18 is a type 1 intermediate filament protein, which is the main component of single-layer and glandular epithelial cells (5).

An antibody against the CK-18 neo epitope has been developed (5, 6). This antibody, called M30- antigen, recognizes CK-18 fragments after the induction of apoptosis by Caspases. This fragment is not present in normal and necrotic cells. The use of different kinds of CK-18 in the serums of patients (apoptosis antigen M30 and necrotic antigen M65) reveals that they can be used to examine in vivo the way of various death of cells (7).

Serum cytokeratin levels are used as tumor markers. Its clinical benefits were demonstrated in lung and breast cancers. They were also partly observed in gastric and oral cancer (8). It is also claimed that they can be useful in nonalcoholic fatty liver disease (NAFLD), differentiating early fibrosis from severe fibrosis and in the distinction between nonalcoholic steatohepatitis (NASH) and simple steatosis (9). Many studies have reported that serum CK-18 levels in patients with NAFLD were significantly higher than nondiabetic controls (10).

It has been shown that pancreatic β-cells are affected by apoptosis in both type 1 and T2D(2,11). It has been suggested that hyperglycemia causes apoptosis in insulin-deficient β-cells, decreasing the production of pancreatic insulin in long term(4). By augmenting oxidative and nitrosative stress, and activating pro-apoptotic Bcl-2 subgroup proteins and caspase cascade, hyperglycemia affects many steps in the apoptotic signaling (2). Unraveling the mechanism behind the apoptosis in hyperglycemia result in a better understanding of diabetic complications, and pave the way for new treatment strategies.

It is unclear whether apoptosis is associated with the hyperglycemia, and subsequently diabetic complications in T2D. This study investigates the possible relationship between these two processes in T2D to examine whether hyperglycemia causes apoptosis or apoptosis causes hyperglycemia.

MATERIAL and METHODS

The study was conducted between January and March 2016 with 169 individuals consisting of prediabetes (PD) (n=28), overt T2D (n=117) and healthy control (HC) (n=24) which were enrolled from the Istanbul Medipol University, Medical Faculty, Department of Internal Medicine outpatient clinics. Subjects between the ages of 40 and 70 were included in the study by monitoring their fasting blood glucose (FBG), hemoglobin A1c (HbA1c) and 75g oral glucose tolerance test (OGTT). Eighty-one of the participants were female and eighty-eight were male. The value of HbA1c was stated as a percentage according to the National Glycohemoglobin Standardization Program (NGSP). Healthy volunteers with the history of any diseases were included in the HC group. The inclusion criteria for the healthy volunteers were; HbA1c levels lower than 5.7%, and normal OGTT level. The people, whose HbA1c values were 5.7% ⩽ HbA1c ⩽ 6.4%, and who had impaired fasting glucose (IFG) [FBG levels 100 mg/dL to 125 mg/dL], and had impaired glucose tolerance (IGT) [2-h values in the OGTT of 140 mg/dL to 199 mg/dL], was recruited to the PD group. The subjects were grouped according to their HbA1c levels as; group A (Gr A) (HbA1c ⩽ 6.4%), group B (Gr B) (6.5%⩽HbA1c⩽ 7.9%), group C (Gr C) (8.0%⩽HbA1c⩽ 9.9%), and group D (Gr D) (10.0% ⩽HbA1c). The patients for Gr A were individuals who have taken one or more than one antidiabetic agents with HbA1c⩽ 6.4% levels. The T2D and PD diagnosis criteria were based on that of American Diabetes Association (ADA)(12). The medical treatment received by the patients had not interfered during the study.

Patients with diabetes were also excluded from the study if they were diagnosed with cancer or were undergoing cancer treatment, were severely malnourished, along with those receiving immunosuppressive treatment. Moreover, patients with cirrhosis, anemia and hemolysis or who were pregnant were excluded from the study because of the interference with the HbA1c levels.

All participants provided written informed consent. The protocol of the study was reviewed and verified by the Ethical Committee of Faculty of Medicine, Istanbul Medipol University.

Screening: The following variables were recorded for each subject; age, gender, and background. A medical history of the patients, including the duration of diabetes, and the treatments were recorded at the outpatient clinic. A detailed physical examination was conducted and the symptoms were examined during the examination, their anthropometric measurements were recorded. The subjects were classified according to the degree of obesity measured by their body mass index (BMI). The standard waist-hip ratio (WHR) for males was set at <1 cm, and for females, it was set at<0.8 cm.

Blood Analysis: Blood and spot urine samples of all study subjects were collected after at least 10 h of fasting overnight. Blood specimens were collected in 8.5 mL vacutainers (Becton Dickinson) tubes for biochemical parameters. HbA1c samples were carved up/divided into 2 mL tubes including ethylene diamine tetra-acetic acid (EDTA). Depending on the results on the HbA1c, FBG, and OGTT tests, patients were classified into study groups. To assess the M30-antigen levels, the samples were centrifuged (Hence - Xiang Yi 500 model) and the supernatant was collected. The samples were then kept at −80°C until further analysis. Two hours after the breakfast, blood samples were collected again for postprandial glucose (PPG) levels. Boronate affinity technology determined (Quo-Lab®, EKF Diagnostics plc, Cardiff, UK) the HbA1c concentration according to standard NGSP. Plasma glucose was measured by the glucose oxidase method for diagnostic criteria. Standard 75-g glucose OGTT determined IFG and IGT. ADVIA Centaur XP (Siemens) analyzed C-peptide fasting and Cobas e411 measured insulin fasting level. To detect the Homeostatic Model of Assessment-Insulin Resistance (HOMA-IR) the following formula was used fasting insulin level (µU/ml) x fasting glucose level (mg/dl)/405. FBG, PPG, triglyceride (TG), total cholesterol (Col), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and uric acid levels were measured by VITROS® 350 Chemical Systems (Ortho Clinical Diagnostics). Low-density lipoprotein cholesterol (LDL-C) concentration was calculated using the Friedewald

equation (LDL-C= [Tcol] - [HDL-c] - [TG] / 5). C-reactive protein (CRP) and microalbumin, that in spot urine, were tested with the use of i-chroma reader device (Boditech).

ELISA: The level of CK-18 M30 neo-epitope was measured through an immunoassay according to manufacturer's instructions using M30-Apoptosense ELISA kit (Peviva AB, Sweden). The M30 neo-epitope (K18Asp396-NE) is a sensitive and integrative indicator-specific for epithelial cell death involving caspase-3, −7 or −9-activation. M30-antigen concentrations were stated as unit per liter (1 U/L = 1.24 pM). The amount of protein in each sample was calculated by plotting a standard curve of the known concentrations that were made for CK-18 neo epitope M30-antigen vs. measured epitope.

Statistical analysis: The primary aim of the study was to classify five patient groups and one HC group, according to HbA1c and to determine whether they differ in terms of M30-antigen. Based on this information, the sample size was calculated by considering the following pre-acceptances: 5% probability of error, 80% of test power, and 70±80 U/L increase on average in M30-antigen compared to healthy individuals, and consequently, at least 160 people had to be included in the study. Since the M30-antigen values of healthy individuals were similar, 24 healthy patients were assigned to the HC group and 28–30 patients were assigned to the other groups.

The appropriateness of numerical variables in a normal distribution that was observed during the study was evaluated by the One-Sample Kolmogorov Smirnov test and the identifying values were calculated as mean and standard deviation (mean±SD). Taking account of the distribution of the values, the one-way ANOVA model and Kruskal Wallis test were used in the comparison of the groups, and then different groups were detected via post-hoc Scheffe test. The relevance between the values was observed by Pearson's correlation coefficient. In addition, the ability of the M30-antigen to diagnose diabetes, according to HbA1c was investigated by ROC analysis. The statistical significance level was approved as $p<0.05$ and analysis was performed using SPSS V22.0 statistic software (SPSS, Inc, USA).

RESULTS

In total, there were 169 participants classified into an HC group (n=24, 14,2%), PD group (n=28, 16,5%), and diabetes group (n=117, 69,3%). According to analysis, a significant difference was observed between participant's ages, durations of diabetes, BMI, WHR, systolic blood pressure (SBP), and diastolic blood pressure (DBP). In the groups, there was a significant difference in terms of biochemical analysis, including HbA1c, M30-antigen, FBG, PPG, fasting insulin, HOMA-IR, CRP, C-peptide, and uric acid. In terms of IFG, ALT, AST, creatinine and T col no difference was observed. The details of all participants are shown in Table 1.

The relationship between the age of the patients, and the levels of the HbA1c and M30-antigen were evaluated. In addition, diabetic duration of the groups were analyzed in relation to HbA1c and M30- antigen. A significant relationship was not found in the groups in terms of chronological age, diabetic duration, HbA1c levels, and M30-antigen levels. When the diabetes group was analyzed in terms of diabetic durations a correlation was found with levels of HbA1c, M30-antigen, and C-peptide (Table 2).

When the differences between the groups were analyzed in terms of M30-antigen; it was observed (Table 1) that M30-antigen levels in HC and PD groups were significantly lower than the ones in Gr C and Gr D diabetes groups (p=0.043) (Table 3). When all individuals were taken into consideration, a positive and significant relationship was found between HbA1c and M30-antigen levels (r=0.231, p=0.002) (Table 4).

The analysis of the effects of FBG and PPG on apoptosis showed a positive and significant correlation with M30-antigen of both the parameters. Furthermore, a positive and significant relationship between HOMA-IR and HbA1c levels was found. Moreover, further analysis observed a positive and significant relation between the HOMA-IR and M30-antigen levels (Table 4).

Obesity in the individuals was analyzed according to BMI, and the descriptive statistics for the M30-antigen and HbA1c levels are presented in Table 5. When all individuals and individual groups are analyzed, the analysis showed no correlation between BMI and the M30-antigen levels. BMI was correlated with HOMA-IR. The results showed no relationship between WHR and M30-antigen concentration. However, when all individuals are analysed positive and significant correlation was found between WHR and HbA1c levels. In individual groups, no correlation was found (Table 6). During this study, no correlation between hyperglycemia and C-peptide level was found. The analysis of the C-peptide level and M30-antigen level showed a positive and significant correlation only in the Gr B; however, none of the other groups showed a correlation. A significant and positive relationship was found between insulin and C-peptide levels. A positive and significant correlation was between HOMA-IR and C-peptide (Table 4). A statistically significant and negative correlation was observed between the duration of diabetes and the C-peptide levels in patients with diabetes. However, no correlation was observed between C-peptide and chronological age order (Table 2).

A positive and significant relation between CRP levels and BMI was observed. However, no significant relation was found between CRP levels and WHR (Table 6). A significant relationship was found between HbA1c and HOMA-IR with CRP levels. Taken together, we observed a significant relationship between M30-antigen and CRP levels (Table 4).

In our study, we found a significant correlation between ALT and AST levels, and M30-antigen values. However, no significant correlation was found among M30-antigen, fasting insulin, uric acid, creatinine, microalbuminuria, T col, HDL-c, LDL-c, and TG (Table 7). Additionally, no correlation was found between SBP and DBP with M30-antigen levels. Blood pressure showed a significant correlation with HbA1c, HOMA-IR, and CRP (Table 6). Although there was not a significant correlation between levels of M30-antigen in the HC and PD groups, HbA1c levels were significantly different (Figure 1a). It was suggested that levels of both M30-antigen and HbA1c could effectively differentiate healthy controls from patients with diabetes. Furthermore, ROC analysis suggested that HbA1c is a better marker than M-30 antigen in distinguishing patients with diabetes from healthy individuals (Figure 1b).

doi http://dx.doi.org/10.36472/msd.v8i4.524

Table 1. General and biochemical features of individuals participated in the study

HbA1c Level (%)	HC HbA1c< 5.7	PD 5.7≤ HbA1c≤6.4 IFG/IGT, IFG+IGT	Gr A HbA1c≤ 6.4	Gr B 6.5≤HbA1c≤7.9	Gr C 8.0≤HbA1c≤9,9	Gr D 10.0≤ HbA1c	GrT	P
Number	24(%14,2)	28(%16,5)	29(%17,1)	30(%17,7)	29(%17,1)	29(%17,1)	169	
Gender (M/F)	13/11	15/14	16/14	14/15	15/14	88/81	-	
Age (year)	47,9±5,75	52,1±8,63	58,1±9,22	56,2±9,55	56,0±7,60	57,1±8,89	54,8±8,98	0.001
Dd (month)	-	-	67±69,8	100±74,8	98,6±83,8	117±80	66±78,7	0.001
BMI (kg/m²)	27,2±3,11	32,1±5,77	32,8±5,47	33,8±5,51	34,8±5,25	35,6±5,85	32,9±5,83	0.001
WHR (cm)*	0,92±0,07	0,96±0,04	0,98±0,59	0,98±0,06	0,97±0,06	1,00±0,05	0,97±0,06	0.001
SBP(mmHg)	115,4±7,79	122,2±7,63	133,6±24,3	134,6±19,8	135,5±20,6	135,1±19,9	129,9±19,5	0.001
DBP(mmHg)	79,5±4,64	81,8±5,57	83,9±7,24	85,6±7,27	84,1±8,56	87,9±10,39	84,0±7,93	0.001
HbA1c (NGSP) (%)	5,09±0,17	5,73±0,45	5,87±0,34	7,01±0,45	8,86±0,65	11,31±1,14	7,39±2,24	0.001
M30 (U/L)	64,3±19,4	82,0±72,51	109,3±86,16	117,4±78,76	73,6±259,14	163,4±201,12	120,2±150,54	0.002
FBG (mg/dl)	84±5,4	103±8,7	116±17,3	138±17,7	182±40,1	264±54,4	150±67,4	0.001
PPG (mg/dl)	88±10,5	123±30,7	131±41	197±44,2	248±76,5	327±117,8	190±103,5	0.001
FI (µU/mL)	7,9±4,4	14,4±7,3	15,0±8,1	17,9±10,5	16,1±14,6	13,9±8,4	14,4±9,9	0.001
HOMA-IR index	1,65±0,92	3,72±2,15	4,38±2,36	6,29±3,77	7,45±9,46	9,06±6,05	5,60±5,65	0.001
FCP (ng/ml)	1,36±0,52	2,38±0,69	2,26±0,92	2,46±1,17	2,12±1,24	2,15±1,28	2,15±1,07	0.001
CRP (mg/L)	2,0±,2,4	2,8±2,4	3,0±2,2	3,8±2,2	5,7±4,4	8,3±8,8	4,3±4,8	0.001
UA (mg/dl)	4,5±0,9	5,2±1,5	5,5±1,2	5,1±2	4,6±1,7	4,1±1,4	4,8±1,6	0.002
ALT (U/L)	29,8±9,7	31,2±12	30,8±17,2	31,9±16,1	34,5±16,8	32,3±13,5	31,8±14,5	0.562
AST (U/L)	23,4±4,8	23,3±4,8	24,8±9,8	26,6±9,8	28,6±14	25,1±9,9	25,4±9,6	0.610
Crn (mg/dl)	0,76±0,15	0,82±0,17	0,72±0,14	0,76±0,18	0,71±0,17	0,70±0,19	0,74±0,17	0.102
MAU (mg)	5,95±4,09	17,8±56,4	29,8±57,1	46,8±65,9	65,6±94,4	53,4±88,2	37,8±70,8	0.001
Tcol (mg/dl)*	176±41	188±36	195±39	212±53	193±43	192±34	193±2	0.072
HDL-c (mg/dl)	51±14	42±9	42±7	43±9	40±10	39±10	43±10	0.009
LDL-c (mg/dl)	93±40	110±34	117±30	132±48	95±44	90±47	107±43	0.014
TG(mg/dl)	123±108	168±97	169±81	211±130	297±523	295±306	214±270	0.001

HC: healthy control; PD: prediabet; Gr A :group A ; Gr B :group B; Gr C: group C; Gr D: group D; Gr T: group Total; Dd: diabetes duration BMI: body mass index; WHR: waist-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycosylated hemoglobin; M30; M30-antigen; FBG: fasting blood glucose; PPG: post prandial glucose; FI:fasting insulin ; HOMA-IR: the homeostatic model of assessment-insulin resistance; FCP: fasting C-peptide; CRP: c-reactive protein; UA: uric acid; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Crn: creatinine; MAU: Microalbuminuria; T col: total cholesterol; HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; TG: triglyceride; Data: were expressed as mean±SD , p values (*) oneway ANOVA, other data were calculated by Kruskal Walls tests

http://dx.doi.org/10.36472/msd.v8i4.524

Table 2. HbA1c, M30-antigen and C-peptide correlation with diabetes duration and chronologic age*

	HbA1c (NGSP) (%)			M30 (U/L)			C-peptide (ng/ml)		
	r	p	N	r	p	N	r	p	N
HC									
age**	0.241	0.257	24	-0.144	0.502	24	0.018	0.936	23
PD									
age	-0.091	0.645	28	-0.265	0.173	28	0.039	0.848	27
GrA									
age	0.159	0.410	29	-0.276	0.148	29	0.340	0.071	29
dd***	0.159	0.410	29	0.056	0.772	29	0.241	0.207	29
GrB									
age	0.227	0.229	30	-0.092	0.630	30	0.275	0.141	30
dd	-0.108	0.578	29	-0.196	0.308	29	-0.353	0.060	29
GrC									
age	-0.419	0.024	29	-0.377	0.050	29	-0.266	0.164	29
dd	-0.018	0.927	29	-0.287	0.131	29	-0.405	0.029	29
GrD									
age	-0.185	0.337	29	0.135	0.485	29	0.170	0.378	29
dd	-0.053	0.787	29	0.152	0.432	29	-0.153	0.428	29
Gr Diabet									
age	-0.055	0.554	117	-0.133	0.153	117	0.131	0.160	117
dd	0.183	0.049	116	-0.057	0.545	116	-0.203	0.029	116

HC: healthy control; PD: prediabet; Gr A :group A ; Gr B :group B; Gr C: group C; Gr D: group D;Gr diabet: All groups of diabetics; HbA1c: glycosylated hemoglobin;dd: diabet duration; (*)Pearson correlation analysis, (**) year, (***) month

Table 3. Distribution of M30-antigen by groups

	M30-Antigen (U/L)			
	N	Mean*	SD	p**
HC	24	64,39 a	19.4	0.043
PD	28	82,07 a	72.51	
GrA	29	109,38 b	86.16	
GrB	30	117,47 ab	78.76	
GrC	29	173,70 bc	259.14	
GrD	29	163,41 bc	201.12	

(*) If the averages have completely different letter from one another, it means that they are significantly different from one another. If they have same or common letters, it means that their difference is not significant. (**)It was assested with One-Way ANOVA and post hoc Scheffe test. Data: mean± SD, HC: healthy control; PD: prediabet; Gr A :group A ; Gr B :group B; Gr C: group C; Gr D: group D

Table 4. Correlation between biochemical parameters*

	HbA1c	C-peptide	HOMA-IR	Insulin(µU/mL)	FBG(mg/dl)	PPG(mg/dl)	CRP(mg/L)
HbA1c (%)(NGSP)							
r	-	0.055	0.413	0.092	0.909	0.781	0.409
p	-	0.483	0.001	0.236	0.001	0.001	0.001
C-peptide (ng/ml)							
r	0.055	-	0.682	0.821	0.089	0.185	0.058
p	0.483	-	0.001	0.001	0.254	0.018	0.460
HOMA-IR							
r	0.413	0.682	-	0.863	0.5	0.455	0.180
p	0.001	0.001	-	0.001	0.001	0.001	0.022
M30-antigen (U/L)							
r	0.231	0.147	0.205	0.167	0.243	0.319	0.457
p	0.002	0.059	0.008	0.031	0.001	0.001	0.001

HbA1c C-peptide HOMA-IR Insulin(µU/mL) FBG(mg/dl) PPG(mg/dl) CRP(mg/L), FBG: fasting blood glucose; PPG: post prandial glucose; HOMA-IR:homeostatic model of assessment-insulin resistance; CRP: c-reactive protein; HbA1c: glycosilated hemoglobin; (*)pearson correlation analysis

Table 5. Distribution of M30-antigen and HbA1c in terms of obesity degrees*

Obesity degree	BMI (kg/m²)	Frequency	M30-antigen(U/L)	p	HbA1c (NGSP) (%)	p
Normal weight	18,5-24,9	8(%4,7)	64,7±18,9	,337*	5,8±1,4	,013*
Overweight	25-29,9	44(%26)	126,8±208,5		6,9±2,4	
1º obese	30-34,9	53(%31,4)	97,1±87,5		7,2±2,0	
2º obese	35-39,9	39(%23,1)	133,2±121,1		8,0±2,0	
Morbid obese	40≤	23(%13,6)	163,5±198,4		8,2±3,0	

BMI: body mass index; HbA1c: glycosilated hemoglobin; *It was examined via One-Way ANOVA and post hoc Scheffe test. Data: expressed as mean± SD

Table 6. Comparison of blood pressure and anthropometric parameters with biochemical parameters*

	BMI(kg/m²)		WHR (cm)		SBP(mmHg)		DBP (mmHg)	
	r	p	r	p	r	p	r	p
M30-antigen (U/L)	0.102	0.191	0.007	0.924	0.056	0.471	0.062	0.424
HbA1c (%)	0.325	0.001	0.219	0.005	0.224	0.004	0.238	0.002
HOMA-IR	0.325	0.001	0.154	0.050	0.166	0.033	0.162	0.037
CRP (ng/ml)	0.187	0.017	0.029	0.713	0.176	0.025	0.237	0.002

HOMA-IR: the homeostatic model of assessment-insulin resistance; CRP: c-reactive protein; HbA1c: glycosylated hemoglobin;
*pearson correlation analysis

Table 7. Relation of M30- antigen with biochemical parameters*

	M30- antigen level (U/L)	
OGTT 2nd hour (mg/dl)	r	p
UA (mg/dl)	0,546	0,002
ALT (U/L)	0,004	0,963
AST (U/L)	0,503	0,001
Creatine (mg/dl)	-0,068	0,386
MAU (mg)	-0,012	0,878
T col (mg/dl)	0,111	0,154
HDL-c (mg/dl)	-0,044	0,569
LDL-c (mg/dl)	0,092	0,238
TG (mg/dl)	0,040	0,606

OGTT 2nd: oral glucose tolerance test 2nd hour ; UA: uric acid ALT: alanine aminotransferase; AST: aspartate aminotransferase;
MAU: Microalbuminuria; T col: total cholesterol HDL-c: high-density lipoprotein; LDL-c: low-density lipoprotein; TG: triglyceride;
*pearson correlation analysis

DISCUSSION

It has been demonstrated through autopsies of pancreatic tissue in patients with T2D that a decrease in β-cell mass is accompanied by an increase in cell apoptosis (13). The study of cellular apoptosis in primary human pancreatic tissue is limited due to the invasive techniques. In this study, this limitation was avoided to some extent by estimating M30-antigen levels in serum (in vivo), which reflects the total apoptosis of single-layer and glandular epithelial cells in the body. This study investigated the relationship between hyperglycemia and apoptosis marker M30-antigen through the comparison of HC subjects and patients with T2D.

Does the increase of hyperglycemia levels escalate apoptosis?

The glucose sensing and subsequent glucose metabolism comprises the mechanism involved in glucose-stimulated insulin secretion by β-cells. Aberrant β-cell functioning results in hyperglycemia by insufficient insulin production(14). ROS is produced because of the exposure of β-cells to glucolipotoxicity that is caused by increased plasma glucose and lipid levels. A perspective claims that ROS produces apoptosis by releasing cytochrome C and activating caspase(15-18). A study observed that apoptotic cell death in diabetic myocardial cells occurs via caspase-3 activation. The study reported that hyperglycemia leads to ROS production in vivo subsequently causing apoptosis of cells in the myocardium (19). Many stimulants such as cytokines, leptin, glucose, sulphonylurea, and free fatty acids can trigger apoptosis in β-cells (4). A second view supports that support the notion that significant and progressive loss in β-cell mass appears before patent hyperglycemia emerges in the patients with the T2D(20). It has been proposed that the loss in β-cell mass is an early stage indicator in the process of T2D development, and it is the primary reason rather than being secondary to hyperglycemia.

They urge that the loss in β cell mass is an early stage in T2D process, and it is a primary reason instead of being secondary for hyperglycemia (13,21). Sulphonylureas have positive insulin secretory effects, they also cause secondary failure decreasing mass and the secretory function of the β-cells because of the continuous increase of Ca^{+2} influx due to a long-term treatment (22).

In a group of patients with morbid obesity selected for bariatric surgery, a significant correlation was observed between HbA1c and M30-antigen levels (23). In a study involving individuals with NAFLD, they claimed that there was not any difference between CK-18 concentrations among the patients having T2D. They also claimed that CK-18 existing in blood will not be affected by apoptotic tendencies in the patients having diabetes(15). Several studies have evaluated the correlation between hyperglycemia and apoptosis, however, it is still unclear whether hyperglycemia induces apoptosis or not. In our study, hyperglycemia level and severity of diabetes were designed on the basis of HbA1c level, regardless of the duration of diabetes (Table 1). When we examined the differences among the groups with reference to M30-antigen, we observed that levels of the M30-antigen in the HC and PD groups were significantly lower than the Gr C and Gr D (p=0.043) (Table 3). Furthermore, when all individuals were taken into consideration, there was a significant correlation among M30-antigen and HbA1c levels (r=0.231, p=0.002) (Table 4). Therefore, it may be concluded that as long as level of HbA1c increases, the level of M30-antigen increases, as well. In other words, when glycemic levels increase, apoptosis increases, too. The PD is a period that hyperglycemia starts, and hyperglycemia severity is lower compared to other groups. In this group, the level of M30-antigen is low in parallel with hyperglycemia that is why it can support the idea that the level of hyperglycemia increases apoptosis.

A significant relationship was found between β-cell mass and FBG concentrations. It has been shown that a decrease in β-cell mass is correlated with an increase in blood glucose level(20). In our study, we report the effects of both parameters FBG and PPG on apoptosis, which revealed a positive correlation with M30-antigen levels (Table 4). Apoptosis may occur as a secondary effect of hyperglycemia and in turn the apoptosis may cause hyperglycemia because of β-cell death. It is thought that uncontrolled hyperglycemia increases M30-antigen levels and in case of emerging of an intense hyperglycemic state, even though as a new-onset, it accelerates the process of apoptosis. Rather than the duration of the diabetes, the level of hyperglycemia in other words its severity may increase the rate of apoptosis.

The relation between insulin resistance and M30-antigen

β-cell hyperplasia develops at the early stage of the T2D, followed by insulin resistance, and impaired insulin secretion, then a decrease occurs in the β-cell mass. In T2D, body produces insulin, but cannot use efficiently, which results in hyperglycemia and subsequently IR. In a study involving morbidly obese patients, a correlation between M30-antigen and HOMA-IR was observed (23). Furthermore, a significant correlation was found between HOMA-IR score and M30-antigen levels in NASH patients (24). In this study, we detected a positive and a significant correlation between HOMA-IR score and HbA1c levels. When the relation between HOMA-IR and M30-antigen is considered, there was also a significant correlation (Table 4). Our results suggest that IR is the key factor causing hyperglycemia. In addition, IR may trigger the process of apoptosis in β-cells.

Relation of the duration of diabetes and chronological age with M30-antigen level

Approximately 90% of individuals over 40 years of age with T2D have early pancreatic β cell loss and a progressive impaired in glucose tolerance (25). Furthermore, it is unclear whether it is a physiological apoptosis period or a pathological one. It was claimed that programmed cell death, in other words, apoptosis are related to the aging effect, as well. On a study focused on patients with breast cancer, it was revealed that apoptosis increases when patient age is higher(26). In another study involving M30-antigen analysis, it was suggested that CK-18 fragments are more sensitive in older patients than younger patients in the diagnosis of NAFLD(27).In our study, we examined whether there is a relation between M30-antigen levels, chronological age, and duration of the diabetes. It was observed that there was no correlation between M30-antigen levels, chronological age, and diabetes duration of the patients (Table 2). Our results provided a contrasting observation to the previous studies that the rate of apoptosis increases with the age. The patient age and the duration of the disease in individuals with diabetes are correlated with HbA1c levels. The duration of diabetes is related to hyperglycemic levels, however, there is no correlation between apoptosis and the duration. When only the diabetics were taken into consideration, a significant, but adverse correlation was observed between the duration and C-peptide levels (Table 2). Furthermore, there was no correlation between HbA1c and C-peptide levels. It can be concluded that in T2D age and duration of the diabetes are not exclusive factors contributing to apoptosis, but hyperglycemia is also correlated with the duration of the disease and the age.

Relation between anthropometric parameters and M30-antigen

In the studies, BMI and WHR, which are accepted as IR parameters, and their relationship with M30-antigen level was evaluated, a significant positive correlation was found only in individuals with NASH(24,28). In our study, we observed that BMI correlates with HOMA-IR and HbA1c levels, however, we did not observe any relationship between M30-antigen, BMI, and WHR (Table 6). Moreover, we detected a significant correlation between WHR and HbA1c levels (Table 6). We could not find a significant difference between the degree of obesity and M30-antigen levels. The concentration of M30-antigen in morbidly obese individuals was found to be significantly higher compared to the individuals with normal weight. Apart from this finding, we did not observe any significant difference (Table 5). Further comprehensive studies are certainly needed to explore the correlation between obesity and M30-antigen.

β -cell reserve and apoptosis

We also investigated whether hyperglycemia causes apoptosis or vice versa with respect to C-peptide. C-peptide is the optimal standard for the β-cell function, in other words, endogenous insulin secretion (29). The effect of cytosolic and mitochondrial ROS production induced with hyperglycemia on apoptosis was explored in a study. It was identified that meditated C-peptide activation of AMPKa inhibited mitochondrial fission and endothelial cell apoptosis that was caused by ROS production induced by hyperglycemia (30,31). Therefore, C-peptide replacement therapy was presented as encouraging among new treatment options (32).

In our study, we found a significant relation between insulin and C-peptide levels. However, we could not find any correlation between hyperglycemia and C-peptide. The reason for inexistence of the correlation may be IR, in the presence of IR, although the initial hyperglycemia, the C-peptide level is normal because β-cell produces insulin but insulin does not function. In fact, there was also a significant correlation between HOMA-IR and C-peptide levels in our study (Table 4). When only the diabetics were taken into consideration, a statistically significant adverse correlation was found between C-peptide levels and diabetes duration (Table 2). It appears that the C-peptide level decreases as the duration of diabetes increases. These results make it possible to infer that endogenous insulin levels decrease as time goes by. A decrease in C-peptide and insulin levels may be observed in the late stages of T2D because of progressive loss of β-cell. In addition, it also explains the hyperglycemia that occurred due to an increase in the duration of the disease and the increase in the cellular apoptosis that occurred due to hyperglycemia.

Subclinical inflammation and apoptosis

Hyperglycemia increases general oxidative load by increasing various pro-inflammatory cytokines in pancreatic β-cells, resulting in apoptosis through extrinsic and intrinsic pathways (4). CRP, an inflammatory marker, promotes the recognition and elimination of pathogens and increases the clearance of necrotic and apoptotic cells (33). The level of CRP increases in obesity, and this increase is related to BMI and IR(23,27).

In a meta-analysis, it was suggested that increased CRP levels were significantly related to increased T2D risk (34). CRP that is an acute phase indicator, correlate with metabolic syndrome components (35). It is thought that this acute phase response reflects subclinical inflammation that is mainly present, and that this process is responsible for progressively developing DM (36). In this study, we observed a significant relation between CRP levels and BMI (Table 6). In addition, a significant correlation was observed between CRP levels and HbA1c levels as well as between CRP levels and HOMA-IR (Table 4). We also observed a significant correlation between levels of M30-antigen and CRP (r=0.471, p=0.001) (Table 4). IR leads to hyperglycemia, and in turn it causes an inflammatory condition. CRP increases as an inflammatory response indicator. In addition, that suggests apoptosis can be triggered because of this developing inflammatory condition. The limitation of this study is that because of financial constraints, the high sensitive CRP was not analyzed.

Aminotransferases and M30-antigen relation

In a study focused on the patients with NAFLD and T2D, a positive correlation was discovered between AST and ALT levels, and serum CK-18 concentrations (28). In addition, during a study which was designed to distinguish NASH with simple steatosis by using CK-18 fragments, a significant correlation was found even if the connection between AST and ALT values and M30-antigen levels was weak (37). In our study, we found a significant correlation between levels of ALT and AST with M30-antigen. However, the study lacks in examining the patients in terms of NAFLD thoroughly.

Will M30-antigen be useful while diagnosing Diabetes Mellitus?

The HbA1C level, which is used in T2D diagnosis, was initially solely during the follow-up on the treatment. International Specialist Committee, constituted by the ADA, The European Association for the Study of Diabetes, and International Diabetes Federation, reported in 2009 that HbA1c test could be used as one of the diagnostic criteria of DM (38, 39). However, illnesses, which shorten the life of erythrocytes, renal failure, and chronic over consumption of alcohol prevent the test to be used for the purposes of screening, diagnosis, and follow up (40). Therefore, the question arises if M30-antigen can be used to diagnose T2D and PD illnesses according to HbA1c. In this study, it was observed that the levels of M30-antigen could not distinguish HC and PD

When we analyze the results of HbA1c and M30-antigen in terms of distinguishing diabetes, from the healthy control group, it is possible to say that these two markers can be used for separating both groups. ; ROC analysis has shown that HbA1c is more effective than M-30 antigen in the segregation of patients with diabetes from healthy individuals (Figure 1a, Figure 1b).

CONCLUSIONS

This study concludes that when there is a rise in glycemic level, M30-antigen level increases. Therefore, when glycemia level increases, apoptosis increases consequently. Hence, it might be appropriate to infer that apoptosis occurs as a secondary manifestation to hyperglycemia, and the apoptosis causes the loss of β-cells and aggravates hyperglycemia. When diabetes is not controlled, it increases M30-antigen levels, and even if the duration of the disease is short, the hyperglycemia accelerates the process of apoptosis.

Considering the findings in this study, it can be stated that IR is the real cause that leads to hyperglycemia. In the IR an inflammatory condition is started and as a response to the inflammatory condition the CRP increases. This inflammatory state triggers apoptosis, concurrently. The C-peptide and insulin deficiencies may be observed in late stages of T2D because of progressive loss of β-cells. Moreover, M30-antigen is less successful than HbA1c in terms of diagnosing diabetes and prediabetes.

As a result, we should develop T2D treatment strategies by focusing on breaking or preventing IR occurrence, which causes hyperglycemia. Apoptosis is not a reason but is observed as a response of hyperglycemia, and our results support this opinion. Further studies should explain the effect of medical treatment on apoptosis and relation of diabetes complications with apoptosis.

Acknowledgments: The authors thank Prof. Dr. Azmi Yerlikaya and Talip Kurumemet for initial collaboration on the manuscript.

Author contributions: IB, TY, HA; Literature search and study design, statistical analyzes, **IB;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

Determination rates of antibiotic resistance, inducible beta-lactamase, and metallo beta-lactamase ratios in *Pseudomonas aeruginosa* isolates in a university hospital in Turkey.

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ABSTRACT

Objective: This study aimed to determine the antibiotic resistance, inducible beta-lactamase (IBL), and Metallo beta-lactamase (MBL) rates in *P. aeruginosa* isolates.

Material and Methods: In our study, 100 *P. aeruginosa* isolates obtained from various clinical samples were used. Antibiotic susceptibility was performed by using the Kirby-Bauer disk diffusion method. Carbapenem resistance to imipenem and meropenem was verified by the E test. The disk induction method was used to determine the IBL production while the Modified Hodge test, MBL E test, and combined imipenem/ EDTA disk were used to determine the production of MBL.

Results: According to the results of antibiotic susceptibility tests, 58% of *P. aeruginosa* isolates were susceptible to all antipseudomonal drugs, while resistance rates to other drugs were as follows; ceftazidime 7%, cefoperazone sulbactam 8%, cefepime 13%, piperacillin 14%, piperacillin-tazobactam 12%, imipenem 9%, meropenem 11%, aztreonam 8%, amikacin 8%, gentamicin 13%, tobramycin 12%, netilmicin 19%, There was a 10% resistance to ciprofloxacin. 8% of the isolates were resistant to at least three drugs, of which two isolates were positive for MBL enzyme production. IBL production was detected in 86% of the isolates with the disk induction method.

Conclusion: The results we obtained in our study are consistent with other researchers globally and in Turkey. It was concluded that there is a need for well-standardized phenotypic tests with defined evaluation criteria and further studies to verify these tests genotypically.

Keywords: Antibiotic susceptibility, inducible beta-lactamase, Metallo beta-lactamase, *Pseudomonas aeruginosa*, multidrug-resistant

Research Article

Received 31-03-2020

Accepted 15-04-2021

Available Online: 18-04-2021

Published 30-04-2021

INTRODUCTION

Pseudomonas aeruginosa, the most pathogenic species in the *Pseudomonadaceae* family, is gram-negative, non-spore, straight or slightly curved, 1.5-5.0 µm long, and 0.5-1.0 µm wide (1,2). The characteristic grape scent from tryptophan 2-aminoacetophenone production is used to differentiate *P.aeruginosa* (3,4).

P. aeruginosa is considered an opportunistic pathogen that causes nosocomial and ventilator-associated infections. It rarely causes infections in healthy individuals, but cysts are observed with high morbidity and mortality in patients with fibrosis and immunocompromised individuals. *P. aeruginosa* is an important infectious agent thanks to its ability to reproduce under minimum conditions, the presence of various virulence factors, and the resistance mechanisms it has developed (5,6). Today, the prevalence of multidrug-resistant *P. aeruginosa* strains is reported to be between 15-30% in different regions, while resistance rates of more than 10% are reported for all antimicrobial groups used (7). The resistance profile of *P. aeruginosa* varies between hospitals and even clinics in the same hospital.

The susceptibility profile of the bacterium should be well known and regularly monitored in the application of appropriate empirical therapy. In this context, determining the resistance status of isolated *P. aeruginosa* strains to antibiotics will help clinicians have an empirical approach and promote rational drug use thus, ensuring that resistance profiles that may develop during the treatment process will be prevented (**8,9**). It has been reported that *P. aeruginosa* is a factor in 10% of hospital infections (**10**).

The present study aimed to determine the resistance status in *P. aeruginosa* infections, to contribute to empirical treatment options and effective treatment approaches. For this purpose, antibiotic resistance, inducible beta-lactamase (IBL), and Metallo beta-lactamase (MBL) ratios were determined in *P. aeruginosa* isolates isolated from various clinical samples in Sanlıurfa, Turkey.

MATERIAL and METHODS

This study was approved by the Ethical Committee of Harran University Faculty of Medicine (Protocol no.05/2015).

1. Sample collection

P. aeruginosa isolates were isolated from various clinical samples sent to the Harran Research and Application Hospital microbiology laboratory. Repeating samples belonging to the same patient were excluded from the study. The isolates were taken into a Tryptic Soy Broth storage media and stocked at -70°C until the study day.

2. Identification of isolates

Isolates were identified by standard microbiological identification techniques. Non-fermentative, oxidase-positive, aerobic, motile, blood agar and eosin methylene blue agar grew at 37° C and 42° C with a characteristic odor, and blue-green pigmentary strains were evaluated as *P. aeruginosa*.

3. Antibiotic susceptibility test

Antibiotic susceptibility profiles of the isolates were determined using a Kirby-Bauer disk diffusion method as per the guidelines of the Clinical Laboratory Standard Institute (CLSI) (11).

3.1. Disc diffusion method

In antibiotic disc diffusion tests, imipenem 10µg (IP), meropenem 10µg (MP), aztreonam 30µg (ATM), ceftazidime 30µg (CAZ), cefepime 30µg (CEP), gentamicin 10µg (GN), amikacin 30µg (AK), tobramycin 30µg (TOB), netilmicin 30µg (NET), ciprofloxacin 5µg (CIP), piperacillin 100µg (PRL), piperacillin/ tazobactam 110µg (TZP), and cefoperazone/ sulbactam 105 µg (CFS) antibiotic discs were used. Antibiotic discs were placed in the plates of Mueller-Hinton agar (MHA) (adjusted to 0.5 McFarland standards). Plates were incubated for 18-20 hours at 35 °C. The inhibition zone diameter was recorded for each isolate and reported as per the CLSI guidelines (**11**). Carbapenem resistance was confirmed by determining the MIC value with the E test method according to disk diffusion test results.

3.2. E-test

E test strips (OXOID, UK) containing 0.02-32 µg/ml IP and 0.02-32 µg/ml MP were used. Briefly, bacterial suspensions with 0.5 McFarland turbidity were prepared from *P. aeruginosa* cultures grown on the MHA plate. Imipenem and meropenem strips were placed on plates and left to incubate for 18-20 hours at 35°C. At the end of the incubation, the E test strips were evaluated according to the manufacturer's recommendations and the MIC values were determined (**12**).

3.3. IBL detection test

IBL production was detected by the disc induction method. To determine the IBL activity on the MHA plate, the IP (10µg) was placed in the middle of the plates containing CAZ (30µg) and ATM (30µg) discs with a distance of 20 mm between the disc centers. After 18-20 hours of incubation at 35° C, the narrowing of the inhibition zone diameters of the CAZ and ATM discs in the MHA plate facing the IP disc (⩾ 4 mm) was evaluated as positive. IBL positivity was evaluated for each isolate relative to IP. The *P. aeruginosa* ATCC 27853 strain was used as a negative control (**13**).

3.4. Determination of MBL production in isolates

MBL production was evaluated by using a Modified Hodge test, IP/ EDTA Combined Disk test, and MBL E test.

3.5. Modified hodge test

For the modified Hodge test, bacterial suspension was prepared according to the 0.5 McFarland of E. coli ATCC 25922 strain (indicator strain) and was left to incubate overnight on an MHA plate. After the plate was dried, the IP (10µg) disk was placed in the center of the plate. IP and/ or MP MIC values were determined by E test, and the cultivation of resistant *P. aeruginosa* isolates was done mutually, linearly from the center to the periphery, and incubated at 35° C for 16-18 hours. The MBL production was evaluated as positive when a clover leaf-shaped deterioration of the IP disc inhibition diameter was sighted. *P. aeruginosa* ATCC 27853 was used as a negative control in the assay (**14,15**).

3.6. IP/ EDTA combined disk test

EDTA was used as an MBL enzyme inhibitor in the IP/ EDTA combined disk test. For 0.5 M EDTA (pH=8.0); 18.61g disodium EDTA.H_2O powder was dissolved in 100 ml of sterile distilled water, and stored at + 4° C.Two IP discs were placed 25 mm apart on one-half of the plate of *P. aeruginosa* isolates. 10 µl of 0.5 M EDTA solution was dropped onto one of the IP discs and then incubated at 35 ^{0}C for 18 hours. When the inhibition zone diameter around the IP/ EDTA disc was ⩾7 mm from the inhibition zone diameter around the IP disc, the MBL was considered positive. *P. aeruginosa* ATCC 27853 was used as the negative control (**16,17**).

3.7. MBL E test

MBL E test strips containing 4-256 µg/ml IP on one side and IP (1-64 µg/ml) –EDTA (IPE) on the other were used. IP/ IPE test strips were placed on the plates containing *P.aeruginosa* and incubated at 35 °C for 18 hours. According to the manufacturer's recommendations, the MIC value for IP was proportioned to the MIC value for IPE. Isolates with ⩾ 8 MIC ratio obtained on both the reagent side of the

http://dx.doi.org/10.36472/msd.v8i4.525

"phantom" zone or deformation in both ellipses was evaluated as MBL positive. *P. aeruginosa* ATCC 27853 was used as the negative control (**18**).

4. Statistical analysis

Statistical analysis of the data obtained from antibiotic susceptibility results was performed using the chi-square test in the SPSS (Incorporated, Chicago) program. One-way Annova was used between multiple groups. The Student T and Pearson Correlation tests were used between single groups.

RESULTS

The most isolated sample type of *P. aeruginosa* was urine with 26.7%, and the least sample type was determined as Cerebrospinal fluid (CSF) with 2%. The distribution of isolates according to clinical samples is shown in Table 1.

Table 1. Distribution of clinical samples in which *P.aeruginosa* was isolated.

Sample type	n	%
Trachea	20	19.8
Sputum	19	18.8
Urine	27	26.7
Throat	4	4.0
Blood	6	5.9
Wound	15	14.9
CSF	2	2.0
Aspiration sample	3	3.0
Swab	5	5.0
Total	**100**	**100.0**

P. aeruginosa isolates were studied with 51 females and 49 males. The ages of these patients were between 1-87 and the mean age was 35 ± 2.8. When the patients from whom *P. aeruginosa* isolates were produced are classified according to their age, they are divided into four groups. Accordingly, the prevalence of *P. aeruginosa* isolates was found to be in 38% of children aged 0-14 (Table 2).

Table 2. The age range of patients from whom *P. aeruginosa* strains were isolated (n: 100)

Clinics	0-5 years	5-14 years	14-50 years	>50 years
ICU	36.7	6.7	10	46.7
Urology	28.6	42.9	21.4	7.1
Endocrine	0	16.7	33.3	50
Chest disease	0	0	56.3	43.8
Dermatology	0	0	100	0
Otolaryngology	0	0	100	0
Pediatric service	75	25	0	0
General surgery	33.3	0	50	16.7
Orthopedics	0	0	50	50
Infection	0	0	57.1	42.9

In our study in which 100 *P. aeruginosa* isolates were evaluated, *P. aeruginosa* isolates were most frequently (30%) isolated from the ICU. The distribution of the isolates according to the clinics where they were isolated is shown in figure 1.

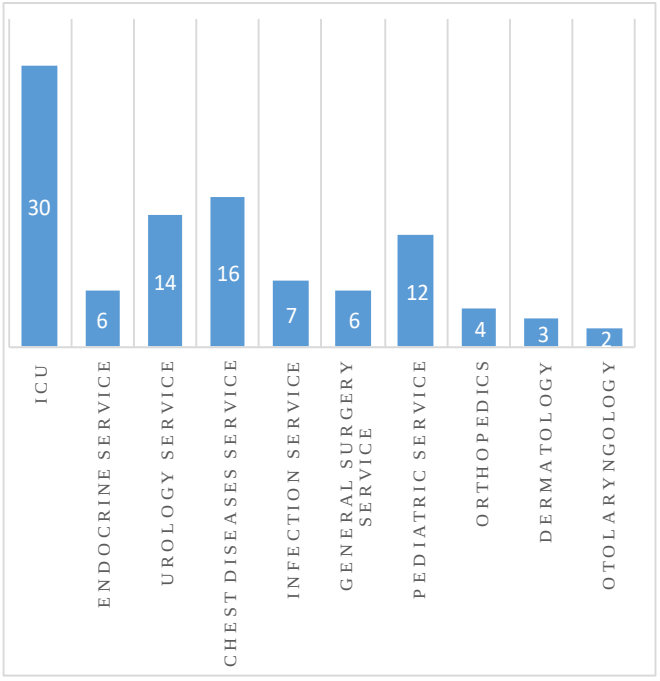

Figure 1. Distribution of *P. aeruginosa* isolates according to the clinics where they were isolated.

1.Antibiotic susceptibility test

According to the results of antibiotic susceptibility tests, 58% of *P. aeruginosa* isolates were susceptible to all antipseudomonal drugs studied; CAZ 7%; CFS 9%; CFP 11%; PRL 12%; TZP 8%; IP 9%; MP 11%; ATM 9%; AK 8%; GN 11%; TOB 9%; NET 19%; and 10% resistance to CIP. The sensitivity of *P. aeruginosa* isolates to antibiotics is shown in Table 3. It was found that 8% of the isolates were multidrug resistance (MDR) to at least three antipseudomonal drug groups. MBL enzyme production was determined in two of these eight MDR isolates. It has been observed that the rate of MBL enzyme production is higher in bacteria with MDR. As a result, colistin with a sensitivity rate of 100% was seen as the most effective antibiotic in *P. aeruginosa* isolates. Apart from colistin, the least resistance was determined in CAZ, followed by AK, IP, and CFS.

Table 3. Sensitivity of *P. aeruginosa* isolates to antimicrobial agents.

Antibiotic agents	Sensitive		Resistant	
	n	%	n	%
Imipenem	91	91	9	9
Meropenem	89	89	11	11
Ceftazidime	93	93	7	7
Cefepime	87	87	13	13
Aztreonam	92	92	8	8
Piperacillin	86	86	14	14
Piperacillin/tazobactam	88	88	12	12
Amikacin	92	92	8	8
Gentamycin	87	87	13	13
Tobramycin	88	88	12	12
Netilmicin	81	81	17	17
Ciprofloxacin	90	90	10	10
Cefoperazone/Sulbactam	92	92	8	8
Colistin	100	100	0	0

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The difference between the resistance rates of colistin and beta-lactam antibiotics IP, MP, CAZ, CEP, CFS, and PRL, was found to be statistically significant ($p < 0.05$). Again, the difference between the resistance rates of beta-lactam antibiotics within themselves was found to be statistically significant also ($p < 0.05$). The sensitivity of nine *P. aeruginosa* isolates resistant to carbapenems to other antibiotics is shown in Table 4.

Table 4. Sensitivity of 9 isolates of *P. aeruginosa* resistant to carbapenems to antibiotics other than carbapenem.

Antibiotic agents	Sensitive		Resistant	
	n	%	n	%
Ceftazidime	6	66.7	3	33.3
Cefepime	3	33.3	6	66.7
Aztreonam	4	44.4	5	55.6
Piperacillin	3	33.3	6	66.7
Piperacillin/tazobactam	4	44.4	5	55.6
Amikacin	5	55.6	4	44.4
Gentamycin	4	44.4	5	55.6
Tobramycin	1	11.1	8	88.9
Netilmicin	1	11.1	8	88.9
Ciprofloxacin	3	33.3	6	66.7
Cefoperazone/Sulbactam	4	44.4	5	55.6
Colistin	9	100	0	0

2. Disk induction test

IBL production in *P.aeruginosa* isolates was determined by the disc induction method as 86% of the isolates were IBL positive and 14% as IBL negative (Fig. 2). All carbapenem-resistant isolates were identified as IBL negative. When the relationship between IBL positivity and color was investigated, it was found to be significant ($p < 0.05$); the green pigment ratio of IBL producing *P. aeruginosa* strains was 68.9%, and the green pigment ratio of *P. aeruginosa* strains that did not produce IBL was 31.1%.

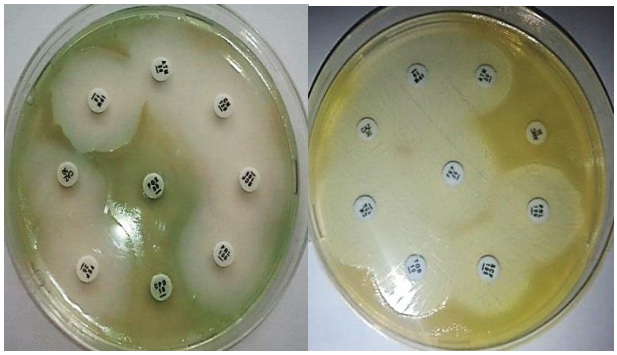

Figure 2. IBL positive (left) and negative (right) *P. aeruginosa* isolates.

3. MBL E combined disc and Modified Hodge tests

MBL production in *P. aeruginosa* isolates was investigated by three different phenotypic methods. MBL production was determined in 2 isolates out of 9 carbapenem-resistant isolates with the MBL E test (Fig. 3).

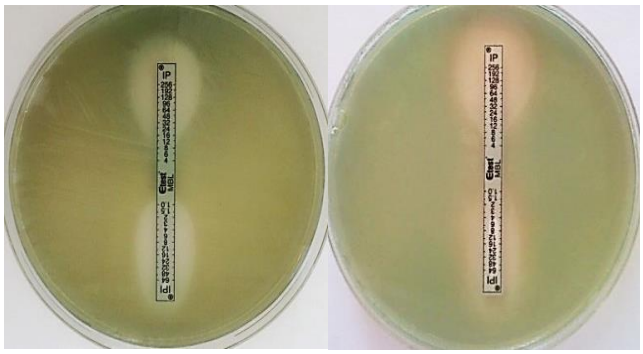

Figure 3. MBL positivity (left) and negativity (right) with the MBL E test.

MBL uremia was again determined in two isolates with the Combined disc test. Two isolates that were found positive with the MBL E test also gave positive results with the Combined disc test. Combined disc test results were also negative in all isolates with negative results with the MBL E test (Fig. 4). With the modified Hodge test, one of the 9 carbapenem-resistant isolates was detected as MBL positive and 8 as MBL negative (Fig. 4). One isolate was detected as MBL positive by all three tests and one isolate detected as MBL positive by the MBL E test, and the Combined disk test was accepted as MBL producing isolates.

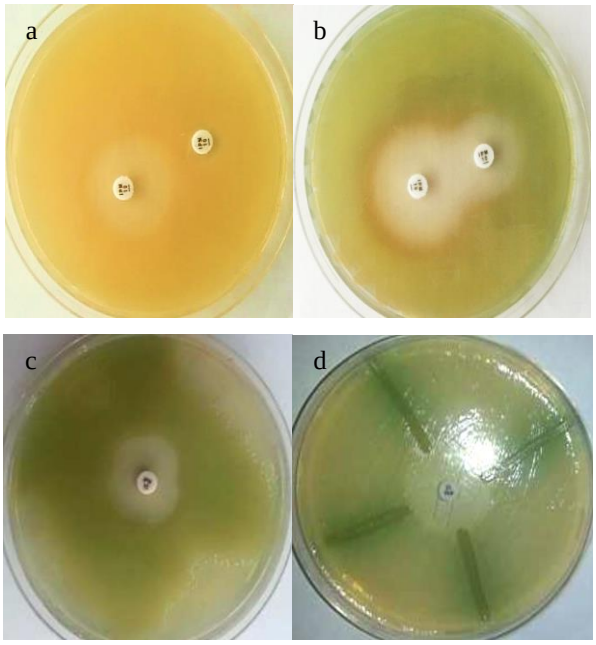

Figure 4. Detection of MBL production with the Combined disc test (**a, b**) and modified Hodge test **(c, d**). **a,c**: MBL positive, **b,d**: MBL negative.

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Table 5. Test results of two MBL positive *P. aeruginosa* isolate by MBL E test and Combined disc test.

MBL positive isolates	MBL E test (MIC value/µg/ml)		MBL with Combined Disk test (zone diameter/mm)	
	IP	IP/EDTA	IP	IP/EDTA
1	12	1	9	19
2	9	1	11	28

Table 6. Results of MBL detection by phenotypic tests in 9 carbapenem-resistant *P. aeruginosa* isolates and standard strains.

Isolates	Phenotypic Methods		
	Modified Hodge Test	Combined Disk Test	Etest
1	1 (11.1%)	2 (22.2%)	2 (22.2%)
2	1 (11.1%)	7 (77.7%)	7 (77.7%)
***P. aeruginosa*ATCC 27853**	Negative	Negative	Negative

The difference between the IP/ EDTA and IP disc inhibition zone diameters of two isolates with MBL production positive with the Combined disk test was19 mm in the first isolate and 28 mm in the second isolate (Table 5).

It was determined that the difference between inhibition zone diameters in 7 isolates determined as MBL negative with the Combined Disk test varied between 1-6 mm.

Among the carbapenem-resistant *P. aeruginosa* isolates, the rate of MBL positive isolates was determined as 22.2%, while the rate of MBL among all isolates was determined as 2%. The results of three phenotypic methods used in detecting the MBL enzyme are shown in Table 6.

The compatibility of MBL E, Combined Disc, and Modified Hodge tests with each other was calculated according to the formula below:

The compatibility rate (%) = a + b / c x 100; where

a: Number of isolates giving positive results with both tests: 1

b: Number of isolates that gave negative results with both tests: 7

c: Total number of isolates: 9

According to this; the compatibility between the MBL E test and the Combined Disc test was 100%, the compatibility between the MBL E and the Modified Hodge tests was 88.8%, and the agreement between the IP/ EDTA Combined Disc and the Modified Hodge tests was 88.8%.

The sensitivity of *P. aeruginosa* isolates positive for MBL by E test and Combined Disc test to other antibiotic agents was evaluated in this study. Accordingly, the MBL positive 1st isolate was resistant to CEP, TZP, GN, TOB, and NET, while the 2nd isolate was resistant to CAZ, CEP, TZP, GN, TOB, NET, and CFS (Data have not shown). Two MBL positive isolates were isolated from the tracheal aspirate of patients hospitalized in the ICU.

DISCUSSION

P. aeruginosa is an opportunistic pathogen and it has been reported to frequently cause nosocomial infections in immunocompromised patients (**19**). It is responsible for 10-25% of hospital infections in the world (**20,21**). *P. aeruginosa* is the most commonly isolated non-fermentative bacterium among nosocomial pneumonia, urinary tract infection, wound infections, and septicemia.

The ability of *P. aeruginosa* to reproduce easily, the β-lactamase enzymes it produces, and its natural and acquired resistance capabilities emerge as an important problem worldwide (**22**). In most of the studies conducted in our country, *P. aeruginosa* strains were most frequently isolated from the ICU (**23**).

In a study conducted between 2010 and 2016, it was observed that the *P. aeruginosa* strain isolated was mostly associated with patients admitted to the pediatric outpatient clinic and hospitalized in the ICU. Outpatient isolates were 58% urine, 24.2% wound, 11.5% external auditory canal, and 7.5% sputum. 37.8% of the patients hospitalized in the ICU had respiratory tract, 25.4% wound, 22.7% urine, and 9.7% blood (**24**).

In our study, *P. aeruginosa* strains were isolated most frequently (30%) from the ICU. These strains were isolated as 26.72% urine, 19.8% tracheal aspirate, 18.8% sputum, 14.9% wound, 5.9% blood, 5% swab, 4% throat, 3% aspiration fluid and 2% CSF. *P. aeruginosa* can develop resistance to antibiotics through various resistance mechanisms. Production of chromosomal and plasmid-derived beta-lactamases, changes in antibiotic targets, decrease in outer membrane permeability as a result of changes in porin proteins, and expulsion of the antibiotic by the efflux pump system are the main resistance mechanisms (**25**). One of the mechanisms to develop resistance during antibiotic therapy is to produce AmpC inducible chromosomal beta-lactamase and OXA type chromosomal beta-lactamase enzymes (**26,27**).

Resistance rates have been reported in various studies conducted in our country. In one study, all 195 *P. aeruginosa* strains (100%) had resistance to CAZ, 90.8% to TZP, 60.5% ATM, 50.2% CEP, 48.2% IP, 47.2% MP, 44.1% PRL, 31.3% levofloxacin (LVX), and 26.2% to CIP, It was also reported that 11.8% was resistant to GN, 8.7% to AK and 6.2% to TOB (**28**).

In a study conducted in our country, IP resistance was detected in 20 (18.5%) of 108 *P. aeruginosa* strains isolated from ICU patients, and MBL production was 14 (70%) in 20 IP resistant strains. The highest MBL production was observed in isolates obtained from sputum (**29**).

In a study in which 72 *P. aeruginosa* isolates from 1443 stool samples were evaluated, ceftazidime resistance was 8%, CEP 7%, ATM 7%, GN 3%, CIPas 1% and IPresistance as 1% (**30**). While 58% of *P. aeruginosa* isolates were sensitive to all antipseudomonal drugs in our study; IP 9%, MP11%,

ATM 8%, CAZ 7%, CFS 8%, CEP 13%, PRL 14%, TZP 12%, AK 8%, GN 13%, TOB 12%, NET 19%, and 10% resistance to CIP was found.

Antimicrobial resistance rates in *P. aeruginosa* isolates in our hospital were determined similar to other studies in our country. The most effective antibiotic has been determined as colistin with a sensitivity rate of 100%. Less resistance except for colistin, beta-lactam antibiotics CAZ; AK was determined from the aminoglycosides Determination of MBL production in Pseudomonas strains in hospitals by phenotypic methods will contribute to infection control with early control measures. In a study conducted in Italy, MBL positivity was found in 12.6% of *P. aeruginosa* isolates by E-test (**31,32**).In our study, MBL production was detected in 22.2% of 9 carbapenem-resistant *P. aeruginosa* isolates. The MBL positivity rate was determined as 2% among all isolates. The rate of MBL was found to be lower than many studies in our country and abroad. Carbapenem-resistant isolates, which we found to be MBL negative, could not be determined by phenotypic methods other than the MBL enzyme or efflux due to OprD porin protein loss. However, it has been concluded that molecular studies are required to confirm this. Various studies on IBL production have been carried out abroad and in our country. In a study conducted in Turkey isolated from various clinical specimens of 87 *P. aeruginosa* strains, 64 (74%) was the determined IBL production rate (**33**).

In our hospital, no research has been conducted on the prevalence of IBL in *P. aeruginosa* isolates in previous years. In our study, IBL production was detected in 86% of *P. aeruginosa* isolates by the disc induction method. While this rate is parallel to IBL rates in studies abroad, it was higher than many studies conducted in our country. Although the production of IBL in the treatment was not specified by the laboratory due to the high rate of IBL in our hospital, it should be accepted that *P. aeruginosa* isolates have this feature. Also, the rate of green pigment was 68.9% in the IBL producing *P. aeruginosa* isolates and 31.1% in the isolates that did not produce IBL. It has been determined that most of the carbapenem-resistant isolates produce green pigment. These results suggest that there may be a relationship between the pigment color produced by *P. aeruginosa* and antibiotic resistance. It was thought that other studies should be conducted on this subject.

There are some limitations to this study. All isolates were obtained from one center in Turkey (Sanlıurfa). The low number in the centers led to limited the spread of the results across the country. Also, resistance mechanisms were not investigated in the study. As future perspectives, further studies with the molecular methods are needed to the identification of the resistance mechanisms.

CONCLUSIONS

In conclusion, for the treatment of cases caused by *P. aeruginosa*, the resistance profile of these isolates should be well known. Accordingly, necessary precautions should be taken to prevent the increase in resistance rates, effective policies regarding the use of antibiotics in hospitals should be developed, and an appropriate infection control program should be implemented. In summary, the study will contribute to the treatment of *P. aeruginosa* infections by determining its resistance profile, inducible beta-lactamase, and Metallo beta-lactamase ratios.

Acknowledgments:

Author contributions: MB, HO, AO, BMSI; Literature search and study design, experimental analysis, data collection, statistical analyzes, **BMSI;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

How does smartphone usage affect the severity of neck pain, neck-pain related disability, and cervical lordosis? A pilot study

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ABSTRACT

Objective: Long-term and repetitive use of smartphones may cause musculoskeletal symptoms such as neck pain. This pilot study aimed to examine the effect of smartphone usage on neck pain, disability, and cervical lordosis.

Material and Methods: Seventy-eight patients were enrolled in this cross-sectional pilot study. The level of smartphone use was evaluated with the Smartphone addiction scale-short version (SAS-SV). Neck Bournemouth Questionnaire (NBQ), Neck Disability Index (NDI), and 100 mm Visual Analog Scale (VAS) were used to assess pain and disability. Cervical lordosis angle was measured using the Cobb method.

Results: A positive significant correlation with low correlation coefficient (r= 0.277, p=0.014; r: 0.295, p=0.009) was determined between SAS-SV and NBQ, and NDI. However, no correlation was found between SAS-SV and cervical lordosis angle (p>0.05). When applying simple linear regression modeling to predict neck pain severity, the SAS-SV total score alone explained 7.7% of the variance of the NBQ and 9.0% of the variance of the NDI total score.

Conclusion: We concluded that it would be beneficial to question the frequency and position of smartphone use, to recommend to use it less, and to avoid prolongation in neck flexion for patients with chronic neck pain.

Keywords: Neck pain, smartphone addiction, radiography, lordosis

INTRODUCTION

Smartphones have been the most popular electronic devices, especially among the young population (1). In recent years, the number of individuals who have a smartphone and the time spent on a smartphone has increased considerably due to the developments in various smartphone models (2). Smartphones are used for multi-purposes including communication, music, media, internet access, games, some applications, and professional fields (3). Until recently, a diagnostic system for smartphone addiction has not been clarified, but it is classified as 'non-substance addictions' as a psychiatric diagnosis in the Diagnostic and Statistical Manual of Mental Disorders. This paved the way for approaching other digital addictions and behavioral addictions (4).

Intensive smartphone usage is thought to cause some problems. Reportedly; using a smartphone can cause sleep disorders, stress, anxiety, decreased academic success, and decreased physical activity in previous studies (5). In addition, prolonged and repetitive use of smartphones can cause musculoskeletal symptoms in various parts of the body, including the neck, shoulders, elbows, wrists, fingers, and back (2, 6, 7). Among smartphone users with the highest prevalence compared to other body parts, the rate of neck pain ranges from 17.3% to 67.8% in many countries, including China, Canada, South Korea, and India (8).

Research Article

Received 01-04-2020

Accepted 13-04-2021

Available Online: 14-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.527

Also, the frequency of neck and shoulder pain between the ages of 20 and 34 has increased in the last 20 years, and the most likely cause is using a computer and smartphone (8).

The cause of neck pain is known to be multifactorial, and incorrect spinal posture is one of the common causes (9). Individuals take their heads down sharply and holding their arms in front of them reading the screen can cause excessive anterior curve in the lower cervical vertebra and excessive posterior curve in the upper thoracic vertebra (1). Prolonged neck flexion together with lack of support for the arms and repetitive movement of the fingers causes static muscular loading on the cervical muscles, especially when only one hand is used (2). In addition, cervical flexion for long time has a negative effect on cervical lordosis (10).

In this pilot study, we wanted to draw attention to the effects of intensive smartphone use on neck pain, the most common musculoskeletal symptom associated with smartphone use. We aimed to investigate not only pain severity, but also disability and radiological changes in the cervical region due to intense smartphone use. Our first hypothesis was that there is a positive relationship between smartphone use and neck pain, and disability. Second, there is a negative relationship between smartphone addiction and cervical lordosis.

MATERIAL and METHODS

Participants:

Patients with chronic neck pain (more than 3 months) in the young population, who applied to our outpatient clinic between January 2020 and April 2020, were included. Patients enrolled in this study were 1-) 18-35 years old, 2-) Eligible smartphone users, 3-) having chronic neck pain, 4-) literate enough to answer the questionnaire. Patients with rheumatologic diseases which may affect the cervical spine, who worked in the cervical flexion posture (desk workers), who had a diagnosis like cervical disc degeneration, cervical disc disease, cervical myelopathy, cervical trauma history, and who underwent cervical spine surgery were excluded from the study.

Study Design:

This study was a cross-sectional study. Before the beginning of this study, ethical approval was obtained from the local ethic community of our hospital (Approval time: 20.04.2020, Approval number: 86/03). All participants were informed about the study and were given an informed consent form to sign.

First; age, gender, other musculoskeletal pain, years of using a smartphone, frequency of using a smartphone, duration of using a smartphone, and position of using a smartphone were answered by the participants.

All participants were evaluated with the Smartphone Addiction Scale- Short Version (SAS-SV), Neck Bournemouth Questionnaire (NBQ), Neck Disability Index (NDI) and 100 mm Visual Analog Scale (VAS).

Smartphone Addiction Scale- Short Version (SAS-SV)

SAS-SV was used to assess the smartphone addiction level.

This 10 self- reported scale items address 5 content domains, such as: (i) daily-life disturbance, (ii) withdrawal, (iii) cyberspace-oriented relationships, (iv) overuse, and (v) tolerance; these domains were responded on a 6-point Likert scale (1= strongly disagree to 6= strongly agree). The SAS-SV score range is 10-60. Higher scores are considered a higher risk of addiction.

The cut-off score is 31 for men and 33 for women evaluating smartphone addiction (11). Noyan et al. translated this scale to Turkish in 2015 (11). In this study, we used this scale to evaluate the intensity of smartphone use, and patients were classified in two groups: Non-addicted and addicted groups.

Neck Bournemouth Questionnaire (NBQ)

The NBQ is a 7-item self-administered questionnaire evaluating pain, physical function, social activity, anxiety, depression, work-related fear avoidance, and pain control. The total score is 70 and it is reached by adding the 7 dimension scores (12). Variability and reliability of this scale were demonstrated by Yılmaz et al. in 2019 (13).

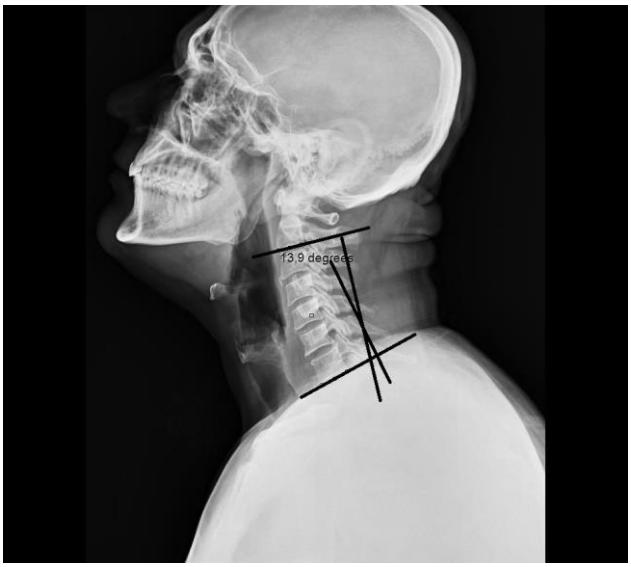
Neck Disability Index (NDI)

The NDI is mostly used to score neck pain related disability. It was published in 1991 and reviewed by the same author in 2008 (14). It includes 10 questions: Self-care, lifting, reading, work, driving, sleep, recreational activities, pain intensity, concentration and headache. Each item is scored on a scale from 0 (no disability) to 5. The total score ranges from 0 to 50: 0-4, minimal disability; 5-14, moderate disability; 15-24, severe disability; 25-34, crippled; 35-50, bedbound. Clinic properties of The Turkish translation of NDI were published in 2012 (15).

Cervical lordosis angle

We measured cervical lordosis angle with the Cobb method using lateral X-ray in a neutral position. In this method; cervical lordosis is measured by drawing two lines parallel to the endplate of C2 and C7, drawing orthogonal lines to these two lines, and measuring the intersection angle between these orthogonal lines (10) (Figure 1).

Figure 1: Measurement of cervical lordosis angle with the Cobb method



http://dx.doi.org/10.36472/msd.v8i4.527

Statistical analysis

Data obtained in the study were analyzed statistically using the Statistical Package for the Social Sciences (SPSS 15.0 for Windows) software. In descriptive statistics, data were expressed as mean ± standard deviation (SD) for continuous and categorical variables, and as frequencies and percentages (%) for nominal variables. The Shapiro-Wilks test and visual methods (histograms, probability plots) were used to assess the normality of data distribution. Comparison results of VAS, NBQ, NDI, and the lordosis angle between the addiction and non-addiction group was assessed with the independent sample t test. Correlations between VAS, NBQ, NDI, cervical lordosis angle, duration, frequency , and daily smartphone use and SAS-SV were analyzed with Pearson's or Sperman's correlation test where required; then, univariate linear regression analysis was performed for statistical significance. A value of $p<0.05$ was considered statistically significant.

RESULTS

Seventy-eight patients were included in this study and the mean age of the patients was 29.64 ± 6.56 years. Fifty-four (69.2%) of the participants were women and 24 (30.8 %) were men. The demographic characteristics of the patients were shown in Table 1. The most common concomitant pain was shoulder pain in patients.

Table 2 shown features of smartphone use. More than half of the patients (79.2%) had been using a smartphone for more than 6 years. The most common position when using a smartphone was the sitting position (55.1%).

Twenty-three patients (32.05%) were in the addicted group, 53 (67.94%) were in the non-addicted group. The comparison of VAS, NBQ, NDI, and cervical lordosis angles between the addicted and non-addicted groups were presented in Table 3. There was a significant difference regarding the NBQ, and NDI scores between the addicted and non-addicted groups ($p=0.03$, $p=0.02$ respectively). But yet, no significant difference was found in VAS and cervical lordosis angle between the addicted and non-addicted groups. ($p>0.05$) (Table 3).

The results of correlation analyses between VAS, NBQ, NDI, cervical lordosis angle, duration of smartphone use, daily smartphone use, and frequency of smartphone use SAS-SV were shown in Table 4.

Positive significant correlations were determined between SAS-SV and NBQ, and NDI with low correlation coefficient ($r= 0.277$, $p=0.014$; r: 0.295, $p=0.009$). Daily smartphone usage, and frequency of smartphone use were correlated with SAS-SV ($p<0.05$).

After the correlation analysis, multiple regression analyzes were used to clarify the relationship. However, there was no correlation between NDI, NBQ and age, and lordosis angle. The two measurements mentioned were only related to SAS-V.

Table 5 showed the results of linear regression analyses. When applying simple linear regression modeling to predict neck pain severity, the SAS-SV total score alone explained 7.7% of the variance of the NBQ, 9.0% of the variance of the NDI total score.

Table 1. Demographic Characteristics of Patients

n=78		N (%)
Age(years) (mean±SD)	29.65±6.46	
Gender	**Female**	54 (69.2)
	Male	24 (30.8)
Concomitant musculoskeletal pain	**Shoulder pain**	49 (62.8)
	Elbow pain	1 (1.3)
	Wrist pain	3 (3.8)
	Thumb pain	0
	Back pain	16 (20.5)
	No other pain	9 (11.5)

Table 2. Features of Smartphone Usage

		N, %
Duration of smartphone usage	*Less than 1 year*	2 (2.6)
	1 to 3 years	2 (2.6)
	3 to 6 years	12 (15.4)
	More than 6 years	62 (79.5)
Daily smartphone usage	*Less than 1 hour*	15 (19.2)
	1 to 3 hours	29 (37.2)
	3 to 5 hours	11 (14.1)
	More than 5 hours	23 (29.5)
Frequency of smartphone usage	*One in a week*	2 (2.5)
	One every three days	1 (1.2)
	One every two days	1 (1.2)
	Everyday	74 (94.8)
Position of smartphone use	*Sitting*	44 (57.1)
	Lying	5 (6.5)
	Standing	4 (5.2)
	All positions	24 (31.2)

Table 3. Comparison of VAS, NBQ, NDI, and cervical angles between the addicted and non-addicted group

	Non-addicted n=53 mean±SD	Addicted n=25 mean±SD	P
VAS (0-100mm)	67.93±15.24	74.01±18.71	0.14
NBQ	37.87±12.58	47.49±12.99	**0.01**
NDI	12.89±7.50	17.37±6.97	**0.02**
Cervical lordosis angle (%)	13.13±9.25	15.30±10.04	0.36

SD: Standard deviation, VAS: Visual Analog Scale, NBQ: Neck Bournemouth Questionnaire, NDI: Neck Disability Index, p values were calculated with independent sample t test, bold values shows statistical significance (p<0.05).

Table 4: Results of correlation analyses between Visual Analog scale, Neck Bournemouth Questionnaire, Neck Disability Index, cervical lordosis angle and Smartphone Usage

n=78	SAS-SV r (p)	Lordosis Angle r (p)	VAS r (p)	NBQ r (p)	NDI r (p)
Lordosis angle	**145 .205**	-			
VAS	**111 .334**	**-.090 .432**	-		
NBQ	**.277 .014**	**-.153 .181**	**.518 .000**	-	
NDI	**.295 .009**	**-.025 .827**	**.408 .000**	**.674 .000**	-
Duration of smartphone usage (years)	**.222 0.053**	**.-.095 0.414**	**.084 0.470**	**.051 0.661**	**.032 0.784**
Daily smartphone usage(hours)	**.479 0.001**	**.223 0.051**	**.056 0.620**	**-.176 0.120**	**-.031 0.783**
Frequency of smartphone usage	**.298 0.008**	**.008 0.944**	**.076 0.513**	**.044 0.718**	**-.200 0.083**

SAS-SV: smartphone addiction scale-short version, VAS: Visual Analog Scale, NBQ: Neck Bournemouth Questionnaire, NDI: Neck Disability Index, Correlations were analyzed with Spearman Correlation test, bold values shows statistical significance (p<0.05).

Table 5. Results of liner regression analyses

	n=78			
	B	**Std. error B**	**Beta (P)**	**R2**
NBQ/SAS-SV	.333	.133	.277 (.014)	.077
NDI/SAS-SV	.204	.074	.300 (.008)	.090

SAS-SV: smartphone addiction scale-short version, NBQ: Neck Bournemouth Questionnaire, NDI: Neck Disability Index.

DISCUSSION

According to the results of this pilot study, the severity of neck pain, and neck-pain related disability were significantly higher in patients with smartphone addiction comparing the participants without smartphone addiction. It was determined that, as the level of smartphone usage increased, the severity of neck pain, and disability increased. However, no effect was found smartphone addiction on cervical lordosis. Daily smartphone usage, and frequency of smartphone use were associated with smartphone addiction. Smartphone addiction was found an independent risk factor on neck pain, and neck-pain related disability.

The frequency of smartphone addiction among the young population has been increasing. The rates of smartphone addiction vary between 36.5 % and 62.4% in different studies (2). In Turkey, this rate was found to be 43.9 % among university students (3). The neck is a common area where problems can arise due to the overuse of smartphones.

Smartphone addiction has been found to be an independent significant factor on neck pain (2) and the question 'Why does use a smartphone cause neck pain?' has been investigated in many studies. The frequency, duration, and purpose of using a smartphone, the degree of neck flexion during use, and repetitive movements of the upper extremities were found to be associated with neck pain (8). Overuse of smartphones can cause the head and neck to move habitually and constantly towards the screen throughout the day. When using smartphones, people often flex their necks down to look at the lowered object and keep their head in a forward position for a long time (2, 16). In a study conducted; cervical flexion postures lasting longer than 10 minutes alter erector spine muscle activation, causing elongation of posterior cervical tissues, decreasing the excitability of receptors and reducing neural conduction activity (17). Such movements were associated with a high risk of chronic neck pain (18).

A study involving 799 smartphone users found that neck pain was the most common musculoskeletal problem and shoulder pain was the second most common problem (9).

In this study, we wanted to answer how much smartphone use affects neck pain, like other authors in the literature. We found a positive correlation between smartphone use and neck pain, and neck pain-related disability. Our findings supported previous studies showing that smartphone addiction causes musculoskeletal problems, especially in the neck and circumference, in young adults (1, 19, 20).

In a meta-analysis including 6 studies, a significant relationship was found between the duration of smartphone use and musculoskeletal symptoms such as shoulder, neck, and low back pain in 3 studies, while no significant relationship was found in other tree studies (8). While some of the cross-sectional studies did not show a significant dose-response relationship (21, 22), others showed that more smartphone use was associated with musculoskeletal symptoms (23-25).

In another study including 249 smartphone users, 65.9% of their participants had neck pain among the last one-year, and they found that; smartphone addiction scores in participants with neck pain were significantly higher than participants without pain. Duration of owing smartphone, and duration of smartphone use a typical day were correlated with smartphone addiction (26).

According to the results of our study, there was a difference between smartphone addiction and both NDI and NBQ in the evaluation made on SAS-SV scales. Consistent with the results in the literature, we found a relationship between intensity of smartphone use and clinical symptoms. The other result our study similar the literature; there was a positive relationship between features of smartphone use like as; frequency or hours used daily, and smartphone addiction.

Cervical lordosis is the first physiological curvature of the human spine and is a dominant disc herniation site due to its load-bearing function. It maintains the stability of the spine and is an essential part of normal spinal biomechanics (27, 28). The average of the cervical lordosis angle is between 20-35 (10). Cobb and Harrison's Tangent methods are two main different methods used to measure the cervical lordosis angle on sagittal radiography (10). In this study, we evaluated cervical lordosis using the Cobb angle measurement method. Cervical lordosis can be affected by many factors and the change in cervical lordosis can cause neck pain. Gao et al. evaluated the images of 3261 patients with cervical spondylosis and 1886 (57.8%) were found with abnormal cervical curvature (29). In other studies, no significant correlation was found between the sagittal alignment of the cervical spine and clinical symptoms (30, 31). In our study, no significant relationship between cervical lordosis and neck pain, and disability was found.

In this pilot study, our first hypothesis was confirmed, and there were a positive relationship between smartphone use and neck pain, and disability.

However, we could not find a significant relationship between cervical lordosis and the intensity of smartphone use. This situation may be related to the age group of our participants, and the effects of smartphone use on the skeletal system may not yet be seen.

Elderly participants in whom degenerative changes can often be seen were not included in this study. So this condition restricted our number of participants. The lower sample size of our study is a limitation of this study. In addition, existing posture disorders, personal habits or the purpose of using the smartphone (long-term activities such as playing games) which could affect the relationship between smartphone use and measured parameters, were not taken into account. Finally, this pilot study is a cross-sectional study, so studies with more participants and follow-up periods are needed to understand the relationship more clearly.

CONCLUSIONS

Neck pain is the most common musculoskeletal problem among smartphone users. As the time spent with the smartphone increased, the concept of smartphone addiction, which is classified as behavioral addictions, emerged. Preventive approaches are as important as therapeutic approaches in the management of neck pain. We think that questioning the frequency, and position of smartphone use, recommending less use of it, and avoiding prolonged neck flexion may be helpful for our patients with chronic neck pain.

Acknowledgments: We thank to our participants and our clinic staff.

Author contributions: DC, ZAY, FY, GC; Literature search and study design, Patient examination and therapies, statistical analyzes, **DC;** Article write up and revisions.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

Evaluation of the prevalence and seasonality of human parainfluenza virus over five year period in pediatric patients

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ABSTRACT

Objective: Human Parainfluenza viruses (HPIVs) cause respiratory tract infections, and the second most common cause of acute respiratory illness-related hospitalizations after the respiratory syncytial virus in children <5 years of age. The aim of the study; determination of HPIVs positivity and common types in pediatric patients with respiratory tract infection; investigation the distribution of HPIV positivity by age groups, months and seasons, respectively.

Material and Method: HPIV results of 1613 pediatric patients who were sent to the molecular virology laboratory from various pediatric clinics of Gazi Hospital between March 2016 and February 2021 (five years period) were investigated. Nucleic acid isolation was performed on the EZ1 Advanced (Qiagen, Germany) device using the EZ1 Virus Mini Kit by the manufacturer's protocol.

Results: HPIV positivity was detected as 4.1% in clinical samples and, the most common HPIV type was found to be HPIV-3 (55%). The distribution of other HPIV types were; HPIV-2, HPIV-4 and HPIV-1 with 26%, 23% and 14%, respectively. HPIV-3 is the most common type in 2016, 2017, 2018 and 2019; however, HPIV-1 is the most common type in 2020. HPIVs co-infection was detected with other respiratory tract viruses in 51% of samples. The highest HPIV co-infection was detected in Rhinovirus. The highest HPIV positivity rate (45%) were determined in the 0-2 age group compared to other age groups ($p<0.05$). The highest positivity rate was in October in the autumn season ($p<0.05$), the lowest was in January and February in winter. The highest rate (8.1%) of HPIV positivity was found in 2016 and the lowest rate (0.7%) was in 2020.

Conclusions: Since it is not possible to diagnose viral etiology of respiratory tract infections based on clinical findings, viral respiratory tract panel and Multiplex real-time PCR test are a fast and useful method in early diagnosis, treatment decision and prevention of unnecessary antibiotic use. HPIVs positivity is seen at higher rates in children aged 0-2 and in autumn months with seasonal differences.

Keywords: Respiratory tract infections, Human Parainfluenza virus (HPIV), Multiplex Real-Time polymerase chain reaction

Research Article

Received 05-04-2020

Accepted 20-04-2021

Available Online: 22-04-2021

Published 30-04-2021

INTRODUCTION

Human parainfluenza viruses (HPIVs) are single-stranded, enveloped RNA viruses in the Paramyxoviridae family. HPIVs can cause upper/lower respiratory tract infections and after respiratory syncytial virus (RSV), the second most common cause of acute respiratory illnesses related hospitalizations in children <5 years of age (**1, 2**). Human parainfluenza viruses with four distinct types known to infect humans: HPIV-1, HPIV-2, HPIV3, and HPIV-4; and included into two genera, Respirovirus (HPIV-1 and HPIV-3) and Rubulavirus (HPIV-2 and HPIV-4). HPIV-1 and HPIV-2 both cause croup, upper and lower respiratory illnesess. HPIV-3 is more often associated with bronchitis, bronchiolitis and pneumonia; HPIV-4 is recognized less often but may cause mild to severe respiratory illnesses (**3**).

Human parainfluenza viruses spread via the air by sneezing or coughing, personal contact, such as touching hands, objects or surfaces that have HPIVs on them then touching nose, mouth or eyes from an infected person to others. Currently, there is no vaccine to protect and specific antiviral treatment for HPIV infection (**4, 5**). Parainfluenza viruses replicate in the ciliated epithelial cells of the respiratory tract. Infection begins in the nose, oropharynx and then spreads to the lower airways 2-5 days after the initial infection. Once epithelial cells of the respiratory tract become infected, inflammatory infiltrates develop and the immune response is thought to contribute to disease pathogenesis. Although these infections are rather mild in healthy individuals, they may lead to serious respiratory diseases in children and immunocompromised individuals (**4, 5**).

In the respiratory system infections caused by viruses are clinically similar and the agent could be determined with only laboratory diagnosis. The Multiplex polymerase chain reaction can be used easily and efficiently to detect and diagnose the HPIV infection. Based on the sensitivity, specificity, and rapidity of the test, it is the most accurate test for HPIV diagnosis. Compared to viral culture and IFA testing, the PCR has been reported to have superior sensitivity and specificity (**6**).

Human parainfluenza virus is one of the main pathogens of respiratory tract infection in children. In the present study, we aimed the determination of HPIVs positivity and common types in pediatric patients who were sent to molecular virology laboratory due to the symptoms of respiratory tract infection; investigation the distribution of HPIV positivity by age groups, months and seasons, respectively.

MATERIAL and METHODS

In the study, the Human Parainfluenza virus results of 1613 pediatric patients, between 0-18 age, who were sent to the molecular virology laboratory due to the symptoms of acute respiratory tract infection from pediatric clinics of Gazi University Medical Faculty Hospital between March 2016 and February 2021 (five years period) were investigated retrospectively.

Collection of Samples

Totally 1648 clinical samples (nasopharyngeal swab, throat swab, nasal swab, and bronchoalveolar lavage (BAL)) of 1613 patients were included in the study. Throat swab, nasal swab and nasopharyngeal swab samples in transport medium (UTM-RT Transport, Copan Diagnostics, Italy); bronchoalveolar lavage samples were sent to the laboratory in a sterile transport container.

Nucleic Acid Isolation and Viral DNA Amplification

Nucleic acid isolation was performed on the EZ1 Advanced (Qiagen, Germany) device using the EZ1 Virus Mini Kit by the manufacturer's protocol. Viral DNAs were stored at -80 °C until amplification.

The amplification of the obtained DNA was performed by using Fast Track Diagnostics Respiratory Pathogen 21 (FTD, Luxembourg) and multiplex Real-Time PCR method. Fast Track Diagnostics® Respiratory Pathogen 21 test detects a total of 21 respiratory tract pathogens, including Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4, Influenza A, Influenza B, Influenza A (H1N1), Coronavirus NL63, Coronavirus OC43, Coronavirus HKU1, Coronavirus 229E, Human metapneumovirus A/B, Rhinovirus, Human bocavirus, Respiratory syncytial virus A/B, Adenovirus, Enterovirus, Human parechovirus and Mycoplasma pneumoniae.

FTD® Respiratory Pathogen 21 is ready to use test kit containing TaqMan probes and primers and results in the measurement of the fluorescence signal. Sample results with a FAM (fluorophore) fluorescence signal as a result of the test are considered positive.

Statistical Analysis: Statistical analysis was performed through SPSS 20.0. The data were evaluated using the Chi-Square and Mann-Whitney U test, and the $p<0.05$ value was considered significant in the analyses.

Ethical permission: This study was approved by Gazi University Medical Faculty Non-Interventional Clinical Research Ethics Committee.

RESULTS

A total of 1648 samples (throat swabs, nasal swabs, nasopharyngeal swabs and bronchoalveolar lavage (BAL) were obtained from 1613 patients; 872 male (%54) and 741 female (%46) were sent to the Molecular Virology Laboratory from pediatric clinics were investigated retrospectively.

Human parainfluenza virus positivity was detected as 4.1% (69/1648) in clinical samples. Among the HPIV positive cases, 50.7% (35/69) were female, 49.3% (34/69) were male. Gender distribution of HPIV positivity did not show any statistically significant differences ($p>0.05$).

The HPIV positive samples sent from Department of Pediatric Infectious Diseases 28/69 (40%), Pediatric Hematology 11/69 (16%), General Pediatrics 7/69 (10%), Pediatric Pulmonology 5/69 (7%), Pediatric Neurology 4/69 (6%), Pediatric Oncology 4/69 (6%), Neonatology 4/69 (6%), Pediatric Metabolism 1/69 (1.5%), Pediatric Endocrinology 1/69 (1.5%), Pediatric Gastroenterology 1/69 (1.5%), Pediatric Cardiology 1/69 (1.5%), Pediatric Nephrology 1/69 (1.5%), Pediatric Rheumatology 1/69 (1.5%) clinics. It was determined that the most frequent reasons for the patients were cough (48.7%) and fever (21.4%).

The distribution of human parainfluenza virus-positive patients were examined by age groups; it was found as 45% (31/69) in the age group 0-2; 27.5% (19/69) in the age group 3-5; 17.4% (12/69) in the age group 6-8; 5.8% (4/69) in the age group 9-12; 4.3% (3/69) in 13-16 years old. HPIV positivity was not detected in patients aged 16-18 years.

It was found that there is a higher rate (45%) of positivity in the 0-2 age group compared to other age groups. When the distribution of human parainfluenza virus positivity among age groups was examined, human parainfluenza virus positivity was found to be statistically significant in 0-2 years of age group patients ($p <0.05$).

The most common HPIV type was found to be HPIV-3 with 55% (38/69). The distribution of other HPIV types was detected HPIV-2, HPIV-4 and HPIV-1 with 26% (18/69),

http://dx.doi.org/10.36472/msd.v8i4.530

23% (16/69) and 14% (10/69), respectively. Co-positivity of the two HPIV types was detected as 18% (13/69) in samples. HPIV-3 was found the most common HPIV type as a percentage, but it was not statistically significant (p>0.05).

Among the HPIV positive cases, HPIV-3 was the most common type in 0-2, 3-5 and 9-12 years of age with 27.5%, 21.7% and 2.9%, respectively. However, HPIV-4 was the most common type in 6-8 and 13-16 years of age with 7.2% and 1.5%. Human parainfluenza virus types positivity rates according to age groups are given in **Figure1**.

The highest positivity rate was in October (13.2%; 15/113) and November (8.7%; 11/126), the lowest was in February (0%; 0/94) and January (0.6%; 1/166). When the distribution of HPIV positivity according to the seasons it was observed that the highest positivity rate was in the autumn season (9.3%) and the lowest rate was in the winter season (2%). In our study, a statistically significant difference was found between the autumn and winter seasons (p <0.05). The distribution of HPIV types positivity according to the months is shown in **Figure2**.

HPIV-1 and HPIV-2 were commonly detected in autumn; HPIV-3 was detected in early summer to autumn and HPIV-4 autumn and winter months. The distribution of HPIV types positivity according to the months and season shows in **Table1**.

Human parainfluenza virus positivity was found as 8.1% (13/160) in 2016, 5.9% (13/220) in 2017, 7.8% (22/281) in 2018, 3.6% in 2019 (17/465) and 0.7% (4/522) in 2020. The highest rate of HPIV positivity was found in 2016, and the lowest rate was in 2020. The distribution of HPIV positivity according to years is shown in **Figure3**. When the distribution of HPIV types according to the years were investigated; it was seen that HPIV-3 is the most common type in 2016, 2017, 2018 and 2019 with 3.1%, 3.1%, 4.9% and 2.3% respectively. However, HPIV-1 is the most common type with 0.3% in 2020. The distribution of HPIV types according to the years are shown in **Figure4**. Among the HPIV positive samples, only HPIV positivity was found as 49% (34/69) and HPIV co-infection with other viruses in 51% (35/69) of samples. The highest HPIV co-infection was detected in Rhinovirus, 37.1% (13/35); the lowest HPIV co-infection was found in Human parechovirus, 2.8% (1/35) (Table 1). Co-positivity of the two HPIV types was detected as 18% in samples. Among human parainfluenza virus-positive samples, co-infection with the triple agent was detected in six samples. HPIV-2/ HPIV3/ Adenovirus association was detected in two samples, HPIV-3/ Bocavirus/ Adenovirus; HPIV-1/ Adenovirus/ Metapneumovirus A/B; HPIV-3/ CoronavirusOC43/ Rhinovirus; HPIV-3/ Adenovirus/ Human parechovirus were detected in each of four sample. The distribution of co-infection with triple agents is given in **Table2**.

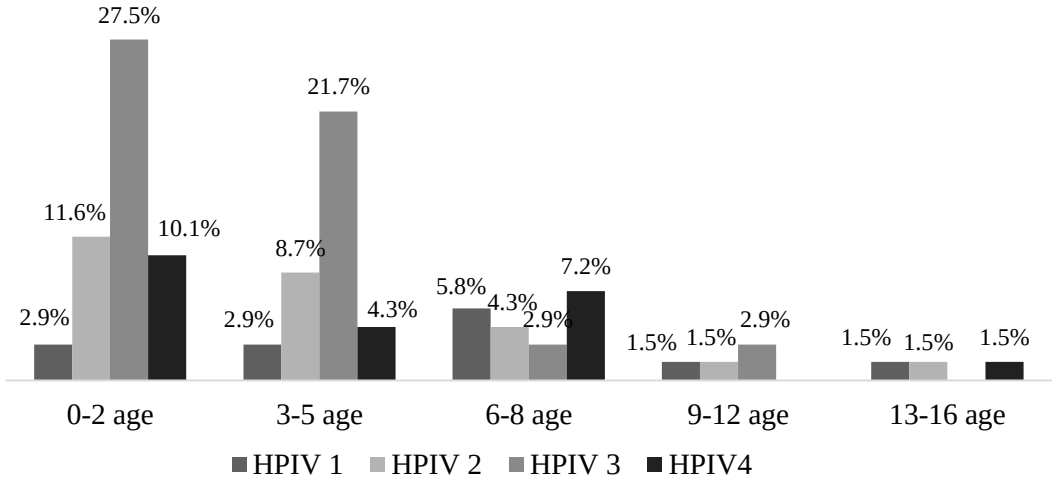

Figure 1: Human parainfluenza virus type's positivity rates according to age groups

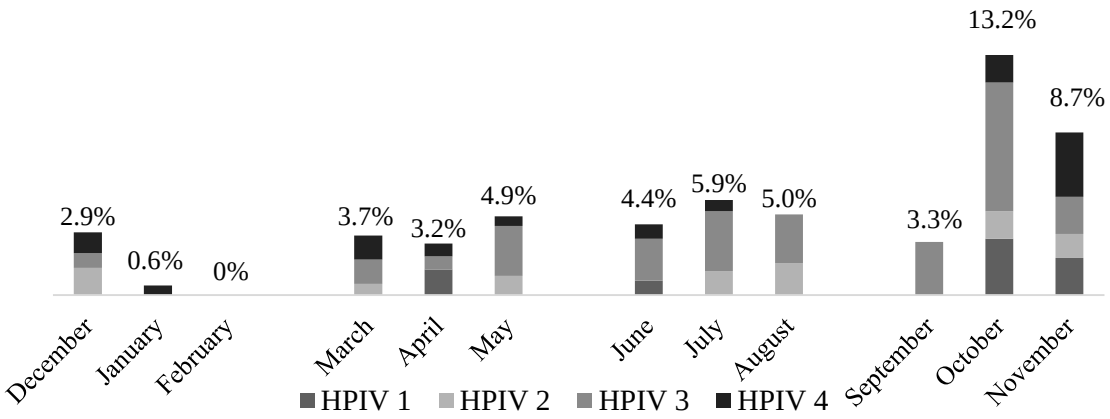

Figure 2: The distribution of HPIV positivity according to the months

http://dx.doi.org/10.36472/msd.v8i4.530

Table 1: The distribution of HPIV types positivity according to the months

%	January	February	March	April	May	June	July	August	September	October	November	December
HPIV-1	0.0	0.0	0.0	1.6	0.0	0.9	0.0	0.0	0.0	3.0	1.7	0.0
HPIV-2	0.0	0.0	0.7	0.0	1.2	0.0	1.5	2.0	0.0	1.6	1.3	1.2
HPIV-3	0.0	0.0	1.5	0.8	3.1	2.6	3.7	3.0	3.3	7.0	1.7	0.8
HPIV-4	0.6	0.0	1.5	0.8	0.6	0.9	0.7	0.0	0.0	1.6	4.0	0.9
TOTAL	0.6	0.0	3.7	3.2	4.9	4.4	5.9	5.0	3.3	13.2	8.7	2.9

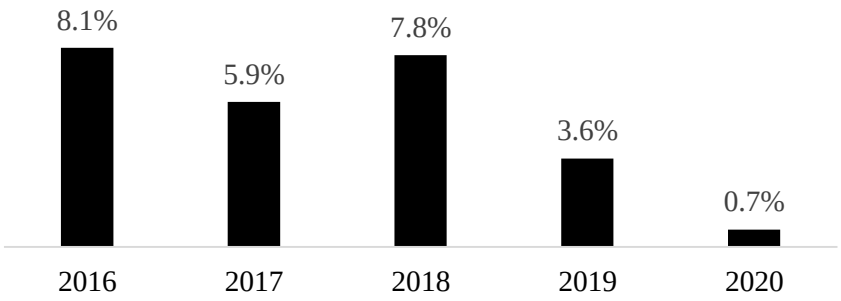

Figure 3: The distribution of HPIV positivity according to years

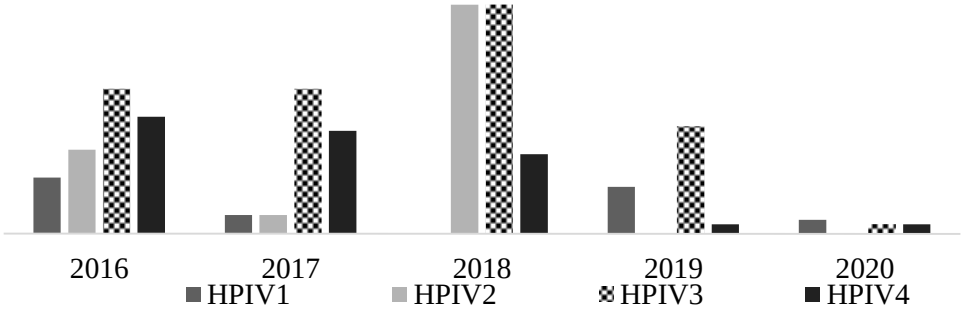

Figure 4: The distribution of HPIV types according to the years

Table 2: The distribution of HPIVs and co-infection with dual and triple agents

Co-infection with HPIVs ***Dual (n:35)***	**Patients n (%)**
Rhinovirus	13 (37.1)
HPIV-2/HPIV3	11 (31.4)
Adenovirus	3 (8.6)
Coronavirus (OC43, 229E, HKU1)	3 (8.6)
Human bocavirus	1 (2.8)
Enterovirus	1 (2.8)
İnfluenza A	1 (2.8)
Respiratory synctial virus A/B	1 (2.8)
Human paraechovirus	1 (2.8)
TOTAL	35 (100)
Triple co-infection agents (n:6)	
HPIV-2/HPIV3/Adenovirus	2 (33)
HPIV-3/Bocavirus/Adenovirus	1 (16)
HPIV-1/Adenovirus/MetapneumovirusA/B	1 (16)
HPIV-3/CoronavirusOC43/Rhinovirus	1 (16)
HPIV-3/Adenovirus/Human parechovirus	1 (16)
TOTAL	6 (100)

DISCUSSION

Acute respiratory infections are one of the most common diseases in children worldwide, and it is known that viruses are responsible for approximately 80% of these infections. After RSV, HPIVs are the second most common cause of acute respiratory illness-related hospitalizations in children <5 years of age (**6, 7**).

In the present study, we investigated the HPIVs positivity, common types and the distribution of HPIVs types positivity by age groups, months and seasons, in pediatric patients. Using multiplex real-time PCR technique we demonstrated that 4.1% of samples were positive for any HPIV types and, the most common HPIV type was HPIV-3 (55%) followed HPIV-2, HPIV-4 and HPIV-1. Aykaç et al. (**8**) investigated the prevalence of respiratory viruses in 1240 pediatric patients by multiplex real-time PCR and HPIV positivity was found as 3.7% (46/1240) in pediatric patients in Turkey. DeGroote et al. (**9**) retrospectively investigated HPIV test results belong to about 3 million pediatric patients during 2011-2019 in the USA. They reported that HPIV positivity was 5% in children and HPIV-3 was the most common type. Similarly, in lots of studies on HPIV epidemiology, HPIV-3 was reported to be the most common HPIV type and our results are in concordance with the literature (**8-12**).

The studies showing that viral co-infections of HPIV and other respiratory viruses are common in respiratory tract infections (12, **13**). In the present study, HPIV co-infection with other viruses detected in 51% of samples, and triple co-infection HPIV and other respiratory viruses were detected in six samples. Rhinovirus and Adenovirus were the most frequently coinfecting virus with 37% and 31%, respectively in our study. GU et al. (**12**) reported that rhinovirus was the most commonly detected virus with HPIV coinfection in children in Korea. In Turkey, it was reported that RSV, Rhinovirus and Adenovirus were the most common viral pathogens in respiratory tract infections in children (**11, 14, 15**). Rhinovirus and Adenovirus are one of the leading causes of respiratory diseases and are often isolated from respiratory tract infections. Also, even though the infection has been recovered clinically, the virus can be detectable for a long time. It is thought to be associated with the sensitivity of the multiplex real-time PCR method and detecting more than one virus simultaneously.

The human parainfluenza virus is one of the pathogens associated with respiratory tract infection and is responsible for substantial morbidity and mortality in children between 0 and 2 years of age. Aykaç et al. (8) found HPIV positivity in <18 years of age and they reported that 41 of 46 HPIV positive children were 0-2 years old. In China, 11,398 respiratory samples were collected from pediatric patients with acute respiratory illness and the median age of HPIV positive patients was detected as 1.7 years (0.6-2.5). In the present study, HPIV positivity was found as 45% in the 0-2 years of age ($p<0.05$). Due to incomplete immunity, children may be more susceptible to infection. The analysis of age distribution according to viral infection shows that the largest number of positive cases of HPIV, occurs in children less than 2 years of age, which is consistent with the international literature (8, **16, 17**).

Human parainfluenza virus infections are seen throughout the year, however, their highest rates were found in autumn. In our study, the highest positivity rate was in October and November (Autumn) the lowest was in February and January (Winter) and statistically, significant difference was found between the autumn and winter seasons ($p <0.05$). HPIV-1 and HPIV-2 were commonly detected in autumn; HPIV-3 was detected early summer to autumn and HPIV-4 autumn and winter. The seasonality of HPIV types differs by region. In literature HPIV-1 is usually detected in the autumn; HPIV-2 does not show seasonal variation but detects more commonly in autumn. There are generally more cases of HPIV-3 in spring and early summer; HPIV-4 usually occurs during late autumn and winter in temperate countries (18-20). Our study supports that although HPIVs are seen all year round, they reach their highest rates in autumn months by showing seasonal variation.

CONCLUSIONS

The human parainfluenza virus positivity was found between 3.6% and 8.1% in 2016-2019; however, in 2020 HPIV positivity was detected as 0.7%. The COVID-19 pandemic and related mitigation strategies and pandemic restrictions such as face mask use, disinfection and social distancing have possibly exerted a strong impact on the circulation of respiratory viruses. Not only HPIV but also all respiratory virus positivity rate was decreased all over the World (21, 22). Nevertheless, interventions used to limit person-to-person transmission of SARS-CoV-2 are the same as those that would be recommended in a parainfluenza pandemic and should therefore be expected to limit the spread of seasonal parainfluenza as well.

Author contributions: MC, SY, AT, HM, KY, HT, GB; Literature search and study design, Patient examination and therapies, experiments, statistical analyzes, **MC;** Article write up and revisions.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

The antibiotic susceptibilities of methicilline-resistant *Staphylococcus Aureus* strains isolated from various clinical samples

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ABSTRACT

Objective: In this study, it was aimed to determine the in vitro susceptibilities of Methicillin-Resistant Staphylococcus aureus (MRSA) strains to fluoroquinolone, linezolid, tigecycline, and quinupristin/dalfopristin as well as the macrolide-lincosamide-streptogramin B (MLSB) resistance phenotype.

Materials and Methods: A total of 94 MRSA strains isolated from various clinical samples in our hospital laboratory between January 2020 and September 2020 were included. The in-vitro susceptibilities of MRSA strains against fluoroquinolone, linezolid, tigecycline, and quinupristin/dalfopristin were determined by Kirby-Bauer disc diffusion assay according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST). The E test assay was used for evaluation of tigecycline susceptibility. The D-zone test was performed with erythromycin (15 µg) and clindamycin (2 µg) discs to determine the MLSB resistance. Besides, bacterial identification, antibiotic susceptibility tests including methicillin resistance and MLSB phenotype determination were performed by using VITEK 2 Gram-positive diagnostic kits (Bio-Mérieux/France).

Results: Results: Among 94 MRSA strains included, resistance rates to ciprofloxacin, moxifloxacin, tigecycline, and quinupristin/dalfopristin were found as 71% (67 isolates) 64% (60 isolates), 17% (16 isolates), and 2% (2 isolates), respectively. Resistance was not detected for linezolid. A total of 36 (49%) isolates showed cMLSB resistance phenotype, while 18(19%) had iMLSB resistance. The methicillin susceptibility (MS) phenotype – strains resistant to erythromycin and susceptible to clindamycin- was not detected.

Conclusion: Very little resistance was found to linezolid, quinupristin/dalfopristin and tigecycline. Therefore, these antibiotics may be beneficial for the proper treatment of infections caused by MLSB-resistant isolates.

Key Words: Methicillin-Resistant Staphylococcus aureus, linezolid, tigecycline

Research Article

Received 09-04-2020

Accepted 24-04-2021

Available Online: 24-04-2021

Published 30-04-2021

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important cause of infections caused by multiple resistant microorganisms, which makes treatment difficult and reduces treatment options (**1, 2**). Resistance to beta-lactam group and fluoroquinolones leads to use of last-option drugs such as vancomycin and teicoplanin, thus increases the resistance rates of these drugs. Therefore, the need for new antimicrobial drugs has come to the fore and various antibiotics have been developed for the treatment of infections caused by this bacterial group (**3, 4**).

Tigecycline (GAR-936) is a semi-synthetic analogue of classical tetracyclines which has activity against both Gram-positive and Gram-negative bacteria (5). Tigecycline prevents the aminoacyl tRNA from entering its target by binding to the 30S ribosomal subunit. This prevents the bacteria's protein synthesis and stops its growth (**6, 7, 8**). Linezolid from the oxazolidinone group is another antimicrobial agent used in the treatment of MRSA infections. Linezolid prevents the formation of the initial complex in protein synthesis by binding to the 50S subunit in ribosomes. The absence of intrinsic resistance gene against linezolid is an advantage for Gram-positive bacteria (**9, 10**).

http://dx.doi.org/10.36472/msd.v8i4.531

Quinupristin/dalfopristin is a combination of semisynthetic streptogramins containing 30:70 ratio of quinupristin and dalfopristin. This macrolide-lincosamide-streptogramin B (MLSB) group antibiotic is effective against Gram-positive bacteria. The drug acts by binding to the 50S ribosomal subunit and inhibits protein synthesis (**11, 12**). Frequent use of MLSB group antibiotics in MRSA infections is important in terms of leading to the increase of the number of resistant strains. Methylase enzymes encoded by methylase genes (erm), which is associated with the development of resistance to erythromycin, play a role in the development of resistance (**13**). MLSB resistance phenotypes are of two types, structural (cMLSB) and inducible (iMLSB). Strains with inducible MLSB resistance are crucial as erythromycin treatment causes enzyme induction in the bacterium, leading to resistance to macrolides and lincosamides (**14**). This study aims to investigate in-vitro susceptibilities of MRSA strains isolated from various clinical samples to fluoroquinolone, linezolid, tigecycline, and quinupristin /dalfopristin and to determine the MLSB resistance phenotype.

MATERIAL and METHODS

This study is a retrospective study and it was conducted in accordance with ethical principles for medical research with the Declaration of Helsinki. A total of 94 MRSA strains isolated from various clinical samples in the laboratory of our hospital between January 2020 and September 2020 were included.

Identification: S. aureus strains were identified by conventional methods -colony morphology, hemolysis type, Gram stain, catalase, and coagulase tests- and VITEK 2 automated system (Bio-Mérieux / France).

Detection of antibiotic susceptibilities: Methicillin resistance and antibiotics susceptibility testing (AST) were investigated by the Kirby-Bauer disk diffusion method according to the recommendations of The European Committee on Antimicrobial Susceptibility Testing (EUCAST 2020) (**15**). Cefoxitin (30 µg) (Oxoid, England) disk was tested for methicillin resistance. Isolates with a cefoxitin inhibition zone diameter of less than 21 mm were defined as methicillin resistant. The MRSA isolates were subjected to the antibiotic susceptibility test with ciprofloxacin (5µg), moxifloxacin (5µg), linezolid (30µg), quinupristin/dalfopristin (15µg) discs (Oxoid, UK) and tigecycline E test strips (Bio-Mérieux / France).

A suspension of 0.5 McFarland fresh bacterial culture in sterile physiological saline was prepared and spread on two separate Mueller Hinton agar (Oxoid, England) plates. Ciprofloxacin (5µg), moxifloxacin (5µg), linezolid (30µg), quinupristin/dalfopristin (15µg) disks (Oxoid, UK) were placed on one plate, and tigecycline on the other one. After incubation at 35±1°C for 18-24 hours, the minimal inhibitor concentration (MIC) of tigecycline and the inhibition zone diameters of other antibiotics were measured and the results were evaluated according to EUCAST criteria.

In addition, D-test was performed with erythromycin (15 µg) and clindamycin (2 µg) disks adjacent to each other in order to detect MLSB resistance (**Figure 1**). The flattening of the clindamycin inhibition zone - defined as the (D) zone- facing the erythromycin disk was evaluated as inducible clindamycin resistance (iMLSB).

Strains without an inhibition zone around the clindamycin and erythromycin disks were defined as constitutive clindamycin resistant (cMLSB). AST was also performed by VITEK 2 Gram-positive diagnostic kits (Bio-Mérieux / France) automatically (**Figure 2**).

Quality control: S. aureus ATCC 25923 and S. aureus ATCC 29213 and S. aureus 43300 were used as quality control strains in the study.

Statistical methods: The results were evaluated in terms of frequency and percentage, in line with the purpose of the study.

RESULTS

Out of 94 MRSA strains included in the study, 67 (71%) were resistant to ciprofloxacin, 60 (64%) to moxifloxacin, 16 (17%) to tigecycline, 2 (2%) to quinopristin/dalfopristin. There was no resistance to linezolid. The sensitivity of MRSA strains to antibiotics is shown in **Table-1**.

Of all the MRSA strains examined, 46 (49%) had cMLSB resistance, 18 (19%) had iMLSB resistance, and 30 (32%) had no resistance. In the strains included in the study, inducible resistance was found in all strains resistant to erythromycin and susceptible to clindamycin (**Table-2**).

VITEK 2 (Bio-Mérieux / France) results were 100% concordant with classical microbiological identification tests and antibiotic susceptibility test results.

Figure 1: Bacterial colonies isolated in sheep blood agar and disk diffusion tests.

http://dx.doi.org/10.36472/msd.v8i4.531

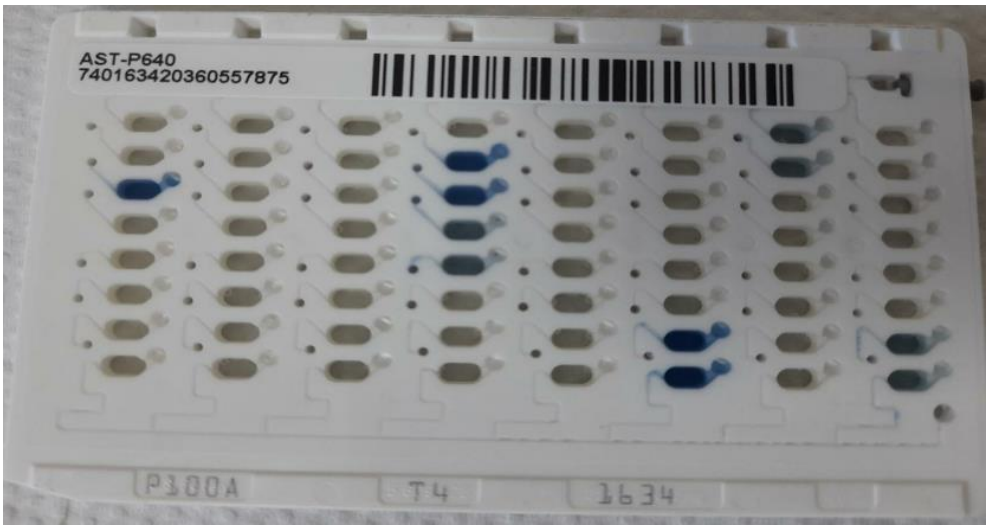

Figure 2: View of used Vitek 2 AST card.

Table 1: Antibiotic Sensitivity of MRSA Strains

	CIP		MXF		TGC		QD		LZD	
	n	%	n	%	n	%	n	%	n	%
Sensitive (S)	27	29	34	36	78	83	92	98	94	100
Resistant (R)	67	71	60	64	16	17	2	2	-	-

CIP: Ciprofloxacin, **MXF:** Moxifloxacin, **TGC:** Tigecycline, **LZD:** Linezolid, **QD:** Quinupristin/dalfopristin

Table 2: Distribution of MLSB Resistance in MRSA Strains

MLSB Resistance	n	%
Number of strains with cMLSB resistance	46	49
Number of strains with iMLSB resistance	18	19

cMLSB : Constitutive macrolide-lincosamide-streptogramin B, **iMLSB :** Inducible macrolide-lincosamide-streptogramin B

DISCUSSION

In recent years, infections caused by multi-drug resistant MRSA have increased worldwide. MRSA strains resistance to various antimicrobials such as fluoroquinolones have led to use of glycopeptide antibiotics as the first and sometimes the only option (2). With the reporting of glycopeptide resistance in MRSA infections, it has brought the use of antimicrobials such as linezolid, tigecycline and quinupristin/dalfopristin in treatment (16, 17).

In this study, a very high rate of fluoroquinolone resistance was found. 71% of 94 MRSA strains were ciprofloxacin-resistant and 64% to moxifloxacin. The fluoroquinolone resistance rate reported for MRSA strains in our country is between 33% and 85.9%; in other countries it ranges from 9.2% to 85%. Dündar et al.; investigated the antimicrobial susceptibility of S. aureus strains in a 3-year period (2005-2007) and reported ciprofloxacin resistance rates as 87%, 90% and 92%, respectively (18). Similar to this study, in a study in which ciprofloxacin resistance rates as 87%, 90% and 92%, respectively (18). Linezolid and tigecycline are reported to be highly effective in MRSA strains. Linezolid resistance has been reported to be less than 0.1% in various surveillance programs since linezolid resistance, which was first published in 2001 (19-22). In this study, no resistance to linezolid was found among the MRSA strains. Similar results have been obtained in various studies, too.

In a study conducted by Dizbay et al. in 2005 on 120 MRSA strains isolated from various clinical samples, all strains were found to be susceptible to linezolid (23). In another study conducted with 1707 MRSA strains between 1997 and 1999, again, linezolid sensitivity was found to be 100% (24). A study conducted in Korea retrospectively examined antibiotic susceptibility tests of a total of 22,067 MRSA isolates over 4 years, and only 110 (0.5%) were found to be resistant to linezolid (25).

In various studies, MRSA strains were found to be highly susceptible to tigecycline and resistance was not reported. Arslan et al. investigated tigecycline and linezolid resistance in 80 MRSA strains isolated from various clinical specimens and found all strains susceptible to linezolid and tigecycline (4). Similarly, Goff et al. found all strains susceptible to tigecycline and linezolid in a study they conducted between January 2004 and September 2005 on 879 MRSA strains (26). Behera et al. found 21 MRSA strains isolated from a hospital in India to be 100% susceptible to tigecycline (27). In a study conducted in Male Malaysia, five isolates (5.6%) were found, tigecycline-resistant, but they were not linezolid resistance in 90 MRSA (28). In this study, 16 (17%) of the MRSA strains were found to be tigecycline-resistant. Similar to this study, in a study by Kaya et al. investigating the in-vitro activity of tigecycline and linezolid in 60 MRSA strains;

while they found all strains susceptible to linezolid, they found resistance to tigecycline in 1 strain (29). Hoban et al. reported tigecycline sensitivity as 98.9% in a study they conducted with 5348 MRSA strains in 2004 (30). The lower rate of tigecycline resistance in various studies conducted in the past years may be attributed to the resistance of MRSA strains to this antibiotic over time.

In a review article published in 2020, quinupristin/dalfopristin resistance was found as 0.7% (0.3%-1%) in MRSA strains (31). Additionally, in some studies investigating the susceptibility of MRSA to quinupristin/dalfopristin abroad, the rate of resistance was reported to be between 0 and 31% (13, 32, 33). Kim et al. did not find resistance to quinopristin/dalfopristin in any of 439 MRSA strains in Korea (13). Baddour et al. found that all 512 MRSA strains in Saudi Arabia were susceptible to quinopristin/dalfopristine (34). Luh et al. determined this rate as 31% in Taiwan (32). In our country, Baysallar et al. and Yavuz et al. found the quinopristin/dalfopristin resistance to be 1% for MRSA strains and it was found as 2.3% by Tünger et al. (35, 36, 37). Kılıç et al. found no resistance in MRSA strains in the study they conducted in 2001 and 2002, while they reported that they found 2% resistance in 2003 (2). In this study, similar to various studies conducted in our country, quinupristin/dalfopristin resistance was 2%.

Although macrolides and lincosamides are used effectively in MRSA infections, they cause problems in treatment due to MLSB resistance recently detected. Among 94 MRSA isolates included in this study, MLSB resistance was determined as 49% and iMLSB as 19%; no resistance was found in 30 MRSA strains (32%). These rates are similar to various studies conducted in our country. For example, in the study conducted by Doğruman et al. on 63 MRSA strains isolated from various clinical samples in Ankara between 2005 and 2006; 32 (50.8%) had cMLSB resistance, 13 (20.6%) had iMLSB resistance and 18 (28.6%) had no resistance (14). In the strains included in the study, no strains resistant to erythromycin, susceptible to clindamycin but not inducible resistance (MS phenotype) were detected. In the study conducted by Azap et al. in Ankara, similar to the results of this study, cMLSB resistance was found as 45% and iMLSB resistance was found as 37% (38). Different resistance rates were found in the studies abroad examining MLSB resistance in MRSA infections. Otsuka et al. In Japan, reported that they found iMLSB resistance is 38.7%, cMLSB resistance is 61.3%; Fiebelkorn et al. reported that they found iMLSB resistance as 29.8% and cMLSB resistance as 34.2% in the USA (39, 40).

Limitations of this study; molecular methods were not used in this study due to technical and financial impossibilities.

CONCLUSIONS

Aims of antimicrobial susceptibility tests are contributing to prescribed appropriate antibiotics and monitoring resistant pathogens. Researches of antibiotic susceptibility, conducted with genotypic or phenotypic methods, contribute to provide epidemiological data, as well as regulation of correct antibacterial treatment regimens. In addition, by collecting data on regional antibiotic susceptibility test results, the types of resistance detected can guide empirical treatment selection.

Determining the resistance to MLSB group antibiotics will be useful in providing appropriate and effective treatment in MRSA infections. Thus, the selection of appropriate and effective drugs before treatment will both prevent the increase in resistance and increase the chance of treatment. Lack of resistance or low rate of resistance to antimicrobial agents such as quinupristin/dalfopristin, linezolid and tigecycline in MLSB-resistant MRSA infections will positively affect the success of the treatment.

Results of this study are shown that there is widespread resistance to other antibiotics besides methicillin resistance in S. aureus strains and it emphasized that to importance of antibiotic susceptibility tests.

Author contributions: EÖ, HT, NÖ, HA; Study design, Literature search, Experimental studies, and statistical analyzes, **EÖ;** Article write up and revisions.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

An opportunistic infection during stay home period in COVID 19 outbreak; Helicobacter Pylori

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ABSTRACT

Objective: Helicobacter pylori infection is a well-established risk factor for gastric cancer, which is more common in developing countries where poor sanitation and domestic overcrowding are common. We tried to analyze the impact of the pandemic on Helicobacter pylori infection during COVID 19 related curfew and stay-at-home order.

Material and Methods: Between 1-30 June 2020 (during stay-at-home order), a total of 172 dyspeptic patients who underwent upper gastrointestinal system endoscopy at our institution following stay-at-home order during COVID pandemic were included in this study along with a control group covering 192 dyspeptic patients who underwent endoscopy between 1-20 June 2019.

Results: When we analyzed gastric biopsy specimens, those who faced stay-at-home order had similar rates of intestinal metaplasia (22% versus 29%; p=0.15) and atrophic gastritis (24% versus 29%; p= 0.396) compared to recent year's data. On the other hand, the rate of H.pylori infection was higher compared to pre-COVID period (59% vs. 46%, p<0.05).

Conclusion: Domestic overcrowding during COVID 19 related stay-at-home order is a risk factor for H.pylori infection.

Keywords: Helicobacter pylori, COVID 19, Stay-at-home order, SAH0

INTRODUCTION

Novel coronavirus (COVID 19) infection is one of the most fearful pandemics in recent decades and is associated with increased rates of mortality with well-established risk factors including diabetes mellitus, ischemic heart disease, and cancer (**1**).

Given established person-to-person transmission of the disease, most countries have ordered stay at home to prevent the spread of the infection (**2, 3, 4**).

More than 80% of individuals in Turkey have faced Stay-at-Home Order (SAHO) under government control since the pandemic began. Governors in Turkey also ruled a wide range of restrictions in addition to SAHO including interior trips and cancellations of public events. Despite substantial evidence that quarantine damages quality of life, increases mortality, and decreases socioeconomic status, stay-at-home order had to be ruled by the governors (**5**).

H.pylori infection is one of the leading causes of gastric cancer and it is still endemic in the Middle East (**6**).

In addition, high serum urea and creatinine levels, useful tools for evaluation of health status, are also associated with increased risk for H.pylori infection (**7**).

The epidemiological dynamics of HP infection during SAHO remains unknown; therefore we analyzed time-dependent changes in HP infection during the SAHO period.

Research Article

Received 10-04-2020

Accepted 26-04-2021

Available Online: 26-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.532

MATERIAL and METHODS

Between 1-30 June 2020 (during stay-at-home order), a total of 172 dyspeptic patients (mean age 56 years) who underwent upper gastrointestinal system endoscopy at our institution following stay-at-home order during COVID pandemic were included in this study.

Of those, 82 (47,7%) were male. Biopsy specimens of gastric mucosa were evaluated by the pathologists of our institution. We also identified data of 192 dyspeptic adults in the hospital data system that underwent endoscopy between 1-30 June 2019.

Patients demographic characteristics and laboratory parameters were also obtained from electronic health records. We excluded those with acute gastrointestinal bleeding, gastric cancer, and sepsis on admission.

They were included in the study after obtaining consent from the patients who participated in the study. Among study subjects, baseline clinical characteristics, biochemical and hematological parameters were compared between pre-COVID period and post-SAHO course.

Statistical Analyzes: Chi-square and t-test were used for statistical analysis and a p-value of lower than 0.05 was considered significant. The study was approved by the Van Yuzuncu Yil University ethics committee(Etical number:2020/03-01)

RESULTS

After SAHO at the entire Turkey, 150 patients were referred to the endoscopy clinic.

There was no difference in age, gender, race, co-morbidities, signs, and symptoms of dyspepsia, renal function, cardiac failure, presence of pulmonary hypertension between SAHO-experienced and non-experienced subjects.

In SAHO group, the mean age was 55 ±14.9 years, 43.8% were male, and 54% were farmers. In the time-matched control group, the mean age was 56.5 ±15 years, 47.7 % were male, and 55% were farmers.

Compared to previous year, SAHO-experienced dyspeptic subjects had higher hematocrit (40 versus 41; p= 0.001), higher urea (28. 2 versus 33.4 mg/dl; p=0.009), higher serum creatinine (0.8 versus 0.89 mg/dl.; p= 0.003) and higher AST (21 versus 23 U/L; p= 0.01) levels.

Interestingly, serum albumin levels did not change after SAHO compared to pre-COVID period (4.5± 0.7 versus 4.72 ± 0.9 mg/ dl; p= 0.574) (Table-1).

When we analyzed gastric biopsy specimens, those who faced stay at home order had similar rates of intestinal metaplasia (22% versus 29%; p=0.15) and atrophic gastritis (24% versus 29%; p= 0.396) compared to recent year's data. On the other hand, the rate of H.pylori infection was higher compared to pre-COVID period (59% vs. 46%, p<0.05) (Table-2).

Table 1: Laboratory data of patients

	n	Before COVID-19***	n	After COVID-19***	p
AGE*	192	56,5 (54,1-58,8)	172	55 (52,4-57,5)	0,395
HEMOGLOBIN**	147	13,1 (12,8-13,5)	171	13,1 (12,8-13,4)	0,890
HTC*	147	40,0 (39,2-40,9)	171	41.1 (39,2-41,0)	0,001
MCV**	147	86,2 (85,3-87,0)	171	86,9 (86,0-87,9)	0,067
WBC**	147	8,20 (6,92-9,48)	171	7,23 (6,89-7,57)	0,410
NEUTROPHIL			171	4,81 (3,89-5,73)	
LYMPHOCYTE	147	2,36 (2,02-2,70)			
PLATELET**	147	251 (240-262)	171	261 (249-272)	0,268
GLUCOSE**	147	111 (105-116)	170	115 (109-122)	0,129
UREA**	192	28,2 (24,4-32,0)	171	33,4 (30,3-36,4)	0,009
CREATININE*	146	0,89 (0,83-0,95)	172	0,80 (0,76-0,84)	0,003
AST**	146	21 (19-22)	170	23 (21-25)	0,011
ALT**	146	21 (18-23)	171	20 (18-23)	0,291
ALBUMIN**	52	4,57 (4,43-4,71)	100	4,72 (4,3-5,14)	0,574

*Independent T-Test, **Mann Whitney U, ***Mean (95% CI)

Table 2: Endoscopic findings of patients

	Before COVID-19	After COVID-19	p*
Male	84 (43,8%)	82 (47,7%)	0,453
Female	108 (56,3%)	90 (52,3%)	
H. Pylori	95 (46 %)	101 (59 %)	0,049
Metaplasia	42 (21,9%)	49 (29,0%)	0,150
Atrophy	46 (24,0%)	48 (28,2%)	0,396

*Pearson Chi-Square test

http://dx.doi.org/10.36472/msd.v8i4.532

DISCUSSION

More than 80% of individuals in Turkey faced SAHO between 1 April 2020 and 31 May 2020, despite substantial evidence on starting SAHO without enough financial status disturbed quality of life, increased mortality, and healthcare costs. Barriers to health access, overcrowded conditions beyond hospital capacity during COVID pandemic and socioeconomic turmoil prior to SAHO initiation have been implicated in this phenomenon (8). This, in turn, led to further lower health parameters in those with low income. Dietary restrictions and diminished social lifestyle might also influence the rate of H. pylori infection amid the COVID-19 pandemic. The distribution of H. pylori is influenced by age, sex, geographical location, ethnicity, and socio-economic factors (9). Thus we conducted this study to reveal what kind of changes occurred after SAHO in terms of histopathological findings on gastric biopsy specimens as well as other laboratory parameters. Epidemiological studies indicate that nutritional alterations and overcrowding increase the risk of HP infection in adults. However, nearly all prior studies in the setting of natural disasters assessed HP as a key sign of the deterioration of the socioeconomic status (10, 11). A recent study demonstrated the association between natural disasters and an increased rate of HP infection (12, 13). However, there was no clinical study investigating the rate of HP infection among dyspeptic patients who faced SAHO at least 2 months. Most of the subjects who faced SAHO during the COVID 19 pandemic had less physical activity in many countries. This sedentary lifestyle in those subjects is associated with higher cardiovascular mortality risk. The restrictions on physical activity could also be a driver of new infections including HP. Due to the action of catecholamine; lymphocytes usually increase in circulation during exercise and return to baseline levels or below within a few hours after exercise. The number and function of natural killer (NK) cell display the most homogenous response to exercise (increase during and decrease after) (14, 15). Renal failure is an established risk factor for morbidity and mortality in adults and children. Adult studies showed an association between renal failure and socioeconomic turmoil in different settings (16). It has been shown that the increasing rate of HP infection has been linked to poor resource settings (17). Although we did not obtain study subjects financial income and dietary patterns, we postulated that disturbed economic and hygienic conditions during the COVID isolation period could be a driver of H.pylori infection. On the other hand, it might be due to a higher rate of emergency admissions of severely ill patients during the isolation period. Recent COVID studies found that a lack of systematic approaches regarding tracking patients through health care providers were mainly responsible for the spreading of the different diseases during the COVID 19 pandemic (18, 19). This study also showed that stay-at-home order had a significant effect on AST levels. In contrast to the pre-COVID group, the SAHO group was more likely to have higher AST levels at baseline. HP infection is the fastest-growing cause of gastric cancer in the world. Treatment for HP infection has averted a huge number of gastric cancers and blunted the increase in mortality from this cancer in the general population. HP infection also remains the leading cause of gastric cancer among subjects who have poor socioeconomic status (20, 21).

Moreover, natural disasters including earthquakes have recently been shown to have a profound epidemiologic impact on the prevalence of HP infection (11). Multiple methods of assessing HP infection are currently being evaluated to determine an optimal approach for the identification of the disease. We chose histopathological examination, the most valuable method to assess the presence of HP infection. In the current study, both of control subjects and study patients underwent gastric endoscopy, and biopsy specimens were obtained as well. Prevalence of HP was higher compared to pre-COVID subjects, suggesting additional investigation is needed to understand the key facilitators of disease development.

We postulated that the prevalence of HP infection is on the rise particularly during the COVID pandemic due to a rise in social isolation, sedentary lifestyles, low income, and poor nutrition. Social isolation and stay-at-home order related to the COVID pandemic pose a substantial health care disease burden in the context of HP infection.

CONCLUSIONS

Stay at home order was identified as a significant modifiable risk factor for both HP infection and renal failure. Early identification of HP infection during the SAHO course may avoid delay inappropriate therapies.

More than 80% of individuals in Turkey faced SAHO in this year, despite substantial evidence on starting SAHO without enough financial status disturbed quality of life, increased mortality and healthcare costs

Despite substantial evidence that quarantine damages quality of life, increases mortality, and decreases socioeconomic status, stay at home order had to be ruled by the governors

The distribution of H. pylori is influenced by age, sex, geographical location, ethnicity, and socio-economic factors

We postulated that the prevalence of HP infection is on the rise particularly during COVID pandemic due to a rise in social isolation, sedentary lifestyles, low income and poor nutrition. Social isolation and stay at home order related to the COVID pandemic pose a substantial health care disease burden in the context of HP infection

Author contributions: MA, SO, YD, ACD; Literature search and study design, Patient examination and therapies, experiments, statistical analyzes, **MA;** Article write up and revisions.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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MSD

Medical Science and Discovery
ISSN: 2148-6832

Examination of impact of Di(2-Ethylhexyl) Phthalate and Dibutyl Phthalate on Rat internal organs by scanning acoustic microscopy and inductively coupled plasma optical emission spectroscopy

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ABSTRACT

Objective: Phthalates, despite their endocrine-disrupting effects, are widely used as plastifiants. Environmental exposure of phthalates was demonstrated to cause fetal death and reproductive toxicity in human beings, as well as in laboratory animals. However, underlying mechanisms are not clear.

Material and Methods: Here, we examine the impact of di(2-Ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) on rat lungs, brain, and heart by scanning acoustic microscopy (SAM) and inductively coupled plasma optical emission spectroscopy (ICP-OES). First, we evaluate tissues of mother rats and we show that the acoustic impedance values of tissues of DEHP and DBP delivered rats differ from those of tissues of the control rat. Then, element level analyses within these tissues are done and element levels within tissues of DEHP and DBP delivered pregnant rats are found to be higher than those within tissues of the control pregnant rat. We then evaluate the tissues of offspring female rats.

Results: It is shown that acoustic impedance values of tissues of offspring rats of DEHP and DBP delivered mother rats are higher than those of tissues of the control offspring rats of the control mother rat. Besides, element analysis reveals higher element levels in the tissues of offspring rats of DEHP and DBP delivered mother rats.

Conclusion: Therefore, we can conclude that phthalates cause structural and functional changes within rat internal organs such as lungs, brain, and heart. In summary, both modalities are confirmatory in a way that tissues of DEHP and DBP exposed pregnant rats and their offspring rats are differentiated by different acoustic impedance values obtained by SAM and higher element levels specified by ICP-OES.

Keywords: scanning acoustic microscopy, inductively coupled plasma optical emission spectroscopy, phthalate exposure

Research Article

Received: 11-04-2020

Accepted: 26-04-2021

Available Online: 26-04-2021

Published: 30-04-2021

INTRODUCTION

Advanced technology provoked the invention of new chemicals, which are used predominantly for the enhancement of agricultural production and also as ingredients in various products. Major types of these chemicals include herbicides, fungicides, insecticides, plastifiants, polychlorinated biphenyls and alkylphenolic compounds. Most of these chemicals are mainly released into the environment as a consequence of industrial production. These substances may mimic natural hormones or present antagonistic effects (**1, 2, 3, 4**).

Therefore, they are named as endocrine disruptors. Bisphenol A (BPA), whose endocrine-disrupting effect has been confirmed, is used in the production of agricultural, industrial and house detergents, pesticide and herbicide formulations, dyes and plastics, industrial products and antioxidant and drug agents. European Union has banned the use of BPA in the production of toys for children under three years old, since high doses of BPA have been linked to infertility and other health problems. However, these substances are still in use in toys for children over three years of age. Other chemicals with proven endocrine-disrupting effects include natural and synthetic hormones, phytoestrogens, and many industrial chemicals such as pesticides and plastifiants. Plastifiants are used for making rigid polyvinyl chloride (PVC) more elastic (**5**). Phthalates, phthalate esters or phthalic acid esters are usually added to plastics to increase flexibility. Phthalate plastifiants are produced industrially and used in PVC applications of artificial leather, electric cables, shoe bases, ground tiles and tubes (such as dialysis tubes), syringes, and gloves used in hospitals. Phthalates are released over time, since they do not tightly bound to PVC and humans get exposed to phthalates via inhalation, ingestion, dermal route or during medical procedures (**6, 7, 8**). Non-PVC applications of phthalate plastifiants include dyes, rubber, glues and baby milk, cheese, margarine, and chips packages. The most studied and most prevalent form of phthalates is di(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP). Animal studies in recent years revealed that accumulation of DEHP in tissues leads to long-term toxicity, causing embryo death, fetal toxicity, and teratogenicity at high doses (1000 mg/kg/day) (**6, 9**). In animals, body weight loss and testicular weight loss were observed and the decrease in testicular weight caused reduced zinc concentrations in the testis, apoptosis, and mass destruction of spermatogenic cells (**10**). Perinatal exposure of DBP was examined and found to induce neurotoxicity in neonatal and immature rats (**11**). Similarly, it was demonstrated that gestational or postnatal DEHP exposure induced adverse effects on rat brain development and function (**12**). Exposure to bisphenol A is directly associated with inflammation, which causes initiation and progression of cardiovascular disease (**13**). Many of the population-based human studies confirm the relationship with phthalates, specifically DEHP, with the increased risk of developing wheeze, asthma, and allergy (**14**). Therefore, examination of the phthalate exposure and the dose at which disruptions occur in living organisms is crucial.

Alterations in tissues with certain limitations can be identified with imaging modalities such as magnetic resonance imaging (MRI), micro-optical coherence tomography (micro-OCT), or positron emission tomography (PET), which are either very expensive or involve ionizing radiation (**15**). On the other hand, ultrasound (US) imaging, which is a very common modality for the observation of soft tissue, has high axial and lateral resolutions of approximately 20-100 µm, a good penetration depth of approximately 5 mm. Although it can only provide morphological information, US imaging has a low cost. Scanning acoustic microscopy (SAM) can give information about the morphology and elastic properties of biological tissues simultaneously at microscopic levels with focused high-frequency ultrasound. Major advantages of SAM are its speed in obtaining the two-dimensional images and immediate installation of the specimen without special preparation and staining. SAM has two modes calculating either the speed of sound (SOS) through tissues (**16,17,18,19,20,21,22,23,24,25**) or acoustic impedance of samples (**26,27**). Spectrometric techniques are largely used for obtaining chemical information about biological samples. Inductively coupled plasma optical emission spectroscopy (ICP-OES) is one of them, which is widely used for element analysis due to its high sensitivity, versatility, high speed, and acceptable cost (**28,29,30**). ICP-OES is an emission spectroscopy that determines elemental composition in a biological material by analyzing emitted electromagnetic radiation characteristic to a particular element. Combining ICP-OES with SAM will enable morphological and chemical information simultaneously with a less expensive system. In this study, we want to investigate the effects of DEHP and DBP exposure during the gestation period by examining internal organs of DEHP and DBP exposed pregnant rats and their offspring female rats. For this purpose, two doses of 61 µg/kg/day and 61 mg/kg/day of DEHP and DBP, which are established according to the observed human exposure, are given to the rats with a gavage. Lung, brain, and heart tissues are characterized by scanning acoustic microscopy (SAM) and inductively coupled plasma optical emission spectroscopy (ICP-OES). While SAM provides micrometer resolution structural and mechanical information regarding the internal organs, ICP-OES provides information about the elements within these organs. Elements examined within all tissues are copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), and zinc (Zn). Acoustic impedance values of tissues of DBP and DEHP delivered pregnant rats and their offspring rats differ from those of tissues of control pregnant and their offspring rats. Similarly, element levels in tissues of DBP and DEHP delivered pregnant rats and their offspring rats are higher compared to those in tissues of control pregnant and their offspring rats. Finally, we can conclude that the toxic agent-induced structural and functional deviations are observed and confirmed by these techniques.

MATERIAL and METHODS

Specimens: The study was approved by the Ethical Committee of Istanbul University (Number: 2013/138). Healthy adult male and female Sprague Dawley rats were provided by Laboratory Animal Center in Istanbul University and housed in one cage in a controlled environment at 22 ± 3 °C and relative humidity level of 50-60 % with a 12/12 h light/dark cycle. 4 groups, composed of pregnant rats of control, low dose prenatal (given a low dose of DEHP and DBP, dissolved in corn oil) and high dose prenatal (given a high dose of DEHP, dissolved in corn oil), together with their female offspring rats, were studied. In the control group, animals received edible corn oil by intragastric administration. Low and high intragastric doses of 61 µg/kg/day and 61 mg/kg/day, respectively, were administered to the low and high dose prenatal groups at the same time of each day from the sixth to the nineteenth day of the pregnancy. The mother rats were anesthetized using ether and cervical dislocation was performed in the twentieth day of pregnancy. The lung, brain, and heart tissues were carefully removed, cleaned and fixed in 4 % paraformaldehyde (PFA). There were 2 female offspring rats of the control mother. There were 3 female offspring rats of DBP (Low) exposed

mothers. There were 6 female offspring rats of DEHP (Low) exposed mothers. There were 3 female offspring rats of DEHP (High) exposed mother.

Scanning Acoustic Microscopy: The methods were carried out in accordance with the Institutional Ethics Committee for the Local Use of Animals in Experiments in Bogazici University. The internal organs of the rats were characterized by scanning acoustic microscope (AMS-50SI) developed by Honda Electronics (Toyohashi, Japan). SAM setup and the principle of SAM, in acoustic impedance mode, can be found in our previous study (**31**). SAM is composed of a transducer with quartz lens, a pulser/receiver, an oscilloscope, a computer, and a display monitor. 80 MHz transducer is used as a generator and a receiver of ultrasonic signals, therefore, it acts as a pulser/receiver. It has a spot size of 17 µm and a focal length of 1.5 mm. Single pulses of a width of 5 ns with a repetition rate of 10 kHz are transmitted to the specimen and the reflected signals are collected. Water is the coupling medium between the quartz lens and the substrate. X-Y stage controlled by the computer scans the transducer. The reflected signals from the reference and the target material are analyzed by the oscilloscope. Finally, intensity and acoustic impedance maps of the region of interest with 300 x 300 sampling points are constructed with a lateral resolution of approximately 20 µm. The target is the tissue under investigation and distilled water is the reference material. The target signal is

$$S_{target} = \frac{Z_{target} - Z_{sub}}{Z_{target} + Z_{sub}} S_0$$

where S0 is the signal generated by the 80 MHz transducer, Ztarget is the acoustic impedance of the tissue and Zsub is the acoustic impedance of the polystyrene substrate (2.37 MRayl). The tissue's acoustic impedance is calculated by comparing the reflected signal from the tissue with the one from the reference. The reflected signal from the reference is

$$S_{ref} = \frac{Z_{ref} - Z_{sub}}{Z_{ref} + Z_{sub}} S_0$$

where Zref is the reference's acoustic impedance (1.50 MRayl). Consequently, the target's acoustic impedance can be written as with a constant generated signal S0 (**26**).

$$Z_{target} = \frac{1 + \frac{S_{target}}{S_0}}{1 - \frac{S_{target}}{S_0}} Z_{sub}$$

Inductively Coupled Plasma Optical Emission Spectroscopy

The element levels in lung, brain, and heart tissues were measured using an inductive coupled plasma optical emission spectrophotometer (iCAP 6000 series, Thermo Fischer Scientific Inc., Istanbul, Turkey) at Trace Element Analysis Laboratory in Department of Biophysics in Cerrahpasa Faculty of Medicine. Tissue samples were placed into tared glassware tubes and the weight of the samples was recorded. The tissue samples were subjected to ashing with 2 mL of nitric acid (65 %) and 1 mL of perchloric acid (70 %) in a drying furnace at 150 °C. The digested samples were left to cool at room temperature and diluted with distilled water then vortexed to get ready to conduct elemental analysis in an ICP-OES device. The analyses of copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), and zinc (Zn) levels were performed with ICP-OES Thermo iCAP 6000 series at Trace Element Analysis Laboratory of Biophysics Department of Cerrahpasa Medical Faculty in Istanbul, Turkey. The wavelengths of 324.754 nm, 259.940 nm, 285.213 nm, 257.610 nm, 196.090 nm, and 206.200 nm were used for the detection of Cu, Fe, Mg, Mn, Se and Zn, respectively, in the device. Measurement of each element level was carried out three times and averaged. The ICP-OES system was operated with incident power of 1.3 kW, 15 L/min plasma argon flow rate, 1.5 L/min auxiliary argon flow rate, 0.7 L/min nebulizer argon flow rate in this study. Transport lines were obtained using 1.25 mm internal diameter polytetrafluoroethylene tubing. The standard concentrations for standard graph calibration were prepared from standard stock solutions of 1000 µg/ml for each analyzed trace element. Trace element levels in serum samples were expressed in micrograms per milliliter (µg/ml).

Statistical Analysis

Statistical analysis was performed using SPSS 21 statistical software for Windows. Results were presented as means ± standard deviation (SD). For comparison of parameters between the 4 groups, ANOVA parametric test was used. The mean and median values were presented along with their 95% confidence interval. All analyses were assumed statistically significant at $p < 0.05$.

RESULTS

Scanning Acoustic Microscopy Results

The lung, brain, and heart tissues, received from pregnant control mother rats and mother rats exposed to DEHP and DBP and their female offspring rats were sliced cross-sectional for SAM studies. **Table1** presents acoustic impedance values within all tissues of the mother rats examined. **Table2** presents average acoustic impedance values within all tissues of the offspring rats examined. The average acoustic impedance values and their standard deviations were calculated considering 2 female offspring rats of the control mother, 3 female offspring rats of DBP (Low) exposed mother, 6 female offspring rats of DEHP (Low) exposed mother and 3 female offspring rats of DEHP (High) exposed mother. In **Table1** and **Table2**, low is for a concentration of 61 µg/kg/day, and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy. Tissue samples, which belong to the rats exposed to DEHP and DBP, are easily distinguishable with higher acoustic impedance values, since, they are quite stiff when compared to tissues with smaller acoustic impedance values. In other words, the tissues have distinctive acoustic impedance values due to the discrepancy in elastic properties. The SAM images, obtained using the acoustic impedance mode of SAM, were constructed by comparing the reflections of ultrasound signals from the reference and front surfaces of the slices. **Figure1** shows the acoustic impedance distribution of a lung sample obtained from an offspring rat of the control mother rat. **Figure2** shows the acoustic impedance distribution of a lung sample obtained from an offspring rat of DEHP (Low) exposed mother rat, with higher acoustic impedance values

http://dx.doi.org/10.36472/msd.v8i4.534

when compared with the one of the control offspring rat, shown in **Figure1**. In **Figure2** an offspring rat of DEHP (Low) exposed mother rat is chosen since the acoustic impedance value of that rat's lung tissue is significantly higher than that of the lung tissue of the control offspring rat.

Inductively Coupled Plasma Optical Emission Spectroscopy Results

We determined Cu, Fe, Mg, Mn, Se, and Zn levels in tissue samples. **Table3** presents element levels within lung tissues of the pregnant rats. Similarly, **Table4** and **Table5** present element levels within brain and heart tissues of pregnant rats, respectively. As can be observed in **Table3**, Cu, Fe, Mg, Mn, Se, and Zn levels are the highest in the lung tissue of DEHP (Low) exposed pregnant rat, when compared to element levels in tissues of control, DBP (Low) and DEHP (High) exposed pregnant rats. Besides, Cu, Mg and Se levels in lung tissue of DBP (Low) exposed pregnant rats are apparently lowered when compared to those in lung tissue of control mother rats. As can be seen in **Table4** and **Table5** element levels within brain and heart tissues of DEHP exposed pregnant rats are higher than those within tissues of DBP exposed mother rats. **Table6** presents average element levels within lung tissues of female offspring rats.

Similarly, **Table 7** and **Table 8** present average element levels within brain and heart tissues of female offspring rats, respectively. The average values and their standard deviations were calculated considering 2 female offspring rats of the control mother, 3 female offspring rats of DBP (Low) exposed mother, 6 female offspring rats of DEHP (Low) exposed mother and 3 female offspring rats of DEHP (High) exposed mother. As can be observed in **Table 6**, Cu, Fe, Mg, Mn, Se, and Zn levels are the highest in the lung tissues of DEHP (Low) exposed offspring rats, when compared to element levels in tissues of control, DBP (Low) and DEHP (High) exposed pregnant rats. Besides, Cu and Se levels in lung tissues of offspring rats of DBP (Low) exposed pregnant rat are apparently lowered when compared to those in lung tissues of offspring rats of control mother rat. As can be seen in **Table7**, the average Mg level within brain tissues of offspring rats of DBP exposed pregnant rats reaches the highest value while, average Fe level within brain tissues of offspring rats of DBP exposed pregnant rats reaches the highest value as can be seen in **Table8**. Besides, levels of most of the elements examined the increase in offspring rats of DBP and DEHP exposed rats when compared with offspring rats of the control mother, together with a higher variance due to variable structural and functional changes in offspring rats of DEHP and DBP exposed mother rats.

Table 1: Acoustic impedance values of lung, brain, and heart tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Tissue	Control Acoustic Impedance (MRayl)	DBP (Low) Acoustic Impedance (MRayl)	DEHP (Low) Acoustic Impedance (MRayl)	DEHP (High) Acoustic Impedance (MRayl)
Lung	1.61 ± 0.19	1.38 ± 0.03	1.74 ± 0.21	1.60 ± 0.04
Brain	1.55 ± 0.04	1.48 ± 0.03	1.83 ± 0.08	1.73 ± 0.10
Heart	1.41 ± 0.07	1.60 ± 0.07	1.66 ± 0.12	1.78 ± 0.18

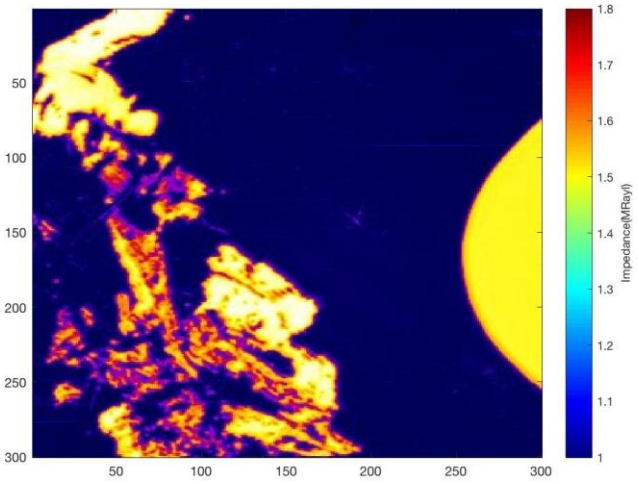
Data are shown as the means ± SD ($p < 0.05$)

Table 2: Average acoustic impedance values of lung, brain and heart tissues of offspring rats of the pregnant rats, which were exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Tissue	Control Acoustic Impedance (MRayl)	DBP (Low) Acoustic Impedance (MRayl)	DEHP (Low) Acoustic Impedance (MRayl)	DEHP (High) Acoustic Impedance (MRayl)
Lung	1.49 ± 0.17	1.56 ± 0.04	1.68 ± 0.06	1.59 ± 0.06
Brain	1.51 ± 0.06	1.56 ± 0.03	1.63 ± 0.03	1.75 ± 0.06
Heart	1.51 ± 0.07	1.60 ± 0.06	1.69 ± 0.07	1.56 ± 0.05

Data are shown as the means ± SD ($p < 0.05$)

Figure 1. SAM image of the lung tissue of an offspring rat of the control mother rat. The image is obtained by comparing reflected ultrasound signals from both surfaces of water and the tissue. Scanning area is 4.8 mm x 4.8 mm.



http://dx.doi.org/10.36472/msd.v8i4.534

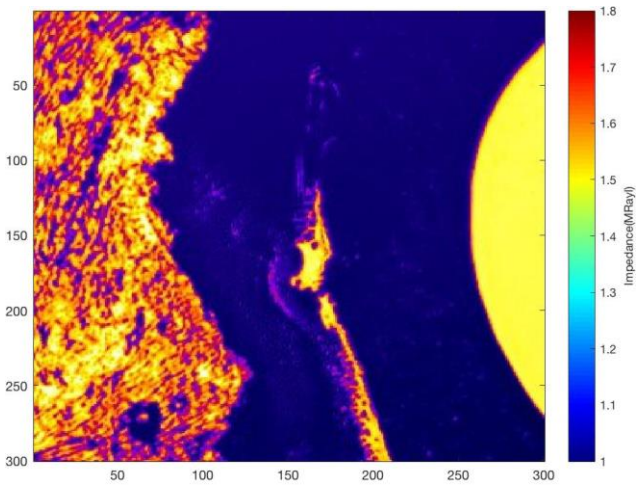


Figure 2. SAM image of the lung tissue of an offspring rat of DEHP (Low) exposed mother rat. The image is obtained by comparing reflected ultrasound signals from both surfaces of water and the tissue. Scanning area is 4.8 mm x 4.8 mm.

Table 3: Element levels within the lung tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	4.48 ± 0.96	2.38 ± 0.02	23.64 ± 0.62	7.21 ± 0.60
Fe	21.66 ± 0.21	35.05 ± 0.72	409.91 ± 1.50	118.50 ± 1.77
Mg	99.07 ± 0.34	53.34 ± 0.54	574.09 ± 2.33	172.99 ± 2.83
Mn	0.37 ± 0.08	0.33 ± 0.09	4.09 ± 0.13	0.95 ± 0.15
Se	3.24 ± 0.86	0.00 ± 0.00	18.83 ± 0.23	10.27 ± 0.88
Zn	14.31 ± 0.74	13.12 ± 0.34	194.93 ± 0.79	36.12 ± 0.95

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc,
Data are shown as the means ± SD ($p < 0.05$)

Table 4: Element levels within the brain tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	15.28 ± 1.23	14.66 ± 2.10	44.72 ± 2.59	29.40 ± 1.38
Fe	104.44 ± 2.84	153.38 ± 2.56	158.978 ± 2.95	187.67 ± 2.59
Mg	120.06 ± 2.22	191.05 ± 3.29	244.49 ± 3.45	324.88 ± 3.34
Mn	0.69 ± 0.07	1.07 ± 0.12	2.99 ± 0.44	5.41 ± 0.75
Se	3.27 ± 0.76	2.07 ± 0.55	13.39 ± 0.83	5.62 ± 0.81
Zn	25.84 ± 1.69	54.94 ± 1.46	112.76 ± 2.13	69.75 ± 1.74

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc,
Data are shown as the means ± SD ($p < 0.05$)

Table 5: Element levels within the heart tissues of the pregnant rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to these rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	8.00 ± 0.78	5.44 ± 0.95	43.80 ± 1.49	7.53 ± 0.68
Fe	108.93 ± 1.88	221.86 ± 1.79	284.00 ± 2.09	501.83 ± 2.55
Mg	41.44 ± 1.58	50.59 ± 1.66	123.60 ± 2.32	131.77 ± 2.41
Mn	0.34 ± 0.06	0.51 ± 0.05	5.84 ± 0.76	1.08 ± 0.06
Se	3.09 ± 0.67	1.70 ± 0.85	10.36 ± 1.15	8.28 ± 1.09
Zn	23.28 ± 1.59	23.53 ± 1.23	65.68 ± 2.05	28.10 ± 1.85

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc,
Data are shown as the means ± SD ($p < 0.05$)

Table 6: Average element levels within the lung tissues of offspring rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	5.02 ± 0.98	3.75 ± 0.46	13.98 ± 3.45	5.87 ± 1.69
Fe	18.11 ± 1.70	19.27 ± 4.10	247.48 ± 94.71	65.55 ± 23.73
Mg	61.14 ± 7.66	86.82 ± 20.7	215.07 ± 92.01	81.91 ± 14.22
Mn	0.12 ± 0.08	0.72 ± 0.20	2.21 ± 0.83	0.49 ± 0.16
Se	1.73 ± 0.79	0.79 ± 0.16	10.19 ± 4.69	3.68 ± 0.43
Zn	14.48 ± 2.92	19.54 ± 6.98	52.44 ± 15.62	24.24 ± 7.17

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc
Data are shown as the means ± SD ($p < 0.05$)

Table 7: Element levels within the brain tissues of offspring rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	10.14 ± 0.82	31.58 ± 17.42	21.60 ± 13.51	24.43 ± 12.02
Fe	73.43 ± 7.54	161.41 ± 41.17	181.28 ± 62.23	153.33 ± 23.74
Mg	69.68 ± 6.88	280.43 ± 97.47	204.31 ± 94.58	173.22 ± 27.11
Mn	0.42 ± 0.03	3.97 ± 1.04	2.07 ± 0.88	1.56 ± 0.93
Se	2.92 ± 0.73	2.38 ± 0.95	6.67 ± 1.28	1.54 ± 0.53
Zn	13.84 ± 0.16	64.92 ± 27.74	60.89 ± 18.89	77.52 ± 25.44

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc
Data are shown as the means ± SD ($p < 0.05$)

Table 8: Element levels within the heart tissues of offspring rats exposed to different concentrations of phthalates of DEHP and DBP. Low is for a concentration of 61 µg/kg/day and high is for a concentration of 61 mg/kg/day, applied to mother rats from the sixth to the nineteenth day of pregnancy.

Elements	Control ($\mu g\ gr^{-1}$)	DBP (Low) ($\mu g\ gr^{-1}$)	DEHP (Low) ($\mu g\ gr^{-1}$)	DEHP (High) ($\mu g\ gr^{-1}$)
Cu	8.70 ± 1.84	5.23 ± 0.55	16.33 ± 7.50	7.95 ± 2.84
Fe	111.54 ± 3.21	435.91 ± 99.67	288.62 ± 43.94	235.22 ± 93.64
Mg	83.17 ± 68.41	147.66 ± 53.80	154.71 ± 27.71	93.59 ± 31.88
Mn	0.67 ± 0.44	1.34 ± 0.21	1.68 ± 0.54	0.81 ± 0.36
Se	2.37 ± 0.16	2.02 ± 0.33	4.47 ± 0.75	4.14 ± 0.26
Zn	18.29 ± 1.29	18.54 ± 3.03	47.26 ± 23.05	29.87 ± 6.46

Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Se, selenium; Zn, zinc
Data are shown as the means ± SD ($p < 0.05$)

DISCUSSION

Even though it has been revealed that accumulation of phthalates in animals and also in humans leads to damages in reproductive organs with high dose exposure, literature lacks the dose at which these disruptions start to occur. It has been reported that an average DEHP exposure dose is between 3 to 30 µg/kg/day for an adult whereas multiple times higher for a baby or an infant (**32**). Average DBP exposure of an adult was determined to be 7 µg/kg/day (**33**).

These doses would be even higher during a medical intervention due to the use of plastic blood and serum bags. In this study, phthalate exposure doses were determined to be 61 µg/kg/day and a much higher value of 61 mg/kg/day for comparison, considering the differences in the metabolisms and sizes of humans and rats. Only female offspring rats are taken into account together with mother rats to avoid parameters depending on the sex.

Examination of lung, brain, and heart tissues of pregnant rats and their offspring female rats by SAM is done for the first time in literature and also supported by ICP-OES in our study.

SAM is successful in determining the alterations within tissue samples, by calculating acoustic impedance values in tissues of mother and offspring rats. In **Table 1** acoustic impedance values of lung and brain tissues of DBP exposed pregnant rat were lower than those of lung and brain tissues of the control mother rat. Besides, acoustic impedance values of lung and brain tissues of DEHP (Low) exposed mother rats were higher than those of lung and brain tissues of DEHP (High) exposed mother rats. The decrease in acoustic impedance value of lung tissue of DBP exposed mother rat can be a result of noticeable Cu, Mg, and Se level decrease as can be seen in **Table3**.

Besides, the highest acoustic impedance value observed in lung tissue of DEHP (Low) exposed mother rat can be a result of the observation of the highest element levels in that tissue as can be seen in **Table 3**. Similarly, the decrease in acoustic impedance value of brain tissue of DBP exposed mother rat can be a result of Cu and Se level decrease as can be seen in **Table4**. In **Table1**, the acoustic impedance value of heart tissue of DEHP (High) exposed mother rat is the highest, confirmed with the elevated element levels in that tissue (**Table 5**).

Table 2 presents average acoustic impedance values within all tissues of the offspring rats examined. Each value in Table 2 is the average of multiple female offspring rats of the same mother. Tissues of offspring rats of DBP and DEHP exposed mother rats have higher acoustic impedance values due to increased element levels. The highest acoustic impedance value of lung tissue is observed in offspring rats of DEHP (Low) exposed mother rat, which may be a result of the apparent increase in Fe, Mg, Mn, Se, and Zn levels as can be seen in Table 6, also, the highest acoustic impedance value of brain tissue, observed in offspring rats of DEHP (High) exposed mother rat, maybe a result of the apparent increase in Zn level as can be seen in **Table 7**. Similarly, the highest acoustic impedance value of heart tissue, observed in offspring rats of DEHP (Low) exposed mother rat, maybe a result of the apparent increase in Cu, Mg, Mn, Se and Zn levels as can be seen in **Table 8**.

Inducing oxidative stress and, therefore, making the oxidant and/or antioxidant mechanisms inactive, is one of the important toxicity mechanisms of phthalate esters and this has been investigated both in in vivo and in vitro studies (**34,35,36,37,38,39**). Phthalates have been shown to cause oxidative stress targeting the endocrine system, and reproductive anomalies by decreasing the levels of steroidogenic enzymes. The levels of essential elements and minerals play an active role in enzyme expression and synthesis. DEHP exposure has been found to affect trace element or mineral levels due to changes in antioxidant enzyme levels (**40,41,42,43,44**). Cell culture studies have also been reported to state that DEHP causes changes in antioxidant enzyme levels and results in intracellular reactive oxygen species (ROS) formation and deoxyribonucleic acid (DNA) damage (**34,35,44**). In our study, we determined Cu, Fe, Mg, Mn, Se, and Zn levels by ICP-OES. Even though element levels in tissues of DEHP exposed rats are increased when compared to those of the tissues of the control group, some element levels, as seen in tissues of DBP exposed rats, are decreased when compared to those of the tissues of the control group. The reason for this discrepancy may be due to the fact that DBP and DEHP esters cause changes in the levels of different proteins.

Limitations: The small number of the samples is the major limitation due to incorporating only the female rats into this study and the loss of some of the offspring rats of DEHP and DBP exposed pregnant rats through resorption. Besides, the number of pregnant rats kept at the minimum level, since it is the priority in an animal study. Therefore, another correlation study, between exposure dose and element levels within tissues, will be conducted in a larger cohort and presented later on.

CONCLUSION

There is a limited number of researches about the impact of DBP and DEHP on levels of elements within lung, brain, and heart tissues. In this study, we tried to evaluate the impact of DEHP and DBP on lung, brain, and heart tissues of both mother rats and their offspring female rats, by SAM and ICP-OES. Effects of high dose (1000 mg/kg/day) exposure are studied and determined in animal reproductive systems. However, the dose at which disruptions start to occur is not known. Imaging of the tissues with a micrometer resolution by obtaining acoustic impedance distributions, predict structural and functional changes, induced due to DBP and DEHP exposure of doses of 61 µg/kg/day and 61 mg/kg/day. ICP-OES confirms this prediction by expressing higher element levels within tissues of DEHP and DBP exposed rats. Consequently, we can state that even with these DBP and DEHP exposure doses, toxic agent-induced deviations within lung, brain, and heart tissues of mother and offspring rats can be observed by these techniques.

Acknowledgments: Scanning acoustic microscopy studies were supported by a grant from the Ministry of Development of Turkey (Project Number: 2009K120520).

Author contributions: BT, GA, MAA, LTS, IA, FAA, NPO, BS, MBU; Study design, Literature search, , experimental studies, statistical analyzes, **BT;** Writing of the article and revisions.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

Platelet to lymphocyte Ratio (PLR) as an indicator of survival in rare histopathological subtypes of Renal Cell Carcinoma

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ABSTRACT

Objective: Inflammatory markers have prognostic significance for renal cell carcinomas (RCC) as in many types of cancer. The prognostic effect of inflammatory markers in the rare histological subtypes of RCC has not been adequately evaluated. In our study, we aimed to evaluate the relationship between basal inflammatory indices (neutrophil to lymphocyte ratio [NLR], platelet to lymphocyte ratio [PLR], lymphocyte to monocyte ratio [LMR], and systemic immune-inflammation [SII]) and survival (progression-free survival [PFS] and overall survival [OS]).

Material and Methods: Patients with metastatic non-clear cell RCC (nccRCC) or RCC with sarcomatoid differentiation (sRCC) were included in the study. The relationship between inflammatory indices, which was calculated before any systemic treatment and survival, were retrospectively assessed.

Results: Thirty patients, predominantly males (n = 20, 66.7%), with a median age of 59.1 (IQR, 52.5-70.3) years, were included in the study. Median PFS achieved with first-line tyrosine kinase inhibitors for patients with a PLR level less or greater than the median value (238) was 12.6 (95% CI 1.4-23.9) months and 4.8 (95% CI, 2.3-7.3) months, respectively (p = 0.021). Median OS for patients with a PLR level less or greater than the median (238) was 16.7 (95% CI, 3.7-29.7) months and 8.6 (95% CI, 4.9-12.3) months (p = 0.008), respectively. In the Cox-regression model (including gender, age, presence of metastasis at diagnosis, NLR, PLR, LMR, SII) only PLR was the independent predictive factor for both PSF (HR = 0.131; 95% Cl 0.028-0.620, p = 0.010) and OS (HR = 0.199; 95% Cl 0.048-0.819, p = 0.025).

Conclusion: In rare RCC subtypes such as nccRCC and sRCC, lower PLR may be associated with better PFS and OS.

INTRODUCTION

Kidney and renal pelvis malignancies constitute 4.1% of newly diagnosed cancers (1). Approximately 85% of kidney tumors are renal cell carcinomas (RCC), mostly with clear cell histopathology (2, 3). Other subtypes are papillary, chromophobe, translocation, Bellini duct (collecting duct) and medullary renal cell carcinomas, the most common of which are papillary carcinomas (2, 3). Besides, sarcomatoid differentiation is also a rare entity accompanying RCC. Sarcomatoid differentiation is defined as exhibiting pronounced cytological atypia and containing malignant spindle cells resembling sarcoma (4). Sarcomatoid differentiation in all RCCs is around 5-8% (5, 6). Since both non-clear cell RCC (nccRCC) and RCC with sarcomatoid differentiation (sRCC) are rare conditions, the prognostic factors have not been clearly defined due to the low representation rate in clinical trials.

Besides being a promoting factor for cancer, inflammation also affects tumour progression and cancer patients' survival (7, 8). It has been reported that inflammatory markers (such as neutrophil to lymphocyte ratio [NLR]) may have prognostic significance for RCC, as in many cancer types (9, 10). The systemic immune-inflammation (SII) index is calculated by using neutrophil, lymphocyte and platelet counts, which is thought to reflect inflammation better (11).

Research Article

Received: 11-04-2020

Accepted: 26-04-2021

Available Online: 27-04-2021

Published: 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.535

SII is associated with poor outcomes in patients with hepatocellular carcinoma (HCC) (**11**). Although the prognostic significance of inflammatory markers has been evaluated in many types of cancer, it has not been adequately examined for rare histological subtypes of kidney tumours such as nccRCC or sRCC (8, 12, 13).

A retrospective analysis has shown that platelet to lymphocyte ratio (PLR) and lymphocyte to monocyte ratio (LMR) were determinants of shorter survival for both progression-free survival (PFS) and overall survival (OS) in metastatic nccRCC (**14**). Similarly, a high preoperative NLR was associated with poor clinical and pathological parameters in papillary RCC (**15**).

In our study, we aimed to evaluate the relationship between basal inflammatory indices (NLR, PLR, LMR and SII), PFS achieved with first-line tyrosine kinase inhibitors (TKIs) and OS in metastatic nccRCC and sRCC.

MATERIAL and METHODS

Patients with metastatic rare histological subtype RCC followed up in the medical oncology clinic of a tertiary referral center between November 2011 and March 2021 were included in the study. Rare histological subtypes were identified as nccRCC and sRCC. Patients' medical records were reviewed retrospectively. Exclusion criteria were as follows: clear cell histological subtype, non-metastatic disease, presence of rheumatologic, immunological and infectious (i.e. acute infections, active tuberculosis, or active chronic viral infections) disease that can affect inflammatory markers, secondary malignancy, use of drugs that can affect inflammatory markers and insufficient medical records.

The baseline demographic characteristics (i.e. age, gender) of the patients, disease characteristics (i.e. tumor histological subtype, presence of metastasis at the time of diagnosis, metastatic sites) were recorded. Before any treatment (interferon or TKIs), the patients' neutrophil, lymphocyte, monocyte, and thrombocyte counts were recorded in the database. Neutrophil to lymphocyte ratio (NLR), PLR, LMR, and SII (platelet count × neutrophil count/lymphocyte count) were calculated. First-line treatment agents, PFS obtained with first-line therapy, and OS were also recorded. Progression-free survival was defined as the time of the start of TKI therapy which was used as the first line (with or without previous interferon) treatment to progression. Overall survival was defined as the time from diagnosis of metastatic disease to death or last follow-up.

The patients were subgrouped according to the calculated inflammatory index (NLR, PLR, LMR, and SII) scores (less or greater than median value), gender, age (less or greater than median value), and presence of metastasis at diagnosis. All subgroups were compared about PFS and OS. Statistical analysis was performed using SPSS software (SPSS for Windows, version 24.0., SPSS Inc., Chicago, USA). The distribution of data was evaluated by using the Kolmogorov–Smirnov test. Non-parametric data were presented as median (interquartile range-IQR), and categorical data were presented as frequency (percentage).

A ROC analysis was performed to determine cut-off values of systemic indexes. However, appropriate cut-off values with high sensitivity and specificity for systemic indexes could not found. Therefore median values of systemic inflammatory parameters were used as cut-off points. Survival rates were estimated by Kaplan–Meier method, and the groups were compared by log-rank test for survival differences. The Cox regression model was carried out using multivariate analyses. All statistical analyses were two-sided, and a p-value <0.05 was considered statistically significant.

RESULTS

Study Population

Thirty patients, predominantly males (n = 20, 66.7%), with a median age of 59.1 (IQR, 52.5-70.3) years, were included in the study. Median follow-up was 7.3 (IQR, 2.4-13.8) months. The most common tumour histological subtype was papillary carcinoma (n = 10, 33.3%). Thirteen (43.3%) patients had metastatic disease at diagnosis. Seventeen (56.7%) patients had an early-stage disease at diagnosis, and metastatic disease developed later. Twenty-one (70%) patients had lung metastasis.

According to the International Metastatic RCC Database Consortium risk classification, the highest number of patients was in the intermediate-risk group (n = 17, 56.7%). Main patient characteristics are shown in table 1. Inflammatory indices (NLR, PLR, LMR, and SII) at diagnosis of metastatic disease are given in table 2. Twenty-three patients (76.7%) had TKIs as first-line therapy. Of the patients who received TKIs, 16 (69.6%) received pazopanib and 7 (30.4%) sunitinib. Partial remission, stable disease and progressive disease on TKIs were observed in 5 (21.7%), 8 (34.8%) and 10 (43.5%) patients, respectively.

Table 1. Main patient characteristics

Characteristics		n (%)
Age, median (IQR)		59.1 (52.5-70.3)
Gender		
	Male	20 (66.7%)
	Female	10 (33.3%)
Histological subtype		
	Papillary	10 (33.3%)
	Sarcomatoid	9 (30.0%)
	Chromophobe	3 (10.0%)
	Bellini duct	1 (3.3%)
	Oncocytic	1 (3.3%)
	Undifferentiated	6 (20.0%)
Metastasis at diagnosis		
	Yes	13 (43.3%)
	No	17 (56.7%)
Metastasis Site		
	Lung	21 (70.0%)
	Bone	17 (56.7%)
	Lymph node	12 (40.0%)
	Liver	5 (16.7%)
	Brain	4 (13.3%)
IMDC risk group		
	Favorable	1 (3.3%)
	Intermediate	17 (56.7%)
	Poor	11 (36.7%)
	Unknown	1 (3.3%)

IMDC, International Metastatic RCC Database Consortium

http://dx.doi.org/10.36472/msd.v8i4.535

Table 2. Baseline inflammatory indices

Index	Median (IQR)
NLR	3.7 (2.9-5.4)
PLR	238 (161-323)
LMR	2.7 (1.7-4.5)
SII	1197 (692-1918)

NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; LMR, lymphocyte to monocyte ratio; SII, systemic immune-inflammation index

Survival

Median PFS was 6.7 (95% CI, 3.8-9.7) months for all patients (n = 23) who received a TKI as first-line therapy. Results of PFS analysis according to subgroups are shown in table 3. In this subgroup analysis, only PLR had a significant effect on PSF obtained by using first-line TKI. Median PFS for patients with a PLR level less (n = 12) or greater (n = 11) than median was 12.6 (95% CI 1.4-23.9) months and 4.8 (95% CI, 2.3-7.3) months, respectively (p = 0.021).

Table 3. Progression-free survival analysis by subgroups

Parameter	Median (Range) (95% CI)	*p*-value
Gender		0.885
Male (n = 15)	6.7 (3.9-9.6)	
Female (n = 8)	3.9 (0.0-10.7)	
Age		0.472
< 59.1 (n = 13)	6.0 (1.4-10.6)	
> 59.1 (n = 110)	2.1 (2.7-10.8)	
Metastasis at diagnosis		0.584
Yes (n = 11)	5.5 (3.3-7.8)	
No (n = 12)	7.4 (0.0-19.8)	
NLR		0.746
< 3.7 (n = 14)	6.7 (3.3-10.2)	
> 3.7 (n = 9)	6.0 (0.0-12.1)	
PLR		**0.021**
< 238 (n = 12)	12.6 (1.4-23.9)	
> 238 (n = 11)	4.8 (2.3-7.3)	
LMR		0.456
< 2.7 (n = 11)	6.7 (3.5-10.0)	
> 2.7 (n = 12)	5.5 (0.0-11.5)	
SII		0.404
< 1197 (n = 13)	7.4 (0.0-15.7)	
> 1197 (n = 10)	4.8 (1.5-8.1)	

NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; LMR, lymphocyte to monocyte ratio; SII, systemic immune-inflammation index

Median OS was 11.5 (95% CI, 9.2-13.8) months for all patients (N = 30). Results of OS analysis according to subgroups are shown in Table 4. In this subgroup analysis, only PLR had a significant effect on OS.

Median OS for patients with a PLR level less (n = 15) or greater (n = 15) than median was 16.7 (95% CI, 3.7-29.7) months and 8.6 (95% CI, 4.9-12.3) months (p = 0.008), respectively.

Kaplan-Meier curves of the OS analysis by grouping the patients based on inflammatory indices (NLR, PLR, LMR, and SII), such as greater or less than the median value, are shown in Figure 1.

In the Cox-regression model (including gender, age, presence of metastasis at diagnosis, NLR, PLR, LMR, SII) PLR was the only independent predictive factor for both PSF (HR = 0.131; 95% Cl 0.028-0.620, p = 0.010) and OS (HR = 0.199; 95% Cl 0.048-0.819, p = 0.025).

Table 4. Overall survival analysis by subgroups.

Parameter	Median (Range) (95% CI)	*p*-value
Gender		0.302
Male (n = 20)	10.6 (8.9-12.4)	
Female (n = 10)	11.6 (2.8-20.4)	
Age		0.503
< 59.1 (n = 15)	8.6 (3.0-14.1)	
> 59.1 (n = 15)	12.3 (10.9-13.7)	
Metastasis at diagnosis		0.744
Yes (n = 13)	10.6 (7.2-14.0)	
No (n = 17)	12.3 (6.4-18.2)	
NLR		0.258
< 3.7 (n = 15)	14.9 (6.8-23.1)	
> 3.7 (n = 15)	11.5 (6.3-16.7)	
PLR		**0.008**
< 238 (n = 15)	16.7 (3.7-29.7)	
> 238 (n = 15)	8.6 (4.9-12.3)	
LMR		0.135
< 2.7 (n = 15)	11.5 (6.4-16.6)	
> 2.7 (n = 15)	11.6 (9.0-14.2)	
SII		0.105
< 1197 (n = 15)	14.9 (7.1-22.7)	
> 1197 (n = 15)	10.6 (3.6-17.7)	

NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; LMR, lymphocyte to monocyte ratio; SII, systemic immune-inflammation index

http://dx.doi.org/10.36472/msd.v8i4.535

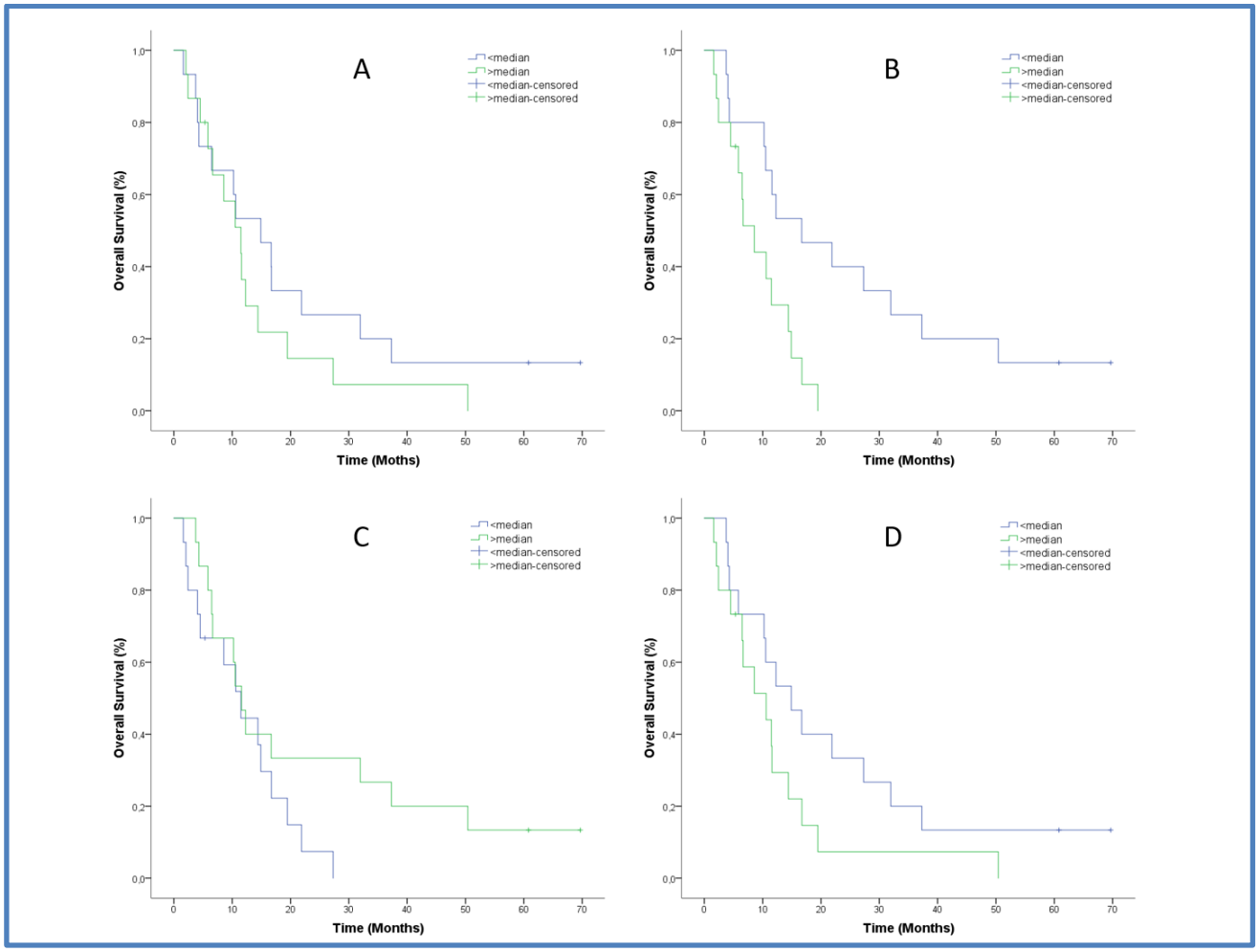

Figure 1. Kaplan-Meier curves of the overall survival analysis based on inflammatory indices; **A.** NLR, **B.** PLR, **C.** LMR, and **D.** SII.

DISCUSSION

In our study, both median PFS with first-line TKI and median OS were statistically better in the PLR lower group. Moreover, there were noticeable numerical differences for PSF of first-line TKI treatment in subgroup analysis performed according to the SII. Similarly, in the subgroup analysis performed according to NLR and SII, numerical differences for OS may have clinical significance. However, there was no statistically significant difference in PFS and OS analysis for either NLR or SII. Numerous prognostic factors and prognostic models have been evaluated for RCC, but none of them was shown to predict prognosis accurately (16). A novel prognostic nomogram for metastatic RCC is promising, but there has not been sufficient clinical experience with this nomogram (17). Although there have been studies evaluating prognostic factors in RCC, the literature data on prognosis for rare histological subtypes (such as nccRCC and sRCC) is quite insufficient.

The Cancer Genome Atlas (TCGA) Research Network showed that a CpG island methylator phenotype (CIMPA) in a distinct subgroup of type 2 papillary RCC (18). This subgroup was characterized by poor survival and mutation of the gene encoding fumarate hydratase (18). Similarly, according to the gene expression data, low tumour suppressor gene CKDN1A mRNA and protein mutation were associated with poor OS for chromophobe RCC (19).

A transcriptomic analysis containing sRCC found that high PD-L1– and CD8-positive cell expression may account for their improved outcomes with immuno-therapies and showed that high MYC targets version 1 gene expression in these tumours was associated with poor outcome (20). The results of these precious trials on the prognostic factors in rare histological subtype RCCs enable us to look to the future with confidence. However, these prognostic parameters are not yet usable since they require high technology and are not easily accessible. Simple, cheap and accessible parameters are needed to predict prognosis in daily oncology practice.

Thrombocytosis was an independent poor prognostic factor in anti-vascular endothelial growth factor (VEGF) treatment-naive metastatic RCC (p = 0.01) (21). A large-scale meta-analysis, in which Gu et al included 25 studies of 11,458 patients with a diagnosis of RCC, have reported that an elevated platelet level was associated with poor OS (HR = 2.24, 95% CI 1.87-2.67, p <0.001) and cancer-specific survival (HR = 2.59, 95% CI 1.92-3.48, p <0.001) (22). Saroha et al in a study including 430 patients diagnosed with RCC, reported that lymphopenia was associated with worse OS regardless of other risk factors (pT and TNM stages, nuclear grade, age, tobacco smoking, and comorbidity index) (23). Evaluating the prognostic importance of PLR in RCC for the first time, Gündüz et al found that high PLR is an

independent negative predictive factor for PFS (p = 0.001) and OS (p = 0.013) (24). In a recent meta-analysis involving 1,528 RCC patients, an elevated PLR was an effective prognostic marker of both OS (HR = 2.10, 95% CI: 1.38-3.19, p = 0.001) and PFS (HR = 3.45, 95% CI: 1.61-7.40, p = 0.001) (25). However, in the metanalysis, including 1528 patients, only 91 patients were reported to have nccRCC histological subtype, but no information was given about the sRCC subtype (25). In our study, a PLR level below 238 was a positive factor for both PFS and OS. These findings are in accordance with the literature data and are important in terms of including nccRCC and sRCC cases, which are a group that has not been adequately evaluated.

In the study by Başal et al evaluating 187 patients with metastatic RCC, it was revealed that SII is an independent prognostic factor about survival for the favourable, intermediate, and poor IMDC groups (26). Similarly, Özbek et al observed an association between SII and increased TNM stage and poor prognosis in RCC patients undergoing radical nephrectomy (27). In our study, there was an absolute difference of 2.6 months in median PFS and 4.5 months in median OS in favour of the group with low SII. However, this difference did not reach a statistical significance.

In a recent meta-analysis, high NLR value was found to be a negative prognostic factor for OS (HR = 1.80; 95% CI 1.61-2.00) and PFS (HR = 1.69; 95% CI 1.42-2.01) in RCC in both metastatic and nonmetastatic patients (28). In three different trials involving patients with non-metastatic nccRCC, it was concluded that a high preoperative NLR was associated with poor DFS (15, 29, 30). In a retrospective analysis including 37 patients with metastatic nccRCC who received pazopanib as first-line therapy, lower basal NLR was associated with both better PFS (p = 0.009) and OS (p = 0.008) (31). In a similar retrospective study, it was stated that in 36 metastatic chromophobe RCC patients who received sunitinib treatment, pretreatment NLR<3 was associated with a better OS (HR = 0.55, p = 0.03) (32). Although we consider that NLR as a prognostic factor in our study, 3.4 months of OS advantage in patients with NLR <3.7 did not reach statistical significance.

Main limitations of our study are its retrospective nature and a small number of patients. It would be more appropriate to evaluate the prognostic significance of inflammatory parameters at homogeneous groups to be formed according to the histological subtypes of nccRCC and sRCC prospectively. However, it is difficult to perform this analysis due to the scarcity of patients. Another limitation is that the C-reactive protein/albumin ratio, whose prognostic importance in RCC is known (33), cannot be calculated since the C-reactive protein level is not routinely evaluated in the pre-treatment period. Nevertheless, we consider that our study has clinical value since it is conducted in a rare patient group that has not been adequately evaluated yet.

CONCLUSION

In rare RCC subtypes such as nccRCC and sRCC, PLR is associated with both PFS and OS. Systemic immune-inflammation index and NLR may also have prognostic significance for survival in this group.

Acknowledgments: None

Author contributions: EE, MD; Study design, Literature search, Patient examinations, Data collection, Statistical analyzes, **EE;** Writing of the article and revisions.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

Basal Cell Nevus Syndrome caused by a new splice site mutation in PTCH1

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ABSTRACT

Objective: Basal cell nevus syndrome is a rare inherited autosomal dominant syndrome characterized by developmental defects and tumor predisposition. There are more than 400 reported PTCH1 mutations, including frameshift, nonsense, missense, deletions, duplications, and splicing mutations. We report a 68-year-old Thai female presenting with multiple basal cell carcinoma scattered on the face and upper back and palmoplantar pits. Molecular diagnosis showed a novel heterozygous mutation in the splice site region c.746+1_746+4delGTAA, localized within the splice donor after exon 5 of PTCH1. Although the clinical manifestations are characteristic, this report adds another splice site mutation to the genotypic variation of BCNS patients and also highlights the importance of a multidisciplinary approach in the management of BCNS patients.

Keywords: Basal cell nevus syndrome, PTCH1, Mutation

INTRODUCTION

Basal cell nevus syndrome (BCNS) (OMIM109400) is a rare, multisystem, inherited autosomal dominant syndrome characterized by developmental defects and tumor predisposition (1). The clinical manifestations of BCNS include multiple basal cell carcinomas (BCCs), palmoplantar pits, odontogenic keratocysts of the jaw, ocular anomalies, skeletal, reproductive systems, and calcification of the falx cerebri (1). PTCH1 is the leading cause of mutations in BCNS (1). To date, there are more than 400 reported mutations, including frameshift, nonsense, missense, deletions, duplications, and splicing mutations (2). Genomic sequencing of 23 exons all showed mutation, yet there was no hot spot identified (2).

CASE

Here, we report a 68-year-old Thai female presenting with multiple progressive dark brownish to blackish lumps and bumps scattered on the face and upper back for about 30 years. Initially, the lesions developed on the periorbital and perioral areas. The patient reported no similar symptoms in family or relatives. Physical examination revealed multiple discrete well-defined dark brown to black papules and plaques with elevated rodent border and some ulcers scattered on both periorbital, perioral areas, temporal areas, forehead, and upper back (Fig. 1a, c). Bilateral symmetrical multiple pitted lesions were also noted her both palms and soles (Fig. 1b, d). Neither hepatosplenomegaly nor lymphadenopathy was observed. The histological exam revealed nodular and infiltrative basaloid tumor connecting from epidermis with peripheral nuclear palisading and peritumoral artificial clefts consistent with basal cell carcinoma (Fig. 1e). Additional laboratory tests for associated developmental anomalies and tumors were investigated, including chest radiograph and CT scan of the brain-skull-neck-oropharynx and whole abdomen. All the results were unremarkable. After obtaining informed consent, Sanger sequencing of all 23 coding exons and flanking introns of PTCH1 was performed using primers previously described (3) and genomic DNA from peripheral blood obtained from the affected individual, which revealed a novel heterozygous mutation, NM_001354919.1 c.746+1_746+4delGTAA, localized within the splice donor after exon (5). A prediction tool suggested alternate splicing leading to possible deleterious effects on the protein (https://franklin.genoox.com/clinical-db/home) (Fig. 1f).

Case Report

Received 22-03-2020

Accepted 30-03-2021

Available Online: 01-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.519

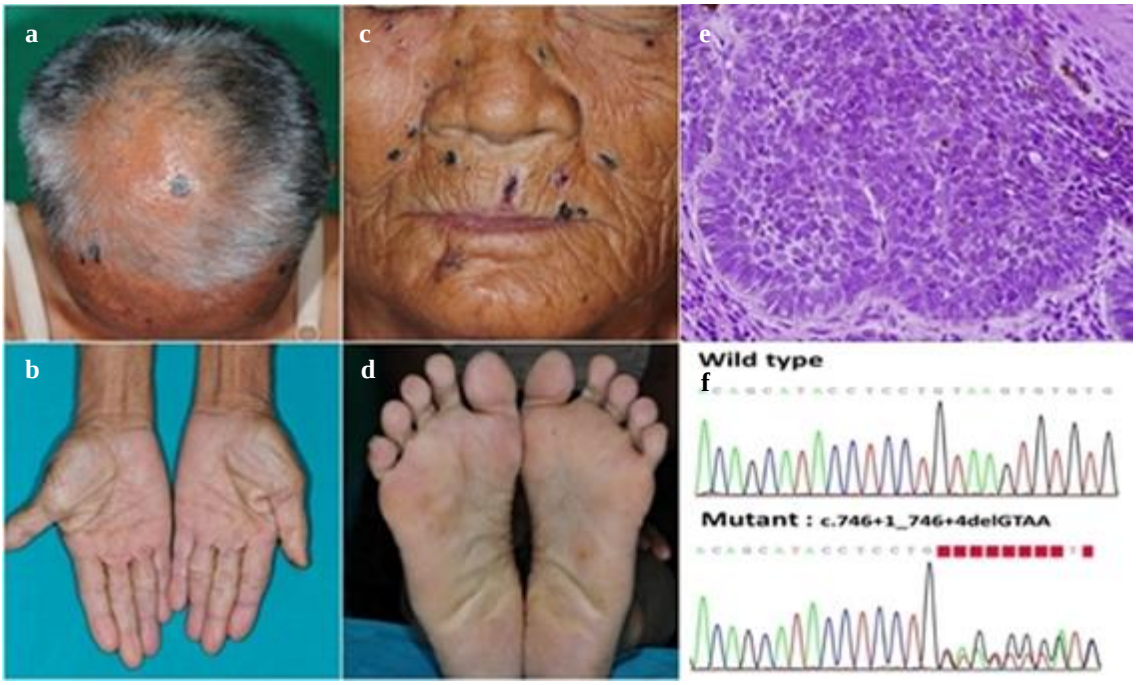

Figure 1. Clinicopathological and molecular findings in basal cell nevus syndrome. (a,c) multiple basal cell carcinoma on the head and neck; (b,d) numerous palmoplantar pits; (e) basal cell carcinoma showing basaloid tumor with peripheral nuclear palisading and peritumoral artificial clefts (hematoxylin&eosin, 40x) (f) Sanger sequencing reveals a four base pairs heterozygous deletion in PTCH1 (NM_001354919.1; c.746+1_746+4delGTAA).

However, further functional studies will be required to confirm experimentally. This mutation does not appear in any lists of genomic databases, such as the gnomAD browser (https://gnomad.broadinstitute.org/). Thus, the molecular diagnosis was consistent with BCNS.

This syndrome exhibits a nearly complete penetrance with variable expressivity. The prevalence is estimated from 1/56,000 up to 1/256,000, in which both sexes are equally affected (4). Pathogenesis of BCNS has been attributed to heterozygous germline mutations in tumor suppressor gene PTCH1, located on chromosome 9q22.3 (4). The human PTCH1 gene encodes a transmembrane glycoprotein, patched 1, which functions as the antagonist receptor for the sonic hedgehog ligand (4). Mutations in PTCH1 have been found in 40-80% of patients with BCNS (5). PTCH1 is the most frequently mutated gene, but mutations in PTCH2, SUFU can also occur5. About 20-30% of BCNS patients can be caused by de novo mutation (2).

The treatment of patients with BCNS requires a holistic approach. For the management of BCCs in BCNS, there are no established standard guidelines. Therefore, surgical excision by standard or micro excisional/Mohs micrographic surgery (MMS) has been the mainstay treatment4. BCCs lesions in our case were operated on by cryosurgery, and oral acitretin 25 mg/day has been prescribed for cancer chemoprevention, as recommended for high-risk non-melanoma skin cancer (4).

CONCLUSION

In summary, we report a case of BCNS in which clinical features fit into the diagnostic criteria with a novel heterozygous mutation in the splice site region. Although the clinical manifestation is characteristic, this report adds another splice site mutation, highlighting no evidence of genotype-phenotype correlation among BCNS patients.

Author contributions: NN, TB, PC, CS; Patient examination, Genetic analysis, Literature search and study design, data collection and analyzes **CS;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

Gastric Mixed Neuroendocrine-Nonneuroendocrine Neoplasm: A rare case report

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ABSTRACT

Objective: The incidence of gastric neuroendocrine neoplasms is less than 1%. They are seen as combined tumors with non-neuroendocrine neoplasms at a rate of approximately 7%. This study aims to share the case of mixed neuroendocrine and non-neuroendocrine cancer with the literature.

Case: Endoscopic biopsies were taken from the tumoral mass detected in the gastric cardia region at endoscopy in a 60-year-old male patient that has complaints of weight loss and epigastric pain. Histopathological examination revealed malignant tumor infiltration that consisting of neuroendocrine cells with large nuclei and narrow cytoplasm in some areas and adenoid structures composed of atypical cells with pleomorphic large nuclei in some areas. Strong staining was observed in neuroendocrine areas with neuroendocrine markers such as synaptophysin and Chromogranin. Ki-67 proliferative index and mitotic activity were high in neuroendocrine neoplasm areas. The case was reported as a high-grade neuroendocrine-non neuroendocrine mixed neoplasm.

Conclusion: Gastric Mixed Neuroendocrine-Nonneuroendocrine neoplasms are rare cases and correct diagnosis and grading are important in the treatment and patient follow-up protocol.

Keywords: Gastric, neuroendocrine, non-neuroendocrine, neoplasm

INTRODUCTION

Gastric cancer is the fifth most common malignancy worldwide and the third cause of death among all malignancies (1). It is rare before the age of 40, most common in the 50 to 70 age group, and more often in men (2). Symptoms such as dysphagia and persistent vomiting can be seen (3). The most common histopathological type, constituting 95% of all gastric malignant tumors, is adenocarcinoma (4). Gastrointestinal neuroendocrine neoplasms originate from neuroendocrine cells located in the mucosa and submucosa (5). Neuroendocrine neoplasms (NEN) are about 1% of digestive system malignancies (6). The incidence among all gastric cancers is approximately 0.1-0.6% (7). They can be seen as a mixed neoplasm approximately 7% with a non-neuroendocrine neoplasm (8).

The aim of this study is to share the case of mixed neuroendocrine and non-neuroendocrine neoplasm (MiNEN) with the literature.

CASE

A 60-year-old male patient was admitted to the hospital with complaints of weight loss and epigastric pain. Endoscopic biopsies were taken from the patient whose endoscopy revealed a mass in the gastric cardia region. 4-micron sections were taken from the paraffin blocks prepared from the tissues belonging to the lesion. The samples were examined by staining Hematoxylin-Eosin. Malignant tumoral infiltration consisting of atypical adenoid structures and neuroendocrine cells with narrow cytoplasm with large nuclei in some areas was detected in histopathological examination (Figure 1). Necrosis areas and high mitotic activity were present in areas containing NEN. Staining was observed with pan-cytokeratin in areas with atypical adenoid structures, and with synaptophysin and chromogranin in neuroendocrine areas. Ki67 proliferative index was 80% in neuroendocrine areas (Figure 2). The case was reported as MiNEN (adenocarcinoma and neuroendocrine carcinoma mixed tumor).

Case Report

Received 02-04-2020

Accepted 16-04-2021

Available Online: 19-04-2021

Published 30-04-2021

http://dx.doi.org/10.36472/msd.v8i4.528

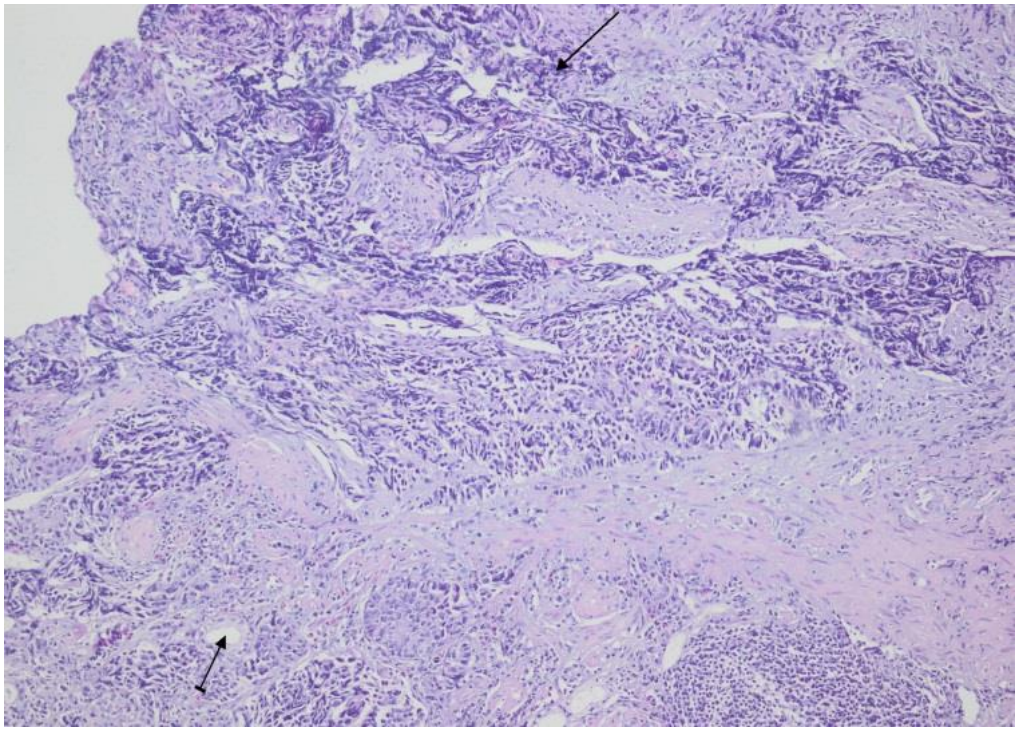

Figure 1. Short arrow: Adenoid areas, long arrow: Neuroendocrine areas (HEx200).

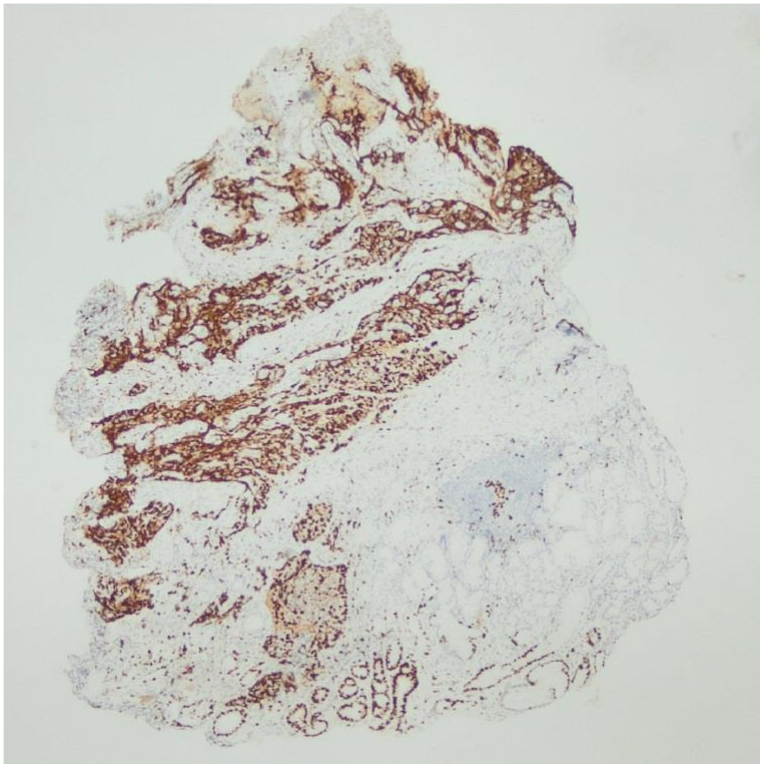

Figure 2. Ki-67 proliferation index (x40).

Table 1. The 2017 World Health Organization classification of neuroendocrine neoplasms

Grade	Mitotic index, mitoses per 10HPF	Ki67, %
Grade 1	<2	<3
Grade2	2-20	3-20
Grade 3	>20	>20

DISCUSSION

NEN is classified as a neuroendocrine tumor, neuroendocrine carcinoma, and mixed neuroendocrine-non-neuroendocrine neoplasms (9). The 2017 World Health Organization classification of NEN was made according to the Ki-proliferation index and mitotic activity (10). In grade 1 NEN, the Ki-67 proliferative index is less than 3 and mitotic activity is less than 2 in 10 high power fields (HPF). In grade 2 NEN, 2-20 mitosis/10 HPF and Ki-67 proliferative index is 3-20%. In grade 3 NEN, Ki-67 proliferative index is higher than 20 and mitotic activity is more than 20 in 10 HPF (Table 1).

High-grade MiNEN is usually in the form of adenocarcinoma and neuroendocrine carcinoma (11). Clinically, NEN can be functional with symptoms due to hormonal hypersecretion (12). The neuroendocrine component has an important role in determining biological behavior.

Chromogranins and synaptophysin markers are used to determine neuroendocrine differentiation in the gastrointestinal system (13). Prognosis and treatment planning should be made according to the most aggressive neoplastic component (14). Medications such as somatostatin analog can be used to control hormonal syndrome in advanced NEN (15). The surgical approach is the most important treatment in early cases. In functional NEN, hormonal stabilization should be provided with therapies before surgery (16).

CONCLUSION

Gastric MiNEN is rare cases and correct diagnosis and grading are important in the treatment and patient follow-up protocol. In this study, the case detected in the stomach cardiac region MiNEN was shared with the literature.

Author contributions: MGB, MT; Patient examination, Literature search and study design, data collection and analyzes **MGB;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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Medical Science and Discovery
ISSN: 2148-6832

Succinyl-CoA: 3-Ketoacid CoA-Transferase Deficiency in a Saudi Girl

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ABSTRACT

Objective: Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency is a rare genetic disorder of ketone utilization and isoleucine catabolism caused by mutations in the OXCT1 gene,

Case: A Saudi girl case of SCOT deficiency confirmed by genetic analysis has been reported in this study. A 5-year-old girl presented to the emergency with the first episode of severe metabolic ketoacidosis after a febrile illness. On admission, she was drowsy lethargic, and severely dehydrated needs to admit in a highly dependent area. Initial investigations were done during the crisis showed refractory severe metabolic acidosis (pH of 7.18, HCO3- of 7.4 mmol/L), normal ammonia, lactic acidosis, and urine organic acid profile revealed elevations in 3-hydroxybutyrate and acetoacetate. Genetic analysis was done by CentoMito Comprehensive (Large extended screening panel), sequencing of OXCT1 gene revealed that the proband is homozygous for the missense likely pathogenic variant c.1402C>T p.(Arg468Cys) confirming the diagnosis of SCOT deficiency.

Conclusion: This is the first Saudi child with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency case report as searched in the literature. This case highlights the importance of suspecting SCOT deficiency in the differential diagnosis of pediatric metabolic ketoacidosis in preventing life-threatening of severe Metabolic ketoacidosis

Keywords: Ketoacidosis, OXCT1, Succinyl-CoA: 3-oxoacid CoA transferase deficiency, ketone

INTRODUCTION

Succinyl-CoA:3-ketoacid CoA transferase (SCOT) is an enzyme needed for ketone body utilization (1). SCOT deficiency is a rare autosomal recessive disorder with an incidence of less than one per1,000,000 newborns (2). Delayed on diagnosis and lack of awareness, many cases have been missed during their initial presentation (2). It usually manifests with acute, recurrent ketoacidosis episodes triggered by ketogenic stress, infection (3). The neonatal presentation has been reported in the first days of life and the other patients usually manifesting the disease within the first two years (4). Patients are typically asymptomatic the intervals between episodes of metabolic ketoacidosis. The laboratory finding is the nonspecific pattern of an anion gap metabolic acidosis with the elevation of urine organic acids; analysis reveals high concentrations of 3-hydroxybutyrate and acetoacetate but patients with a mild mutation do not (4) and no abnormalities in ammonia, lactate, and amino acid.

To date approximately 37 affected individual and 24 different mutations of the OXCT1 have been reported as causative for SCOT since the first case was reported in 1972 (5).

In this study, we report on the Saudi child case as of SCOT deficiency confirmed by molecular analysis and characterize a missense mutation in OXCT1 gene.

CASE

A 5-year-old girl who is the fourth child of healthy, Saudi, consanguineous parents, there was no family history of similar or metabolic diseases. presented to the emergency room with a two days' history of vomiting, lethargy, tachypnea, and tachycardia no fever has been documented. She has been previously well with uneventful birth and neonatal periods and She had normal growth and development prior to the recent crisis.

Case Report

Received 10-04-2020

Accepted 19-04-2021

Available Online: 20-04-2021

Published 30-04-2021

She was drowsy, unresponsive to painful stimuli and had decreased reflexes. A febrile, no skin rash, no meningeal sign and she had acidotic breathing. Laboratory investigations showed severe high anion gap metabolic acidosis (pH 7.13, pCO2 10.6 mmHg, HCO3 7.2 mmol/L). Plasma glucose was normal, Ammonia 56 (mcg/dl), Lactic acid 3.1(mmol/L) and with 3+ ketonuria. Septic screening workup including blood culture, urinalysis and culture were unremarkable. Radiographic imaging of the chest and abdomen was unremarkable. She has required intubation and was managed with intravenous fluids, repeated doses of intravenous bicarbonate therapy, inotropes, and broad-spectrum antibiotics. Further investigation was requested by the metabolic team tandem mass spectrometry, urine organic acid analysis. GC/MS analysis revealed the presence of acetoacetate and 3-hydroxybutyrate. Normal serum and urine amino acid. To confirm the diagnosis, CentoMito Comprehensive (Large extended screening panel), was sent and revealed a homozygous pathogenic variant was identified in the OXCT1 gene. The test was performed on dried blood spots on a filter paper at Centogene AG, Germany. The child was homozygous for a missense variant c.1402C>T p. (Arg468Cys) causes an amino acid change from Arg to Cys at position 468. According to HGMD Professional 2018.4, this variant has previously been described as a disease-causing for 3-oxoacid CoA transferase deficiency (**6**). The patient was bound to ventilation for 48 hours and ketoacidosis improved, and she was weaned off mechanical ventilation and discharged on day 7days in her normal state of health

Table 1: Biochemical parameters of the patient

Investigations	Result of patient
Plasma glucose	Normal
VBG	Severe high anion gap metabolic acidosis (pH 7.13, pCO2 10.6 mmHg, HCO3 7.2 mmol/L).
Serum and urine amino acid	Unremarkable
Urine organic acid analysis. GC/MS	Acetoacetate and 3-hydroxybutyrate
Metabolic tandem mass spectrometry	Unremarkable
Urine dipstick	3+ ketonuria
Blood and urine cultures	Normal
Lactic acid	3.1(mmol/L)
Radiographic imaging of the chest and abdomen	Unremarkable

MATERIAL and METHOD

Peripheral blood samples (5 ml) were collected in EDTA tubes from the patient Written informed consent was obtained before the samples' collection. CentoMito Comprehensive (Large extended screening panel), was performed by Centogene using Next Generation Sequencing

Technologies. The entire coding region of nuclear genes including 10 bp of intronic flanking sequences were amplified and sequenced. Raw sequence data analysis, including base calling, demultiplexing, alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37) and variant calling were performed using validated in-house software.

Due to the limitations of the method, the target sequences of the requested panel might not be covered 100%.

For mitochondrial genome analysis, the genes were amplified and sequenced, and raw sequence data analysis, including base calling, demultiplexing, alignment to the Revised Cambridge Reference Sequence (rCRS) of the Human Mitochondrial DNA (NC_012920), and variant calling were also performed using validated in-house software.

Following the base calling and primary filtering of low-quality reads, a standard Bioinformatics pipeline was implemented to annotate detected variants and to filter out

probable artifacts. The pipeline confidently detects heteroplasmy levels down to 15%. All identified variants are evaluated with respect to their pathogenicity and causality, and these are categorized into classes 1 - 5.

All variants related to the phenotype of the patient, except benign or likely benign variants are reported. Variants of relevance identified by NGS are continuously and individually in-house validated for quality aspects: those variants which meet our internal QC criteria (based on extensive validation processes) are not validated by SangerCentoMito Comprehensive (Large extended screening panel), the list of analyzed genes (nuclear and mitochondrial) Analysis does not include copy number variations (CNV) or large deletion/duplications.

RESULT and DISCUSSION

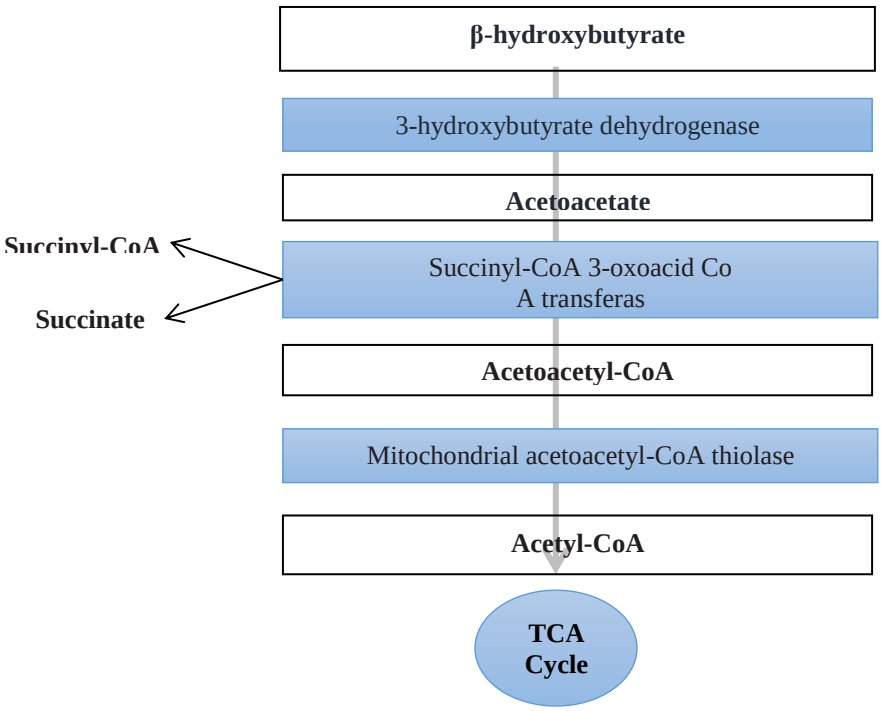
SCOT protein is a homodimer of the 56.2-Kd subunit (7). SCOT protein is found in the tissue of the brain, heart, and kidney except for the liver (8). Human SCOT cDNA is about 3.2 kb and encodes for 520 amino acids (7). The OXCT1 gene is located on chromosome 5p12-p13 and spans more than 100 kb, includes 17 exons (9).This gene is a member of the CoA transferase family I, which has a role in catalyzing the transfer of CoA between carboxylic acid groups (10)

SCOT catalyzes the first, step in ketosis by transferring the CoA from succinyl-CoA to acetoacetate, to produce acetoacetyl-CoA. Then acetoacetyl-CoA converted by mitochondrial acetoacetyl-CoA thiolase into acetyl-CoA,

Acetyl-CoA is then entering the tricarboxylic acid TCA cycle to generate the energy so (succinyl-CoA:3-ketoacid-CoA transferase, is the key reaction that enables ketone body utilization as energy during starvation (**Fig. 1**)

The typical presentation is in early childhood, with vomiting, hyperpnea, drowsiness, lethargy and in severe cases coma evoked by ketogenic stress such as fasting, infection and physical exertion. Though rare, neonates can present with vomiting, poor suckling, and lethargy. Episode intensity and frequency is variable and severe attacks are fatal. All clinical presentation of SCOT due to Permanent ketosis or persistent ketonuria which is pathognomonic feature of SCOT deficiency (**11**). SCOT deficiency may mimic diabetic ketoacidosis if associated with hyperglycemia; or even organic acidemia such as propionic, methylmalonic acidemias, congenital lactic acidosis, salicylate and mitochondrial acetoacetyl-CoAthiolase deficiency (**12**).

Figure 1: Pathway of Succinyl-CoA 3-oxoacid CoA transferase



Individuals with mitochondrial acetoacetyl-CoA thiolase, T2 deficiency, can mimic attacks of succinyl CoA:3-oxoacid CoA transferase SCOT deficiency but owing to specific finding in urinary organic acid profile revealed high levels of tiglylglycine (TIG) 2 methylacetoacetic acid (2MAA) and 3-OH-2-methyl-butyric acid (2M3HB). was effectively excluded. In SCOT deficiency, a blood acylcarnitine profile by tandem mass spectrometry unremarkable only elevated levels of ketone bodies in the blood and urine, patients which is consider is considered a pathognomonic feature of SCOT deficiency (**11**)

Patients are reported to be healthy between episodes, even if the initial episode or crisis was severing which has been documented (**14**). Most patients make a full recovery following episodes of acidosis with a good prognosis and tend to decrease in ketoacidosis episodes after mid-age (**15**). But in some patients with SCOT deficiency may develop Cardiomegaly or congestive heart failure (16) and the cases of neurological deficit and mortality are rare (**18**).

Definitive diagnosis of SCOT deficiency is by molecular genetic studies. And enzyme assays on lymphocytes or cultured fibroblasts. But we proceed to genetic test and no enzyme assay was done for our patient SCOT-deficient patients either have a residual activity or no will have the same the of clinical presentation severity and frequency of metabolic ketoacidosis crises (**13**) but the patient with residual enzyme or mild mutation may have no permanent ketosis (**11**).

The proband was homozygous for the c.1402C>T p.(Arg468Cys) causes an amino acid change from Arg to Cys at position 468. According to HGMD Professional 2018.4, this variant has previously been described as disease-causing for 3- oxoacid CoA transferase deficiency by Fukao et al., 2011(**11**), and Sulaiman et al., 2018 (**17**)).

It is classified as pathogenic (class 1) according to the recommendations of Centogene and ACMG

Homozygous or compound heterozygous pathogenic variants in the OXCT1 gene have been associated with succinyl-CoA:3-oxoacid-CoA transferase deficiency (SCOTD), an autosomal recessive disorder (OMIM® 245050).

There are no data about the presence of common Mutations of OXCT1 gene and there has been no obvious relation between the severity of the disease and the genotype.

The family members screening is crucial to identify asymptomatic individuals and genetic counseling should be provided.

The management of an acute crisis includes hydration with normal saline and dextrose, intravenous sodium bicarbonate bolus to corrected acidosis followed by infusion if needed, and avoid rapid correction and hypernatremia correcting hypoglycemia and peritoneal dialysis in severe acidosis Treated underlying infection with broad-spectrum antibiotic and further septic workup.

CONCLUSION

SCOT deficiency should be suspected in patients presenting with severe metabolic ketoacidosis with or high anion gap metabolic acidosis preceded by an acute infection or fasting. And have non-specific laboratory findings. The patients have favorable outcomes with time

Acknowledgments: We are grateful to the patient, their family and CENTOGENE GmbH (Rostock, Germany). For their dedicated assistance and support

Author contributions: MA; Patient examination, Genetic analysis, Literature search and study design, data collection and analyzes **MA;** Writing article and revisions

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical issues: All authors declare originality of research.

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International Journal of
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