СПИСАНИЕ НА МАКЕДОНСКОТО ЛЕКАРСКО ДРУШТВО
Мак. мед. преглед, 2017;71(1)

JOURNAL OF THE MACEDONIAN MEDICAL ASSOCIATION
Mac. Med. Review, 2017;71(1)

UDK: 61+061.231=866=20 CODEN: MKMPA3 ISSN: 0025-1097

МАКЕДОНСКИ МЕДИЦИНСКИ ПРЕГЛЕД

MACEDONIAN MEDICAL REVIEW

Основано 1946
Founded 1946

www.mld.mk

1/17

Мак Мед Преглед

MMP

Списание на Македонското лекарско друштво

Journal of the Macedonian Medical Association

Наслов на Редакцијата и издавачот / Address of the Editorial Office and Administration:
1000 Скопје, Даме Груев 3, Градски ѕид блок 2
тел. 02/3162 577
www.mld.org.mk / mld@unet.com.mk
Жиро сметка / Bank Account
300000000211884 - Комерцијална банка Скопје

Печати: Бранко Гапо графичко производство - Скопје

Македонски медицински преглед се печати три пати годишно. Претплатата за списанието изнесува 10 евра за лекари, 50 евра за установа, странство 80 евра.

Основано 1946 Founded 1946

Содржина/Contents

DE GRUYTER
OPEN

Mac Med Review 2017; 71(1): 1-4

MMR

DOI:10.1515/mmr-2017-0001
Review article

LASER IN THE TREATMENT OF STRESS URINARY INCONTINENCE AND SYNDROME OF RELAXED VAGINAL WALLS

LASER VO LEKUVAWE NA STRES INKONTINENCIJA NA URINA I NA SINDROM NA OPU[TENI VAGINALNI ZIDOVI

Simonida Kotlarova Poposka and Afrodita Stomnaroska Jovanoski

PZU ReMedika, Skopje, Republic of Macedonia

Abstract

The aim of this paper is to present the new laser technology using erbium laser JAG various minimally invasive, non-surgical procedures in gynecology. The laserhas its effect through photothermal action, leading to reconstructtion and stimulation of new collagen synthesis in the vaginal walls and fascia of the pelvic floor muscles. This results into hardening and tightening of the vaginal walls, which contributes to better leverage of the bladder and urine retention. In gynecology this laser treatment is used for treatment of vaginal walls failure, stress urinary incontinence, pelvic organ prolapse and vaginal atrophy. From 2010 to 2014 several clinical studies were conducted, all of them about the use of laser in the treatment of these 4 conditions and the aim was to confirm the efficacy and safety of this technology. The results showed that SMOOTH mode erbium laser is an effective and safe method for treatment of vaginal walls weakness, stress urinary incontinence, pelvic organ prolapse and vaginal atrophy.

Keywords: laser, stress incontinence of urine, relaxed vaginal walls

---

Апстракт

Целта на овој труд е презентирање на новата ласер-технологија, со користење ербиум ЈАГ ласер за различни минимално инвазивни, нехируршки процедури во гинекологијата.
Својот ефект, ласерот го има преку фототермалното дејство, кое води до реконструирање на постојниот и стимулација на синтезата на новиот колаген во вагиналните ѕидови и фасции на мускулите на карличното дно. Тоа води до зацврстување и затегнување на вагиналните ѕидови што придонесува за подобра потпора на мочниот меур и задржување на урината.

Во гинекологијата се применува за третман на слабост на вагинални ѕидови, стрес уринарна - инконтиненција, пролапс на карличните органи и за третман на вагинална атрофија.
Во периодот од 2010 до 2014 година се спроведени неколку клинички студии за употребата на ласерот во третман на овие четири состојби и целта беше потврдување на ефикасноста и на сигурноста на оваа технологија.
Резултатите покажаа дека SMOOTH mode ербиум ласерот претставува ефективна и сигурна метода за третман на слабост на вагинални ѕидови, стрес уринарна инконтиненција, пролапс на карличните органи и вагинална атрофија.

Клучни зборови: ласер, стрес инконтиненција на урината, опуштени вагинални ѕидови.

Introduction

Back in 2000 Dr. Claudia Pidal and her collaborators published a paper on the use of erbium YAG laser technology in the treatment of vaginal tissue and presented an impressive result [1]. Over the course of time we have followed the rapid development of this technology including treatment of HPV infection, cervical ectropion, vulvar intraepithelial neoplasia, dystrophic changes, melanosis and other similar conditions. For all these interventions a great success rate is reported, with only minor complications. An interesting and unexpected side effect of treatment reported by many patients was that after the intervention they felt tightness of the vaginal walls and enhanced sexual experience. This discovery initiated further research aimed at the development of minimally invasive, non-surgical treatment of vaginal mucosa syndrome of relaxed vaginal walls.
Early studies of thermal treatment with Er: YAG laser on human tissue using precisely controlled sequential pulsing bursts of photon laser (smooth mode) were performed by Majaron and coworkers in 2002 and by Drnovsek and his colleagues in 2004. Studies showed that during treatment with smooth mode deep remodeling of collagen and synthesis of new collagen was

Correspondence to: Simonida Kotlarova Poposka, 16-ta Makedonska brigada 18, Skopje 1000, R. Macedonia; Mob. Phone: 072 44 38 66; E-mail: skotlarova@remedika.com.mk

going on. When collagen gets exposed to the appropriate temperature, reacts with a sharp contraction of its fibers, leading to shortening and contracting of the irradiated tissue [2]. The thermal effect of collagen is not only instant during exposure to the increased temperature, but continues in the process of remodeling and collagen neocolagenosis, resulting in the generation of new collagen and generally improving the strength and elasticity of the treated tissue. Based on the previously described findings, smooth mode ER: YAG laser began to be used on mucous membranes. The first such experience was the oral mucosa and soft palate and its subsequent contracting in the treatment of snoring and sleep apnea. In 2008 and 2009 smooth erbium laser was used onto the vaginal mucosa.

These studies revealed another significant effect of smooth mode Er: JAG treatment, and that is improving the accidental leakage of urine (stress incontinentio urinae-SUI) in women. Since then, special applicatorhas beendesigned for the treatment of this condition; an applicator which provides a uniform and well-controlled irradiation along the entire length and volume of the vaginal canal. There have been two protocols of two minimally invasive, non-surgical and non-ablative procedures and the same clinical validation: IntimaLase-syndrome relaxed vaginal walls and IncontiLase -for SUI [3-5].

In the last 4 years, the use of technology for smooth vaginal tightening and incontinence quickly spread worldwide and many studies have started further processing of results and proof of the effect of this technique. General use of Er: YAG smooth technique led to new discoveries-in 2013 the work of Bizjak-Ogrinc and Sencar was published about reduction of prolapse of pelvic organs, and the same year Gaspar presented his paper about the treatment of vaginal atrophy [6,7].

Nowadays, in addition to the foregoing, Er: YAG SMOOTH modality is used for ablative procedures on the cervix and vagina, to remove genital warts, remodeling, whitening and tightening of the vulvar region, as well as labioplasty, lichen sclerosus and atrophicans, and many other procedures.

Materials and methods

IntimaLase and IncontiLase protocols are based on heating the vagina to about 65°C and include two treatments with an interval of 4-6 weeks. The time needed to perform IntimaLase protocol is about 8 minutes, and for IncontiLase is 15 min.

Protocol for the treatment of pelvic organ prolapse (ProlapLase) is based on the same principle of hyperthermiaof the collagen as the protocols for incontinence and vaginal tightening, and the difference in the intensity of treatment. Here it uses increased intensity. Another difference is the location-here larger zone is treated-prolapsed part of the vaginal wall. ProlapLase protocol requires three to five treatments for a period of 4-6 weeks. The number of treatments depends on the intensity or degree of prolapse.

Protocol for vaginal atrophy (RenovaLase) is based on a slightly different concept of mild hyperthermia, while the mucosa is heated to about 45°C thereby causing stimulation of cell proliferation by heat shock activating the protein, the production of collagen increases and causes anti-inflammatory response. This protocol consists of three treatments at an interval of 3 weeks.

Results of clinical evaluation

Measurements of average shrinkage of the vaginal canal after one treatment with IntimaLase, as measured by Rivera in 27 women showed 17% narrowing, while Bezmenko measured an average 56% thickening of the vaginal wall 6 months after two IncontiLase treatments in 77 patients [8]. By using MRI measurements to reduce the vaginal canal, Bezmenko and collaborators also showed a significant reduction in the vaginal canal treated with erbium smooth technology.

Several subjective and objective evaluation methods were used to measure the effectiveness of treatment with IntimaLase to enhance sexual pleasure by tightening the vaginal canal, like prolapse questionnaire of pelvic organs/questionnaire SUI (PISQ-12) and the Index of Female sexual function (FSFI), Likert scale with 4 points and perineal measurements. For each evaluation mode, a significant improvement was reported.

Fistonic and colleagues showed an average improvement of 12 PISQ-value of 5.5 points (increased average 33.8 to 39.3) for a period of 6 months after a single treatment [9]. Guimaraes interviewed male partners on a scale of 4 degrees, and 4 months after a single treatment 69% of partners said it had an excellent improvement, 27% good and 4% moderate. Using the FSFI questionnaires, Garcia found that 96.6% of patients reported improvement after two sessions of IntimaLase [10]. The first results from Rivera found that better results were achieved after the second session of erbium smooth treatment and his findings were confirmed by the results of Garcia's study [10].

Different assessment tools are used to measure the efficiency of erbium treatment for SUI. Several researchers used the questionnaire from the Association of International Consultation on Incontinence-Urinary Incontinence (ICIQ-UI) and all found a significant reduction of ICIQ-UI score. Fistonic and colleagues reported a decrease of more than 6 points at 6 months after a single treatment [11]. Lukanovic reported improvement of 3.7 points in just 3 months in one session [12] while Gambacciani and Levancini reported more than 6 points of improvement after 3 months and three sessions of erbium therapy [13]. Sencar and Bizjak-

Ogrinc used ICIQ-IU to define the index of severity of incontinence according to Klovning: after treatment, the index decreased by 2.6±1.0 points in patients diagnosed with mild urinary incontinence before treatment, 3.6±1.4 points in those with moderate incontinence, 5.7±1.8 points in those with severe stress incontinence and 8.4±2.6 points for those with very severe SIU. They stated that even 77% of patients with SUI were cured (dry) after 12 months [14].

Fistonic also measured the residual urine volume and type Q-angle. His results showed a significant reduction in residual urine, which on average decreased by 9±12.1, 1.6 ±1.9 ml, 6 months after treatment. Q-type angle at a pressure drop from 61.3±24.to 47.2±23.7 degrees [11]. Bezmenko with colleagues hatched urodinamic measurements of urethral pressure when opening and closing before and 6 months after treatment with erbium JAG and found a large increase in the two pressures: the pressure of opening changed from 3.7±13.1 to 25.1±4.8 mmHg and closing pressure of up to 12.2±3.5 25.2±4.9 mmHg [8].

They also analyzed some biochemical parameters of the metabolism of connective tissue and significantly reduced oxilizin collagen amino acids were found (from 8.4± 0.4 to 5.3±0.5 μmol/g) as well asoxyproline (from 5.4±0.3 to 3.7±0.6 μmol/g) [15]. A reduction of both amino acids means a reduced level of collagen degradation and thus more vital and stable collagen.

From the histological point of view, changes that occur during erbium therapy are increasing the amount and activity of fibroblasts, increasing the density of the connective tissue and appearance of neoangiogenesis. Gaspar used 3-day diaries for evaluating the effectiveness of the procedure for IncontiLase and observed decrease in the frequency and amount of leakage in the follow-up windows at 2, 6, 8 and 12 months. Frequency leakage was reduced by 89.8%, 77.5%, 73.5% and 59.2% at 2, 6, 8 and 12 months, respectively [16].

The efficiency of non-ablative Er: YAG laser in the treatment of SUI and improvement of sexual function in premenopausal, multiparous patients was also evaluated as compared to placebo treatment in a randomized, controlled study by Lukanovic, which indicated that the laser led to significantly improved SUI and symptoms of sexual dysfunction compared to placebo treatment [12].

For evaluating the effectiveness of erbium smooth treatment of vaginal atrophy, Gaspar used the following tools: Visual Analog Scale (VAS) (0-no symptoms, 1-mild, 2-moderate and severe-3) symptoms to assess the severity of atrophy (pain, dryness, irritation and leukorrhea) value of maturity and pH. He also made a histological analysis of the vaginal mucosa 3 months after treatment and found significant improvement in all parameters observed: pain decreased from 2.4±0.5 to 1.8±1.00, dryness from 2.24±0.60 to 1.04±0.89, irritation from 2.04±0.79 to 0.96± 0.93 and leukorrhea from 2.28±0.83 to 0.83±0.79 at 6-month follow-up.

The maturity improved (20.8 to 47.9 points) in six months, while the pH decreased (5.0±0.4 to 4.1±0.4) in 3 months, indicating restoring of the vaginal acidity. Histology showed significant changes in tropisms of the vaginal mucosa. Gambacciani and colleagues [17] also analyzed atrophy symptoms-pain and dryness and determined the so-called Score index of vaginal health (VHIS). After Renova Lasetreatment, they reported a reduction in VAS score (on a scale of 0 to 10) and dyspareunia and dryness of more than 5 points and increase in VHIS by 9 points.

In the studies of Gaspar and Gambacciani, laser treatment of vaginal atrophy was compared with hormone replacement therapy (HRT). Gaspar showed that laser treatment compared with HRT provided significantly greater and longer-lasting improvement of the value of maturity, pH value and the signs and symptoms of vaginal atrophy [7]. Similarly, the results of Gambacciani referring to dryness, dyspareunia and VHIS showed that the Er: YAG laser treatment was significantly better than HRT [17].

Evaluation of the effectiveness of laser treatment for pelvic organ prolapse using the ProlapLaseprotocol was made by assessing the degree of prolapse, measured on a scale of Baden-Walker. Bizjak-Ogrinc and Sencarpr esented 61 patients with prolapse of second or higher degree [18]. Before treatment there were 40 patients with cystocelelevel II, 15 with III and 6 with IV degree. The final check showed a large numberof patients (58 or 95%) with reduced prolapse of at least one degree, 27 of them (44%) of two grades and 8(13%), even of three grades. After 12-month follow-up, 85% of patients had either stage 0 or I of prolapse and the rest 15% of patients had prolapse level II.

Treatment discomfort from these four protocols was very low (maximum score was3 on a scale of 10 points) and a large number of patients judged their improvement as very positive. There were no side effects reported from these treatments.

Discussion

There are many approaches to the treatment of vaginal relaxation, urinary incontinence, pelvic organ prolapse and vaginal atrophy, ranging from conservative methods to the most complex surgical procedures. However, none of the existing procedures is ideal and the majority offer more challenges. This situation led to searching for new therapeutic methods for all four mentioned indications.

Erbium smooth technology for minimally invasive treatments in gynecology is based on the concept of controlled heating of the vaginal mucosal tissue. Precisely controlled sequence (s) of sub-ablative Er: YAG laser pulses are delivered to the mucosa in order

to achieve controlled heating of collagen in the deeper layers of the mucosal surface without overheating the lining. Published data show that reduction of collagen fibers without ireversible denaturation of their structure requires temperatures which do not exceed the optimum temperature of 60-70°C [19].

Er: YAG laser radiation acts almost like a heater surface due to the extremely shallow penetration of its optical infrared radiation, and relies on the diffusion of heat that affects the deeper layers of the mucosa. Collagen exposed to appropriate temperatures gives the effect of increasing the contraction of its fibers, leading to contraction and tightening of the mucosal tissue exposed to irradiation. Thermal effect of collagen lasts not only during the exposure to increased temperature, but it continues the process of collagen remodeling and neocollagenosis, resulting in the production of new collagen and improves the overall strength and flexibility of the treated tissue. After the current contracting of the tissue, the process of neocollagenosis can take up to 6 months until completed.

Conclusion

This study indicates that the non-ablative erbium: YAG laser is an effective and safe new method for treatment of vaginal relaxation, stress urinary incontinence, prolapsed pelvic organs and vaginal atrophy and can be used in treatment of these conditions.

Conflict of interest statement. None declared.

References

1. Mansilla EC, Pidal CM, Rios VE, San Martino JA. Erbium YAG Laser. Nuevo Aporte Terapeuticoen Tracto Genital Inferior. Presentedat the *11th International Congress of Cervical Pathologyand Colposcopy*, Barcelona, Spain, 2002; 9-13.
2. Goldberg DJ. *Ablative and Non-ablative Facial Skin Rejuvenation* London, New York: MartinDunitz, 2003; 1-8.
3. Vizintin Z, Rivera M, Fistoni I, *et al.* Novel minimally invasive VSPEr: YAG laser treatments in gynecology. *J LA & HA* 2012; 1: 46-58.
4. Fistonic I, Findri-Gustek S, Fistonic N. Minimally invasive laserprocedure for early stages of stress urinary incontinence (SUI). *J LA & HA* 2012; 1: 67-74.
5. Gaviria JE, Lanz LA. Laser vaginal tightening (LVT)-evaluationof a novel noninvasive laser treatment for vaginal relaxation syndrome. *J LA & HA* 2012; 1: 37-45.
6. Bizjak Ogrinc U, Sencar S. Non-surgical minimally invasive Er: YAG laser treatment for higher-grade cystocele. Presented at *NDP 373, 38th Annual Meeting of International Urogynecological Association*, Dublin, 2013.
7. Gaspar A. Comparison of new minimally invasive Er: YAG laser treatment and hormonal replacement therapy in the treatmentof vaginal atrophy. *Climacteric* 2014; 17(Suppl 1): 48-108, P 124 (Abstr).
8. Bezmenko AA, Schmidt AA, Koval AA, Karpishchenko ZM. Clinical and morphological study of efficacy of Inconti Lase and Intima Lase in treatment of female stress urinary incontinence. Study report, Military Medical Academy Kirov, St. Petersburg, Russia, February 2014.
9. Fistonic N, Fistonic I, Findri-Gustek S, *et al*. Laser treatment for stress urinary incontinence (SUI)in women. Presented at the *44th International Continence SocietyCongress*, Rio de Janeiro, Brazil, 20-24 October 2014.
10. Vizintin Z, Rivera M, Fistoni I, *et al*. Novel minimally invasive VSP Er: YAG laser treatments in gynecology. *J LA & HA* 2012; 1: 46-58.
11. Fistonic N, Fistonic I, Findri-Gustek S, *et al*. Laser treatment for stress urinary incontinence (SUI)in women. Presented at the *44th International Continence Society Congress*, Rio de Janeiro, Brazil, 2014; 20-24.
12. Lukanovic A, *et al*. Non-ablative Er: YAG laser therapy effect on SUI related quality of life and sexual function: a randomized controlled trial. Presented at the *44th International Continence Society Congress*, Rio de Janeiro, Brazil, 2014; 20-24.
13. Gambacciani M, Levancini M. Short-term effect of vaginalerbium laser on the genitourinary syndrome of menopause. *Minerva Ginecol* 2015; 67: 1-2.
14. Bizjak-Ogrinc U, Sencar S, Vizintin Z. Minimally invasive non-surgical ErYAG laser treatment for female stress and mixedurinary incontinence-one year follow-up. Proceedings of *20th World Congress on Controversies in Obstetrics, Gynecology &Infertility*, Paris, France, 4-7 December 2014.
15. Bezmenko AA, Elkin GI, Schmidt AA, *et al*. Treatment of stressurinary incontinence in women with Er: YAG laser: some biochemicalparameters of connective tissue metabolism. [Russian].
16. Gaspar A, Vizintin Z. Assessment of efficacy of Er: YAG laser treatment for female SUI using 3 days voiding diary-pilot studywith 12 months follow-up. Presented at *the 2nd Annual Meetingof the Mediterranean Incontinence and Pelvic Floor Society*, Nimes, France, 26-29 November 2014. http://www.jowd.ru/arhiv/JOWD_3_2014/JOWD_3_2014.html
17. Gambacciani M, Levancini M, Cervigni M. Vaginal erbiumlaser as second-generation thermotherapy for the genitourinary syndrome of menopause. *Climacteric* 2015; 18: 757-763.
18. Sencar S, Bizjak-Ogrinc U, Vizintin Z. Evaluation of Er: YAG lasertreatment for higher grade pelvic organ prolapses-1 year followup.Presented at the *20th World Congress on Controversies in Obstetrics, Gynecology & Infertility*, Paris, France, 4-7 December 2014.
19. Hayashi K, Thabit G, Massa KL, *et al*. The effect of thermalheating on the length and histologic properties of the glenohumeraljoint capsule. *Am J Sports Med* 1997; 25: 108-112.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 5-9

MMR

DOI:10.1515/mmr-2017-0002
Original article

RHEUMATOID FACTOR AS A POTENTIATOR OF ANTI-CITRULLINATED PROTEIN ANTIBODY-MEDIATED INFLAMMATION IN EARLY UNDIFFERENTIATED SERONEGATIVE OSTEOARTHROPATHY

РЕВМАТОИДЕН ФАКТОР КАКО ПОТЕНЦИАТОР НА АНТИТЕЛА КОН ЦИТРУЛИНИРАНИОТ ПЕПТИД КАЈ РАН, НЕДИФЕРЕНЦИРАН СЕРОНЕГАТИВНА ОСТЕОАРТРОПАТИЈА

Dejan Spasovski[1], Sonja Genadieva-Stavric[2], Tatjana Sotirova[2] and Julijana Brezovska-Kavrakova[3]

[1]University Clinic of Rheumatology, [2]University Clinic of Hematology, [3]Department of Clinic Biochemistry, Faculty of Medicine, University "Ss Cyril and Methodius", Skopje, Republic of Macedonia

Abstract

Introduction. To compare the values and accuracy of the test in anticyclic citrullinated peptides antibodies, rheumatoid factor (RF), C-reactive protein (CRP) and disease activity index in early diagnosis of untreated psoriatic arthritis (PsA).
Methods. Using the ELISA method of DIA-STAT™ Anti CCP (Axis-Shield Diagnostics), sera of 70 participants were examined (35 untreated patients with PsA and 35 subjects from the healthy control group). RF and CRP were determined with the agglutination test (latex test). At the same time the sensitivity, specificity, predictive value for positive and negative testsand accuracy were determined.
Results. Of 35 patientswith PsA, 1 patient showed presence of anti-CCP antibodies (sensitivity test 2.86%), while RF was foundin 0 patients(sensitivity test 0%). In the healthy control group positivevalues for RF, CRP and erythrocyte sedimentation rate were detected in 1 patient.
Conclusion. ACPA antibodies have low sensitivity, but high specificity in PsA.

Keywords: anti-cyclic citrullinated peptide, psoriatic arthritis, rheumatoid factor

Апстракт

Вовед. Да се евалуираат и споредат вредностите и точноста на тестот кај антицикличните цитрулинирани пептидни (Anti-CCP/ACPA) антитела, ревматоидниот фактор (RF) и Ц-Реактивниот протеин (CRP), индекс за активност на болеста (PASI), во раната дијагноза, кај нетретиран псоријатичен артритис (PsA).
Методи. Користејќи ја ELISA техниката на DIA-STAT™ Anti CCP (Axis-Shield Diagnostics) се испитани серуми на 70 испитаници (35 PsA нетретирани, 35 контолна здрава група). RFi CRP е одреден со тест за аглутинација (Латекс тест) кај истите партиципанти, при што е одредена сензитивноста, специфичноста, предикторната вредност за позитивен и негативен тест, како и точноста.
Резултати. Од испитаните 35 пациенти со PsA, еден пациенти покажа присуство на Анти CCP антитела (сензитивност на тестот 2,86%), додека RF беше застапен кај 0 пациенти (сензитивност на тестот 0%). Кај контролната здрава група, по еден испитаник е детектиран со позитивни вредности за RF, CRP, седиментација.
Заклучок. ACPA антителата имаат мала сензитивност, но висока специфичност кај PsA.

Клучни зборови: (Анти-CCP), цикличен цитрулиниран протеин, (PsA), псоријатичен артритис. (RF), ревматоиден фактор

Introduction

Anti-cyclic peptide antibodies (CCP/ACPA) are antbodies directed towards synthetic citrullinated peptides and are specific markers in diagnosis of rheumatoid arthritis (RA). They belong to the group of protein/peptide antibodies. There are several generations of these antibodies in their evolution. Antibodies like APF (anti-perinuclear factor) and AKA (anti-keratin antibodies) detected by indirect fluorescence using buccal epithelium or rat's esophagus [1], have great specificity for RA.
Absence of donors for buccal cells limits the use of APF as a routine laboratory test. Antigen for these anti-filaggrin antibodies (AFA) is identified as an epidermal filaggrin which is intermediary filament involved in the epidermal cornification [2,3]. Profilaggrin, present in keratohyalin granules of the buccal cells is proteolytically released in filaggrin subunits during cell differentiation. In this stage, the protein is dephospho-

Correspondence to: Dejan Spasovski, University Clinic of Rheumatology, "Vodnjanska" 17, 1000 Skopje, R. Macedonia; Phone: +389 2 31 47 668; E-mail: sdejan36@yahoo.com

rylated and some arginine residues are converted in citrullines from enzyme peptidyl-arginine deaminase (PAD) [4]. Their reactivity depends on epitopes which contain amino acid citrulline. In 1998, Schellekers *et al*. reported about autoantibodies that reacted in linear synthetic peptides which contained unusual amino acid citrulline. So two types of CCP assay were developed with peptides A and B. They are present in 76% in RA, with 96% specificity. Antibodies in patients with RA are predominantly of IgG type and have relatively high affinity [5]. The ELISA test, based on these cyclic citrullinated peptides (CCP), has superior characteristics in detection of RA [6], with different sensitivity and specificity [7]. Sensitivity of the ACPA test in different populations ranges between 64% and 74%, while specificity ranges between 90% and 99%. There are PsA patients in whom these antibodies are detected.

The aim of this study wasto determine the diagnostic value of ACPA antibodies in PsA.

Material and methods

In patients included in the study the disease diagnosis was based upon revised diagnostic criteria for classification of psoriatic arthritis from 2005, proposed by the American Association of Rheumatism (ARA) [8]. Clinical evaluation of disease activity and disease diagnosis was performed by a subspecialist in the field based upon the diagnostic criteria of Moll-Wright for classification of psoriatic arthritis [9]. They were dermatologically tested, including examination of the psoriatic changes of the nails, psoriatic areas, disease activity index (PASI) and evaluation of the peripheral and axial joints [10]. Oligoarthritis is taken in consideration when <5 joints are involved and polyarthritis when >5 joints are involved. Symmetric arthritis is considered when there is bilateral involvement and when >50% of joints are seized.

The study included 35 patients (pts) (18 women, 17 men) with PsA and 35 pts (19 women, 16 men) from the healthy control group. Mean age was 47.18 years (±9.08) (35-65 years) in the group with PsA, while 40.2 years (±9.21) (29-65 years) in the healthy control group. Mean disease duration was 6.27 months (±8.22) (1-36 months). None of them received disease modification drugs. The others negated drug use before entering the study, especially drugs from the baseline such as methotrexate, leflunomide or sulphasalasine. Specimen were collected in the period of 2 years.

Inclusion criteria

Patients with psoriatic arthritis,aged 18-65, newly diagnosed and previously untreated were included in the study.

Exclusion criteria

Patients with diseases or conditions that could influence on the results directly or indirectly were excluded from the study, as follows:

1. Patients younger than 18 years.
2. Patients with previous history of disease of the spleen, thyroid gland, liver, kidneys, hematological, cardiovascular, neurological, autoimmune and lung diseases.
3. Patients with diabetes mellitus, febrile conditions, acute infections,neoplasms.
4. Patients with uric arthritis, SLE, mixed connective tissue disease, vasculitis.
5. Patients with history of blood transfusion and patients with body overweight.
6. Patients with history of use of drugs from the baseline.
7. Patients that in 0 point had increased level of glucose, serum and urine urea and creatinine, blood hypertension, smokers and blood and enzyme disorders.
8. Patients previously treated with salycilates, antibiotics, golden salts or diuretics.

All patients took part in this study voluntarily, so the ethic criteria for this study werefulfilled.

Laboratory evaluation

For clinical evaluation of the disease, the following parameters were necessary: complete blood count (CBC) and differential, reactants of the acute phase, ACPA antibodies, C-reactive protein (CRP), rheumatoid factor (RF) and erythrocyte sedimentation rate (ESR), alkaline phosphatese (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine kinase (CK), lactate dehydrogenase (LDH), serum urea and serum creatinine.

Serum creatinine was determined with the “Jaffe" method.

Reference values: serum creatinine 45-109 mmol/L; urine creatinine 7-17mmol/dU.

CRP was determined with the agglutination test (latex CRP test) (BioSystems S.A. Reagens&Instruments Costa Brava 30, Barcelona, Spain). Reference values: <6 mg/L CRP in serum.

RF was determined with the agglutination test (latex CRP test) (BioSystems S.A. Reagens&Instruments Costa Brava 30, Barcelona, Spain).

Reference values: < 8 IU/ml RF in serum.

Westergrenwas the quantitative methodused for for determination of ESR.

Reference values: 7-8mm for women, 11-16mm for men.

DIA-STAT^{TM}anti CCP (Axis-Shield Diagnostics) test is semiquantitative/qualitative ELISA test, based on the detection of IgG autoantibodies in human plasma or serum, directed towards synthetic cyclic citrullinated

peptides (CCP), which contains modified arginine residues. This test was an additional tool.

Principles of work

The walls of the microtiter have highly purified synthetic cyclic peptide which contains modified arginine residues. During the first incubation, the specific autoantibodies from diluted serum or plasma are connected to the surface antigen. It is washed then in order to eliminate the unconnected components. During the second incubation, the conjugate, which is an enzyme of the monoclonal autoantibody for human IgG, is connected to the surface autoantibody. After the second wash, the specific autoantibodies are incubated with the substrate. After adding the stop solution, the reaction is interrupted and this results in a colored end-result. The amount of the absorbed conjugate is expressed in absorption units. In the quantitative protocol, the amount of the conjugate connected tothe sample is compared with the same connected tothe reference control. In the semi-quantitative protocol the anti-CCP autoantibody concentration could be estimated with interpolation of the curve based on the standard. Fresh serum or plasma is used.
Calculation and interpretation of the results for the quailtative protocol are estimated from the absorbed value (optic density) from the positive and negative control as well as for every sample.

Statistical analysis

For testing the significance of differences between two arithmetical means, i.e. proportions the Student'st-test wasused to compare the mean parameters of certain numerical parameters between groups, as well as Willcoxon-matched test for independent samples. Sensitivity and predictivity for positive and negative tests of the examined markers weredetermined with the test for sensitivity and specificity. P-value between 0.05 and 0.1 wasconsidered statistically significant. Analysis of the data wasperformed with the statistical package Statistica 7.0.

Results

Ofthe 35 pts with PsA, 1 patient (2.86%) showed presence of ACPA antibodies, while RF was present in 0 pts (0%).

Table 1. ACPA autoantibodies in PsA and healthy control group

	Untreated PsA Group No 35 Value (M ± SD) Positive/negative	**Healthy control group No 35 Value (M ± SD) Positive/negative**
ACPA + ≥ 1.26	1/34	0/35
RF +30 ≥ IU/ml	0/35	1/34
CRP +12 ≥ mg/L	16/19	1/34
ESR + ≥16	18/17	1/34

Table 2. Diagnostic performances of ACPA and other laboratory variables inPsA

	ACPA PsA No 35	RF PsA No 35	CRP PsA No 35	ESR PsA No 35
Sensitivity (%)	2.86	0	45.71	51.43
Specificity (%)	100	97.14	97.14	97.14
Predictive value for positive test (%)	100	0	94.12	94.74
Predictive value for negative test (%)	50.72	49.28	64.15	66.67
Accuracy (%)	51.42	48,57	71.42	74.28

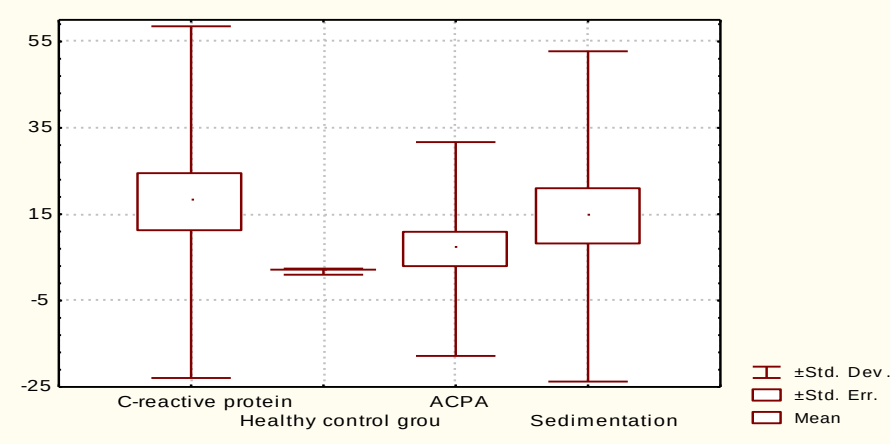


Fig. 1. Distribution of ACPA, ESR and CRP in PsAand healthy control group

In the healthy control group 0 pts (0%) showed ACPA positivity, while 1 patient (2.86%) had positive RF (Table 1).

Diagnostic value of ACPA autoantibodies in psoriatic arthritis

ACPA autoantibodies and reactants of the acute phase in PsA, sensitivity, specificity, predictive value for positive and negative tests and their accuracyare shown in Table 2. ACPA autoantibodies had equal diagnostic performances as RF (sensitivity 2.86% vs 0%, specificity 100% vs 97.14% in detection of untreated PsA (Figure 1).

1. Using the Wilcoxon-matched test we found a statistical correlation between ACPA in PsA and healthy control group for $p<0.05$ ($p=0.01$). In the PsA group there was a statistical correlation between ACPA and RF for $p<0.05$ ($p=0.00$); ACPA andCRP ($p=0.00$).
2. Using the Wilcoxon-matched test we found no statistical correlation in the PsA group between ACPA and age, disease duration in months, PASI index, RF and CRP for $p< 0.05$ (ACPA vs age $p=0.04$; ACPA vs disease duration in months $p=0.07$; ACPAvs PASI $p=0.08$; ACPA vs RF $p=0.02$; AAP vs CRP $p=0.05$; AAP vsESR $p=0.06$).
3. Using the Wilcoxon-matched test we found a statistical correlation in the PsA group between disease duration in months, PASI index, RF and CRP for $p<0.05$ (CRPvs age $p=0.00$;CRP vs disease duration in months $p=0.00$; CRP vs PASI $p=0.00$;CRP vs RF $p=0.02$;CRP vs ESR $p=0.00$).

Discussion

When introducing a new diagnostic method, it is necessary to estimate its quality i.e. to find out the utility of information compared to the risk for the patient and the price of the test. This notion has become more interesting recently when many methods have been introduced due to the developments in technology. Although the subjective estimation of the doctor who is responsible for the patient can be crucial in the choice of the available diagnostic methods, objective quantitative estimation of every method would help him in the most rational approach.

ACPA autoantibodies are specific markers in diagnosis of RA and have role in the disease pathophysiology. Psoriasis vulgaris is found in 3% of the common population. PsA in psoriasis vulgaris is found in 7%. ACPA autoantibodies in PsA have greater prevalence of 8% in comparison with the common population [11]. Isolation of ACPA autoantibodies could be from synovial fluid also [12]. Unlike RA, the presence of ACPA autoantibodies in PsA could be explained only as an epiphenomenon in this disease [13]. It is noted in the literature that these antibodies have significance in PsA for the evaluation of osteoporosis, erosive changes and bone destructions-fractures). The ACPA autoantibody as an isolated laboratory variable does not dominate with its performances in diagnosis of early, undifferentiated PsA [14-19].

However, it should be mentioned that values obtained in this study are equal and do not deviatefrom the values obtained bythe producer DIA-STAT™ Anti CCP (Axis-Shield Diagnostics) for PsA (sensitivity for anti ACPA 5%, specificity 100%).

Conclusion

ACPA autoantibodies have low sensitivity and high specificity in diagnosis of PsA. Every positive result should be interpreted together with the clinical evaluation of the disease and diagnostic procedures designated for it. Elevated values of these antibodies canappear in persons without clinical signs ofthe disease.

The concentration of these antibodies does not always correlate with the disease severity.

They could be applied in pediatric cases with great caution, because they are not designated for them.

Conflict of interest statement. None declared.

References

1. Nakamura RM. Progress in the use of biochemical and biological markers for evaluation of rheumatoid arthri- tis. *J Clin Lab Anal* 2000; 14: 305-313.
2. Simon M, Girbal E, Sebbag M, *et al*. The cytokeratin filament-aggregating protein filaggrin is the target of the so-called »antikeratin antibodies«, autoantibodies specific for rheumatoid arthritis. *J Clin Invest* 1993; 92: 1387-1393.
3. Sebbag M, Simon M, Vincent C, *et al.* The antiperinuclear factor and the so-called antikeratin antibodies are the same rheumatoid arthritis-specific autoantibodies. *J Clin Invest* 1995; 95: 2672-2679.
4. Girbal-Neuhauser E, Durieux JJ, Arnaud M, *et al.* The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin auto-antibodies are posttranslationally generated on various sites of (pro) filaggrin by deimination of arginine re- sidues. *J Immunol* 1999; 162: 585-594.
5. Schellekens GA, de Jong BA, van den Hoogen FH, *et al*. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest* 1998; 101: 273-281.
6. Schellekens GA, Visser H, de Jong BA, *et al*. The diagnostic pro- perties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 2000; 43: 155-163.
7. Van Boekel MAM, Vossenaar ER, van den Hoogen FHJ, van Venrooij WJ. Autoantibody systems in rheumatoid arthritis: specificity, sensitivity and diagnostic value. *Arthritis Res* 2002; 4: 87-93.
8. Helliwell PS, Taylor WJ. Classification and diagnostic criteria for psoriatic arthritis. *Ann Rheum Dis* 2005; 64 Suppl 2:ii3-ii8.

9. Moll JMH, Wright V. Psoriatic arthritis. *Semin Arthritis Rheum* 1973; 55-78.
10. Schmitt J, Wozel G. The psoriasis area and severity index is the adequate criteria to define severity in chronic plaque-type psoriasis. *Dermatol* 2005; 210: 194-199.
11. Vander Cruyssen B, Hoffman IE, Zmierczak H, *et al*. Anti-citrullinated peptide antibodies may occur in patients with psoriatic arthritis. *Ann Rheum Dis* 2005; 64: 1145-1149.
12. Spadaro A1, Riccieri V, Scrivo R, *et al.* Anti-cyclic citrullinated peptide antibody determination in synovial fluid of psoriatic arthritis. *Clin Exp Rheumatol* 2007; 25: 599-604.
13. Huynh D, Etzel C, Cox V, *et al*. SAT0268 Anti Citrullinated Peptide Antibody (ACPA) in Patients with Psoriatic Arthritis (PSA): Clinical Relevance. *Ann Rheum Dis* 2013; 72: A673 doi:10.1136/annrheumdis-2013-eular.1993.
14. Palazzi C, Olivieri I, Petricca A, Salvarani C. Rheumatoid arthritis or psoriatic symmetric polyarthritis? A difficult differenttial diagnosis. *Clin Exp Rheumatol* 2002; 20: 3-4.
15. Helliwell PS. Relationship of psoriatic arthritis with the other spondyloarthropathies. *Curr Opin Rheumatol* 2004; 16: 344-349.
16. Alenius GM, Berglin E, Dahlqvist SR. Antibodies against cyclic citrullinated peptide (CCP) in psoriatic patients with or without joint inflammation. *Ann Rheum Dis* 2006; 65: 398-400.
17. Korendowych E, Owen P, Ravindran J, *et al*. The clinical and genetic associations of anti-cyclic citrullinated peptide antibodies in psoriatic arthritis. *Rheumatology (Oxford)* 2005; 44: 1056-1060.
18. Inanc N, Dalkilic E, Kamali S, *et al*. Anti-CCP antibodies in rheumatoid arthritis and psoriatic arthritis. *Clin Rheumatol* 2007; 26: 17-23.
19. Abdel Fattah NSA, Hassan HE, Galal ZA. Antibodies to cyclic citrullinated peptides in patients with psoriatic arthritis. *Egypt J Dermatol Venereol* 2008; 28: 13-23.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 10-14

MMR

DOI:10.1515/mmr-2017-0003
Original article

TREATMENT OF SEVERE AUTOIMMUNE DISEASES WITH AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

ТРЕТМАН НА АВТОИМУНИ ЗАБОЛУВАЊА СО АВТОЛОГНА ТРАНСПЛАНТАЦИЈА НА МАТИЧНИ ХЕМАТОПОЕТСКИ КЛЕТКИ

Zlate Stojanoski[1], Anzelika Karadzova-Stojanoska[2], Aleksandra Pivkova-Veljanovska[1], Sonja Genadieva-Stavrik[1], Lazar Cadievski[1], Martin Ivanovski[1], Oliver Karanfilski[1], Lidija Cevreska[1] and Borce Georgievski[1]

[1]University Clinic of Hematology, [2]University Clinic of Rheumatology, University "Ss Cyril and Methodius", Medical Faculty, Skopje, Republic of Macedonia

Abstract

Introduction. Autoimmune diseases are a family of more than 100 heterogeneous conditions that affect 5 to 8% of the world's population. The etiology is still unknown but the disregulation of the regulatory T-lymphocytes play a central role inthe autoimmunity and the success of the long-term remission. Although conventional immunosuppression and new biological agents can provide disease control in severely affected patients, such treatments are rarely curative and alternative strategies are needed. Indeed, severe forms of systemic autoimmune diseases, such as multiple sclerosis (MS), systemic sclerosis (SSc), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), juvenile idiopathic arthritis (JIA), hematologic immune cytopenia (HIC) and Crohn's disease are difficult to be treated. High-dose immunosuppressive therapy followed by autologous stem cells transplantation is reliable option for a successive treatment of this group of patients.
Aim. To determine the safety of the procedure of autologous stem cell transplantation in patients with autoimmune diseases and concomitant malignant hematological disorders.
Methods. During a period of 15 years (from September 2000 to September 2015) at the University Clinic of Hematology in Skopje we have treated 6 patients with autoimmune disease and concomitant hematological neoplasm. None of the patients was treated for primary autoimmune diseases. Two men and 4 women, with median age of 47 years were treated. Sjogren syndrome and multiple myeloma were found in 2 patients, polyartheritis nodosa and multiple myeloma in 1 patient, rheumatoid arthritis and acute myeloblastic leukemia in 1, systemic lupus erythematosus and non-Hodgkin lymphoma in 1; severe psoriasis and acute myeloblastic leukemia in 1 patient.
Results. All treated patients are alive after transplanted procedure, with transplant related mortality day +100: 0.
Conclusion. Autologous stem cell transplantation is safe and recommended option for treatment ofpatients with autoimmune disease and hematologic neoplasm.

Keywords: autoimmune diseases, stem cell transplantation

Correspondence to: Stojanoski Zlate, University Clinic of Hematology, Clinical Center "Mother Teresa", 1000 Skopje, R. Macedonia; E-mail: stojanoskiza@t.mk

Апстракт

Вовед. Автоимуните заболувања претставуваат група со повеќе од 100 хетерогени состојби, афектираат од пет до осум проценти од светската популација, а се карактеризираат со аберантна активација на имунолошкиот систем и пореметено одржување на адаптираната толеранција [1]. Ваквиот третман не може да доведе до излекување, иако конвенционалната имуносупресија и новите биолошки агенси можат да ја контролираат болеста, кај тешките форми. Затоа, алтернативна стратегија за лекување на овие болести е неопходна, особено за тешките форми на мултипла склероза, системска склероза, ревматоиден артритис, системски лупус еритематозус, хематолошки имуни цитопении и Кронова болест. Високодозна имуносупресивна терапија, со автологна трансплантација на матични клетки претставува опција за лекување голем број тешки автоимуни болести.
Цел на трудот. Прикажување на нашето искуство во третманот на автоимуни болести, со конкомитантни малигни хематолошки болести, со автологна трансплантација на матични хематопоетски клетки.
Методи. Во период од септември 2000 до септември 2015 година на Клиниката за хематологија ниту еден пациент не е третиран со трансплантација заради основното автоимуно заболување. Третирани се вкупно шест пациенти: двајца мажи и четири

жени, со средна возраст од 47 години. Со Сјогренов синдром и мултипен миелом: двајца пациенти; Ревматоиден артритис и акутна миелобластна леукемија: еден пациент; Псоријаза и акутна миелобластна леукемија: еден пациент; Полиартритис нодоза и мултипен миелом: еден пациент; Т-клеточен не-Хочкинов лимфом и Системски лупус еритематозус: еден пациент. Пациентите се третирани со високодозна хемиотерапија, во зависност од основното хематолошко заболување.

Резултати. Сите третирани пациенти се живи, најмалку 100 дена по извршената трансплантација, што ја потврдува безбедноста на овој тераписки модалитет кај автоимуните болести.

Заклучок. Автологната трансплантација на матични клетки претставува безбедна метода за трансплантација на пациенти со тешки автоимуни болести и конкомитантни малигни хематолошки болести.

Клучни зборови: автоимуни болести, стем клеточна трансплантација

Introduction

Autoimmune diseases are a heterogeneous group of diseases that affect 5% to 8 % of the world's population. Although the etiology is still unknown, the latest researches blame the dysregulation of the regulatory T-lymphocytes as the key to the autoimmunity in many autoimmune diseases. The key role in the success of reaching a long-term remission is given to the abovementioned lymphocytes. The implementation of the new immune-modulating drugs, the biological agents, the monoclonal antibodies (anti tumor necrosis factor-alpha blockers, anti-interleukin-6 receptor antibodies, anti-CD20 monoclonal antibodies) and many more give promising results in the autoimmune disease treatment, yet there is a group of patients who remain immune even to this kind of treatment. The cellular therapy, mainly used in the treatment of malignant diseases, has been later tested and successfully used in the treatment of severe autoimmune diseases. The transplantation concept derives from a large number of experimental data, which were obtained as a genetically predisposed autoimmune disease models (systemic lupus erythematosus and diabetes mellitus), as well as through animmunization against foreign antigens (acute arthritis and encephalitis) testedon experimental animals. This experiment has shown an opportunity for a full recovery through a tolerance induction after an allogeneic or syngeneic transplanttation of hematopoietic stem cells.

The autologous transplantation of hematopoietic stem cells is a treatment option in thecase of very severe autoimmune diseases, in which the prognosis is very poor, such as the severe cases of scleroderma, multiple sclerosis and the systemic lupus erythematosus, in which the conventional therapy has a little or barely any effect. The conditioning regimen improvement almost eliminates the transplant related mortality factor, making the transplantation a relatively safe choice of treatment for these patients. The European Society for Blood and Marrow Transplantation (EBMT) and the European League against Rheumatism (EULAR) had their first official meeting in September 1996, in Basel, Switzerland, where it was decided to establish the right place for a stem cell transplantation in the treatment of autoimmune diseases and to further study the immunologic reconstitution after the transplantation had been performed. The minutes of this meeting were published in 1997, i.e. Consensus indications/guidelines for an autoimmune disease treatment with a transplantation of hematopoietic stem cells.

The only possible candidates for this complex immunobiological method treatment are patients who qualify under these criteria:

1. Those diagnosed with a severe autoimmune disease followed by a high mortality or irreversible invalidity risk.
2. The autoimmune disease does not match the given conventional therapy.
3. Positive clinical benefits are expected from the transplantation (before irreversible damages to the organs appear).

Until now, the autologous transplantation has been extensively used, first and foremost because of its safety, but also for its high effect tostop the disease. Many scientific disputes still debate whether the expected immune reconstitution, after the transplantation will be performed, will grant us the "re-education" and the full recovery. The transplantation is a non-specific therapy and it is clear that the effectiveness of the therapy is not the result of a high-dose immunosuppressant, but a "reset" of the abnormal immuneregulation of the primary autoimmune ailment. The practices of allogeneic transplanttation are still in an early stage for us to draw a conclusion. The full recovery of the autoimmunity with this procedure has still not been reached, even though the graft-versus-autoimmunity effect has been obtained both in the experimental and in the clinical conditions.

Materials and methods

In the period from September 2000 to September 2015 at the University Clinic of Hematology in Skopje, 351 patients with different malignant and non-malignant hematological diseases were treated. Eighty-eight (88) patients were treated with allogeneic transplantation from an HLA identical compatible donor, while 263 patients were treated with autologous transplantation. Three hundred and twenty-four (324) patients were treated with peripheral stem cells, while 27 transplan-

tations were performed with bone marrow. The most common indication for thetreatment wasacute myeloblastic leukemia. None of the patients was treated for a primary autoimmune disease. Six (6) patients were treated for hematological malignancy and for concomitant autoimmune diseases: Sjogren syndrome and multiple myeloma-2 patients; polyartheritis nodosa and multiple myeloma-1 patient; scute myeloblastic leukemia and rheumatoid arthritis-1 patient; non-Hodgkin lymphoma and systemic lupus erythematosus-1 patient; acute myeloblastic leukemia and severe psoriasis-1 patient.

The median age was 47 years (31-60); there were 2 male: 2, and 4 female patients. All patients were treated with autologous hematopoietic stem cell transplantation.

The autologous stem cell mobilization was carried out with a prior stimulation using a colony-stimulating factor, at a 10 mcg/kg TT dosage, in a period of 5 days. The harvest was made on a Bakster 3000 CS separator used in 4 patients, while 2 patients had the harvest on a Cobe Spectra separator. All patients had gone through 2 apheresis procedures. The median harvest was$3.3x10^6$/ kg.TT CD34+ cells (from 2.45 to $5.67x10^6$ kg.TT CD34 + cells). The cryopreservationwas made in a Nicole PC Air Space Liquid cooling system, using dimethyl sulfoxide (DMSO) and autologous plasma serving as cryoprotectants. Before the cooling and the application, the cells were kept at -172°C temperature. Prior to the application, the stem cells were thawed in a wet bathroom at +37°C temperature. The conditioning regimen was applied in line with the primary hematological disease. Patients with multiple myeloma were treated with high doses of Melphalan ($200mg/m^2$) during the first and with a dose of $140mg/m^2$ during the tandem transplantation. Patients with acute leukemia underwent a myeloablative regimen with Busulfan/Cyclophosphamide. Non-Hodgkin lymphomas were conditioned according to the BEAM regimen. The median dose of CD34+ applied cells was $3.3x10^6$/kg TT. Transplantations were performed in sterile units equipped with HEPA filters, in strict-isolating conditions and a low-bacteria diet. Ciprofloxacin (2x500 mg) was given as an anti-infectious prophylaxis. Fluconazole 200 mg as antifungal prophylaxis. A daily dose of 600mg Acyclovir was given until the day of the engraftment. The intravenous immunoglobulin of 0.1mg/kg TT 1 was given every 14 days in the first 4 weeks. The granulocyte colony-stimulating factor was included from the day of the lowest neutropenia (+ day 5) until the day of receiving the graft. The whole group reached the engraftment on the 12^{th} day from the transplantation for a **veno-occlusive disease of the liver prophylaxis**. All patients were alive in a period of 100 days after the performed transplantation. The transplant mortality rate was 0.

Results

Chart 1.

Patient	Hematological Dx	Autoimmune disease
V.Z.	Multiple myeloma	Sjogren syndrome
T.S.	Multiple myeloma	Sjogren syndrome
N.O.	Multiple myeloma	Polyarteritis nodosa
K.K.	AML	Rheumatoid arthritis
A.R.	AML	Psoriasis vulgaris
N.K.	T-NHL	Systemic lupus erythematosus

Chart 1. Epidemiological features of the patients

Patient	Dx 1	Dx 2	Age	Gender	Prior treatment
V.Z.	MM	Sjogren	52	F	Corticosteroids
T.S.	MM	Sjogren	46	F	Corticosteroids
N.O.	MM	Polyarteritis	49	M	Cyclophosphamide
K.K.	AML	Rheumatoid	60	M	Methotrexate
N.K.	NHL	SLE	43	F	Cyclophosphamide
A.P.	AML	Psoriasis	31	F	Corticosteroids

Chart 2.

Patient	CD34+ cells	Regimen	Engraftment	Complications	G-CSF	PLT	RBC
V.Z.	3.02	Melphalan	+10	/	6	6	/
T.S.	2.74	Melphalan	+11	Mucositis	7	6	/
N.O.	5.67	Melphalan	+12	/	7	12	2
K.K.	3.42	BuCy	+15	Catheter	9	12	2
N.K.	2.53	BEAM	+13	/	7	12	2
A.P.	2.45	BuCy	+14	/	8	20	2

Patient no. 1 (V.Z.), with Sjorgen syndrome, hypothyroidism, chronic viral hepatitis B, treated with corticosteroids, developed multiple myeloma. The patient was treated with a high dose of Melphalan and a tandem autologous transplantation, using peripheral hematopoietic stem cells. Two years after the completion of the second transplantation, CC-A and CC-B antibodies developed. The patient does not receive therapy for the Sjorgen syndrome, but Thalidomide maintenance therapy.

Patient no. 2 (T.S.), with Sjorgen syndrome, treated with corticosteroids and antimalarial medication (Resochin), developed multiple myeloma. The patient was treated with a high dose of Melphalan and an autologous trans-

plantation, using peripheral hematopoietic stem cells. Nine years after the transplantation, the patient does not receive any therapy for both diseases.
Patient no.3 (N.O.), with a severe form of polyarteritisnodosa, had to go through severe mutilating operations, such as: right nephrectomy, intestinal resection and a below-knee amputation. The patient was treated with corticosteroids and high doses of Cyclophosphamide. A multiple myeloma developed that required administration of high doses of Melphalan and a tandem autologous transplantation, using peripheral hematopoietic stem cells. Five years after the transplantation, the patient has no signs of the diseases and receives a Thalidomide maintenance therapy.
Patient no.4 (K.K), with rheumatoid arthritis, was treated with Methotrexate, corticosteroiids and nonsteroidal anti-inflammatory medications. Acute myeloblastic leukemia (M2) developed, leading to autologous transplantation, using peripheral stem cells according to the Busulfan/Cyclophosphamide regimen.
Patient no.5 (N.K.), with systemic lupus erythematosus and with no signs of visceralization was treated with corticosteroids and antimalarial medication. A T-cell non-Hodgkin lymphoma developed. The patient was treated with a high dose of BEAM regimen and an autologous transplantation, using peripheral stem cells. Four years after the transplantation, the patient is in complete remission and receives Interferon 3.0 MIE maintenance therapy, three times weekly.
Patient no.6 (A.P.), with a severe form of psoriasis, was treated with psoralen ultraviolet light treatment. Acute myeloblastic leukemia (M2) developed. The patient was treated with the Busulfan/Cyclophosphamide regimen and an autologous transplantation, using peripheral stem cells. A complete regression of the psoriatic changes occurred in the early post-transplantation period. After three months from the transplantation, mild-form changes re-developed, with a moderate intensity. Six years after the transplantation, the patient is in a complete remission.

Discussion

Our understanding regarding the immunobiology of the autoimmune diseases and their treatment by using hematopoietic stem cell transplantation has significantly improved. The reconstructed immune system of the patients suffering from an autoimmune disease after lymphoablation and autologous transplantation goes through quality changes both on the immune defects and the modification of the adaptive immune response. The fruitful experiments made on animals have shown that the allogeneic or the autologous transplantation can stop the advancement of the disease and the organ damaging in the hereditary (genetic) or acquired (antigen induced) autoimmune diseases. The data gathered from the studies made on animals and humanshave shown that after the performed transplanttation, using hematopoietic stem cells, the immune system is normal and reset. This occurs through a replacement of the reset repertoire, after the completion of the transplanttation. Nowadays, we study whether and to which extent the inflammation suppression is due to T or B platelets' function regulation, after the performed transplantation. The common results obtained by the European League Against Rheumatism (EULAR) and the European Society for Blood and Marrow Transplantation (EBMT), have added experience to the treatment of more than 2000 patients with autoimmune diseases, who were treated with a hematopoietic stem cells transplantation, including the three most randomized studiesregarding: systemic sclerosis (ASTIS) [2], multiple sclerosis (ASTIMS) [3] and Crohn's disease (ASTIC) [4]. The completed II phase of the studies in USA, for stem cells transplantation in SLE, systemic sclerosis and multiple sclerosis, gives promising results. The third phase of the studies is still ongoing for the above-mentioned diseases, as well as the immune, genome and molecular studies, sponsored by the National Institute of Health in USA (NIH). They are expected to provide an answer regarding the molecular mechanisms of the autoimmunity, the immune regulatory mechanisms of the autoimmune diseases and the response from the treatment.
There is no curative treatment for the chronic inflammatory diseases. It would require a necessary depletion of the expanded pool of the auto-reactive B and T platelets, and at the same time retention of the memory cells, needed to handle the pathogens from the **region**. The experiences about the allogeneic transplantation in autoimmune diseases are gradually increasing, even though modest for us to draw a conclusion. Although the graft-versus-autoimmunity effect has been reached both in the experimental and in the clinical conditions, the full recovery from the autoimmunity, using this phenomenon, has not been reached. However, the implementation of this procedure has proven to be safe, without fatal outcome in the first 100 days and with no serious complications, which will cause high mortality from the transplantation procedure, even in patients with autoimmune disease and concomitant malignant hematological diseases. In our group of patients, none has been treated for his/her primary autoimmune disease.
Below is the number of performed transplantations by the European Society for Blood and Marrow Transplantation until 2015 (2052 transplantations in autoimmune diseases).
Distribution according to the diagnosis:
Multiple sclerosis: 801, other neurological diseases: 16 (myasthenia gravis: 3, other: 13).
Connective tissue diseases: 595.

(Systemic sclerosis: 443, SLE: 113, Poly/dermatomyositis: 18, Sjorgen syndrome: 3, Antiphospholipid syndrome: 5, other: 23).
Arthritis: 184 (rheumatoid arthritis: 85, juvenile chronic arthritis: 66, psoriatic arthritis: 4, other: 18).
Inflammatory bowel disease: 188 (Crohn's disease: 155, ulcerative colitis: 4, other: 29).
Vasculitis: 49 (Behchet's syndrome: 9, Wegener granulomatosis: 12, Takayasu's disease: 2, polyarteritisnodosa: 5, Churg-Strauss syndrome: 2, other: 20).
Hematological diseases: 97 (ITP: 31, AIHA: 25, Evans syndrome: 20, Pure red cell aplasia: 7, Pure white cell aplasia: 2, other: 21).
Insulin-dependent diabetes: 20, other: 32.

Conclusion

The autologous hematopoietic stem cell transplanttation is established as a method of choice for patients with severe autoimmune diseases, which are **refracttory** to the state-of-the-art biological therapy. Although none ofthe patients in our group has undergone transplantation for his/her autoimmune disease, the safety of the method is confirmed in patients with hematological malignant diseases and concomitant autoimmune diseases. The results of our study and the major randomized studies should encourage the specialists, who work with severe autoimmune diseases, to recruit patients who are candidates for a cell therapy treatment.

Conflict of interest statement. None declared.

References

1. Tyndall A, and Gratwohl A. Blood and marrow stem cell transplants in auto-immune disease: a consensus report written on behalf of the European League against Rheumatism (EULAR) and the European Group for Blood and marrow transplantation (EBMT). *Bone Marrow Transplantation* 1997; 19: 643-645.
2. van Laar JM, Farge D, Sont JK, *et al.* EBMT/EULAR Scleroderma Study Group.: Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. *JAMA* 2014; 311(24): 2490-2498.
3. Mancardi GL, Iacopino P, Farge D, Saccardi R. ASTIMS Haemato-Neurological Collaborative Group, On behalf of the Autoimmune Disease Working Party (ADWP) of the European Group for Blood and Marrow Transplantation (EBMT); ASTIMS Haemato-Neurological Collaborative Group On behalf of the Autoimmune Disease Working Party ADWP of the European Group for Blood and Marrow Transplantation EBMT. Autologous hematopoietic stem cell transplantation in multiple sclerosis: a phase II trial. *Neurology* 2015; 84(10): 981-988.
4. Christopher J Hawkey, Matthieu Allez F, *et al*. Riccardo Saccardi, Autologous Hematopoetic Stem Cell Transplantation for Refractory Crohn Disease: A Randomized Clinical Trial. *JAMA* 2015; 314(23): 2524-2534.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 15-19

MMR

15

DOI:10.1515/mmr-2017-0004

Original article

ANTI-VEGF IN MANAGEMENT OF MACULAR EDEMA IN RETINAL DISEASE

АНТИ-VEGF ВО ТРЕТМАН КАЈ МАКУЛАРЕН ЕДЕМ КАЈ РЕТИНАЛНИ ЗАБОЛУВАЊА

Natasha T Shekerinov[1], Vesna Dimovska Jordanova[1] and Milco Bogoev[2]

[1]University Eye Clinic, Faculty of Medicine, University "Ss Cyril and Methodius" Skopje, [2]PZU D-r Bogoev, Skopje, Republic of Macedonia

Abstract

Aim. To present new opportunities, clinical implications and benefits of the available VEGF therapy as a treatment of macular edema, which is a result of venous vascular occlusions, diabetic macular edema in diabetic retinopathy and age- related macular degeneration.

Background. The pathophysiology of macular edema is complex and various processes are involved in its development. It is actually an abnormal retinal capillary permeability and a disorder in the blood retinal barrier, which only increases the vascular permeability. This causes an expansion of the extracellular spaces, which leads to fluid accumulation, which additionally leads to macular thickening and eventual vision loss.

Methods. The studies included 40 patients, of whom17 was diagnosed with macular edema in diabetic retinopathy and were treated with anti-VEGF therapy. Also, there were 11 patients diagnosed with wet form of AMD, and 12 cases diagnosed with macular edema secondary to vein occlusion. This retrospective study of 18 months monitored the effects of visual acuity on Snellen chart and the effects of macula anatomy using Optical Coherent tomography /OCT/. All patients received intravitreal injection of Bevacizumab /Avastin/ of 1.25mg /0.04ml/ and were evaluated monthly or every 4 to 8weeks. We monitored the potential ocular and systematic side effects in all our cases.

Results. In the first group which included patients with edema due to venous vascular occlusion improvement of visual acuity in 58.33% patients, 25.0% showed no change in visual acuity and 16.66% showed slight worsening of 0.029 and regression of CMT entirely to 393.22 after 4.6 intravitreal injections on average. In the second group there was no improvement of VA 0.172 and reducing central macular thickness for 218.34μm by 5.6 intravitreal applications. The third group, 17 patients with macular edema due to diabetic retinopathy had stabilization of visual acuity, i.e. slight improvement in 8 of them by 0.14; and, in 9 and improvement of 0.21 and regression CMT, an average of 174.3 μm.

Although it has been shown that benefit of intravitreal use of Bevacizumab and improvement of visual acuity has not been always change hand in hand with the reduction of macular edema, the need for this kind of treatment in certain cases are needed to maintain stable CMT and VA in such patients.

Conclusion. Over the last few years monoclonal antibodies have become a standard therapy in treatment of wet form of AMD. Switch on anti-VEGF drugs has shown significant results in clinical and visual outcomes in patients with changes of the macula as a result of other disease. In fact, they caused a revolution in the treatment of refractory macular edema.

Keywords: anti-VEGF, macular edema, age-related macular degeneration (ARMD), retinal vein occlusion (RVO), optical coherence tomography (OCT)

Correspondence to: Natasha T. Shekerinov, University Eye Clinic, "Vodnjanska" 17, 1000 Skopje, R. Macedonia;
E-mail: n_trpevska@yahoo.com

Абстракт

Цел. Да се прикажат новите можности, клиничките импликации и користа од достапната анти VEGF терапија како третман на макуларниот едем, резултат на венски васкуларни оклузии, дијабетичен макуларен едем кај дијабетичната ретинопатија и кај сенилната дегенеарција на макулата.

Вовед. Патофизиологијата на макуларниот едем е комлексна и различни процеси се вклучени во неговото формирање. Во принцип се работи за абнормална ретинална капиларна пермеабилност и нарушување на хематоретиналната бариера, што ја зголемува васкуларната пермеабилност при која екстрацелуларните простори се шират и доведуваат до акумулација на течност доведувајки до макуларно пропуштање и нарушување на видот.

Методи. Во трудот се презентираат 40 случаи на пациенти и тоа 17 пациенти со дијабетичен макуларен едем кај дијабетична ретинопатија во кои секундарно се активира примената на анти VEGF терапијата, 11 пациенти со влажна форма на сенилна дегенерација на макула (ARMD Wet) и 12 па-

циенти со макуларен едем, секундарен од оклузија на вена.
Во оваа ретроспективна студија за пероид од 18 месеци се проследени ефектите од третманот со анти VEGF препарат, влијанието врз видната острина според Shellen chart и промените врз макуларната анатомија користејќи Оптичка кохерентна томографија (OCT). Сите пациенти примаа интравитреално Bevacizumab (Avastin) 1,25мг/0,04мл и промените се евалуираа на секој 4-8 недели. Беа проследувани и сите потенцијални очни и систематски негативни појави кај поединиите групи паценти.
Резултати. Во првата група каде се вбројуваат пациенти со едем резултат на венска васкуларна оклузија има подобрување на видната острина кај 58,33% пациенти, кај 25,0% нема промена во видната острина, а кај 16,66 % е со незначително влошување од 0,029., и со регресија на CMT во целост за 393,22 по 4,6 прмени инекции во просек. Во втората постои подобрување на видната острина за 0,172 и намалување на централната макуларна дебелина за 218,34 μm, по 5,6 интравитреални апликации; а кај третата група, со 17 пациенти со макуларен едем резултат на дијабетична ретинопатија имаше стабилизација на видната острина, т.е. незначително подобрување кај 8 од нив за 0,14; а, кај 9 има подобрување за 0,21 и регресија на CMT во просек за 174,3 μm.
Од прикажаното се акцентира бенефитот од интравитреалната примена на Авастин и подобрувањето на видната острина, но не секогаш промените одат па- ралелно со редукцијата на макуларниот едем.
Заклучок. Последните неколку години, моноклоналните антитела станаа стандард во третманот на влаж-ната форма на сенилната дегенерација на макула (ARMD Wet). Преминот кон истите покажа значителни резултати како во клиничките така и во визуелните резултати кај пациентите со промени на макулата резултат на бројни заболување. Практично тие предизвикаа револуција во третманот на рефрактерниот макуларен едем.

Клучни зборови: анти-VEGF, макуларен едем, сенилна дегенерација на макула (ARMD), ретинални венски оклузии (RVO), оптичка кохерентна томографија (OCT)

Introduction

Retinal vasculo-occlusive disorders are the most common cause of visual impairment and blindness in the world. Diabetic retinopathy is with the highest prevalence, and has a leading place of blindness in the working-age population. The circumstances that lead to these pathophysiological conditions can be described through three principles, namely: conditions that vary the speed of blood flow, conditions that are based on physical modification of the blood vessels and the blood structures [1].
Diabetic macular edema (DME) comes as a result of the thickening of the basement membrane, and the loss of pericytes leading to increased permeability of blood ve-ssels of the retina. This disorder of the hematoretinal barrier causes leakage of plasma constituents and subsequent changes, edema, and retinal hypoxia, which stimulate the production of vascular endothelial growth factors, i.e. the occurrence of neovascularization.
Cystoid macular edema (CME) is relatively painless co-mmon condition associated with reduced vision, and occurs in almost 10% of the diabetic population. It practically means an accumulation of fluid around the macula, creating intraretinal lipid deposits which completely disrupt the architectonic of retinal tissue forming cystic spaces [2].
Retinal vein occlusion (RVO) is the second most common retinal vascular disease after DR which significantly affects the central vision and has a prevalence of 0.7% -1.6% by the appearance of macular edema, vitreous hemorrhage, epiretinal membrane, macular ischemia, retinal detachment and neovascular complications.
Macular edema is generally visualized by routine diagnostic of posterior segment of the eye, but a correct diagnosis is reached by measuring the central retinal thickness and presentation changes with OCT.
Exudative senile macular degeneration or "wet form" is cause for irreversible vision loss in more than 2 million people in the US by 2008 and this incidence will rise to almost 3 million by 2020. In Western countries, the pre-valence in those over 55 years is 1.6% and increases to about 13% in those over 84 years [3]. It is characterized by neovascularization in macular area due to the formation of choroidal neovascular membrane, the appearance of abnormal blood vessels that leak, accumulation of fluid and blood with progression of irreversible changes that impair the central vision.

Material and methods

This is a retrospective analysis of 40 randomized patients, 22 men (55.0%) and 18 women (45.0%) for a period of 18 months.
According to the etiology and pathophysiology of the patients included, we separated them into three groups: the first group of 12 patients had macular edema due to venous retinal occlusion, the second group of 11 patients with senile macular degeneration and group3 consisted of 17 patients with diabetic macular edema from diabetic retinopathy.
In Inclusion criteria are incorporated swelling in the region of the macula, visual acuity BCVA <0, 3 sc and therapeutic treatment application of Avastin (Bevacizumab) intra-vit-real 1.25 mg / 0.04 ml.

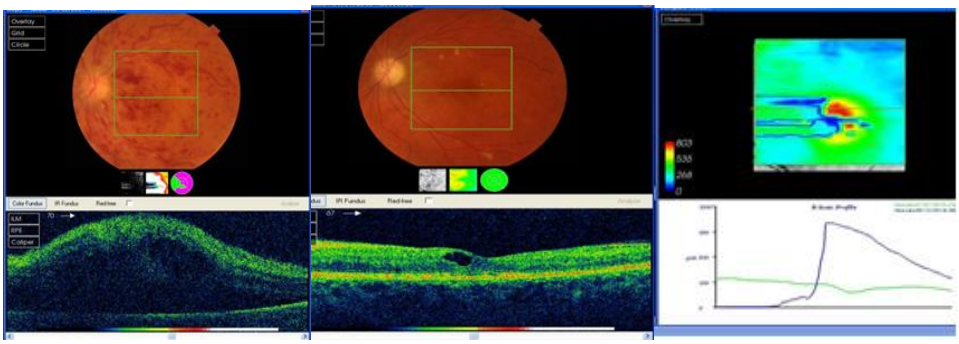


Fig. 1. Image of comparative OCT findings of a patient with OVCR OS, before and after treatment, fundus camera and comparative curve of the values of CMT

Case 1:

P.S., 51-y. woman;
OS: CRVO / Th: anti-VEGF NIII;
CMT 872µm → CMT 288 µm
BCVA OS: 0.01 → BCVA OS: 0.32 sc

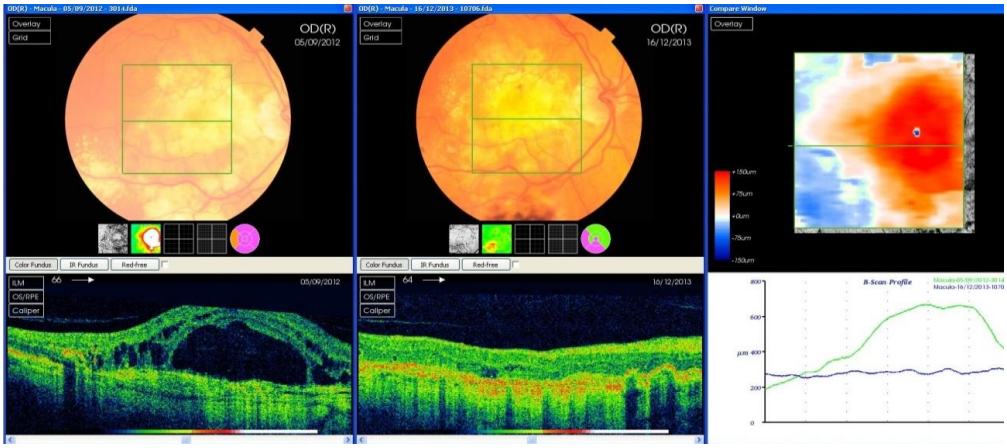


Fig. 2. Displaying comparative OCT findings of a patient with ARMD Wet OD, before and after treatment, fundus camera and comparative curve of the values of CMT (µm)

Case 2:

A.A., 73-y. woman;
OD ARMD Wet;
Anti VEGF NVI; CMT CMT 682µm → 381µm
BCVA OS: 0.02 → BCVA OS: 0.05

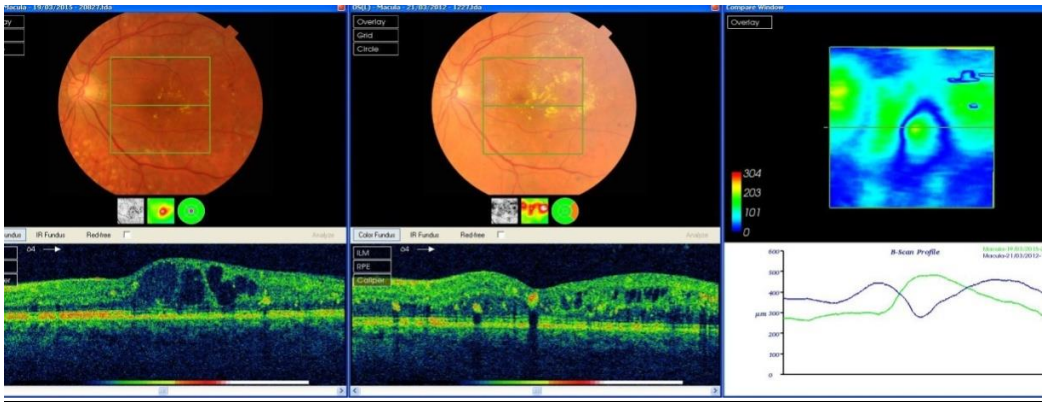


Fig. 3. Image the comparative OCT findings of a patient with RDNP, MD primarily made with LFC, then anti-VEGF, before and after treatment, fundus camera and comparative curve of the values of CMT

Case 3:

D.D., 58-y. man; OS: RDNP, MD;
Th: LFC, then anti-VEGF NVI;
CMT 468µm → CMT 256 µm
BCVA OD: 0.14 → BCVA OD: 0.04

Patients were followed with complete ophthalmologic examinations, by best corrected visual acuity (BCVA), measurement of intraocular pressure, examination on dilated pupil review 78Dpt, and evaluation with OCT imaging method. OCT is a procedure that uses reflection of low-coherence interferometry typically employing near-infrared light of the retinal layers, making cross-sections; it provides a picture of the retina and retinal pigment epi-thelium (RPE) in 3D resolution, centered on the macula region; noninvasive, fast and simple method. Macular thickness was measured using calipers (macula thickness caliper, CMT) while analyzing certain cystic structures.

Results

Given the different pathophysiological and etiological genesis of patients we divided and analyzed them in three groups. The first group, patients with macular edema due to central retinal vein occlusion, included seven females (58.33%) and five males (41.66%) with an average age of 63.4 years. After average 4, 6 applied intravitreal injections there was improvement of visual acuity in 58.33% patients, in 25.0% there was no change in visual acuity, in 16.66% with negligible deterioration of 0.029, and regression of CMT entirely to 393.22.

The second group included patients with randomized changes in the macula as a result of wet senile macular degeneration; there were 8 women (72.73%) and 3 men (27.27%) with an average age of 67.3 years. On average, they all received 5.6 injections; there was a decrease in central macular thickness of 218,34μm, and improve of the visual acuity of 0.172.

The third group of 17 patients with DME in diabetic retinopathy previously treated with laser photocoagulation of the retina, but for the cystoid macular edema they all needed treatment with Avastin. The average age of patients was 60.01 years; the average applied intravitreal injections was 3.7 and the statistical analysis confirmed visual acuity stabilization or slight improvement in 8 of them by 0.14; while 9 improved to 0.21 and had regression CMT on average of 174.3 μm.

Today, the need for this kind of treatment in certain cases is unquestionable, primarily for maintenance of a stable central macular thickness and improvement in visual acuity. Anti-VEGF treatment is an actual treatment in macular edema and retinal neovascularization.

Discussions

Otani *et al*. in (1999) presented a classification based on the morphological change of retinal OCT-findings like sponge edema, cystoid macular region, diffuse macular edema and significant retinal detachment [4,5].

All the efforts in recent years have been aimed to finding medicines which will act competitively and will inhibit the production of active vascular prolixferative substances and will regressively act on angiogenesis. Thus, there are already found preparations and numerous ongoing clinical studies [6-10].

The therapeutic protocol involves direct application of the drug intravitreal under aseptic conditions according to a standardized protocol, at monthly intervals depending on the clinical findings of the patient and the control OCT [8,9].

In fact, although the mechanism of retinal diseases is not fully determined, it is generally accepted that the regulation of vascular functions of a healthy retina is maintained through balance of the expression of angiogenic stimulators and angiogenic inhibitors, VEGF i.e. against Angiostatin and murine factor of pigment epitel- PEDF [10-13].

The application of anti-VEGF therapy started in 1948, with Michelson hypothesis that hypoxia induces angiogenic "factor X" responsible for retinal neovascularization. Decades later, glycoprotein was partially descrybed and named as a factor of vascular permeability, and further studies were aimed at identifying it and change the endothelial level [12].

VEGF as the most important inductor in the process of angiogenesis as result from hypoxia is a homodimeric glycoprotein and molecule important for early neovascularization and increased permeability, described by HF. Dvorak and D. Sanger [14], purified and cloned in 1989 by N. Ferrara [15].

Bevacizumab: (Avastin, Genentech Inc., San Francisco, LA) is a human monoclonal antibody (Full length ab) for all forms of VEGF-A which selectively binds to VEGF and specifically block VEGF receptor ligand and receiver forming protein complex, disables further ties of VEGF receptor sites which in turn initiate neovascularization. [12,13]; molecular weight of 149 kDa; has two antigen-binding domains of its receptor Flt-1 and KDR [14].

Hypoxia is an inducer that triggers upregulation of growth factors, integrins and proteinases leading to endothelial cell proliferation and migration. Hypoxia regulates VEGF mRNA in the retinal endothelial cells, the retinal pigment epithelium (RPE), perycites, Miller cells and ganglion cells. Induction of VEGF is by hypoxia-inducible factor-1 (HIF-1), low pH, inflammatory cytokines (IL-6), growth factors (fibroblast), sex hormones (androgens and estrogens), chemokines, and oncogene activation and decreased activity of the tumor suppressor gene [12-17].

The intravitreal administration of substances started in 1911, with the application of air as a tamponade of the retina ablation, later started applications of drugs, including penicillin in 1940 for the treatment of endophthalmitis.

In 2004 FDA approved intravenous antiangiogenic treatment with Bevacizumab for metastatic colorectal cancer, ocular application initially started in 2004 for Wet

form of senile macular degeneration in which Bevacizumab has shown promising results, but today the use of Bevacizumab is still "off label" with signed informed consent from the patients.

Other preparations, Ranibizumab (Lucentis) and Aflibercept (Eylea), have been for approved intravitreal application in 2006 or 2013, but today main limiting factor is that they are not present on the Positive Drug List which makes them inaccessible to the patients from public institutions.

Application of Bevacizumab is related to neovascularization, the wet form of ARMD and DME, premature retino-pathy (ROP), central serous chorioretinopathy (CSH), vascular occlusions, rear uveitis with macular edema and neovascular glaucoma, as well as preoperatively to remove the vitreous hemorrhage in order to facilitate surgery/peeling of the membrane and macular vitrectomy (PPV) [18,19].

In 2013 Avastin (Bevacizumab) and Lucentis (Ranibizumab) were on the 9th and 19th place, respectively, in terms of worldwide sales of pharmaceutical products according to their impact on medicine in general [19].

A recently published study (May 2016) has only confirmed the benefits of regular treatment. Of the included 650 patients in a 5-year-period with wet form of AMD, almost half retained 20/40 visual acuity, which is good enough for daily life [3].

Conclusion

Undoubtedly, the use of inhibitors of angiogenesis in the treatment of patients in the posterior segment of the eye, improve the visual function most of them,but most bene-ficial is stabilization of the visual acuity, regretssion in clinical diagnosis and prevention of neovascularization.

Currently, the intravitreal application of these preparations is in the spotlight as first-line treatment of conditions such as ocular neovascularization and macular edema due to numerous retinal diseases.

Conflict of interest statement. None declared.

References

1. Ding J, Wong TY. Current epidemiology of diabetic retino-pathy and diabetic macular edema. *Current diabetes reports* 2012; 12(4): 346-354. 10.1007/s11892-012-0283-6.
2. Khan BU, Lam W-C. Macular Edema, Diabetic. Available at: http://www.emedicine.com/oph/topic399.htm#target1. Accessed December 3, 2006.
3. Anti-VEGF Therapy for Macular Degeneration; https://www.nih.gov/news-events/age-related-macular-degeneration.
4. Kang SW, Park CY & Ham DI. The correlation between fluorescein angiographic and optical coherence topographic features in clinically significant diabetic macular oedema. *Am J Ophthalmol* 2004; 137: 313-322.
5. Panozzo G, Parolini B, Gusson E, *et al*. Diabetic macularo edema: an OCT-based classification. *Semin Ophthalmol* 2004; 19: 13-20.
6. Boyer DS.The pathophysiology of Macular Edema Retina Today, September 2011 supplement available at http: //bmctoday/net/retinatoday/2011/09/
7. Pelosini L, Hull CC, Boyce JF, *et al*. Optical Coherence Tomography May be used to Predict Visual Acuity in patients with macular edema. IOVS available at: http: //www.iovs. org/content/52/5/2741.full.
8. Koleva-Gjorgjieva D. Optical Coherence Tomography Findings in Diabetic Macular Edema.
9. Dimovska-Jordanova V. Morphological and functional processes involved in the pathogenesis of diabetic retinopathy. *Mак Med Pregled* 2010; 64 (I): 1-6.
10. Zhang S, Wang JJ, Guoguan G, *et al.* Pigment epithelium derived factor downregulateds Vascular Endothelial Growth Factor (VEGF) expression and inhibits VEGF-VEGF recaptor 2 binding in diabetic retinopathy. *Journal of molecular Endocrinology* 2006; 37: 1-12.
11. BOLT Study, a Prospective Randomized Trial of Intravitreal Bevacizumab or Laser Therapy in the Management of Diabetic Macular Oedema Received: December 23, 2009; Received in revised form: March 15, 2010; Accepted: March 18, 2010; Published Online: April 22, 2010.
12. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor, micro-vascular hyperpermeability and angiogenesis. *Am J Patrol* 1995; 146(5): 1029-1039.
13. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; 25(4): 581-611.
14. Carey VJ, Walters EE, Colditz GA, *et al*. Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study. *American Journal of Epidemiology* 1997; 145: 614-619.
15. Matsunaga N, Chikaraishi Y, Izuta H, *et al*. Role of soluble vascular endothelial growth factor-1 in the vitreous in proli-ferative diabetic retinopathy. *Ophthalmology* 2008; 115: 1916-1922.
16. Murugeswari P, Shukla D, Rajendran A, *et al.* Proinflammatory cytokines and angiogenic and anti-angiogenic factors in vitreous of patients with proliferative retinopathy and eales' disease. *Retina* 2008; 28: 817-824.
17. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; 9: 669-676.
18. Rich RM, Rosenfeld PJ, Puliafito CA, *et al*. Short term safety and efficacy of intravitreal Bevacizumab (Avastin) for neovascular age related macular degeneration. *Retina* 2006; 26: 495-511.
19. Miriam Karmel. Avastin new hopes and hesitations, Eye net, January 2010.
20. https://www.aao.org/eyenet/article/avastin-new-hopes-hesitations?january-2010.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 20-26

MMR

DOI:10.1515/mmr-2017-0005
Original article

MUTATION ANALYSIS OF THE COMMON DEAFNESS GENES IN PATIENTS WITH NONSYNDROMIC HEARING LOSS IN REPUBLIC OF MACEDONIA

МОЛЕКУЛАРНА КАРАКТЕРИЗАЦИЈА НА НАСЛЕДНАТА ГЛУВОСТ КАЈ ПАЦИЕНТИ СО НЕСИНДРОМСКО ОШТЕТУВАЊЕ НА СЛУХОТ ВО РЕПУБЛИКА МАКЕДОНИЈА

Emilija Sukarova Stefanovska[1], Gjorgji Bozhinovski[1], Ana Momirovska[2], Marina Davceva Cakar[3], Elena Sukarova-Angelovska[4] and Dijana Plaseska-Karanfilska[1]

[1]Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov", Macedonian Academy of Sciences and Arts, Skopje, [2]Association of Deaf and Hard of Hearing, Skopje, [3]University Clinic of Otorhinolaryngology, Medical Faculty, Skopje, [4]University Pediatric Clinic, Medical Faculty, Skopje, Republic of Macedonia

Abstract

Hearing impairment is the most common sensory disorder, which occurs in 1 of 1000 newborns. It is caused by heterogeneous conditions with more than a half due to genetic etiology. Although hundreds of genes are implicated in hearing process and have been found to be associated with nonsyndromic hearing loss, pathogenic variants in *GJB2* gene have been considered as the main cause of deafness among nonsyndromic hearing loss (NSHL) population worldwide. Pathogenic variants in *MT-RNR1* or *mtDNA12SrRNA* gene were also implicated predominantly in postlingual progresive deafness.

The aim of this study was to analyze the implication of *GJB2* and *MT-RNR1* genes in the molecular etiology of deafness among 130 NSHL patients in the Republic of Macedonia. The presence of the del (*GJB6-D13S1830*) was also analysed. We performed SSCP and/or sequence analysis of *GJB2* and identified sequence variants in 62 out of 130 patients (47.7%); (51 homozygous or compound heterozygous and 11 with only one variant allele). We found 8 different allelic variants, the most prevalent being c.35delG (65.49%), and p.W24*(23.01%), followed by other less frequent alleles (p.V27I, p.V37I, p. P175T and cd. delE120 or delGAG at 360). In addition, two polymorphic substitutions in the *GJB2* gene with no clinical significance (p.V153I and p.R127H) were detected. No del(*GJB6-D13S1830*) was found.

SNaPshot analysis was used to screen for the five most frequent allelic variants in the MT-RNR1 gene. Two MT-RNR1 mutations (A827G and T961G) were detected in three patients where only one GJB2 pathogenic variant was found. A new MT-RNR1 gene variant G1303A was also detected.

In conclusion, MT-RNR1 mutations were not a significant contributor to the etiology of deafness in Macedonia, although could be considered as a modifier gene affecting the expression of deafness in patients carrying one GJB2 variant. On the other hand, the high percenttage of *GJB2* pathogenic variants identified among NSHL cases indicates the necessity of molecular newborn screening for the two most common *GJB2* variants (c.35delG and p.W24*) in the Republic of Macedonia.

Keywords: Nonsyndromic hearing loss, *GJB2* gene, pathogenic variants

Correspondence to: Emilija Sukarova Stefanovska, Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov", Macedonian Academy of Sciences and Arts, Blv. Krste Misirkov 2, 1000 Skopje, R. Macedonia; Phone: +389 2 32 35 411 / 262; Mob. Phone: +389 70 587 288; E-mail: emilija@manu.edu.mk

Апстракт

Губењето на слухот е најчесто сензорно пореметување, кое се јавува со инциденца од 1 на 1000 новородени деца. Постојат бројни причини за глувоста, но наследните фактори се повеќе го заземаат приматот во нејзината етиологија. Во остварувањето на слушниот процес се инволвирани бројни гени и промени во овие гени, кои водат кон оштетување на слухот. Но, и покрај извонредно хетерогената генетска основа, патолошките варијанти во *GJB2* генот, во светски рамки, се сметаат за главна причина за појава на несиндромска наследна глувост (НСНГ). Патолошки варијанти во *MT-RNR1* генот, исто така, се имплицирани, главно, во постлингвалната прогресивна глувост.

Целта на оваа студија е утврдување на инциденцата и типот на патолошки промени во *GJB2* и *MT-RNR1* гените во молекуларната етиологија на наследна глувост кај 130 лица со НСНГ во Република Македонија. Исто така, е испитувано и присуство на делецијата-del (*GJB6-D13S1830*). Спроведени се анализи на едноверижен конформациски полиморфизам (SSCP) и/или директно секвенционирање на

егзонските секвенци од *GJB2* генот. Утврдени се варијанти во секвенцата на *GJB2* генот кај 62 од испитувани 130 лица или 47.7 проценти. Кај 51 лице се утврдени промени во хомозиготна форма или комбинирана хетерозиготна форма, додека кај 11 пациенти е утврдена промена во *GJB2* генот само на еден алел. Идентификувани се осум различни генски варијанти, од кои најчеста е c.35delG, со застапеност од 65.49%, како и p.W24* со застапеност од 23.01%. Други помалку чести варијанти, утврдени во текот на студијата се: p.V27I, p.V37I, p.P175T и cd.119/120delGAG. Дополнително се утврдени и две полиморфни замени во *GJB2* генот, кои немаат клиничка сигнификантност: p.V153I и p.R127H. Кај ниту еден пациент не е утврдено присуство на делецијата del (*GJB6-D13S1830*).
Воведена е и SNaPshot анализа за утврдување на петте најчести промени во *MT-RNR1* генот. Утврдено е присуство на две *MT-RNR1* патолошки варијанти (A827G and T961G) кај тројца пациенти, каде е откриена само по една патолошка варијанта во *GJB2* генот. Исто така, е утврдено присуство и на нова варијанта G1303A во *MT-RNR1* генот.
Како заклучок-*MT-RNR1* мутациите немаат значителен продонес кон молекуларната етиологија на глувоста во Република Македонија, иако нивното присуство може да се смета за модификатор, кој ја афектира тежината на оштетувањето на слухот кај лицата носители, само на една *GJB2* патолошка варијанта. Од друга страна, високиот процент на застапеност на патолошките варијанти c.35delG и p.W24* во *GJB2* генот кај лицата со НСНГ во нашата земја, укажува на потреба од скринирање за присуство на патолошки варијанти во *GJB2* генот кај сите лица со несиндромско оштетување на слухот, а воедно и воведување неонатална скрининг програма за детекцијата на овие две варијанти.

Клучни зборови: несиндромска наследна глувост, ГЈБ2 ген, патолошки варијанти.

Introduction

Hearing loss is the most prevalent sensory defect. According to WHO 5% of the population or 360 million people are affected worldwide. Both genetic and environmental factors are associated with deafness but the inherited causes are exposed as the most prominent etiological factor in developed countries. Deafness has dramatic effects on language acquisition seriously compromising the quality of life and leads to social isolation [1].
The genetic basis of hearing loss is complex. To date, more than 70 genes (http://hereditaryhearingloss.org) have been associated with hearing loss. At least 70% of all cases are classified as nonsyndromic hearing loss (NSHL) manifested with isolated hearing loss without other associated clinical features. Of all NSHL, 75%-80% are autosomal recessive disorders, 15%-20% are autosomal dominant, 5% are X-linked, and 1% is inherited by mitochondrial genes. [2].
Despite the enormous genetic heterogeneity, pathogenic variants in only one gene, *GJB2*, located at the DFNB1 locus (13q12) are responsible for approximately half of all cases with NSHL [3]. The locus contains three genes, *GJB2*, *GJB6* and *GJA1*, encoding for the transmembrane gap junction proteins connexin 26, connexin 30 and connexin 31, respectively, responsible for creating hexameric hemichannels (connexions) implicated in the maintenance of K^+ homeostasis in the inner ear [4,5]. Other connexin genes *GJB6* and *GJA1* have also been associated with deafness, but more rarely.
Pathogenic variants in *GJB2* gene (OMIM 121011) are the most common causes of sporadic and recessive NSHL, in many populations worldwide. More than 200 different pathogenic variants in this gene (davinci.crg.es/deafness/) have been described with specific prevalence in differrent ethnic groups and geographic regions. c.35delG predominates in Caucasians [6], 176delT in Ashkenazi Jews, 235delC in Japanese, while p.W24X in Indian and p.R142W in African population [7,8].
Due to the high influence of *GJB2* in deafness, molecular testing for *GJB2* pathogenic variants has rapidly become the standard of care for the diagnosis and counselling of patients with nonsyndromic hearing impairment of unknown cause.
Interestingly, in many studies 10% of patients with prelingual nonsyndromic deafness were found to carry a single heterozygous recessive mutation in the *GJB2* gene. Presence of the deletion in DFNB1 locus affecting *GJB6* and the promoter region of *GJB2*, a del(*GJB6-D13S1830*), as a digenic effect provided an explanation for the deafness in as many as 30% of affected *GJB2* heterozygotes in some populations [9]. But, the molecular etiology of nonsyndromic sensorineural hearing loss (SNHL) in subjects with only one detectable autosomal recessive GJB2 pathogenic variant is still unclear. Various studies searching for other modifier genes within DFNB1 or elsewhere have been performed in order to answer this question [10].
Nonsyndromic deafness can be caused by mutations in mitochondrial genes as well. *MT-RNR1* (Mitochondrially Encoded 12S RNA) is an RNA Gene, and is affiliated with the non-coding RNA class. Several mutations in the mitochondrial *MT-RNR1* gene have been found to be responsible for both aminoglycoside-induced and nonsyndromic hearing loss. The most common mutations in 12S rRNA (*MT-RNR1*) gene causing nonsyndromic hearing impairment are: A1555G, T961deT/ insC, T961G, T1005C, T1095C, A1116G, C1494T and A827G. These mutations make the

human mitochondrial ribosome more bacteria-like and alter binding sites for aminoglycosides [11].
Aminoglycoside antibiotics such as gentamycin, streptomycin, kanamycin and tobramycin, are commonly used in the treatment of patients with aerobic Gram-negative bacterial infections. These drugs have well-documented adverse reactions such as ototoxicity and nephrotoxicity. The nephrotoxicity is usually reversible but the ototoxicity, which is most likely due to damage of the sensory hair cells and the stria vascularis in the cochlea, is permanent [12].
In order to determine the molecular etiology of deafness in our country, we have analyzed the type and frequentcy of *GJB2* pathogenic variants among NSHL patients. The prevalence of mitochondrial *MT-RNR1* pathogenic variants was also analyzed using SNaPshot method for the five most common mutations. In addition, complete 12S rRNA sequencing in patients with only one detected *GJB2* mutation was performed in order to examine the modifier effect of the mtDNA pathogenic variants on severity of hearing loss. The presence of del (*GJB6*-D13S1830) was also analyzed.

Materials and methods

This study was conducted on 130 unrelated cases with NSHL of different ethnic origin [Macedonians (75), Albanians (20), Gypsies (31) and Turks (4)]. They were referred to our laboratory by the Audiology Center, University Clinic of Otorhinolaryngology, University Pediatric Clinic or the specialized units for speech rehabilitation in our country where audiologic examination and detailed family history analyses were performed. A total of 120 patients had a moderate-to-profound sensorineural hearing loss, while ten patients progressively lost their ability to hear during early childhood. No other clinical features were detected in the analyzed group.
After obtaining an informed consent from all participants and/or members of their families, peripheral blood was taken and total DNA was extracted using standard phenol-chlorophorm extraction, ethanol precipitation method [13].
Molecular studies included screening for: mutations in *GJB2* gene using Single Strand Conformation Polymorphism analysis (SSCP) and/or direct sequencing, large deletions in chromosome 13p region by Multiplex Ligation Probe Amplification (MLPA) method for and del (*GJB6*-D13S1830) mutation by specific PCR analysis. Single base extension or SNaPShot method was introduced for analysis of five common mutations in mithochondrial DNA connected with inherited deafness.
Amplification of non-coding (exon 1), coding (exon 2) and flanking intronic regions of the *GJB2* gene was conducted by PCR on an ABI2720 thermalcycler (Life Technologies). Oligonucleotide primers 5'- CCGGGAAG-CTCTGAGGAC-3' and 5'-GCAACCGCTCTGGGTCTC-3' were used for amplification of exon 1 [14] while exon 2 was amplified in two different PCR reactions, one for amplification of 286 bp PCR fragment of the 5' end of exon 2 using the GJB5- TCT TTC CAG AGC AAA CCG C and GJB8-GAC ACG AAG ATC AGC TGC AGG primers, and the other for amplification of a 270 bp fragment belonging to the 3' end of the GJB2 gene using GJB10 5'-GCA GCA TCT TCT TCC GGG T-3' and GJB6 5'-GGG CAA TGC GTT AAA CTG GC-3' primers.
The SSCP was performed on BioRad DeCode System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were loaded onto a nondenaturing 12% acrylamide/Bisacrylamide (39:1) gel. Electrophoresis was performed at constant power of 25W, at 4ºC for about 20 hours. PCR fragments were visualized with silver staining of the gel. To identify the nucleotide substitutions responsible for altered electrophoretic mobility detected by SSCP analysis, the PCR fragments were sequenced by BigDye sequencing kit v.1 (Life Technologies) and separated on an Applied Biosystems 3500 Genetic Analyzer (Life Technologies).
MLPA analysis using SALSA MLPA kit P163-C1 (MRC Holland, The Netherlands) was performed in order to determine the presence of deletions/duplications in the 13q region. This analysis allows detection of the three common GJB2 mutations: c.35delG, splice site mutation IVS1+1G>A or NM_004004.5(GJB2):c.-23+1G>A (HGVS), and 313del14.
The 309-kb del(*GJB6*-D13S1830) or NC_000013.10: g. 20797176_21100550del (HGVS) was also studied by multiplex PCR as previously described [9]. Namely, a set of three primers was used for simultaneous amplification of the normal *GJB6* allele and the allele with the del (*GJB6*-D13S1830) (primer1F: 5' AGT GAT CCA TCT GCC TCA GC; primer 2RN: 5' GTC TGT GCT CTC TTT GAT CTC and primer 3RD 5' GGA AGG TGT GGA TCA CAG TC).
In order to screen our patients for the presence of the five most common mitochondrial mutations associated with deafness (A827G, 961delT+Cn, T1095C, C1494T and A1555G), a SNaPshot method was designed according to Bardien *et al.* [15]. A 1124bp fragment of the MT-RNR gene was amplified using the following primers: MT-RNR-For: CAA CCA AAC CCC AAA GAC AC и MT-RNR-Rev: GCT CAG AGC GGT CAA GTT AAG. The PCR fragment was cleaned up with 1 unit of Illustra ExoProStar 1-Step (GE Healthcare, Life Sciences). The SNaPshot PCR reaction was performed in multiplex format by adding specific primers for each variant in concentration of 1.8 μM each, except A1555G in concentration of 2.9 μM (Table 1). The reaction was performed on a thermal cycler using the following conditions: 25 cycles at 96ºC/10 seconds, 50ºC/30 seconds and 60ºC/30 seconds. The SNaPshot reaction was cleaned

up with one unit of Shrimp alkaline phosphatase for an hour at 37ºC, followed by capillary electrophoresis on 3130 Genetic analyzer (Life Technologies). The representative electrophoregram of the five *MT-RNR1* normal variants is given in Figure 1a.

Table 1. Specific oligonucleotides used for the SNaPshot analysis of the five most common MT-RNR1 deafness causing variants

Variant	Primer sequence 5'-3'	Orientation	Size	Label (N / M)
A1555G	TTG GCA TTT ATA TAG AGG AG	F	20bp	A / G
C1494T	CGT ACA CAC CGC CCG TCA	F	19bp	C / T
T1095C	CTG GGA TTA GAT ACC CCA CTA TGC T	F	25bp	T / C
961delT+C(n)	ACA GGT GAG TTT TAG CTT TAT TGG GG	R	26bp	A / G
A827G	GCT TAG TTA AAC TTT CGT TTG TTG CTA AAG G	R	31bp	T / C

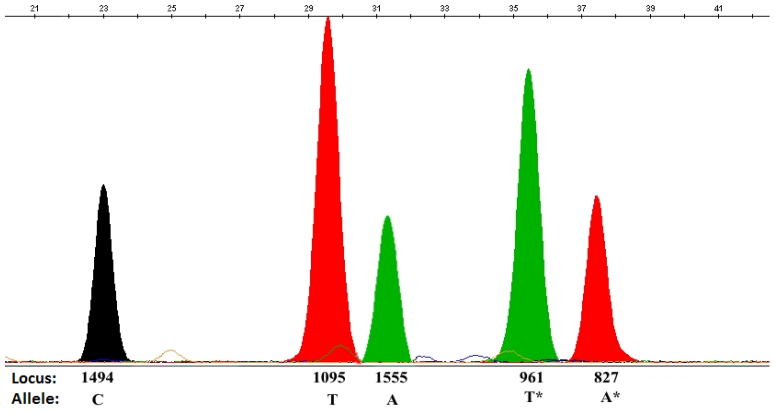


Fig. 1a. Representative electropherogram of the SNaPshot analysis for the five most common mitochondrial DNA mutations (C1494T, T1095C, A1555G, 961delT+C(n) and A827G) causing hearing loss. All shown fragments represent normal allele pattern

The sequencing data of mtDNA *12S rRNA* gene were compared with the Revised Cambridge Reference Sequence ("rCRS") (No. NC_012920) to identify mtDNA variants.

Results and Discussion

In this study we have analysed the type and frequency of *GJB2* variants among 130 unrelated NSHL cases from Macedonia, with different ethnic background. The presence of the five most common *MT-RNR1* pathogenic variants causing hearing loss was also analyzed.

GJB2 pathogenic variants were found in 62 patients (47.7%); 51 were homozygous or compound heterozygous while in 11 only one variant allele was detected (Table 2). We found 8 different allelic variants, the most prevalent being c.35delG (65.49%), found among Caucasians (Macedonian and Albanian patients), followed by p.W24* (23.01%), found only among Gypsy patients (Table 3). Other *GJB2* pathogenic variants: p.V27I, p.V37I, p.P175T and cd.119/120 delGAG were less frequent, found with allelic frequency of 0.88%, 1.77%, 0.88% and 0.88%, respectively. In addition, two polymorphic substitutions in the *GJB2* gene, which do not have clinical significance (p.V153I and p.R127H), were detected with a frequency of 1.77% and 5.31%, respectively. These findings confirm our earlier reported data on the prevalence of the *GJB2* pathogenic variants among the deaf population from the Republic of Macedonia [16]. The pathogenic variant c.-23+1G>A in exon 1, although frequent among Slavic population [17] was not detected in our group of patients.

We found a Trp24Stop (p.W24*) pathogenic variant exclusively among deaf patients of Roma ethnic origin. This is the most frequent *GJB2* pathogenic variant in India [18], and is common among Roma/ Gypsy patients in Spain [19] as well. This finding is indicative that this mutation was brought by Romani people to Europe from their Indian homeland, but this assumption should be confirmed by DNA polymorphic haplotype analysis.

We have identified a high percentage of patients (11 out of 130 or 8.5%) carrying only one *GJB2* pathogenic variant. Detection of only one pathogenic variant in the *GJB2* gene is a common finding. Seeman *et al.* also found that approximately 10% of the analyzed patients carry only one pathogenic variant [20**]**. This indicates the posiblilty of a digenic effect, influence of other modifier genes, or could be a result of an incidental finding of a *GJB2* variant in deafness due to other etiology [21].

Deletions/duplications in DFNB1 locus, in regions that regulate the expresion of the *GJB2* gene, could be also a possible explanation of the severity of hearing loss in GJB2 heterozygous patients. For determination of the possible digenic effect among 11 cases carryng only one *GJB2* pathogenic variant, analysis for the presence of the del (*GJB6*-D13S1830) and/or other deletions in the locus using SALSA MLPA probemix P163-D1 GJB-WFS1, were performed. None of the analyzed patients carried del (*GJB6-D13S1830*), or other deletion in the DFNB1 locus.

Table 2. GJB2 genotypes determined among 130 NSHL patients from different ethnic background in Macedonia

Genotype	Total No.	Ethnicity			
		Macedonian	**Albanian**	**Roma**	**Turks**
c.35delG / c.35delG	33	23	10	/	/
c.35delG / p.V37I	1	1	/	/	/
p.W24* / c.35delG	4	/	/	4	/
p.W24* / p.W24*	10	/	/	10	/
p.W24* / Cd120delGAG	1	/	/	1	/
p.R127H / p.R127H	1	/	/	1	/
p.R127H / p.V153I	1	/	/	1	/
c.35delG / N	3	2	1	/	/
p.W24* / N	1	/	/	1	/
p.V27I / N	1	1	/	/	/
p.V37I / N	1	/	/	/	1
p.P175T / N	1	1	/	/	/
p.R127H / N	3	/	/	3	/
p.V153I / N	1	1	/	/	/
Total	62	29	11	21	1

Table 3. Frequency of pathogenic variants in *GJB2* gene determined among NSHL patients from Macedonia and distribution according to their ethnicity

Variant	Chromosomes with *GJB2* variant		Frequency of GJB2 variants according to ethnical background			
	No.	**%**	**Macedonian**	**Albanian**	**Roma**	**Turks**
c.35delG	74	65.49	32.7%(49/150)	52.5%(21/40)	6.5%(4/62)	/
p.W24*	26	23.01	/	/	42.2%(27/62)	/
c.109G>A, p.V37I	2	1.77	0.7%(1/150)	/	/	12.5%(1/8)
p.R127H	6	5.31	/	/	9.7%(6/62)	/
p.V153I	2	1.77	0.7%(1/150)	/	1.6%(1/62)	/
Cd119/120delGAG	1	0.88	/	/	1.6%(1/62)	/
p.P175T	1	0.88	0.7%(1/150)	/	/	/
c.79G>A, p.V27I	1	0.88	0.7%(1/150)	/	/	/
Total	113	100	32.6%(45/138)	43.3%(13/30)	59.4%(38/64)	12.5%(1/8)

Molecular determination of *GJB2* gene variants is of high clinical significance. Biallelic pathological variants were found only in cases with profound deafness. None of the cases with progressive hearing loss carried pathological variants in *GJB2* gene.

Early diagnosis of deafness by identification of the 35delG or W24* variants would greatly improve genetic counseling, treatment and management of deafness in our country. Determination of a biallelic *GJB2* variant in a patient could allow an early decision for cochlear implantation and start of rehabilitation process earlier in the childhood when the success rate for speach development is higher [22]. Biallelic variants in *GJB2* are associated with variable clinical manifestation of hearing loss, mainly profound deafness, but moderete or mild forms could also be determined. Generally, phenotypic variability has been attributed to unknown modifier genes or environmental factors. On the other hand, the clinically most important point is that these cases always have normal development of vestibular apparatus and they are never accompanied with cochlear defects [23].

Pathogenic variants in mitochondrial DNA are associated potentially with nonsyndromic and aminoglycoside-induced hearing loss. Several nucleotide changes associated with hearing impairment were described, with variable distribution among different ethnic populations [15]. A SNaPshot method for determination of the five most common mitochondrial variants was performed in all 130 analyzed cases. Also, we have performed a systematic and extended sequencing of the mitochondrial *12S rRNA g*ene in 11 hearing loss patients where only one *GJB2* variant was detected. Mitochondrial DNA analysis revealed the presence of two deafness-associated variants, m.827A>G, and m.961T>G (Figure 1b). A sequence variant m.1303G>A was also found in one patient with only one *GJB2* mutation. According to UCSC Genome browser this G1303A variant is a highly

conserved variant in 100 species analyzed. Comparison with the reference sequence NC_ 012920.1 in MitoMap indicated a polymorphic variant. The frequency of this variant in full length sequence is 0.11%.

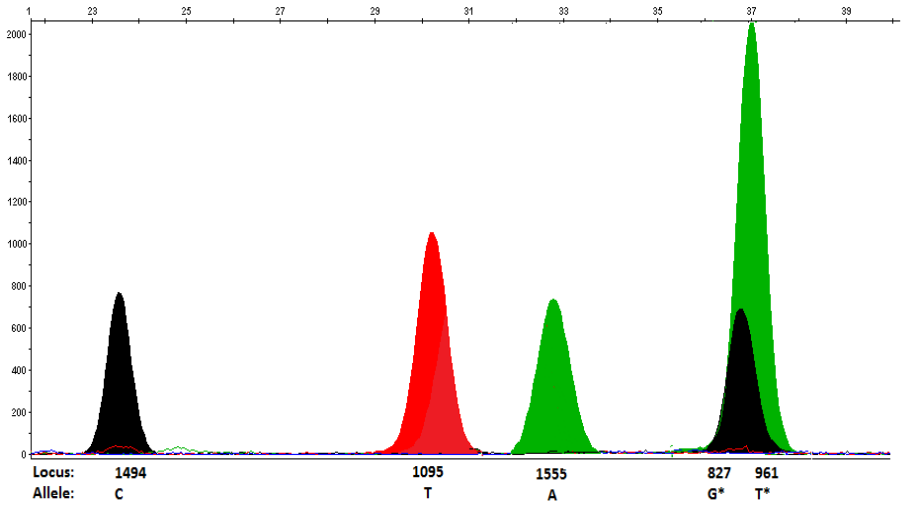


Fig. 1b. Patient with homoplasmic mutation 827A>G

The putative pathogenic mutation at position of 961 (m. 961 T>C) was detected in two cases with profound deafness, both carrying only one *GJB2* variant. One patient had the c.79G>A p.V27I pathogenic variant, while in the other a p.P175T pathogenic variant was determined. In the first case a history of aminoglycoside antibiotics usage during infancy was recorded. The mutation was confirmed by direct sequencing. This mutation was first described by [24] in five patients with distinct sets of mtDNA polymorphisms. Insertion or deletion at this position has been found to be associated with aminoglycoside-induced deafness in several genetically unrealted families as well indicating the pathological effect of nucleotide change at this position.

In conclusion, the high prevalence of c.35delG and p.W24* mutations among our patients (Caucasians and Gypsies, respectively) warrants screening for these two mutations among the deaf population in our country. The introduction of a newborn screening programme should also be considered. Also, our results suggest that mitochondrial DNA mutations do not represent a substantial risk factor for sensorineural deafness in Macedonian population, but mtDNA variants could influence the severity of deafness in cases with only one *GJB2* variant.

Conflict of interest statement. None declared.

References

1. Cohen MM, Gorlin RJ. Epidemiology, etiology and genetic patterns. In: Gorlin RJ, Toriello HV, Cohen MM, editor. Hereditary Hearing Loss and its Syndromes. *Oxford University Press, Oxford*; 9-21.
2. Petersen MB, Willems PJ. Non-syndromic, autosomal-recessive deafness. *Clin Genet* 2006;69:371-392.
3. Kelsell DP, Dunlop J, Stevens HP, *et al.* Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* 1997; 387: 80-83.
4. Zhao HB, Kikuchi T, Ngezahayo A, White TW. Gap junctions and cochlear homeostasis, review. *J Membr Biol* 2006; 209(2-3): 177-186.
5. Maeda S, Tsukihara T. "Structure of the gap junction channel and its implications for its biological functions". *Cellular and Molecular Life Sciences*, 2011; 68(7): 1115-1129.
6. Gasparini P, Rabionet R, Barbujani G, *et al.* High carrier frequency of the 35delG deafness mutation in European populations. Genetic Analysis Consortium of GJB2 35delG. *Eur J Hum Genet* 2000; 8: 19-23.
7. Chan DK, Chang KW. GJB2-associated hearing loss: systematic review of worldwide prevalence, genotype, and auditory phenotype. *Laryngoscope* 2014; 124: 34-53.
8. Tsukada K, Nishio SY, Hattori M, Usami S. Ethnic-Specific Spectrum of GJB2 and SLC26A4 Mutations. Their Origin and a Literature Review. *Ann Otol Rhinol Laryngol* 2015; 124(1): 61S-76S.
9. del Castillo I, Moreno-Pelayo MA, del Castillo FJ, *et al.* Prevalence and evolutionary origins of the del (GJB6-D13S1830) mutation in the DFNB1 locus in hearing impaired subjects: a multicenter study. *Am J Hum Genetics* 2003; 73: 1452-1458.
10. Grillo A-P, de Oliveira F-M, de Carvalho G-Q, *et al.* Single Nucleotide Polymorphisms of the *GJB2* and *GJB6* Genes Are Associated with Autosomal Recessive Nonsyndromic Hearing Loss. *BioMed Research International* 2015; 2015: 318727.
11. Guan MX. Mitochondrial 12S rRNA mutations associated with aminoglycoside ototoxicity. *Mitochondrion* 2011; 11: 237-245.
12. Fischel-Ghodsian N. Genetic factors in aminoglycoside toxicity. *Pharmacogenomics* 2005; 6: 27-36.
13. Efremov G, Dimovski AJ, Plaseska-Karanfilska D, *et al.* LABORATORY MANUAL 3rd Edition, ICGEB, *MANU*, 1999; 1-2.
14. Sirmaci A, Akcayoz-Duman D, Tekin M. The c.IVS1+1G>A mutation in the GJB2 gene is prevalent and large deletions involving the GJB6 gene are not present in the Turkish population. *J Genet* 2006; 85: 213-216.

15. Bardien S, Human H, Harris T, *et al*. A rapid method for detection of five known mutations associated with aminoglycoside-induced deafness. *BMC Medical Genetics* 2009; 10: 2.
16. Sukarova Stefanovska E, Momirovska, A, Cakar M, Efremov GD. GJB2 mutations in non syndromic hearing loss in the Republic of Macedonia. *BJMG* 2009; 12(2): 11.
17. Seeman P, Sakmarvova I. High prevalence of the IVS 1+1 G to A/GJB2 mutation among Czech hearing impaired patients with monoallelic mutation in the coding region of GJB2. *Clin Genet* 2006; 69(5): 410-413.
18. Adhikary B, Ghosh S, Paul S, *et al*. Spectrum and frequency of GJB2, GJB6 and SLC26A4 gene mutations among nonsyndromic hearing loss patients in eastern part of India. *Gene* 2015; 573(2): 239-245.
19. Alvarez A, del Castillio I, Villamar M, *et al*. High prevalence of the W24X mutation in the gene encoding connexin-26 (GJB2) in Spanish Romani (Gypsies) with autosomal recesive non-syndromic hearing loss. *Am J Med Genet* 2005; 137A(3): 255-258.
20. Seeman P, Malikova M, Raskova D, *et al*. Spectrum and frequencies of mutations in the GJB2 (Cx26) gene among 156 Czech patients with pre-lingual deafness. *Clin Genet* 2004; 66(2): 152-157.
21. Kim SY, Kim AR, Kim NK, *et al*. Unraveling of Enigmatic Hearing-Impaired GJB2 Single Heterozygotes by Massive Parallel Sequencing: DFNB1 or Not? *Medicine (Baltimore)* 2016; 95(14): e3029.
22. Davcheva-Chakar M, Sukarova-Stefanovska E, Ivanovska V, *et al.* Speech Perception Outcomes after Cochlear Implantation in Children with GJB2/DFNB1 associated Deafness. *Balkan Med J*. 2014; 31(1): 60-63.
23. Snoeckx RL, Huygen LM, Feldman D, *et al*. GJB2 Mutations and degree of hearing loss: A multicenter study. *Am J Hum Genet* 2005; 77: 945-957.
24. Li R, Greinwald JH Jr, Yang L, *et al*. Molecular analysis of the mitochondrial 12S rRNA and tRNASer(UCN) genes in paediatric subjects with non-syndromic hearing loss. *J Med Genet* 2004; 41(8): 615-620.

Mac Med Review 2017; 71(1): 27-31

MMR

DOI:10.1515/mmr-2017-0006
Original article

FLAG-IDA, POSSIBLE BREAKTHROUGH IN TREATMENT OF REFRACTORY ACUTE MYELOID LEUKEMIA IN THE CONTEXT OF HEMATOPOIETIC STEM CELL TRANSPLANTATION: SINGLE CENTER EXPERIENCE

FLAG-IDA ПРОТОКОЛОТ - МОЖЕН ИЗБОР ВО ЛЕКУВАЊЕ РЕФРАКТОРНА АКУТНА МИЕЛОИДНА ЛЕУКЕМИЈА, ВО КОНТЕКСТ НА ТРАНСПЛАНТАЦИЈАТА НА ХЕМАТОПОЕТСКИ МАТИЧНИ КЛЕТКИ

Lazar Chadievski, Sonja Genadieva Stavric, Aleksandra Pivkova Veljanovska, Zlate Stojanoski, Dijana Miloska, Lidija Cevreska and Borce Georgievski

University Hematology Clinic, Skopje, Republic of Macedonia

Abstract

Introduction. Acute myeloid leukemia (AML) represents an entity well defined among the hematological malignnant diseases from diagnostic and therapeutic point of view. Still, big concern remains for those patients where the induction therapy fails. Classified in the group of refractory AML these patients are with poor prognosis. There are numerous attempts in providing the best surviving results by administration of appropriate therapy. Our center presents its experience in treating patients with refractory AML by administration of FLAG-Ida regimen, followed by hematopoietic stem cell transplanttation, autologous or allogeneic, depending on the availability of HLA matched sibling donor of hematopoietic stem cells.
Methods. In patients with refractory AML, administering FLAG-Ida chemotherapy we have achieved complete remission in 22 patients (47%). Average age of the treated group of patients was 36.6 years (17-53). All of them proceeded to high-dose chemotherapy and underwent hematopoietic stem cell transplantation (HSCT). We performed autologous HSCT in 13 patients, and allogeneic HSCT in 9 patients. Median time to HSCT was 6.6 months (4-10), and in most of the patients we used myeloablative conditioning (MAC).
Results. The disease-free survival in our group of patients is 74 months (22-148). The longest overall survival was 148 months and was registered in a patient with allogeneic sibling HSCT. We can conclude that FLAG-Ida regimen is an appropriate and suitable salvage chemotherapy protocol for patients with refractory AML especially when it is used in the context of preparation for HSCT.

Keywords: acute myeloid leukemia; refractory; FLAG-IDA; hematopoietic stem cell transplantation

Correspondence to: Lazar Chadievski, University Hematology Clinic, "Vodnjanska" 17, 1000 Skopje, R. Macedonia; E-mail: laze_cad@yahoo.com

Апстракт

Вовед. Акутната миелоидна леукемија (АМЛ) претставува добро дефиниран ентитет во спектарот на малигни хематолошки заболувања, и од дијагностичка и од тераписка гледна точка. Сепак, голема загриженост предизвикува групата пациенти со ова заболување каде не се постигнува тераписки успех по иницијалната индукциска терапија. Пациентите класифицирани во групата на рефракторни форми на АМЛ се одликуваат со лоша прогноза во однос на стапките на преживување. Регистрирани се бројни обиди за подобрување на стапките на преживување на оваа група пациенти со ординирање соодветна терапија, односно дефинирање адекватен тераписки пристап. Ги презентираме сопствените искуства од лекувањето на пациенти со рефракторна АМЛ со администрација на цитостатска хемотерапија по FLAG-Ida протоколот, а потоа реализирање на трансплантација на хематопоетски матични клетки, автологна или алогена, во зависност од постоењето на ХЛА-ДНК компатибилен сроден дарител на хематопоетски матични клетки.
Методи. Извршивме евалуација на 22 пациента (47%) со рефракторна форма на АМЛ, каде не е постигнат тераписки успех со ординирање стандардна индукциска хемотерапија и со администрирање хемотерапија по FLAG-Ida протоколот, постигната е комплетна ремисија, во периодот од 2001 до 2014 година. Просечна возраст на испитуваната група изнесуваше 36.6 години (од 17 до 53). Кај сите, лекувањето е продолжено со високодозна хемотерапија и со трансплантација на хематопоетски матични клетки (ТХМК). Автологна ТХМК е извршена

кај 13 пациенти, додека алогена ТХМК кај 9 пациенти. Средно време до реализирање на ТХМК изнесуваше 6.6 месеци. Кај поголемиот број пациенти беа применети миелоаблативни протоколи за кондиционирање.

Резултати. Времето на преживување без болест, во нашата група пациенти, изнесуваше 74 месеци (од 22 до148). Најдолгото преживување изнесуваше 148 месеци и е регистрирано кај пациент со алогена ТХМК од сроден, компатибилен дарител. Нашиот заклучок е дека FLAG-Ida протоколот претставува адекватен и соодветен "salvage" хемотераписки протокол за пациентите со рефракторна АМЛ, особено доколку се применува во контекст на подготовка на пациентите кон ТХМК.

Клучни зборови: акутна миелоидна леукемија; рефракторна; FLAG-Ida; трансплантација на хематопоетски матични клетки

Introduction

Acute myeloid leukemia (AML) is a disease that we know so much of, but so little that we can actually do about it.. AML represents about 80% of all acute leukemias in adults, and is slightly more common in men. The incidence is age dependent, and it is in the range of 1 case in 100.000 until the age of 25, and rises to 25 cases per 100.000 persons after the age of 65-70, which shows exponential rise in the incidence rates [1]. The modern scientific medicine has entered the micro-universe of the malignant cell, describing signaling pathways, but still no magic cure is defined, knowing that for a long time, and still the best way to treat de novo AML, is induction with DA (7+3) [2]. This well-established chemotherapy regimen still provides acceptable survival rates, expanding in the range of 50 to 90% achieved complete remissions [3]. Of course, it is highly understandable that the percent is strictly related to the risk profile of every patient defined by the performance status, comorbidity, age, and by the risk profile of the disease itself. No superior cure that can block the cell cycle of malignant cells and provide superior survival rates is brought to light till present days. Therefore, a big problem arises when we have to confront the patient to a situation of refractory AML. The disease is characterized by fast kinetics giving us little time to choose the best therapeutic approach, and at the same time to make the most benefit of life. So, what shall we do with refractory AML, or better to say how to treat patients with this form of AML, giving them chance of better survival? This question is the hallmark of our attempt to make a small contribution to the battle of defining the treatment option that is most appropriate and most acceptable with these rules of the "game play".

Materials and methods

We made an attempt to identify a possible management solution for patients diagnosed with refractory AML by treating them with salvage chemotherapy regimen, that is FLAG-Ida, but only as an introduction to hematopoietic stem cell transplantation (HSCT), either autologous or allogeneic, depending on the availability of a matched sibling donor.

Our main inclusion criteria for evaluation were patients with refractory AML, confirmed with reevaluation of bone marrow after standard induction therapy (7+3) where we administered FLAG-Ida regimen (fludarabine 30 mg/m^2, AraC 2 g/m^2 for 5 days, idarubicin 10 mg/m^2 for 3 days, and G-CSF 5 micro g/kg from day 0 until neutrophil recovery) and achieved a complete remission as preparation for hematopoietic stem cell transplantation (HSCT) as mandatory further treatment of the disease. We managed to achieve a complete remission in 22 patients, out of 46 analyzed, with refractory AML (47%), and all of them underwent HSCT. Although there is no standardized regimen that will provide a significant benefit for this group of patients [4], still we present our ambitious reports on treating these patients with FLAG-Ida regimen, but only as an induction to HSCT, because we all know that the best and only curative solution can be obtained by performing this procedure, mainly the allogeneic type.

Results

We evaluated 22 patients, diagnosed with refractory AML, or failure to standard induction therapy in the period 2001-2014, 15 males and 7 females (Figure 1). Average age of patients was 36.6 years (17-53) (Figure 2).

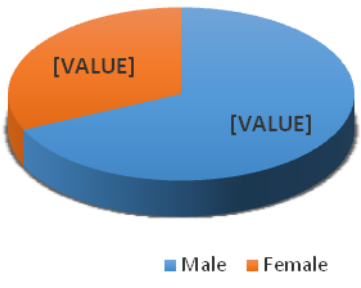


Fig. 1. Sex distribution

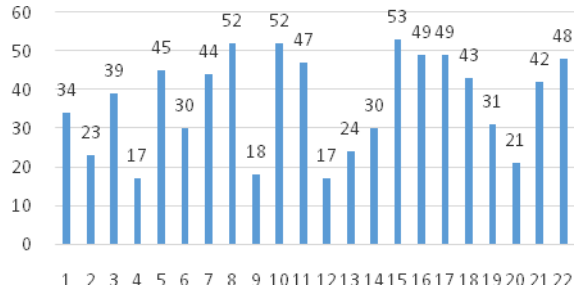


Fig. 2. Distribution by age

Thirteen patients received one course of FLAG-Ida, achieved CR and proceeded directly to HSCT, and 9 patients received two courses. Since no response was received after 1 course in 7 patients for achieving CR, and in 2 patients the chemotherapy was administered as consolidation after achieving CR with the first course. We performed autologous HSCT in 13 patients, and allogeneic in 9 patients (Figure 3).

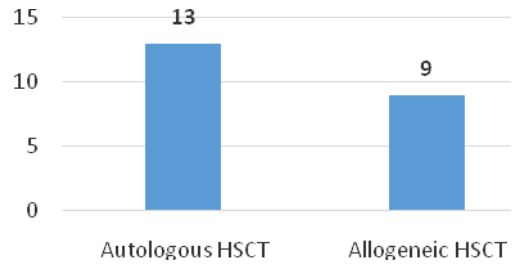


Fig. 3. Distribution of HSCT

In the autologous setting, mobilization of peripheral blood stem cells was performed with etoposide + G-CSF in 5 patients, and G-CSF only in 8 patients. The average number of collected mononuclear cells was 2.9x 10^8/KgTT (1.5-6.5). We used peripheral blood as source of stem cells in the majority of patients, particularly in 16 patients, and bone marrow in 6 patients (Figure 4).

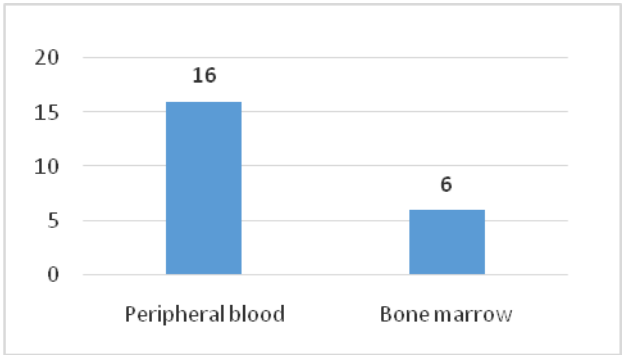


Fig. 4. Source of stem cells

Median time to HSCT was 6.6 months (4-10). In most of the patients we used myeloablative conditioning (MAC) with busulphan+cyclophosphamide (13 patients) and BEAM (8 patients). One patient was conditioned with melphalan high doses (Figure 5). Median time to engraftment was 13.1 days (9-22). No significant increase in supportive treatment with blood products was registered in these patients, and the transplant-related mortality was 0.

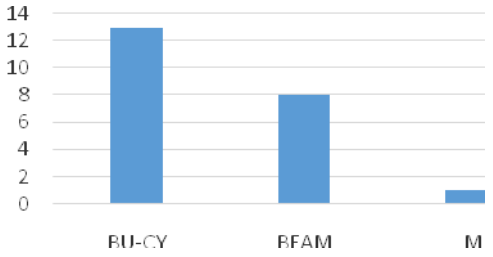


Fig. 5. Conditioning regimens

In the posttransplant period relapse was confirmed in 9 patients. Median time to relapse was 9 months after HSCT. All patients died despite another attempt with chemotherapy, mainly of infectious complications and heart failure. Thirteen patients are still alive.

Of the evaluated relapsed patients, 7 relapses occurred during the first year. Thus, in the follow-up period, during the first year posttransplant, 15 patients (61%) were still in CR (Figure 6).

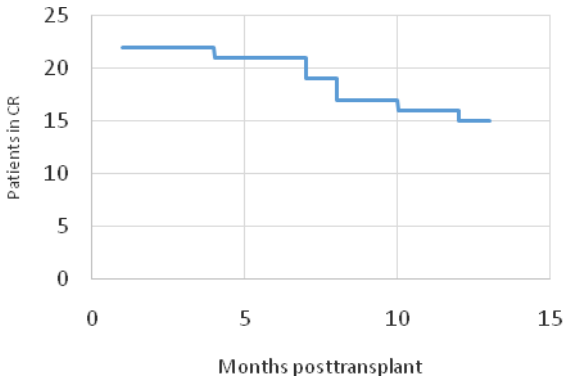


Fig. 6. CR during the 1-st year posttransplant

The disease-free survival (DFS) in our group of patients is 74 months (22-148). The longest period of CR posttransplant is 148 months in a patient with allogeneic HSCT, and registered GvHD. In the autologous setting all relapsed patients were mobilized with G-CSF only. The fastest relapse, or maybe we should say early progression to fatal outcome, was in a patient transplanted in the active disease (4 months).

In the group of relapsed patients after HSCT, the majority received BEAM as conditioning. The de-

cision to proceed with BEAM in this setting was mainly based on the toxicity profile of the planned chemotherapy and the performance status of the patient.

Discussion

We can conclude that FLAG-Ida regimen is an appropriate and suitable salvage chemotherapy protocol for patients with refractory AML especially when it is used in the context of preparation for HSCT.

The statistical analysis of other reports states that the percent of achieving second remission is much lower, and it is in the range of 50% in younger, and 25% in older patients [5]. If a complete remission (CR) is accomplished, then it`s duration is characterized by shortage. To be more accurate, the possibility of sustaining a long and durable remission is very low, and the risk of relapse with more unfavorable outcome is very high in the first year [6].

FLAG-Ida is well tolerated, and it is best to start as soon as possible with the administration of the regimen avoiding further intoxication of the patient. It is reasonable to expect that younger patients will tolerate the regimen better, so we should register better responses. The main point of this analysis is to emphasize the role of HSCT.

If we achieve complete remission in these patients, we have the ground on defending our/hypothesis/statement to administer high-dose therapy and proceed with HSCT. The role of this approach would be to eliminate any possible minimal residual disease. It is highly recommendded to choose allogeneic HSCT, from either sibling, or matched unrelated donor (MUD). Unfortunately, we couldn`t perform MUD allogeneic HSCT in our transplant center, however, the longest disease-free survival was registered in a patient where we performed matched sibling allogeneic HSCT. It is important to state that we also had GvHD in that same patient that was successfully treated in its acute form, but still the patient has skin GvHD of low intensity. This information surely goes in favor of the well-established statement that recognizes the role of Graft versus leukemia effect. The existence of GvL effect may have big influence on the primary objective of allogeneic HSCT that classifies this procedure as the only curative option for patients with hematological malignancies [7].

Maybe someone will still have doubts about the effect of autologous HSCT in this setting. But, using it as a type of consolidation, after achieving CR in this group of patients in order to prolong and sustain the therapeutic response justifies this approach. The main concern is the possibility of contaminating the graft with malignant cells during the mobilization and apheresis of hematopoietic stem cells. In our group of patients, the earliest relapse was in the setting of autologous HSCT, where apheresis of peripheral blood stem cells was done. This graft had the highest probability of contamination with malignant cells that were not eliminated during the induction of remission. That is why it might be more appropriate to perform the mobilization of HSC with chemotherapy plus gowth factor, which can reduce the chance of graft contamination, but this method is not always applicable to all our patients. Unfortunately, we don`t have a 100% accurate technique that can detect single, remained malignant cell. Maybe we should focus on more effective ways of purging the graft in these patients. Maybe the answer is in the new molecular, targeted therapy, or immunological approach [8] that can provide more effective transplantations of HSC for the benefit of the patients.

Regarding the toxicity of this approach we registered no significant increase in the intoxication profile of the patients. Neutropenia in the induction setting and in the posttransplant setting was in the range of the expected duration. We performed infective prophylaxis, and treated accordingly the neutropenia fever based on our center experience and the microbiome that characterizes our environment. We performed the standard supportive treatment with blood and blood products. In patients receiving allogenic grafts we used standard CyA+MTX prophylaxis for GvHD. One major drawback in our analysis is that we did not perform risk stratification of our patients based on their cytogenetic profile. This risk stratification surely would add a lot to the optimal selection in further management of patients [9]. But, we have started from the point that our patients, who have disease refractory to standard induction therapy, are with high probability to be in the high risk profile [10]. Hence, proceeding with an aggressive therapeutic approach, surely balanced on the performance status of the patient and his/her comorbidity profile, may be the only chance of providing survival benefit knowing the fact of poor responses of this group of patients registered in the reports for treating refractory AML.

If another relapse is confirmed after HSCT, then the chance of inducing another remission is very low. In our experience nearly all our attempts were fatal for the patients. The main reasons were heart failure, hemorrhagic or infective complications. This again emphasizes the need of taking with high grip the given chance of achieved CR in this group of patients and making an attempt to sustain this response as long as possible.

Conclusion

Performing autologous or allogeneic HSCT in these patients, combined with a good supportive treatment may provide prolonged survival rates that surely is a significant benefit for patients with this form of the disease, which otherwise is categorized as an entity with poor prognostic features and no therapeutic approach can be recommended as standard of care.

Conflict of interest statement. None declared.

References

1. Jemal A, Thomas A, Murray T, Thun M. "Cancer statistics, 2002". *CA Cancer J Clin* 2002; 52(1): 23-47.
2. Wiernik PH, Banks PLC, Case DC Jr, *et al.* Cytarabine plus idarubicin or daunorubicin as induction and consolidation therapy for previously untreated adult patients with acute myeloid leukemia. *Blood* 1992; 79: 313-319.
3. Flasshove M, Meusers P, Schutte J, *et al*. Long-term survival after induction therapy with idarubicin and cytosine arabinoside for de novo acute myeloid leukemia. *Ann Hematol October* 2000, 79(10): 533-542.
4. Estey EH. Treatment of relapsed and refractory acute myelogenous leukemia. *Leukemia* (08876924). 2000; 14(3): 476-4.
5. Estey E, Kornblau S, Pierce S, *et al*. A stratification system for evaluating and selecting therapies in patients with relapsed or primary refractory acute myelogenous leukemia. *Blood* 1996; 88: 756.
6. Lee S, Tallman MS, Oken MM, *et al*. Duration of second complete remission compared with first complete remission in patients with acute myeloid leukemia. *Leukemia* 2000; 14(8): 1345-1348.
7. Biggs JC, Horowitz MM, Gale RP, *et al*. Bone marrow transplants may cure patients with acute leukemia never achieving remission with chemotherapy. *Blood* 1992; 80: 1090.
8. Feller N1, van der Pol MA, Waaijman T. Immunologic purging of autologous peripheral blood stem cell products based on CD34 and CD133 expression can be effectively and safely applied in half of the acute myeloid leukemia patients. *Clin Cancer Res* 2005; 11(13): 4793-4801.
9. Revesz D, Chelghoum Y, Le QH, *et al.* Salvage by timed sequential chemotherapy in primary resistant acute myeloid leukemia: Analysis of prognostic factors. *Ann Hematol* 2003; 82(11): 684-690.
10. Pastore D, Specchia G, Carluccio P, *et al*. FLAG-IDA in the treatment of refractory/relapsed acute myeloid leukemia: Single-center experience. *Ann Hematol* 2003; 82(4): 231-235.

DE G DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 32-37

MMR

DOI:10.1515/mmr-2017-0007
Original article

DETERMINATION OF FETAL MATURITY USING SIGNAL TRANSFORMATIONS OF FETAL THALAMUS ESTIMATING FETAL MATURITY BY ULTRASOUND

ДЕТЕРМИНИРАЊЕ НА ФЕТАЛНА ЗРЕЛОСТ ПРЕКУ УТВРДУВАЊЕ НА ТРАНСФОРМАЦИИ НА СИГНАЛОТ НА ФЕТАЛНИОТ ТАЛАМУС

Elena Dzikova[1], Goran Dimitrov[1], Olivera Stojceva-Taneva[2] and Gligor Dimitrov[3]

[1]University Clinic for Gynecology and Obstetrics, [2]University Clinic for Nephrology, Medical Faculty, University "Ss.Cyril and Methodius", Skopje, PZU Remedika, Skopje

Abstract

Introduction. The complications associated with preterm birth are still the primary cause of death in children below 5 years of age, leading to nearly 1 million death cases in 2013. We performed our study to examine a new non-invasive method for prediction of fetal maturity.
Methods. The study was designed asa prospective observational-interventional clinical study, conducted at the University Clinic for Gynecology and Obstetrics, Medical Faculty, University Ss. Cyril and Methodius Skopje, Republic of Macedonia.
Ninety pregnant patients were examined, 48 with preterm birth used as examined cases and 42 women above 37 completed weeks of gestation and delivered at term, used as control cases. The investigation was performed before and 72 hours after administration of the therapy protocol for fetal lung maturation. The measurement was done with an ultrasound histogram software, measuring the density of thalamus and surrounding brain tissue. The results were followed up to 72 hours and then compared with the postpartum respiratory distress syndrome (RDS). If the patient was not delivered within 72 hours of measurement, she was excluded from the study.
Results. In the first and in the second gestational age group, we noticed significant fetal maturation. All groups according to nationality and religionshowed high significance before and after treatment. The correlation among the thalamus density vs. surrounding brain tissue and postpartum RDS in all three groups according to gestational age, nationality and religion was high.
Conclusion. Measuring the density of fetal thalamus vs. surrounding brain tissue may become the new non-invasive technique for determination of fetal maturity.

Keywords: ultrasound, fetal maturity, thalamus, density respiratory distress syndrome

Correspondence to: Dzikova Elena, Bul. Ilinden No.59, 1000 Skopje, R. of Macedonia Phpone: +389 70 58 52 22; E-mail: elena.dzikova@gmail.com

Апстракт

Вовед. Компликациите поврзани со предвремено породување се сè уште примарна причина за смрт кај деца под петгодишна возраст, до неодамна, водејќи со речиси еден милион смртни случаи во 2013 година. Нашата метода ја изведовме со цел испитување на новата неинвазивна метода за фетална зрелост.
Методи. Студијата беше дизајнирана како проспективна опсервациско-интервенциска клиничка студија на Универзитетската клиника по гинекологија и акушерство, Медицински факултет, Универзитет "Св. Кирил и Методиј"-Скопје..
Во тек на студијата беа испитани 90 бремени жени, 48 со предвремено породување, кои се користеа како испитувани случаи и 42 над 37 гестациска недела, породени во термин и користени како контролни случаи. Испитувањето се направи пред, како и 72 часа по дадената терапија, по протокол за зреење на феталното белодробие. Мерењето се изведе со ултразвучен хистограмски софтвер, при што дензитетот на феталниот таламус се спореди со дензитетот на околното мозочно ткиво.
Резултатите се следат до 72 часа по завршување на терапијата и потоа се споредуваа со постпарталниот респираторен дистрес синдром (РДС). Пациентките се исклучуваа од студијата, доколку не беа породени до 72 часа од мерењето.
Резултати. Во првата и во втората група беше забележана сигнификантна матурација. Во сите групи, според националност и религија е забележана висока сигнификантна разлика и пред и по третманот. Исто така, постои сигнификантна корелација меѓу ултразвучниот сигнал на феталниот таламус, околното мозочно ткиво и постпарталниот респираторен дистрес синдром (РДС), во сите 3 групи и според г.н., национална и верска припадност.
Заклучок. Ултразвучната техника за мерење фетална зрелост со употреба на сигнална трансформација на феталниот таламус, наспроти околното мозочно ткиво, може да се користи како неинвазивна метода за одредување зрелост на плодот.

Клучни зборови: ултразвук, фетална зрелост, таламус, дензитет, респираторен дистрес синдром

Introduction

According to the World Health Organization, the estimated number of preterm born babies (before 37 completed weeks of gestation) is 15 million, and the number is still rising. Through 184 countries, preterm birth is ranging from 5-18% of born babies.
Preterm refers to babies delivered before completed 37 weeks of pregnancy. Still, there are huge differences in morbidity and mortality rate according to gestational age. The lower the age, the bigger are the complications and morbidity.
The complications associated with preterm birth are still the primary cause of death in children below 5 years of age, leading to nearly 1 million death cases in 2013. Using the current medical knowledge and techniques, ¾ of them could have been saved [1]. Many survivors face a lifetime of disability, including learning disabilities and visual and hearing problems.
The concept of prematurity involves biological immaturity for extra-uterine life. So, maturity means full fetal growth and physical and neurological development. Degree of maturity is the major determinant of mortality and morbidity (the short- and long-term complications) of preterm birth. Therefore, defining the degree of fetal development is very important due to accessible medical knowledge and techniques to save extremely immature babies, aiming to decrease infant morbidity and mortality. Until now, invasive intra-uterine techniques have been used to determine fetal maturity.
The objectives of our study were to examine a new method for prediction of fetal maturity that would be non-invasive, inexpensive, accessible, simple, repeatable, sensitive, specific, and can be performed in routine use in any hospital facility where a good ultrasound device and educated staff are available, thus providing timely prediction of fetal maturity and timely treatment; to determine in which group according to gestational age of the fetus treatment works the best, and in which cases it is necessary to repeat it to get a good fetal maturity, and thus reduce fetal mortality and morbidity, which are among the main objectives of the action Plan of the World Health Organization from 2012 [2] and Healthy people 2020 [3].

Material and methods

In our study we examined 90 pregnant patients. Forty-eight of them had preterm birth, and 42 were delivered at term and were used as control cases. The first 48 patients were divided in three groups. The first group were very preterm 28-30 weeks of gestation, the second were very preterm from 30^{+1}-32 weeks, and the third group were moderate preterm from 32^{+1}-34 weeks of gestation. The control group of 42 patients were 37 and above completed weeks of gestation. The study was conducted at the University Clinic for Gynecology and Obstetrics, Medical Faculty, University Ss. Cyril and Methodius in Skopje, Republic of Macedonia in one-year period.
Inclusion criteria in the study: patients with premature ruptured membranes (PPROM), pain and expected early preterm birth (PPI), or with an expected early caesarean section (PSC). Patients with abnormalities of the fetus, multiple pregnancy, and the presence of a disease in the mother were excluded from the study.
The first group of women from 28-30 weeks of gestation consisted of 10 women, the second group from 30^{+1}-32 weeks included 12 women, and the third group from 32^{+1}-34 weeks involved 26 women. According to nationality, our study examined 24 Macedonian patients, 18 Albanian, and 6 other nationality female patients. In the control group according to nationality there were 30 Macedonian women, 6 Albanian, and 6 other nationality female patients. According to religion we included 30 Christian women, and 12 Muslim female patients.
The protocol for the study was approved by the Ethics Committee of the Medical Faculty, University Ss. Cyril and Methodius and it conformed to the provisions of the Declaration of Helsinki.
The ultrasound machine Voluson Expert E8 was used for examination. Semi-convex ultrasound probe of 3.5 MHz was used trans-abdominally, in the transthalamic plane of the biparietal diameter which was measured in millimeters. The state of echogenicity of the thalamus was measured in comparison with the surrounding brain tissue, which is echogenic through out pregnancy. We used the Histogram software program included in the ultrasound machine to examine the density of the fetal thalamus and we compared it with the density of the surrounding brain tissue. Then we waited our patient to deliver and afterwards we compared our results with the degree of the postpartum respiratory distress syndrome (RDS). If the density of the fetal thalamus was closer to the density of the surrounding brain tissue, the fetus was with better maturation. All measurements were made in patients selected according to the inclusion and exclusion criteria.
The examination was performed before and 72 hours after administration of the therapy, Amp. Betamethasone a 14 mg/II dose/24h, a protocol for fetal lung maturation. The results were followed for up to 72 hours and then compared to the extent of postpartum respiratory distress syndrome (RDS). If the patient was not delivered within 72 hours of measurement, she was excluded from the study!
Statistical analyses were done using standard statistical procedures for data processing, mean, standard deviation, correlation for comparing two groups with numerical variables, analysis of variance for comparing nominal data, and Student's t-test for paired analysis comparing numerical data in the same group before and after

treatment and determination of p value for statistical significance. The value of p <0.05 was considered to be statistically significant. The correlation was made using Spearman's Rho Calculation test.

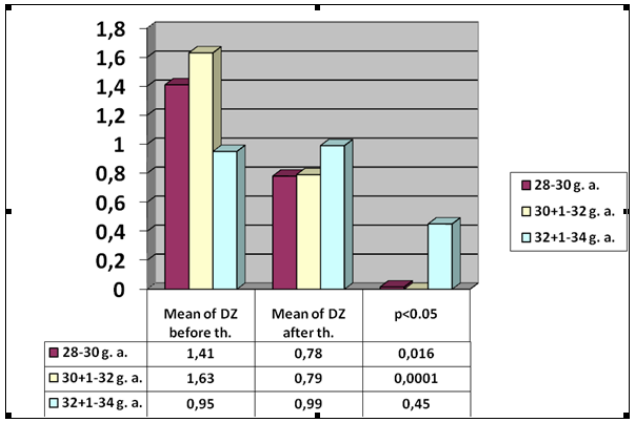
Results

We examined 90 pregnant patients; 48 had imminent preterm birth, and 42 were in term pregnancy (completed 37 weeks of gestation and above). The 48 pregnant patients that had imminent preterm birth and after wards prematurely delivered according to inclusion criteria, were divided into 3 categories:

1. 28-30 weeks of pregnancy (10 women);
2. 30^{+1} – 32 weeks (12 women);
3. 32^{+1} – 34 weeks (26 women).

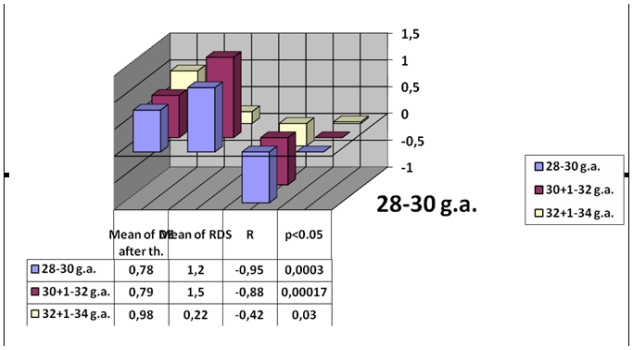
In each group we examined the fetal maturity before treatment and 72h after treatment, and we correlated our results with the postpartum degree of respiratory distress syndrome of the newborn, only if the baby was born in a maximum of three days after the last ultrasound measurement. We also examined the nationality and religion of the patients to see if there was a difference in response to therapy or in the respiratory distress after birth according to that criteria.

Our results presented in Figure 1, showed that in the first (28-30 g.a.) and in the second group (30^{+1}-32 g. a.) a significant fetal maturation was noticed with one course of treatment, p=0.016; and 0.0001, respectively. Opposite to that, there was no significant change in the measurement of the fetal maturity before and after one course of treatment (Amp. Betamethasone a 14 mg/II dose/24h) only in the third group (32^{+1}-34 g. a.) p=0.45.



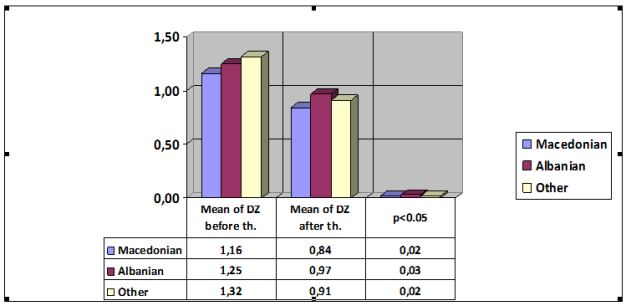
	Mean of DZ before th.	Mean of DZ after th.	p<0.05
28-30 g. a.	1,41	0,78	0,016
30+1-32 g. a.	1,63	0,79	0,0001
32+1-34 g. a.	0,95	0,99	0,45

Fig. 1. Comparing intensity of density in pairs of groups divided according to gestational age before and after treatment
Legend: RDS-respiratory distress syndrome; Th.-treatment; R-correlation; DZ-intensity of density; p-significance; g. a.- gestational age.



	Mean of DZ after th.	Mean of RDS	R	p<0.05
28-30 g.a.	0,78	1,2	-0,95	0,0003
30+1-32 g.a.	0,79	1,5	-0,88	0,00017
32+1-34 g.a.	0,98	0,22	-0,42	0,03

Fig. 2. Correlation of DZ after therapy and postpartum RDS
Legend: RDS-respiratory distress syndrome; Th.-treatment; R-correlation; DZ-intensity of density; p-significance; g. a.-gestational age.

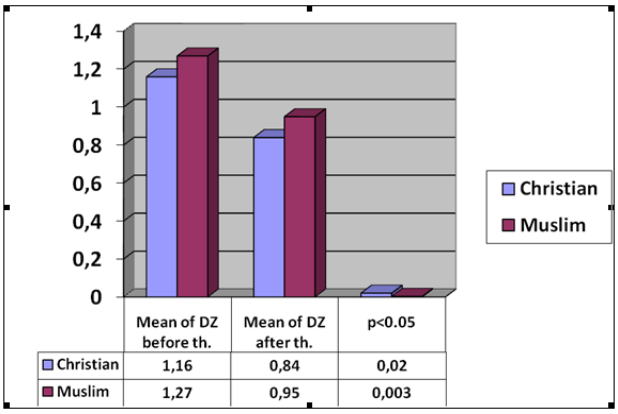


	Mean of DZ before th.	Mean of DZ after th.	p<0.05
Macedonian	1,16	0,84	0,02
Albanian	1,25	0,97	0,03
Other	1,32	0,91	0,02

Fig. 3. Comparing pairs of groups before and after treatment according to nationality
Legend: Th.-treatment; DZ-intensity of density; p-significance.

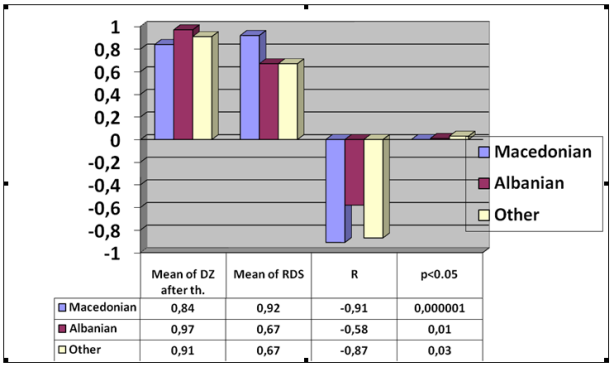
There was a strong correlation between the intensity of thalamus density vs. surrounding brain tissue, and the postpartum RDS in all three gestational groups (p< 0.05) (Figure 2).

There was a significant change in the measurement of fetal maturity before and after one course of treatment (Amp. Betamethasone a 14 mg/II dose/24h) in all three groups according to nationality (Macedonian, Albanian and other), p=0.02; p=0.03; and p=0.02, respectively (Figure 3).



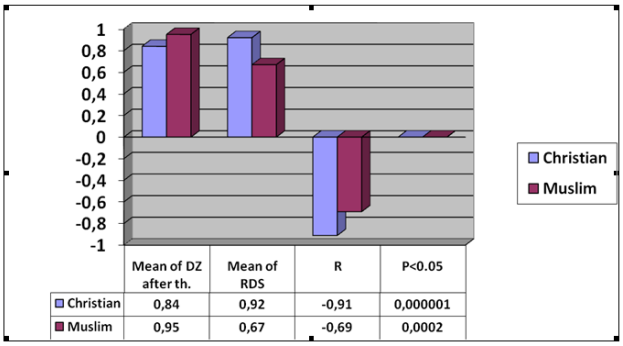
	Mean of DZ before th.	Mean of DZ after th.	p<0.05
Christian	1,16	0,84	0,02
Muslim	1,27	0,95	0,003

Fig. 4. Comparing pairs of groups before and after treatment according to religion
Legend: Th.-treatment; DZ-intensity of density; p-significance.



	Mean of DZ after th.	Mean of RDS	R	p<0.05
Macedonian	0,84	0,92	-0,91	0,000001
Albanian	0,97	0,67	-0,58	0,01
Other	0,91	0,67	-0,87	0,03

Fig. 5. Correlation of DZ after Th. with postpartum RDS according to nationality
Legend: RDS-respiratory distress syndrome; Th.-treatment; R-correlation; DZ-intensity of density; p-significance.



	Mean of DZ after th.	Mean of RDS	R	P<0.05
Christian	0,84	0,92	-0,91	0,000001
Muslim	0,95	0,67	-0,69	0,0002

Fig. 6. Correlation of DZ after Th. with postpartum RDS according to religion
Legend: RDS-respiratory distress syndrome; Th.-treatment; R-correlation; DZ-intensity of density; p-significance.

Also, the results presented in Figure 4, when the patients were divided according to religion, show that there was a significant change in the measurement of fetal maturity before and after one course of treatment in both groups (Christian group, p=0.02; and Muslim group p=0.003).
Furthermore, a strong correlation between the intensity of thalamus density vs. surrounding brain tissue, and the postpartum RDS with a p value of<0.05, according to nationality and religion was observed (Figures 5 and 6).

Discussion

It would be very useful to detect fetal maturity prenatally with a non-invasive technique that is inexpensive, accessible, simple, reproducible, sensitive, specific, and can be performed in routine use in any hospital facility with a good ultrasound device and educated staff, thus providing timely prediction of fetal maturity and timely treatment. It would be also very convenient to predict in which group according to gestational age of the fetus, the treatment works the best, and in which cases it would be necessary to repeat it to achieve good fetal maturity. Such observations inspired several scientists to make different studies to reach these goals.

In 1970, Gluck and Kulovich started to investigate fetal maturity by analyzing the fetal amniotic fluid. They evaluated the Lecithin/Sphingomyelin ratio (L/S) that are glycoproteins consisting the Surfactant. The Lecithin increases the effect of the Surfactant, that is why ratio bigger than 2 is necessary for achieving fetal lung maturity [4-7].
Furthermore, in 2001, Wijnberger *et al.*, and in 2003 Torday and Rehan, introduced lamellar body count as a better laboratory test for detecting fetal maturity in fetal amniotic fluid [8,9]. Yet, these are invasive techniques carrying a risk of 0.5-1% for fetal loss.
In 1997, Duncan *et al.* showed the effect of magnetic resonance to determine the density of fetal liver during pregnancy [10]. It is known that the density of the organs and structures depends on the quantity of fluid stored in them. That is why histogram of the fetal liver was first used for measurement standardization of the fetal lungs intensity signal, presented also in the study of Li *et al.* in 2013 [11].
The idea for performing our study was based on today's knowledge about fetal embryology and neuroscience. Maturational changes in the brain using neuroimaging like magnetic resonance and cranial ultrasound have been noticed first in infants, and then prenatally. Neural development starts very early in pregnancy, 3rd and 4th week of pregnancy, continuing throughout whole pregnancy and is completed after birth. The diencephalon is formed at the 5th week of pregnancy [12]. Myelination is the most important process in the neural development. Myelin is a white fatty matter that wraps the neural axons and has the leading role in cognitive functions and learning. Myelination starts at the spinal cord at about 12 week of gestation. It continues at the brain stem at 14 weeks, thalamic axons at 20 weeks and finally at the cortex at 35 weeks and continues further on throughout decades in life. Severe maternal stress could inhibit intrauterine myelination, and thus, create risk for neurodevelopmental problems and psychic impairment [13-15].
Our study was based on the myelination as a leading process of brain maturation and various density signals in different gestational ages in thalamus vs. surrounding brain tissue.
Since this is a new technique that is still developing, we found a few studies that used echogenicity of thalamus vs. surrounding tissue for determination of fetal maturity.
In the study of Rosier-van Dunne (2007) [16] "echogenicity in thalamus area was seen in 28%. Also, changes in echogenicity were seen throughout the entire gestational-age period studied and at least 32% of the basal ganglia and thalamus echo-densities persisted after delivery. Of course some of them were seen due to maturational process, but others were connected with a risk of pathological processes, such as edema, hemorrhage and gliosis". So, further investigations should

be made to differentiate the physiological and pathological processes.

"The presence of echogenic thalamus as a sign of fetal lung maturity with a specificity of 86.53% which is higher than the three other signs of lung maturity" was seen in the study of Rasheed (2012) [17], "the positive predictive value was (89.6%) which is also higher than the three other signs, but the sensitivity was 63.33% and negative predictive value was 57.69% which is lower than the presence of vernix in the amniotic fluid, 86.66 and 67.56 respectively". He concluded that "evaluation of echogenic thalamus is beneficial, and can be considered as a new marker of fetal lung maturity; however, further studies are required to strengthen such idea".

Our results are similar to those presented in the study of Rasheed. The main findings of our study showed that there was a strong correlation between the intensity of thalamus density vs. surrounding brain tissue and postpartum RDS in all three groups for $p < 0.05$. Also, we noticed a strong correlation between the intensity of thalamus density vs. surrounding brain tissue and postpartum RDS for $p < 0.05$, according to nationality and religion.

On the other hand, in our study there was no significant change in the measurement of fetal maturity before and after one course of treatment (Amp. Betamethasone a 14 mg/II dose/24h) only in the third group (32^{+1}-34 g. a.) p=0.45. In the first (28-30 g.a.) and in the second group (30^{+1}-32 g. a.) a significant fetal maturation was noticed with one course of treatment, p=0.016; and 0.0001, respectively. Then again, our results showed that there was a significant change in the measurement of fetal maturity before and after one course of treatment in the both groups (Christian group, p=0.02; and Muslim group p=0.003).

The strength of our study is that this is a non-invasive technique that is inexpensive, accessible, simple, reproducible, sensitive, specific, and can be performed in routine use in any hospital facility with a good ultrasound device.

Of course, there are limitations, especially if we examine extremely obese patients or if there is oligo/anhydramnos and the ultrasound visibility of the fetal brain structures is restricted.

Conclusion

The ultrasound technique by measuring density of fetal thalamus vs. surrounding brain tissue may become the new non-invasive technique for determination of fetal maturity, and in time, with further extensive investigations, could replace today's invasive techniques in everyday routine practice.

Ethical approval: The protocol for the research project was approved by the Ethics Committee of the Medical Faculty, University Ss. Cyril and Methodius and conformed to the provisions of the Declaration of Helsinki. The Ethics board approval number is 03-5515/5.

Conflict of interest statement. None declared.

References

1. WHO. Preterm birth. Fact sheet N°363 [Internet]. WHO, Media Centre; 2016 [Accessed on: 2016 Aug 5]. Available from: http://www.who.int/mediacentre/factsheets/fs363/en/.
2. WHO. Newborns: reducing mortality Fact sheet N°333 [Internet]. WHO, Media Centre; 2012 [Accessed on: 2015 Mar 25]. Available from: http://www.who.int/mediacentre/factsheets/fs333/en/.
3. Healthy People 2020. Maternal, Infant, and Child Health [Internet]. Washington DC 20201, USA: Office of Disease Prevention and Health, Department of Health and Human Services, USAPromotion; 2014 [Accessed on: 2015 Mar 25]. Available from: https://www.healthypeople.gov/2020/topics-objectives/topic/maternal-infant-and-child-health/objectives.
4. Gluck L, Kulovich MV. Fetal lung development. Current concepts. *Pediatr Clin North Am* 1973; 20(2): 367-379.
5. Whitfield CR, Chan WH, Sproule WB, Stewart AD. Amniotic Fluid Lecithin: Sphingomyelin Ratio and Fetal Lung Development. *BMJ* 1972; 2(5805): 85-86.
6. Wijnberger LDE, de Kleine M, Voorbij HAM, *et al*. Prediction of fetal lung immaturity using gestational age, patient characteristics and fetal lung maturity tests: a probabilistic approach. *Archives of Gynecology and Obstetrics* 2010; 281(1): 15-21.
7. Spillman T, Cotton DB, Painter PC. Current Perspectives in Assessment of Fetal Pulmonary Surfactant Status with Amniotic Fluid. *Critical Reviews in Clinical Laboratory Sciences* 1989; 27(4): 341-389.
8. Wijnberger LD, Huisjes AJ, Voorbij HA, *et al*. The accuracy of lamellar body count and lecithin/sphingomyelin ratio in the prediction of neonatal respiratory distress syndrome: a meta-analysis. *BJOG* 2001; 108(6): 583-588.
9. Torday JS, Rehan VK. Testing for fetal lung maturation: a biochemical "window" to the developing fetus. *Clin Lab Med* 2003; 23(2): 361-383.
10. Duncan KR, Baker PN, Gowland PA, *et al*. Demonstration of changes in fetal liver erythropoiesis using echo-planar magnetic resonance imaging. *Am J Physiol* 1997; 273(4 Pt 1): G965-G967.
11. Li X, Brugger PC, Huang M, *et al*. Signal intensity changes of the fetal liver on MRI in-phase and out-of-phase sequence: Fetal liver signal intensity changes. *Prenatal Diagnosis* 2013; 1-5.
12. Hill MA. Embryology Neural-Thalamus Development. In: Embriology [Internet]. 2016 [accessed 2016 Aug 5]. Available from: https://embryology.med.unsw.edu.au/ embryology/index. php/Neural_-_Thalamus_Development.
13. Miller JH, McKinstry RC, Philip JV, *et al*. Diffusion-tensor MR imaging of normal brain maturation: a guide to structural development and myelination. *AJR Am J Roentgenol* 2003; 180(3): 851-859.
14. Welker K, Patton A. Assessment of Normal Myelination with Magnetic Resonance Imaging. *Seminars in Neurology* 2012; 32(01): 015-028.
15. Linderkamp O, Janus L, Linder R, *et al*. Time table of normal foetal brain development. *International Journal of Prenatal and Perinatal Psychology and Medicine* 2009; 21: 4-16.
16. Rosier-van Dunne FMF, van Wezel-Meijler G, Odendaal HJ, *et al*. Changes in echogenicity in the fetal brain: a

prevalence study in fetuses at risk for preterm delivery. *Ultrasound Obstet Gynecol* 2007; 29(6): 644-650.
17. Rasheed FA, Al-Sattam ZM, Hussain SA. Evaluation of thalamus echogenicity by ultrasound as a marker of fetal lung maturity. *Open Journal of Obstetrics and Gynecology* 2012; 2(3): 270-275.

DE GRUYTER
OPEN

Mac Med Review 2017; 71(1): 38-43

MMR

DOI:10.1515/mmr-2017-0008
Original article

CROSSOVER ALTERNATIVES OF DEFAULT RIGHT RADIAL ARTERY ACCESS FOR ACUTE MYOCARDIAL INFARCTION INTERVENTION

АЛТЕРНАТИВИ НА РАДИЈАЛНИОТ АТЕРИСКИ ПРИСТАП ЗАИНТЕРВЕНЦИЈА ПРИ АКУТЕН МИОКАРДЕН ИНФАРКТ

Oliver Kalpak, Hristo Pejkov, Gjorgji Kalpak, Marija Jovanoska, Biljana Zafirovska and Sasko Kedev

University Clinic of Cardiology, University "Ss Cyril and Methodius", Medical Faculty, Skopje, Republic of Macedonia

Abstract

Introduction. Being a default transradial access center we have encountered the need for alternative to the right radial artery. Determining the most frequent alternative access strategy for PCI in STEMI patientswasthe focus of our study. We sought to analyze whether the wrist access strategy impacts procedure time and success rate particularly for the STEMI interventions where time is of paramount importance.
Methods. During four years, in our Center 2624 consecutive all-comers STEMI patients underwent urgent coronary intervention. TRA was used as the first-choice access strategy. We sought to assess crossover rate and safety of preferable access strategy. Crossover occurred according to the operator's decision. Primary outcomes were: access site crossover rate and In Lab time, secondary outcomes were PCI time, X-ray time, mortality and MACE at 30 days and at 6 months.
Results. Overall crossover rate from default radial was 5.4% (144 out of 2624 patients). We treated 98.7% (2589) patients by wrist access and only 1.3% (35) patients with TFA. Crossover towards left radial occurred in 47.9% (69 out of 144 patients), towards ulnar 27.8% (40 patients) and towards TFA only 24.3% (35 patients). The meanIn-Lab time 40.4±17.7 minutes, PCI time was 21.4±7.4 min, X-ray time 9.2±4.7 minutes. Survival outcomes at 30 days were: MACE rate of 6.6% (174 patients), mortality rate of 5.0% (131 patients). At six months MACE rate was 8.6% and mortality rate was 5.6%.
Conclusions. Default radial access is associated with alow crossover rate. Crossover towards femoral occurred less frequent than ulnar artery access.Complete wrist access strategy is safe and feasible for STEMI interventions with low mortality and MACE rate in unselected all-comers cohort.

Correspondence to: Oliver Kalpak, University Clinic of Cardiology-Skopje, "Vodnjanska" 17, 1000 Skopje, R. Macedonia; Phone: 070 27 09 23; E-mail: kalpakoliver@gmail.com
Keywords: TRA,TUA, TFA, access crossover, STEMI, myocardial infarction, PCI

Апстракт

Вовед. Трансрадијалната стратегија е наш прв избор за пристап, затоа се наметнува потребата за алтернативен пристап при неуспешна рутинска стратегија при интервенција за акутен миокарден инфаркт. Постои недостиг од податоци за стратегијата на кросоверод примарниот радијален пристап кон алтернативниот. Цел на оваа студија е одредување на насоката и времето потребно за алтернативана ТРА пристапот.
Методи. Во тек на четиригодишниот период 2010 до 2014 година беа извршени коронарни интервенции кај 2.624 СТЕМИ пациенти. Податоците од регистарот за интервенции на клиниката ги анализиравме во поглед на користениот артериски пристап и неговата алтернатива, времето потребно за пристап како примарни цели, додека секундарни цели беа морталитетот и несаканите кардиоваскуларни настани по првите 30 и 180 денови.
Резултати. Преминот кон алтернативен пристап од примарниот десен радијален беше со ниска рата од 5.4% или кај 144 од 2.624 пациенти. Кросовер кон користење на левата радијална артерија беше најчест со 47% (69 од 144 пациенти). Улнарната артерија беше користена кај 27,8% (40 од 144 пациенти), што е почесто во споредба со феморалната 24,3% (35 од 144 пациенти). Времето потребно за да се премине кон алтернативен пристап не надминува 10 минути. Средното вкупно време за интервенција од 17,7 минути, времето за ПКИ 21,4±7,4 минути, зрачење 9,2±4,7 минути. Секундарните цели, по 30 дена беа 6.6% за ратата на мајорни кардиоваскуларни настани, додека стапката на морталитетот изнесуваше 5.0%. По шест месеци следење, несакани настани забележавме кај 8.6%, додека морталитетот изнесуваше 5.6%.
Заклучок. Користењето на артериски пристап преку десната радијална артерија рутински е асоциран со ниска стапка на потреба од кросовер. Левата радијална артерија и улнарната артерија се значително

почести, побрз избор во однос на феморалната артерија, како алтернативен пристап кај СТЕМИ интервенциите.

Клучни зборови: трансрадијален, трансулнарен, артерискипристап, ПКИ, СТЕМИ.

Introduction

Urgent percutaneous coronary intervention (PCI) in acute phase of myocardial infarction with ST elevation (STEMI) has been established as the most effective treatment, consequently, modalities for improvement and development still remain a challenge for research. One such step is the proposal for routine use of transradial access (TRA) as opposed to the traditional transfemoral (TFA) access strategy for PCI in STEMI [1,2]. Inseveral recent studies, it was suggested that this simple and elegant change in arterial approach for intervention can improve PCI outcomes and save human lives [3,4]. Access for urgent PCI through smaller radial artery instead of the large and less accessible femoral artery has shown to reduce the risk of bleeding, increase the mobility of the patient immediately after the PCI and thereby improve treatment outcome [4-6].
During the past ten years, we experienced a gradual shift from the TFA to TRA, namely right radial artery, as the first-choice artery access for STEMI patients. Being the oldest and largest Cath-lab in the country we made most interventions nationwide. The change towards radial approach puts the Clinic on the map as the leader in the region for TRA in STEMI PCI as well asin elective PCI. The shift towards TRA was conducted in order to improve the clinical outcome of the coronary intervention, reduce bleeding complications and reduce the hospital stay of patients [7,8]. In order to preserve the improved outcomes, we continue to choose the small wrist arteries as default without resorting to the traditional femoral access strategy. The transition to TRA as thedefault strategy reached a near 100% in our Cath-lab, while TFA became rarely used access strategy [9-11]. Our study represents a retrospective exploration of the data extracted from the Registry during the period of four years, from 2010 to 2014. Without any exception, we included all patients with PCI for STEMI in acute phase of myocardial infarction. There are a few studies that use a near 100% TRA as a first-choicestrategy in the regular routine. As we moved towards a TRA default approach we started to encountera certain number of patients where the right radial artery cannot be cannulated. The main reason for the right radial access failure can be anatomical variation or pathological due to previous transradial puncture [4].The alternative access are the remaining arteries of the wrist consisting of the right ulnar, left radial and left ulnar artery as well as the groin femoral artery [5]. Wesought to analyze whether the wrist access strategy impacts procedure time andsuccessrate particularly for the STEMI intervenetions where time is of paramount importance.

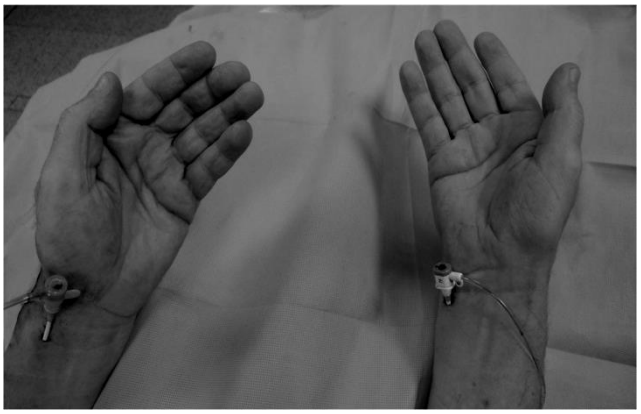


Fig. 1. Example ofsimultaneous alternative access; Left radial and Right ulnar

Objectives

1. To determine the wrist alternatives to default TRA.
2. To assess outcomesof using TRA as the default approach for STEMI intervention.
3. Compare our data with data from other TRA Registers.

Materials and methods

We analyzed data from the Registry of the Department of Interventional Cardiology atthe University Clinic of Cardiology in Skopje between 2010 and 2014. During this period of four years a total of 2624 STEMI patients had a coronary intervention with stent placement. The registry data was analyzed regarding successful default access and the alternative access, time needed for access, door to balloon time, the rate of major adverse cardiovascular events (MACE) observed at 30 days and 6 months respectively as well as the rate of mortality. The decision of access strategy lies solely on the operator judgement. At the beginning of the procedure, once access is secured, an angiography of the wrist arteries isacquired. This routine angio-road map helps us to diagnose possible anatomical and pathoanatomical findings. The procedures and data related to this research are entirely in accordance with established standards of the Clinic, which are under constant monitoring bythe Clinic's expert collegium. The data are open to audit and evaluation by the health administration and authorities.
Criteria for inclusion are:

1. Intervention in the first 12 hours of chest pain symptoms.
2. ST elevation of at least 1mm shown in two ECG leads.
3. Absence of contraindications for antiplatelet and anticoagulant treatment before, during and after the intervention.

Exclusion criteria: 1. Refusing the medical and interventional treatment by the patient in the acute phase. 2. Disproving and rejecting the MI diagnosis. 3. Active bleeding or bleeding diathesis.
Percutaneous vascular access is the main focus ofthis study; however, we must emphasize that the treatment and intervention inall STEMI patients was done in accordance with thecurrent standards and well established recommendations of good medical practice. Intensive anticoagulation and antiplatelet medication package is used by default. Loading before PCI with intravenous bolus of unfractionated heparin (70-100 IU/kg) along with full dose of aspirin (acetylsalicylic acid 300 mg) and clopidogrel (600mg), followed by a sustainment regimen of dual antiplatelet therapy (DAPT) [7,12,13]. The methods and materials for PCI are equal for all participants regardless of the arterial access. The operator selects which of the standard types of guide-catheters will be used for the procedure, standard shapes like Judkins, Amplatz, EBU, JCL with size of 6Fr, depending from the anatomy of the patient and the artery in question. The choice of stent type DES (drug eluting stent) or BMS (bare metal stent), nor the dimensions of the stent affect the choice of access. Percutaneous access to the artery is in the focus for this study. The standard use of introducer set for support is equal in function, size and composition regardless of chosen access artery. The introducer or sheath presents a system made from polyethylene tube with additional valve and drain tube through which the necessary catheters and guidewires can pass safely. The puncture and access procedure is performed by the Seldinger's technique [14-16].
Right radial artery was default first choice. In absence of palpable right radial pulsation, the operatorchooses an alternative access. Alternatives for the right radial artery are right ulnar artery, left radial artery and left ulnar artery. Access through the wrist arteries allows for an immediate removal of the introducer after the procedure and achieving hemostasis with a simple compression bandage or specific inflatable compression track similar to default TRA [15-18].Whether the access is on the right, left radial artery or ulnar artery the compression is gradually relieved over a period of two hours and completely removed 4 hours after its application. In case of bleeding from the wrist access site the compression is kept on for an additional one to two hours. Conversely, the femoral artery hemostasis is associated with prolonged immobility, carries a greater risk for bleeding, and in the case of bleeding is far more difficult to manage. [17,18]

Study endpoints

Primary end point. Our primary endpoint was to determine access crossover site and crossover rate from default right radial artery. Furthermore, we assessed the success of the default access strategy, and the proportion of the right radial alternatives.
Secondary endpoints were clinical outcome measures, rates of MACE and mortality after 30 day and 6 months.
MACE represents a composite endpoint that includes events of death, recurrent MI, stroke, major hemorrhage event not specificto the arterial access, and a repeat revascularization of an intervened coronary artery. Death was defined as every death event that happened within the follow-up periodthat may or may not be causal to the intervention [19].

Statistical analysis

The numerical variables are expressed in values of frequency or represented as a percentage; the said values are medians taken from the minimum and maximum values that did not fit into the symmetric normal distribution and consequently are compared to the non-parametric x2 test. The variables with normal distribution are correlated with Pearson correlation coefficient and compared with Student's t-test. Categorical variables are presented in terms of frequency or percentage and compared with Spearman's correlation, because of the asymmetric distribution curve. SPSS package was used for data analysis [20,21].

Results

Over the course of 4 years our interventional Center treated 2624 patients with acute myocardial infarction.

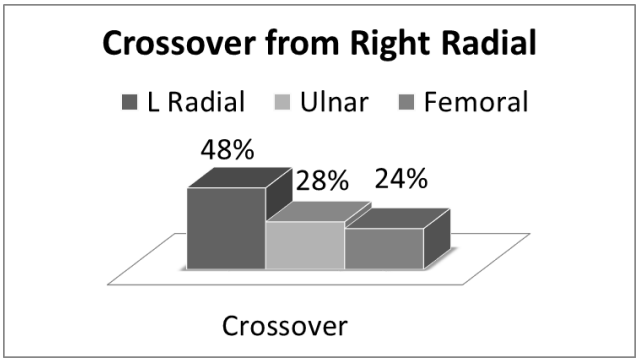


Fig. 2. Primary endpoint

All patients underwent a standardized attempt to puncture the right radial artery as first-choice access for this intervention and it was successful for waste majority. The need of alternative access is represented

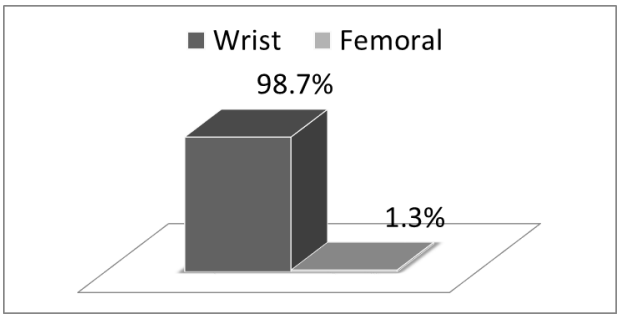


Fig. 3. Wrist access in2589 patients vs. femoral in35 patients

with relatively low rate-5.4% (144) patients. When the primary access was inaccessible we turned to the other wrist arteries, contralateral radial artery or ipsilateral ulnar as well as contralateral ulnar artery. The patients where wrist access was not feasible we turned to groin access TFA.

We treated 98.7% (2589) patients by wrist strategy compared to 1.3% (35) treated through TFA. The cross-over from the primary access, the right radial artery, left radial artery was used in 2.6% (69). The ulnar artery was used in 1.6% (40). The additional time for alternative access change ranges on average from 5 to 10 minutes but never exceeds 20 minutes.

The reasons for change from the primary access of the right radial artery are anatomical variations rendering the artery unusable for uncomplicated intervention as well as inability to puncture the artery.

Anatomical variation was the most common reason that consists of 43% of all changes to alternative artery.

Table 1. Our Registry compared to others

Center/Author	N	% Radial	% Crossover
Macedonia/Kalpak	2624	94.5	1.3
UK/Mammas	48603	39.1	5.1
UK/Hetherington	1051	54.2	7.7
Holland/ Vink	2209	96.1	3.8
Belgium/Dangoisse	3600	46.4	1.3
Italy /Vaglimigli	11068	27.7	7.7-3.5

The anatomically complex tortuous radial artery was visualized after successful puncture and radiographic image. Anatomical variation of the intersection of the artery ranging up to 360 degrees was the most frequent reason for failure to advance the catheter safely without severe artery spasms and pain. This characterizes a valid reason to change the approach and try a different artery.

Impalpable right arterial artery was the second reason for alternative access strategy (34% of total access changes). We speak about impalpable pulsation above the radial artery with inability to puncture. In this group of patients there is no successful puncture made in the right radial artery. After the alternative access was made we proceededwith angiography to conclude with certainty the reasons for being unable to use the primary access strategy. The most often encountered reasons were: a proximal or distal anatomical variation of the radial artery, high pressure in the radial artery, occluded radial artery, as well as low blood pressure. In a clinical setting, a STEMI patient with the afore mentioned reasons urgesus to look for an alternative access strategy.

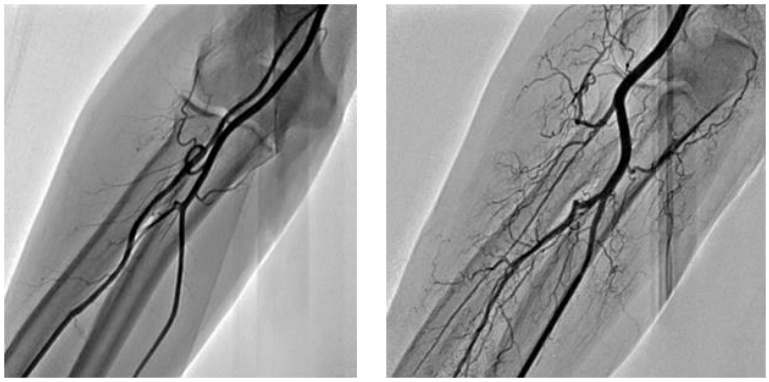


Fig. 3. Major crossover reasons, radial artery loop and occlusion

Table 2. Secondary endpoints

N=2624	n	%
30 d mortality	131	5.0
30 d MACE	174	6.6
6 m mortality	147	5.6
6m MACE	226	8.6

The secondary objectives of the clinical outcome after 30dayswere6.6% rate for MACE while the mortality rate was 5%. At6 months'follow-up, we had 8.6% rate for MACE while the mortality rate was 5.6%.

Discussion

This study includes a large study sample from our clinical intervention register for acute myocardial infarction over the period of four years. We analyzed the data from the register and compared the alternative access strategy with the primary access strategy. The comparison of our results with the relevant studies that examined this field of study showed a significantly

high percentage in radial access use as primary access strategy in STEMI interventions [22]. Our analysis represents a descriptive overview of the current practice in our clinical center which in turn results in several important findings.

The descriptive analysis was done using non-parametric statistical methods because of the asymmetry of distribution, including a relatively low percentage of changing the primary right radial access strategy with the other accessible arteries of the wrist. The x2 test was used for comparing the medians of the variables, which carries a burden of low statistical power. This is underlined when considering that it only accounts for 5.4% of the patients for whomthe access strategy change was made. Moreover, the objecttives ofthis studyare dimensioned appropriately for this statistical power with the idea of descriptively describing the alternative access strategy without measuring them separately with consequently ascertaining which one of them is better as an approach. Therefore, the statistical power for that kind of analysis in the study is low.

Using the arteries of the wrist as a viable alternative access strategy of the primary right radial arteryis in accordance with the well-established and scientifically proven strategy in the relevant literature. The arteries are smaller in dimension which makes them safer for intervention in patients athigh risk of having and/or developping MI. The findings of our study are comparable with the observations inrelevant studies made on this topic [22,23]. Namely, Heterington *et al.* (2009) observed MACE rates of 7.3% in 571 TRA patients compared to 13.3% in 480 TFA group of patients, which is in agreementwith our rate of 6.6% with a similar procedural success [15]. The results from the first major randomized study RIFLE STEACS (Radial versus Femoral Investigation in ST Elevation Acute Coronary Syndrome) with a sample of 1001 patients where group of 500 TRA and 501 TFA were compared showed that the radial strategy wasassociated with a lower rate of MACE as well as lower mortality rate in comparison with the femoral access strategy (30 day point mortality 5.2% vs. 9.2% 95% CI; OR=2.4; 0.8-7.3; p=0.02). This research can confidently show a finding of 5.6% early mortality risk, considering the data is from a register opposed to a randomized study model. It is common that selected study findings have relatively low MACE and mortality rates in comparison tothe registry-based studies where the cases are not selected [24,25].

The four-year practice at our interventional Center dominantly handles TRA interventions in STEMI with a relatively favorable outcome in both MACE and mortality rate, which is achieved with negligible use of TFA access strategy. This suggests that we can safely use the wrist arteries over the more traditional groin approach in everyday practice. The estimated 5.6% early mortality riskin our study is comparatively lower than half of the registry-based studies. Analysis report by the American Register on 90.879 patients from the database of NCDR (North American National Cardiovascular Data Registry, Cath-PCI Registry), has showed that TRA is independently associated with a lower intrahospital mortality rate from 8.3% to 6.7% for TFA and bleeding (OR=0.62; 95% CI=0.53-0.72). Therefore, the authors are committed topromoting TRA as a strategy to reduce complications and improve outcome [23]. Analysis report of the British Register of 46.128 patients of whom 30% TRA, suggests an independent association of radial strategy with a lower mortality rate (HR=0.75; $p<0.05$) as well as a lower MACE rate (HR=0.73; $p<0.05$) [26].

Ulnar access strategy in STEMI patients represents a hot topic for discussion in the interventional academic cardiology society, as well as a potential significant subject for additional scientific research. Therefore, weassumethis study sheds some light on this topic and contributes to the scientific body of knowledge.

It is important to emphasize that our descriptive study examines the correlation between the successful use of the specific alternatives on the right TRA in order to promote the use of wrist arteries as an access strategy in STEMI patients as a possible safe and reliable choice. However, it is imperative to note that the objective of this study is not to determine whether the ulnar artery is a better choice over the use of femoral artery as an access strategy. There was a very small number of patients accessed by femoral artery to make any reasonable comparison.

Conclusion

Our findings suggest that the use of the right radial artery as a first choice foraccess is associated with a low rate of crossover. If the need of an alternative access strategy arises the wrist alternatives offer better patient outcome compared to the traditional femoral access strategy.

Conflict of interest statement. None declared.

References

1. Cannon CP, Braunwald E, McCabe CH, *et al*. The Thrombolysis in Myocardial Infarction (TIMI) trials: the first decade. *J Interv Cardiol* 1995; 8(2): 117-135.
2. Bahit MC, Cannon CP, Antman EM, *et al*. Direct compareson of characteristics, treatment, and outcomes of patients enrolled versus patients not enrolled in clinical trial at centers participating in the TIMI 9 Trial and TIMI 9 Registry. *Am Heart J* 2003; 145: 109-117.
3. Hartzler GO, Rutherford BD, Mc Conahy DR, *et al*. Percutaneous transluminal coronary angioplasty with and without thrombolytic therapy for treatment of acute myocardial infarction. *Am Heart J* 1983; 106: 965-973.
4. Mamas MA, Ratib K, Routledge H, *et al*. Influence of arterial access site selection on outcomes in primary percutaneous coronary intervention. Are the results of randomized trials

achievable in clinical practice? *JACC Cardiovasc Interv* 2013; 6(7): 698-706.
5. Doyle BJ, Ting HH, Bell MR, *et al*. Major femoral bleeding complications after percutaneous coronary intervenetion: incidence, predictors, and impact on long-term survival among 17,901 patients treated at the Mayo Clinic from 1994 to 2005. *JACC Cardiovasc Interv* 2008; 1(2): 202-209.
6. Hamon M, Pristipino C, Di Mario C, *et al.* Consensus document on the radialapproach in percutaneous cardiovascular intervenetions: position paper by the EAPCI and Working Groups on Acute Cardiac Care and Thrombosis of the European Society of Cardiology. *EuroIntervention* 2013; 8(11): 1242-1251.
7. O'Gara PT, Kushner FG, Ascheim DD, *et al.* 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American Collegeof Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol* 2013; 61: e78-140.
8. Bernat I, Horak D, Stasek J, *et al*. STEMI-RADIAL: a prospective randomized trial of radial vs. femoral access in patients with ST-segment elevation myocardial infarction. Paper presented at: Transcatheter Cardiovascular Therapeutics (TCT) Scientific Symposium; October 26, 2012; Miami, FL.
9. Kedev S. Radial or femoral approach for patients with acute coronary syndrome. *Cardiology International* 2012: 45-49.
10. Mehran R, Pocock S, Nikolsky E, *et al*. Impact of bleeding on mortality after percutaneous coronary intervention results from a patient-level pooled analysis of the REPLACE-2 (Randomized Evaluation of PCI Linking Angiomax to Reduced Clinical Events), ACUITY (Acute Catheterization and Urgent Intervention Triage Strategy), and HORIZONS-AMI (Harmonizing Outcomes with Revascularization and Stents in Acute Myocardial Infarction) trials. *J Am Coll Cardiol Intv* 2011; 4: 654-664.
11. Kiemeneij F, Laarman GJ, Odekerken D, *et al.* A randomized comparison of percutaneous transluminal coronary angioplasty by the radial, brachial and femoral approaches: The ACCESS study. *J Am Coll Cardiol* 1997; 29: 1269-1275.
12. Mehta SR, Tanguay JF, Eikelboom JW, *et al.* Double-dose versus standard-dose clopidogrel and high-dose versus low-dose aspirin in individuals undergoing percutaneous coronary intervention for acute coronary syndromes (CURRENT-OASIS 7): a randomized factorial trial. *Lancet* 2010; 376: 1233-1243.
13. Kaev M, Kalpak O. Pharmacological Treatment of Acute Myocardial Infarction. *Mak Med Pregled* 2000; 44: 1-135.
14. Popma JJ, Bashore TD. Qualitative and quantitative angiography. In: Topol E, editor. Textbook of interventional cardiology. *Philadelphia: WB Saunders* 1994: 1052-1068.
15. Hetherington SL, *et al*. Primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction: changing patterns of vascular access, radial versus femoral artery. *Heart* 2009; 95: 1612-1618.
16. Rao SV, Cohen MG, Kandzari DE, *et al*. The transradial approach to percutaneous coronary intervention: historical perspective, current concepts, and future directions. *J Am Coll Cardiol* 2010; 55: 2187-2195.
17. Mangin L, Bertrand OF, De la Rochelliere R, *et al*. The trans-ulnar approach for coronary intervention: a safe alternative to transradial approach in selected patients. *J Invasive Cardiol* 2005; 17(2): 77-79.
18. Andrade PB, Tebet MA, Andrade MV, *et al.* Primary percutaneous coronary intervention through transulnar approach: safety and effectiveness. *Arq Bras Cardiol* 2008; 91(4): e49-52.
19. Mehran R, Rao SV, Bhatt DL, *et al*. Standardized bleeding definitions for cardiovascular clinical trials: a consensus report from the Bleeding Academic Research Consortium. *Circulation* 2011; 123: 2736-2747.
20. Altman DG. Relation between several variables. In: Altman DG, ed. Practical statistics for medical research. London: *Chapman and Hall* 1991; 325-361.
21. Moher D, Dulberg SC, Wells G. Statistical Power, Sample Size, and Their Reporting in Randomized Controlled Trials. *JAMA* 1994; 272: 122-124.
22. Jolly SS, Amlani S, Hamon M, *et al*. Radial versus femoral access for coronary angiography or intervention and the impact on major bleeding and ischemic events: a systematic review and meta-analysis of randomized trials. *Am Heart J* 2009; 157: 132-140.
23. Rao SV, Ou FS, Wang TY, *et al*. Trends in the prevalence and outcomes of radial and femoral approaches to percutaneous coronary intervention: A report from the National Cardiovascular Data Registry. *J Am Coll Cardiol Intv* 2008; 1: 379-386.
24. Romagnoli E, Biondi-Zoccai G, Sciahbasi A, *et al*. Radial versus femoral randomized investigation in ST-segment elevation acute coronary syndrome: the RIFLE-STEACS (Radial Versus Femoral Randomized Investigation in ST-Elevation Acute Coronary Syndrome) study. *J Am Coll Cardiol* 2012; 60: 2481-2489.
25. Jolly SS, Yusuf S, Cairns J, *et al*. Radial versus femoral access for coronary angiography and intervention in patients with acute coronary syndromes (RIVAL): a randomized, parallel group, multicenter trial. *Lancet* 2011; 377(9775): 1409-1420.
26. Mamas MA, Ratib K, Routledge H, *et al.* Influence of access site selection on PCI-related adverse events in patients with STEMI: meta- analysis of randomized controlled trials. *Heart* 2012; 98(4): 303-311.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 44-49

MMR

DOI:10.1515/mmr-2017-0009
Original article

LANDMARK VERSUS ULTRASOUND-GUIDED SUBCLAVIAN CENTRAL VENOUS CATHETERIZATION WITH A COMBINED SHORT AND LONG AXIS APPROACH IN AN INTENSIVE CARE SETTING

АНАТОМСКА НАСПРОТИ УЛТРАЗВУЧНО ВОДЕНА ЦЕНТРАЛНА ВЕНСКА КАТЕТЕРИЗАЦИЈА НА ПОТКЛУЧНА ВЕНА СО КОМБИНИРАН НАДОЛЖЕН И ПОПРЕЧЕН ПРИСТАП ВО ЕДИНИЦА ЗА ИНТЕНЗИВНО ЛЕКУВАЊЕ

Darko Sazdov[1], Marija Jovanovski Srceva[2] and Zorka Nikolova Todorova[3]

[1]Clinical Hospital AcibademSistina-Skopje, General Intensive care Unit, [2]University Clinic of Traumatology Orthopedics Anesthesia and Resuscitation and Intensive Care Medicine, Skopje, [3]European Eye Hospital, Skopje, Republic of Macedonia

Abstract

Introduction. Central venous catheterization of the subclavian vein can be achieved with a landmark and an ultrasound-guided method. Using ultrasound the vein can be catheterized with a long axis in plane or a short axis out of plane approach and a combined approach. The aim of the study was to compare the success, average number of attempts and mechanical complication rate between the landmark and the combined ultrasound-guided method.
Methods. A total of 162 adult patients from the Intensive Care Unit at Clinical Hospital Acibadem-Sistina, Skopje were included in this prospective study. Patients randomized in the examined group (n=71) were catheterized with real-time ultrasound guidance with a combined short axis out of plane and long axis in plane method. Patients randomized in the control group (n=91) were catheterized with the landmark method. Subclavian vein was catheterized in both groups. Overall success, success on first attempt, number of attempts and complications at the moment of catheterization were the main outcome measures.
Results. Catheterization using the landmark method was successful in 94.5% of patients, 65.9% of which during the first attempt. Cannulation using real-time ultrasound guidance was successful in all patients with a first pas success of 83.1%. The complication rate in the ultrasound group was 2.82% and 16.5% in the landmark group (p=0.004404).
Conclusion. Real-time ultrasound guidance with a combined short axis out of plane and long axis in plane approach improves success, decreases number of attempts, and reduces mechanical complications rate.

Correspondence to: Darko Sazdov, Clinical Hospital Acibadem Sistina-Skopje, General Intensive care Unit, street Skupi 5a, Skopje 1000, R. Macedonia; Phone: 070 27 86 20; E-mail: dr.sazdov@gmail.com

Keywords: landmark, ultrasound, subclavian vein

Апстракт

Вовед. Централна венска катетеризација на потклучната вена секојдневно се спроведува со помош на методата на анатомски маркери или ултразвучно водена метода. При ултразвучната метода може да се примени надолжен или попречен пресек на крвиот сад. Целта овој труд е да се спореди успехот, просечниот број обиди, и механичките компликации при катетеризација меѓу овие две методи.
Методи. Во оваа проспективна студија беа вклучени 162 возрасни пациенти од единицата за интензивно лекување (ЕИЛ) во Клиничката болница Ацибадем Систина, Скопје. Пациентите во испитуваната група (n=71) беа катетеризирани со ултразвучно водена метода со комбинација на надолжен и попречен пресек. Кај пациентите во контролната група (n=91) централен венски катетер беше поставен со помош на надворешни анатомски обележја т.н. слепа катетеризација. Кај двете групи пациенти беше катетеризирана потклучната вена и беа следени, успешноста, бројот на убоди до успешна катетеризација и појава на компликации во моментот на катетеризација.
Резултати. Катетеризацијата со помош на слепата метода беше успешна кај 94,5% и тоа кај 65,9% при првиот обид, а успешноста со ултразвучно воденатa метода беше 100% и тоа 83,1% при првиот обид. Процентот на компликации во ултразвучно воденатa група беше 2.82%, додека во анатомската метода беше 16.5% (p=0.004404).
Заклучок. Ултразвучно воденатa метода со комбинација на надолжен и попречен пристап за катетеризација на потклучната вена, ја зголемува успешноста, го намалува бројот на обиди за катетеризација како и механичките компликации асоцирани со оваа постапка.

Клучни зборови: слепа катетеризација, ултразвук, потклучна вена

Introduction

The use of subclavian vein (SCV) for central venous catheterization was firstly described by Aubaniac, 60 years ago. Since then it has been widely used to gain access to the central venous circulation with a landmark and an ultrasound-guided method [1].
Most practitioners prefer subclavian venous access versus other commonly used sites for central venous catheterization, due to the lower incidence of complications (thrombosis, infection), better patient comfort and especially because it can be safely used in hypovolemic patients [2-6]. But, subclavian vein catheterization is associated with mechanical complications such as arterial puncture, hematoma, pneumothorax, hemothoraxandnerve injury. The rate of these complications with the landmark or "blind" method is 18.8% and is correlated to operator experience [2]. Although ultrasound guidance during subclavian vein catheterizationcan improve success rate, and reduce the rate of mechanical complications, complications still occur [4-7]. Other studies on the same topic do not show a significant improvement in these outcomes for ultrasound-guided subclavian vein catheterization when compared to the landmark method [8,9].
Ultrasound-guided subclavian vein catheterization can be achieved either with short axis out of plane approach or long axis in plane approach [7-9]. Both approaches have advantages and pitfalls and complications are still encountered. For thisreason during ultrasound-guided catheterization of the subclavian vein we use a combination of a short axis out of plane approach and a long axis in-plane approachin an attempt to overcome the pitfalls of both approaches. In our daily practice we use both landmark and ultrasound for subclavian vein catheterization.
The aim of this study was to compare the successfulness of central vain catheter placement in the subclavian vein with thiscombinedultrasound method (short axis-SAX out of plane approach and a long axis-LAX in-plane approach) to the landmark method. Additionally we recorded the average number of attempts and mechanical complication ssuch as arterial puncture, pneumothorax and hematoma formation.

Materials and methods

The study was prospectively conducted in the general intensive care unit at the Clinical Hospital Acibadem-Sistina, Skopje, from January 2016 to January 2017 and included 162 adult (18-70 year old) mechanically ventilated patients (EngstrÖmCarestation GE). After the approval of the ethical board at our Hospital, patients with an indication for central venous catheterization wereincluded in the study. Patients were divided into two groups, examined group (n=71) where for the CVC placement the combined ultrasound technique was used and control group (n=91) where CVC insertion was done with the landmark method. In both groups of patients the subclavian vein was used to get access to the central venous circulation. The placement of the CVCineach group was done by 5 experienced doctors for both procedures and under sterile technique (sterile cap, mask and gown).
A normal chest radiography was used to assess the placement of the catheter's tip after the procedure and to check for pneumothorax [10,11]. Mechanical complications were defined as arterial puncture, hematoma greater than 1 centimeter in diameter, and pneumothorax. Arterial puncture was recognized by the pulsatile flow of bright red blood from the needle, hematoma formation by the presence of a bruise and swelling greater than 1 centimeter in diameter, and pneumothorax by chest x-ray done 6 hours after the procedure. The assessed outcomes were: the success of placement overall and on first pass, the average number of attempts needed for successful placement (defined asa number ofskin punctures), and the rate of mechanical complications, arterial puncture, pneumothorax and hematoma formation.

Landmark technique

For the landmark technique, the patient was placed in a Trendelenburg position with the skin of the anterosuperior part of the chest prepared and draped in a sterile fashion with disinfection solution (povidone–iodine or chlorhexidine). After administration of 1% lidocaine with a 22- gauge needle in the designated area the needle for central venous catheterization was inserted 1 centimeter inferior and 1 centimeter lateral to the junction of the middle and lateral two thirds of the clavicle thus using the infraclavicular approach[3]. The return of venous blood into the syringe attached to the needle confirmed entry into the vessel. Then, the central venous catheter was placed using the Seldinger's technique[11-14].

Ultrasound method

For the ultrasound-guided subclavian vein catheterization we also used the infraclavicular approach [4].
Before starting the procedure an ultrasound exam of the infraclavicular area was done on both sides to confirm the position, relation to the subclavian artery, diameter and patency of the subclavian vein. The area on the selected side was prepared (skin disinfection with 2% chlorhexidine, local anesthesia with lidocaine 1%) and draped as described above. An e-Logic General Electric ultrasound machine equipped with a high-

resolution 5-10-MHz transducer was used. The transducer was covered with ultrasonic gel and wrapped in a sterile manner.

We used a combination of a short axis out of plane approach and a long axis in plane approach. First the ultrasound probe was oriented to give the subclavian artery and vein in a transverse short axis view. The differentiation between artery and vein was done by applying pressure and watching for size reduction and collapsibility, and by using Doppler. Then the probe was manoeuvred to get the vein in the center of the screen. The needle was inserted at the middle of the long axis of the probe using the out of plane approach and was advanced slowly to the center of the anterior wall of the vein. When the anterior wall of the vein wasap proached the probe was redirected for 90 degrees to depict the vein and needle in its long axis (long axis in plane approach). The needle was then further advanced to puncture the anterior wall of the vein with continuous aspiration through the syringe. When the anterior wall was punctured and venous blood aspirated the needle tip was positioned in the center of the vein and the guidewire advanced under ultrasound-guidance. After that the procedure was the same as with the landmark method using the Seldinger's technique.

Results

There were 162 patients included in the study. Patients were divided in two groups, ultrasound-guided group with 71 patients and landmark group with 91 patients. The two groups were homogenous regarding age and sex. Patients characteristics and side of catheterization are shown in Table 1.

The main outcomes in both examined and control groups are shown in Table 2.

Table 1. Study population characteristics

	Examined group n=71	**Control group n=91**	**t value**	**p value**
Age (M/±SD)	60.56±11.75	58.96±13.19	0.8070816	0.420816
Right side (side cannulated, %)	136/68%	168/84%	Chi-square:4.73911 p=0.029484	
Left side (side cannulated, %)	64/32%	32/16%		
Male/female	42(59.2%)/29(40.8%)	55(60.4%)/36(39.6%)	Chi-square:0.027396 p=0.968537	

Table 2. Outcome measures in examined versus control group

	Examined group n=71	**Control group n=91**	**p value**
Success (n/%)	71/100%	86/94.5%	ChiSquare: 4.0253 0.044822
Success on first attempt (n/%)	59/83.1%	60/65.9%	ChiSquare: 6.0265 P=0.014093
Average number of attempts (n/±SD)	1.21±0.5	1.52±0.855	t=2.76 P=0.0066
Arterial puncture(n/%)	0/0%	6/6.593%	p=0.03542
Pneumothorax(n/%)	0/0%	7/7.64%	p=0.018498
Hematoma(n/%)	2/2.82%	12/13.29%	p=0.023557
Overall complications(n/%)	2/2.82%	15/16.5%	P=0.004404

n-numberofpatients

Ultrasound-guided subclavian vein catheterization had an overall success of 100% with a first pass success of 83.1%. The reason for this was the inability to insert the guidewire. In thesecases (12 patients) another skin puncture was needed. In 6 patientsthe cause for the failed guidewire insertion was not discovered. In three patients it was due to coiling of the guidewire at the insertion site, in one due to vein puncture at the valve, and in 2 due to the low cross-sectional area of the vein as a result of severe dehydration. Success in the landmark group was 94.5% with 65.9% patients being successfully catheterized on the first attempt. In the landmark group thesecond and third attempts were needed since the vein could not be locatedin 18 patients and in 8 patients it was difficult to insert the guidewire. The difference between the two groups wasstatistically significant for overall success and success on first attempt for $p<0.05$ ($p= 0.044822$ and $p=0.014093$, respectively). Ultrasound guidance resulted in a reduction of the average number of attempts needed for successful catheterization (1.52±0.5 to 1.21±0.855) ($t=2.76$ $p=0.0066$).

Mechanical complications were also reduced using ultrasound guidance. The complication rate in the ultrasound group was 2.82% and 16.5% in the landmark group ($p=0.004404$).

There were no arterial punctures and no pneumothorax in the ultrasound-guided group and two patients had a hematoma formation on the puncture site. In the landmark group 6/6.593% patients had an arterial puncture (p=0.03542), 7/7.64% patients had a pneumothorax (p=0.018498) and 12/13.29% had a hematoma (p=0.023557).

Discussion

The subclavian vein is 3-4 cm long. It lies posterior to the medial third of the clavicle; anterior to the anterior scalene, the brachial plexus, and the subclavian artery; and superior to the first rib [5].

Recent studies have compared the use of ultrasound to the landmark approach in subclavian vein catheterization. A prospective randomized control trial by Fragou *et al*. and recent meta-analyses showed improved success rate, lower number of attempts, and lower rate of mechanical complications including arterial puncture, hematoma formation and pneumothorax [9,17-19].This was also true for less experienced surgeons (92% vs 44%) [6]. In the study by Fragou ultrasound guidance was found to improve success rates, 100% vs. 87.5%, and reduce the rate of mechanical complications, including arterial puncture hematoma formation, and pneumothorax (4.9%) using ultrasound.

Ultrasound-guided subclavian vein catheterization can be learned on simulation models quickly when compared to the landmark technique. In a study by Tokumine *et al*., 20 medical trainees received instruction on both landmark and ultrasound-guided subclavian vein catheterization. Ultrasound-guided subclavian vein catheterization was achieved with three attempts compared to nine for the landmark technique [7].

Ultrasound-guided central venous catheterization of the subclavian and axillary vein is being done with a short axis out of plane approach and a long axis in plane needle approach [8]. By putting the ultrasound probe perpendicular to the course of the vessel a short axis view is obtained (Figure 1).

This view allows visualization of the targeted vein, artery and surrounding structures, and offers the surgeona good midline orientation, but it does not give a good appreciation of needle depth. Using the "out-of-plane" needle-guided approach, the needle is shown as a hyperechoic dot on the ultrasound screen. This can be the needle tip or any part of the needle shaft [9].

Alternatively, the longitudinal, or long axis view, is obtained with the transducer and vessel axes in parallel (Figure 2).

This view shows the targeted vessel along its length. When this view is coupled with an "in-plane" needle tip approach, visualization of both needle tip and needle shaft during catheterization is achieved [10].

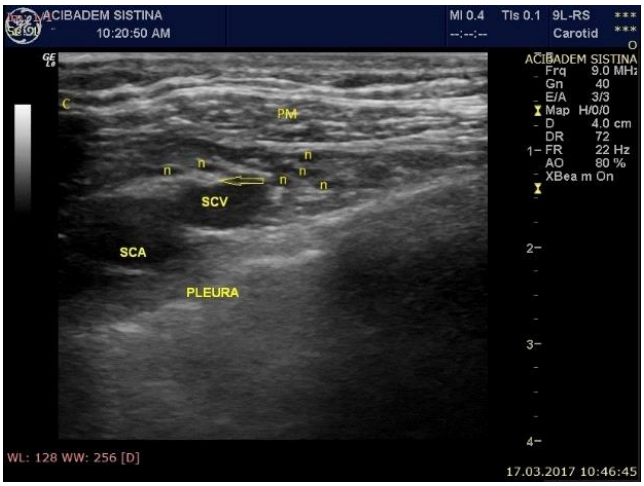


Fig. 1. Short axis view of the subclavian vein and needle C-clavicle, n-branches of the brachial plexus, PM-pectoralis muscle, SCV-subclavian vein, SA-subclavian artery, Pleura, the arrow points the position of the needle

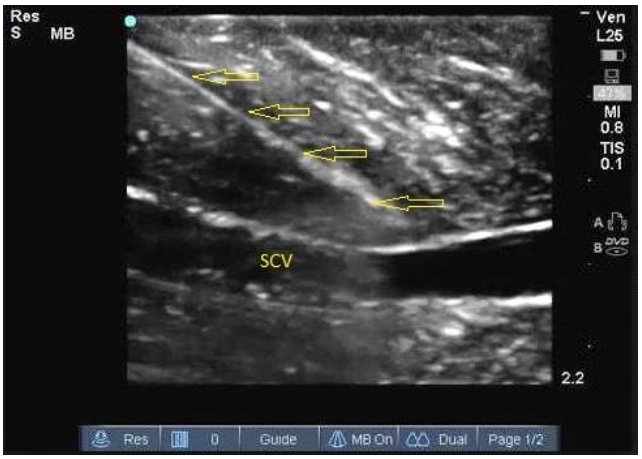


Fig. 2. Long axis view of the subclavian vein with an in plane needle approach SCV-subclavian vein, the arrows point the needle as it approaches the vein

The needle is controlled directly when puncturing the anterior wall and entering the target vessel. Also, the posterior wall of the vessel and deep surrounding structures including the pleura, and the guidewire direction of travel are shown. This can reduce the posterior vessel wall puncture and consequently the risk for pneumothorax [11]. The approach also has been demonstrated to decrease the rate of arterial puncture and hematoma formation and to significantly increase overall success rate, with fewer attempts, redirections, or malpositioned catheters [9,17,18].

The challenge with the "in-plane" technique is to have the needed dexterity to line up the one millimeter thickness of the sound beam with the one millimeter thickness of the needle. All of this should be done in the axis that goes through the centre of the vein. But, in longitudinal in-plane view it is difficult to precisely locate the center of the vein. In that case, the off-centre pressure on the anterior wall of the vein may result in a dimple that is pushed on the lateral wall next to it

which may result in a double wall puncture [12]. When using the longitudinal approach usually one cannot see the vein and the artery at the same time like in short axis view. Due to sliding of the probe a misidentification of the vein with the nearby arteryis possible because they look similar in long axis view especially in a no compressible area. Another problem with the longitudinal in-plane approach is the 'side-lobe' artefact. If the needle is slightly out of the plane of the ultrasound beam, the needle appears to be in the plane of the sonographic beam [13]. The consequences of these problems may be a failure of the procedure or an injury to the nearby structures [12].

Emergency medicine residents and attending physiccians with different experience in ultrasound catheterization compared the short and long axis approach for axillary vein catheterizationusing a torso phantom model in a prospective crossover study [14]. They preferred the long axis approach. This approach led to better first pass success rate, fewer needle redirections and complications. In another prospective study the ability of medicine students to get an adequate view for catheterization of the subclavian vein was assessed. The long axis view led to quicker access time, reduced redirections, and significantly fewer posterior wall penetrations compared to the short axis orientation [15].

The short and long axis were also compared in 83 cardiac surgery patients and it was found that the first-pass success rate was significantly higher in the short-axis group (73%) compared tothe long-axis group (40%) ($P = 0.005$). The procedure time, number of attempts, needle redirection, and skin and vessel punctures were significantly lower in the short-axis than long-axis group ($P < 0.05$). There was no significant difference in the overall number of complications between groups although arterial puncture and hematoma were seen more in the long axis in plane approach.Also, the need to allocate the patient in the other group was more frequent with the long-axis approach [16].

In an attempt to overcome the pitfalls with the short and long axis approach we use a combination of these two. The short axis is better for side to side orientation and for targeting the center of a vessel. Using this approach in the beginning we avoid potential nerve and artery damage and target the centre of the vein. After approaching the proximity of the anterior wall of the vein we use the long axis view to prevent posterior wall punctureand to visualize the guidewire while entering the vessel. There was a difficulty to position the probe in the longitudinal view but all participants managed to get a good view of the vein and needle and to successfully complete the procedure.

Using the landmark method, we hadan overall success rate of 94.5% and an incidence of mechanical complications of 16.5% that were both comparable to previous studies [5,6,10,11].

The overall success of the procedure with our technique was 100% and the first pass success was 83.1%, which is comparable or slightly better than in other studies. The complication rate was 2.82% with no arterial puncture and no pneumothorax, which is also in line or better than previous reports [4,18,30].

Conclusion

Ultrasound-guided central venous catheterisation in intensive care patients of the subclavian vein is superior to the landmark with better success rate and fewer complications than the latter.

Conflict of interest statement. None declared.

References

1. Aubaniac R. Subclavian intravenous injection; advantages and technic. *La Presse medicale* 1952; 60(68): 1456.
2. Ruesch S, Walder B, Tramer MR. Complications of central venous catheters: internal jugular versus subclavian access-a systematic review. *Critical care medicine* 2002; 30(2): 454-459.
3. Mansfild PF, Hohn CD, Fornage DB, *et al*. Complications and failures of subclavian-vein catheterization. *New England Journal of Medicine* 1994; 331(26): 1735-1738.
4. Shiloh AL, Eisen LA, Yee M, *et al*. Ultrasound-guided subclavian and axillary vein cannulation via an infraclavicular approach: in the tradition of Robert Aubaniac. *Critical care medicine* 2012; 40(10): 2922-2923.
5. Fortune JB, Feustel P. Effect of patient position on size and location of the subclavian vein for percutaneous puncture. *Archives of Surgery* 2003; 138(9): 996-1000.
6. Gualtieri E, Deppe SA, Sipperly ME, Thompson DR. Subclavian venous catheterization: greater success rate for less experienced operators using ultrasound guidance. *Critical care medicine* 1995; 23(4): 692-697.
7. Tokumine J, Matsushima H, Lefor K, *et al*. Ultrasound-guided subclavian venipuncture is more rapidly learned than the anatomic landmark technique in simulation training. *The journal of vascular access* 2014; 16(2): 144-147.
8. Pirotte T. Ultrasound-guided vascular access in adults and children: beyond the internal jugular vein puncture. *Acta anaesthesiologica Belgica* 2007; 59(3): 157-166.
9. Troianos CA, Hartman GS, Glas KE, *et al*. Guidelines for performing ultrasound guided vascular cannulation: recommendations of the American Society of Echocardiography and the Society of Cardiovascular Anaesthesiologists. *Journal of the American Society of Echocardiography* 2011; 24(12): 1291-1318.
10. Rezayat T, Stowell JR, Kendall JL, *et al*. Ultrasound-Guided Cannulation: Time to Bring Subclavian Central Lines Back. *Western Journal of Emergency Medicine* 2016; 17(2): 216.
11. Blaivas M AS, Blaivas M, Adhikari S. An unseen danger: frequency of posterior vessel wall penetration by needels during atemts to place central catheters during ultrasound guidance. *Critical Care Medicine* 2009; 37: 2345-2349.
12. Tokumine J, Lefor AT, Yonei A, *et al*. Three-step method for ultrasound-guided central vein catheterization. *British journal of anaesthesia* 2013; 110(3): 368-373.

13. French JL, Raine-Fenning NJ, Hardman JG, Bedforth NM. Pitfalls of ultrasound guided vascular access: the use of three/ four-dimensional ultrasound. *Anaesthesia* 2008; 63(8): 806-811.
14. Sommerkamp SK, Romaniuk VM, Witting MD, *et al*. A comparison of longitudinal and transverse approaches to ultrasound-guided axillary vein cannulation. *The American journal of emergency medicine* 2013; 31(3): 478-481.
15. Vogel JA, Haukoos JS, Erickson CL, *et al*. Is long-axis view superior to short-axis view in ultrasound-guided central venous catheterization? *Critical care medicine* 2015; 43(4): 832-839.
16. Corradi F, Manca T, Brusasco C, *et al*. Real-time ultrasound-guided subclavian vein cannulation in cardiac surgery: comparison between short-axis and long-axis techniques. *Critical Care* 2014; 18(1): 1.
17. Fragou M, Gravvanis A, Dimitriou V, *et al*. Real-time ultrasound-guided subclavian vein cannulation versus the landmark method in critical care patients: a prospective randomized study. *Critcal Care Medicine* 2011; 39(7): 1607-1612.
18. Brass P, Hellmich, Kolodziej L, *et al*. Ultrasound guidance versus anatomical landmarks for subclavian or femoral vein catheterization. *Cochrane Database Syst Rev* 2015; (1).
19. Lalu MM, Fayad A, Ahmed O, *et al*. Ultrasound-guided subclavian vein catheterization: a systematic review and meta-analysis. *Critical care medicine* 2015; 43(7): 1498-1507.
20. Aslamy Z, Dewald CL, Heffner JE. MRI of central venous anatomy: implications for central venous catheter insertion. *CHEST Journal* 1998;114(3): 820-826.
21. Karakitsos D, Nikolaos L, De Groot E, *et al*. Real-time ultrasound-guided catheterisation of the internal jugular vein: a prospective comparison with the landmark technique in critical care patients. *Critical Care* 2006; 10(6): R162.
22. Seldinger IS. Catheter replacement of the needle in percutaneous arteriography: a new technique. *Acta radiologica* 1953; 39(5): 368-376.
23. Marik PE, Flemmer M, Harrison W. The risk of catheter-related bloodstream infection with femoral venous catheters as compared to subclavian and internal jugular venous catheters: a systematic review of the literature and meta-analysis. *Critical care medicine* 2012; 40(8): 2479-2485.
24. Shah A, Smith A, Panchatsharam S. Ultrasound-guided subclavian venous catheterisation-is this the way forward? A narrative review. *International journal of clinical practice* 2013; 67(8): 726-732.
25. McGee DC, Gould MK. Preventing complications of central venous catheterization. *New England Journal of Medicine* 2003; 348(12): 1123-1133.
26. Merrer J, De Jonghe B, Golliot F, *et al*. Complications of femoral and subclavian venous catheterization in critically ill patients: a randomized controlled trial. *Jama* 2001; 286 (6): 700-707.
27. Lane P, Waldron RJ. Real-time ultrasound guided central venous access via the subclavian approach. *Anesth Intensive care* 1995; 23: 728-730.
28. Bodenham AR. Ultrasound-guided subclavian vein catheterization: beyond just the jugular vein. *Critical care medicine* 2011; 39(7): 1819-1820.
29. Palepu GB, Deven J, Subrahmanyam M, Mohan S. Impact of ultrasonography on central venous catheter insertion in intensive care. *Indian Journal of Radiology and Imaging* 2009; 19(3): 191.
30. Randolph AG, Cook DJ, Gonzales CA, Pribble CG. Ultrasound guidance for placement of central venous catheters: a meta-analysis of the literature. *Critical care medicine* 1996; 24(12): 2053-2058.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 50-55

MMR

DOI:10.1515/mmr-2017-0010
Original article

ROLE AND CLINICAL SIGNIFICANCE OF IL-33 IN PATIENTS WITH ASTHMA

УЛОГАТА И КЛИНИЧКОТО ЗНАЧЕЊЕ НА IL-33 КАЈ ПАЦИЕНТИ СО АСТМА

Elena Jovanovska Janeva[1], Angelko Gjorchev[1], Biserka Kaeva[1], Gorica Breskovska[1], Zlatica Goseva[1], Marija Zdraveska[1], Deska Dimitrievska[1], Zoran Arsovski[1], Sava Pejkovska[1], Jordan Minov[2], Dejan Trajkov[3], Magdalena Genadieva Dimitrova[4] and Beti Zafirova [5]

[1]PHI University Clinic of Pulmonology and Allergology, [2]Institute for Occupational Health of RM, [3]Institute of Immunobiology and Human Genetics, [4]PHI University Clinic of Gastroenterohepatology, [5]Institute of Epidemiology and Biostatistics, Faculty of Medicine, University "Ss Cyril and Methodius" Skopje, Republic of Macedonia

Abstract

Introduction. Asthma is a chronic inflammatory disease of the airways in which many cells play a role with secreting a variety of mediators responsible for the clinical manifestation of asthma. It is assumed that IL-33 is one of the earliest-released mediators and can orchestrate the immune cascade of the disease.
The aimof this study was to examine the role and clinical significance of IL-33 as a new and insufficiently explored mediator of inflammation in patients with uncontrolled moderate asthma.
Methods. The study included 87 patients with asthma. Serum IL-33 was measured in all patients by ELISA method. The obtained data were statistically analyzedusing the Kolmogorov-Smirnov and Shapiro-Wilk's test. Qualitative data were presented in absolute and relative numbers, and quantitative data were presented with measures of descriptive statistics. Statistically significant values were considered forp <0.05.
Results. Majority of included patients were female (75.86%). The average age of patientswas 42.3±15.9 years. The results of IL-33 in all patients were significantly increased compared to the reference value of IL-33 which is 0pg/ml. The average values of IL-33 ranged from 6.47±29.3 and they were insignificantly higher in the group with female patients compared to males (p=0.27), and insignificantly correlated with age (p=0.26).
Conclusion. Even though a limited number of studies haveexplored the IL-33, results have shown higher serum level of IL-33 in asthma patients compared to healthy people, emphasizing the factthat IL-33 is an attrac tive candidate for targeted therapy and prognosis inasthma patients.

Correspondence to: Elena Jovanovska Janeva, University Clinic of Pulmonology and Allergology, 1000 Skopje, Republic of Macedonia; Phone: 072 23 25 13; E-mail: eli.janeva@yahoo.com

Keywords: asthma; IL-33; ST2; IL-1 family

Апстракт

Вовед. Астмата е хронично инфламаторно заболување на дишните патишта во кое учествуваат многубројни клетки, кои излачуваат мноштво медијатори одговорни за клиничката слика на болеста. Се претпоставува дека IL-33 е еден од првите медијатори, кои се излачуваат на самиот почетокот и ја оркестира целата имунолошка каскада на болеста.
Целтана трудот е дефинирањенаулогата и на клиничкото значењето на медијаторот на инфламацијата IL-33, како нов и недоволно истражен медијатор кај пациенти со неконтролирана средна астма.
Методи. Во студијата се вклучени 87 пациенти со астма и кај сите е измеренсерумски IL-33 со ELISA метод. Добиените податоци беа статистички обработени со Kolmogorov-Smirnov и Shapiro-Wilk'stест. Квалитативните податоци беа презентирани со апсолутни и со релативни броеви, квантитативните податоци беа прикажани со мерките на дескриптивна статистика. За статистички сигнификантни беа земени вредностите на p<0.05.
Резултати. Во истражувањето, во поголем број беа вклучени испитаниciод женски пол (75.86%). Испитаниците беа на просечна возраст од 42.3±15.9 години. Добиените резултати за IL-33 кај сите пациенти беа сигнификатно зголемени, во однос на референтната вредност за IL-33, која изнесува 0 pg/ml, додека просечните вредности на IL-33 се движеа од 6.47±29.3 и покажаа дека се несигнификантно повисоки во групата на женски испитаници, споредено со машките (p=0.27), и дека несигнификантно корелираат со возраста на испитаниците (p=0.26).
Заклучок. И покрај лимитираноста на бројот на студии кои го истражуваат IL-33, сепак, резултатите укажуваат дека е зголемно нивото на серумскиот IL-33 кај астматичарите, во однос на здравите

лица, истакнувајќи дека IL-33 е атрактивен кандидат за таргетирана терапија и прогноза на астмата.

Клучни зборови: астма, IL-33, ST2; IL-1 фамилија

Introduction

Asthma is a worldwide problem and is one of the most common chronic diseases which affect more than 334 million people worldwide [1-3]. It is estimated that the number of people with asthma will grow by more than 100 million by 2025. Women were more likely than men and boys more likely than girls to have asthma [4-6]. Approximately 500,000 annual hospitalizations are due to asthma, and 250 000 deaths annually [3,7,8]. In the Republic of Macedonia 100,000 or 5% of the population suffer from asthma [9,10].

Asthma is a chronic inflammatory disease of the airways in which many cells and cellular elements play a role (mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and dendritic cells). The chronic inflammation is associated with airway hyperrsponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment [11].

Chronic inflammation of the asthmatic airway leads to epithelial desquamation, infiltration of the airway wall with T cells especially dominated Th2 helper-CD4+ lymphocytes, **smooth muscle** hypertrophy and hyperplasia, vascular congestion, edema due to plasma leakage and mucus plugging [12-17]. All these changes could lead to thickening of the airway walls due to subepithelial fibrosis and reduction of their lumen [18,19].

In this chronic inflammation are involved more than 100 mediators and the cytokines take central place in this inflammation (they are proteins with low molecular weight produced by almost all eukaryotic cells and act through specific receptor "cell surface"). Cytokines are often produced in cascades, as one cytokine stimulates its target cells to produce additional cytokines. Different cell types may secrete the same cytokine, or for a single cytokine may act on several different cell types. Cytokines can also act synergistically with two or more cytokines acting together or antagonistically with cytokines causing opposing activities. Cells that produce cytokines are B cells, T cells, dendritic cells, NK, Tc, Th, Th1, Th2, endothelial cells, mast cells, plasma cells, progenitor, bone marrow, thymus and tumor cells together with fibroblasts, leukocytes, monocytes and macrophages [20-21].

The interleukins are cytokines that stimulate the proliferation and differentiation of immune cells. IL-1 activates T cells, IL-2 stimulates the proliferation of antigen-activated T and B cells; IL-4, IL-5 and IL-6 stimulated proliferation and differentiation of B cells; interferon-gamma (IFNγ) activates macrophages while IL-3, IL-7, and (GM-CSF) stimulate hematopoiesis [22,23].

T cells play a key role in coordinating the immune response in asthma. The key to the functioning of T cells is a molecule that binds to the antigen: T cells receptor. Generally the T cells which have the CD4+ act as helper cells (Th2-Ly), and CD8+ act as cytotoxic cells (Tc-Ly). CD4+ helper cells, differentiate into subpopulations of T cells in Th1, Th2, Th9, Th17, Th22 and T follicular effectors cell. Th2 cellsproduce, IL-4, IL-5, IL-9, IL-13, GM-CSF andIL-25, IL-31, IL-33 that are responsible for chronic eosinophilic inflammation, inflammation in allergic diseases, including asthma [22-27].

Interleukin-33 (IL-33) is a novel cytokine which was found in 2005. It belongs to the IL-1 family consisting of 11 members, high proinflammatory cytokines which play a key role in the early asthmatic responses. IL-33 is a potent type 2-inducing cytokine. It can bind to receptors ST2, which is highlyexpressed on some cells, including mast cellsand Th2 cells. It is found in various cells including fibroblasts, bronchial and epithelialcells, endothelial cells, and some immune cells, as well asma crophages and dendritic cells [28-31].

It is assumed that IL-33 is one of the earliest-released mediators and can orchestrate the immune cascade of asthma and stands out as an attractive candidate for discovering various therapeutic modalities, especially a new targeted therapy (Figure 1).

The limited numbers of studies which have investigated IL-33 with their results have shown an increased level of serum IL-33 in asthmatics compared to healthy subjects and pointed out that IL-33 is an important cytokine for correct diagnosis, evolution, treatment and prognosis of asthma.

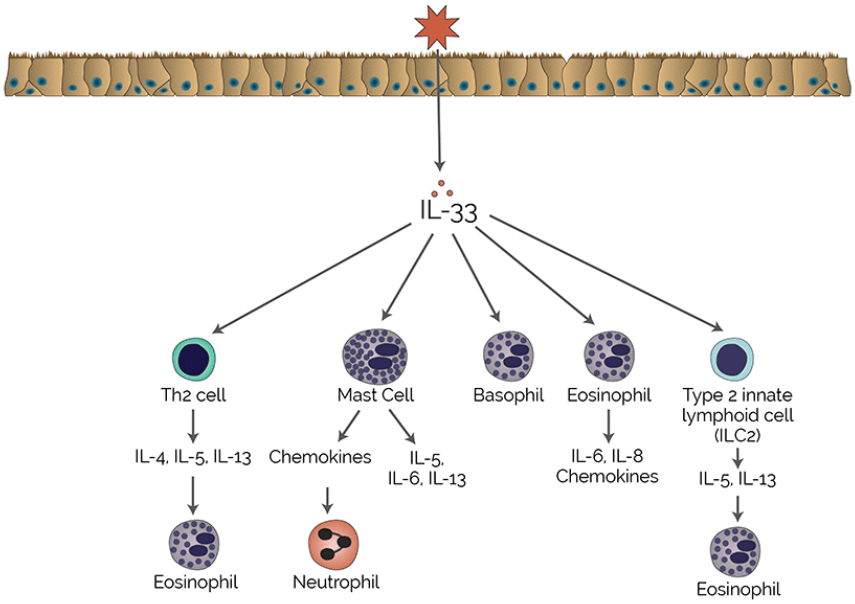


Fig. 1. IL-33 is an upstream cytokine that functions as a central mediator in asthma

The aim of this study was to examine the role and clinical significance of IL-33, as a new and insufficiently explored mediator of inflammation in patients with uncontrolled moderate asthma.

Materials and methods

The study included 87 patients with diagnosis of uncontrolled moderate persistent asthma treated at the Clinic of Pulmonology and Allergology in Skopje. Serum IL-33 levels were measured in all patients by ELISA method at the Institute of Immunobiology and Human Genetics, Faculty of Medicine, Ss Cyril and Methodius University of Skopje. The reference values for IL-33 were 0 pg/ml.

Inclusion criteria: patients who were diagnosed and classified in uncontrolled moderate persistent asthma at PHI University Clinic of Pulmonology and Allergology according to the actual version of the GINA guidelines (Global Initiative for Asthma) [11] and Guidelines for the Diagnosis and Management of Asthma (EPR-3) of National Asthma Education Prevention Program (NAEPP) [32].

Uncontrolled asthma defined as at least one of the following [33]:

1) Poor symptom control: ACQ consistently >1.5, or ACT <20 (or "not well controlled" by NAEPP/ GINA guidelines over 3 months of evaluation.
2) Frequent severe exacerbations: two or more bursts of systemic CS (3 days each) in the previous year.
3) Serious exacerbations: at least one hospitalization, ICU stay or mechanical ventilation in the previous year.
4) Airflow limitation: after appropriate bronchodilator withhold FEV1 <80% predicted (in the face of reduced FEV1/FVC defined as less than the lower limit of normal).

All patients had allergic stable asthma because there was no increase in symptoms or need for additional medication for at least 4 weeks. Eosinophils in peripheral blood in all patients were counted at the University Clinic of Clinical Biochemistry. Some of the patients had arterial hypertension, but had no other comorbidities that could increase IL-33 level. The age of the patients was 20-71 years.

Exclusion criteria: pregnancy, severe diseases of the immune, endocrine, haematological cardiac, renal, gastrointestinal, neurologicalsystem, psychiatricdisorders, and neoplastic diseases**.**

Statistical analysis

The results were statistically analyzed by the statistical program SPSS for Windows 17.0. For testing the normal distribution of data Kolmogorov - Smirnov and Shapiro-Wilk's test and Spearman Rank Order Correlations-test were used. The qualitative data were presented in absolute and relative numbers, and the quantitative data were presented with the measures of descriptive statistics (mean ±SD, median with IQR). Statistical significance was defined as a P value<0.05.

Results

The study included 87 patients with uncontrolled moderate persistent asthma; the majority were females-66 women (75.86%), and 21 were men (24.14%). The average age of patients was 42.3±15.9 years (Table 1).

The results of IL-33 (average values 6.47±29.3 pg/ml) in all asthma patients were significantly increased com-

pared to healthy people which results were in range of reference value of IL-33 (0 pg/ml).
The average values of IL-33 ranged from 6.47±29.3 and were insignificantly higher in women compared to men (p=0.27) (Table 2).
The obtained values of IL-33 showed an insignificant correlation with the age of patients (p = 0.26) (Figure 2).

Table 1. Characteristics of patients with average age of patients

Characteristics of patients		
Sex		**n (%)**
Women		66 (75.86)
Man		21 (24.14)
Age		
mean±SD	(42.3±15.9)	Min-max (20-71)

Table 2. Descriptive statistics of IL-33

Normal level IL-33=0pg/ml IL-33	**mean±SD**	**Median**	**Q25 - Q75**	**p-level**
Women	7.91±33.5	1.83	1.68-2.05	
Men	1.95±0.9	1.76	1.68-1.87	p=0.27
Total	6.47±29.3	1.79	1.68-1.98	

p (Mann-Whitney test)

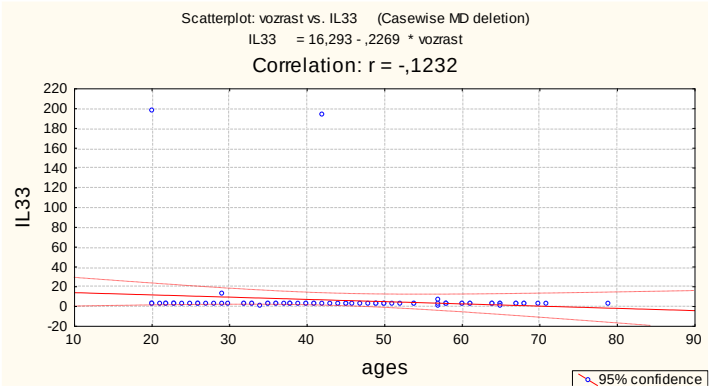


Fig. 2. Correlation between IL-33 and age in asthma patients (r =-.1232, p=0.26)

The value of eosinophils in peripheral blood was significantly increased in all asthma patients compared to healthy people, and the analyzed correlation between values of IL-33 and eosinophils showed a statistically insignificant correlation (IL-33/Eo: r<0.05, R=0.0563). It means that patients who have an increased level of IL-33 have increased concentrations of eosinophils in peripheral blood, but statistically insignificant; perhaps there were patients with neutrophilic asthma among other patients.

Discussion

IL-33 is generally released from damaged immune cells and signals through its receptor ST2 and plays important roles in type-2 innate immunity, and functions as an "alarmin" or a danger signal for cellular damage or cellular stress [34]. The role of IL-33 in lung injury was first identified mainly in lung inflammation and allergic diseases such as asthma [35,36].
In mouse models, transgenic over-expression or administration of IL-33 generates airway eosinophilia, up-regulated Type 2 cytokine expression, elevated serum IgE, AHR and mucus hypersecretion. Conversely, neutralization of IL-33 leads to reduction of airway inflammation, IgE levels, Type 2 cytokine expression, goblet cell hyperplasia, and AHR [37-38].
A recent study by Kaur D et al. showed that bronchial epithelium, airway smooth muscle (ASM), and mast cells expressed IL-33 and correlated with airway hyper responsiveness (AHR) in latter asthma [34,39].
More recent researches have implicated additional roles of IL-33 in asthma, but unfortunately, thereare a limited number of studies which have investigated IL-33. Their results showed an increased level of serum IL-33 in asthmatics compared to healthy subjects as demonstrated in our study, too. The results of meta-analysis which included four studies and evaluated 222 patients with asthma revealed that serum level of IL 33 was higher in asthma patients compared to that in healthy people [34, 37,40-44].
IL-33 have been shown to exert their effects on progenitor cells, mast cells, granulocytes, lymphocytes and dendritic cells. In human studies of allergic asthma, IL-33 and ST2 expression in serum, lung tissue and BALF have found to be higher in asthmatics compared to healthy controls and correlated with asthma severity [37,45-47].
Studies which analyzed correlation between serum levels of IL-33 and asthma severity found an increased serum level of IL-33 in all three forms of asthma (severe, moderate and mild asthma) [34,37,40,44-51].

However, the results of meta analysis which analyzed level of IL-33 in sputum, showed that the sputum IL-33 were increased in severe asthma, but not higher in moderate asthma patients than that in healthy people [44,49,51] Hamzaoui A. *et al*. revealed that IL-33 and ST2 were increased in young and adult asthmatic patients, emphasizing the fact that there were a series of factors influencing the IL-33 and ST2 expression level in asthma patients, including year, sex, races, severity of the disease [51]. But, our study showed no significant differrence between women and men with higher value of serum IL-33, and an insignificant correlation with the age of patients. Endobronchial biopsies from Préfontaine D. et al. proved increased levels of IL-33 in endothelial and epithelial cells in patients with asthmatic lungs, but they were absent in control samples; positive correlation with severity of asthma was also shown [44,46,47].

Bartosz Stolarski *et al*. in their study demonstrated that the IL-33/ST2 signaling pathway activates airway eosinophils that exacerbate airway inflammation in an autocrine and paracrine manner. However, in our study this correlation between IL-33 and eosinophils has proved to be insignificant [52]. Eosinophils are significant effector cells involved into the late and chronic stage of the allergic inflammatory response, and the explanation could be that eosinophils and IL-5 are cytokines which are detected during the late phase response and it is not detectable in early response to antigen provocation like IL-33, which are high proinflammatory cytokines with a key role in the early asthmatic responses [53,54].

Conclusion

IL-33 is new and insufficiently explored mediator of inflammation with assumption that is one of the earliest-releasedsignaling mediators secreted at the beginning in the allergic and non-allergic asthma and can orchestrate a whole immunologic cascade in asthma.

IL-33 stimulates immune activity especially Th2 immune response, and stands out as an attractive candidate for detection of other therapeutic modalities such as the new targeted therapy which would suppress IL-33, and is significantly increased in patients with asthma. By acting on the immune response by reducing the excretion of markers of inflammation could help us to make correct diagnosis, therapy, evaluation and prognosis of asthma.

Conflict of interest statement. None declared.

References

1. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 2004; 59(5): 469-478.
2. Beasley R. The Global Burden of Asthma Report, Global Initiative for Asthma (GINA). Available from http://www.ginasthma.org 2004.
3. World Health Organization. Global surveillance, prevention and control of chronic respiratory diseases: a comprehensive approach, 2007.
4. Eder W, Ege MJ, Von Mutius E. The asthma epidemic. New England Journal of Medicine 2006; 355: 2226-2235.
5. Centers for Disease Control and Prevention, Vital Signs, May 2011.
6. Centers for Disease Control. National Surveillance for Asthma-United States, 1980-2004. *MMWR* 2007; 56(SS08); 1-14; 18-54.
7. American Lung Association. Epidemiology & Statistics Unit, Research and Program Services. Trends in Asthma Morbidity and Mortality, November 2007.
8. Akinbami, L. Asthma prevalence, health care use and mortality: United States 2003-05, CDC National Center for Health Statistics, 2006.
9. Gjorcev A. Makedonski nacionalen plan i program za dijagnostika i lekuvanje na bronhilajnata asthma-realnost i vizija, 1996.
10. Vladimir Cvetanov. *Alergiskite bolesti vo R. Makedonija* 2006; 151-174.
11. Global Initiative for Asthma (GINA), GINA Report 2016, Global Strategy for Asthma Management and Prevention, updated 2016; http://www.ginasthma.org.
12. Holgate ST. Genetic and environmental interaction in Allergy and asthma. *J Allergy Clin Immunol* 1999; 104(6): 1139-1146.
13. Burke W, Fesinmeyer M, Reed K, *et al*. Family history as a predictor of asthma risk. *Am J Prev Med* 2003; 24: 160-169.
14. Thomas MJ, Kemeny DM. Novel CD4 and CDS T-cell subsets. *Allergy* 1998; 53: 1122-1132.
15. Heard BE, Hossain S. Hyperplasia of bronchial muscle in asthma. *J Pathol* 1973; 110: 319-331.
16. Shiels IA, Bowler SD, Taylor SM. Airway smooth muscle proliferation in asthma: the potential of vascular leakage to contribute to pathogenesis. *Med Hypotheses* 1995; 45: 37-40.
17. Brewster CE, *et al*. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3: 507-511.
18. Dirkje S Postma, Wim Timens. Remodeling in Asthma and Chronic Obstructive Pulmonary Disease. *American Thoracic Society* 2006; 3(5): 434-439.
19. Christopher L, *et al*. Effect of bronchoconstriction on Airway Remodeling in Asthma. *N Engl J Med* 2011; 364: 2006-2015.
20. Barnes PJ. Pathophysiologu of asthma. *Eur Respir Mon* 2003; 23: 84-113.
21. Kuipers H, Lambrecht BN. The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Curr OpinImmunol* 2004; 16(6): 702-708.
22. Abbas AK, Lichtman Ah, Pober JS. *Cellular and Molecular Immunology*, 4nd ed. 2000; 235-69.
23. Roitt IM, and Delves PJ. *Essential Immunology*, 10th ed. 2001; 177-199.
24. Park SJ, Lee YC. Interleukin-17 regulation: an attractive therapeutic approach for asthma. *Respir Res* 2010; 11: 78.
25. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003; 111: 450-463.
26. Miyahara N, Swanson BJ, Takeda K, *et al*. Effector CD8(+) T cells mediate inflammation and airway hyper responsiveness. *Nat Med* 2004; 10: 865-869.
27. Zhu J, Hidehiro Y, William EP. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol* 2010; 28: 445-489.

28. Borish L, Steinke JW. Interleukin-33 in asthma:how big of a role does it play? *Curr Allergy Asthma Rep* 2011; 11: 7-11.
29. Schmitz J, *et al*. IL-33, an interleukin-1-like cytokinethat signals via the IL-1 receptor-relatedprotein ST2 and induces Thelper type 2-associatedcytokines. *Immunity* 2005; 23: 479-490.
30. Kakkar R and Lee RT. The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nat Rev Drug Discov* 2008; 7: 827-840.
31. Miller AM. Role of IL-33 in inflammation and disease. *J Inflamm* 2011; 8: 22.
32. National Institutes of Health. National Asthma Education and Prevention Program Expert Panel Report 3: Guidelines for the Diagnosis and Management of Asthma. Accessed: January 1, 2009.
33. Kian Fan Chung, *et al*. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *European Respiratory Journal*, April, 2014.
34. Jing Chang, *et al*. IL-33 Signaling in Lung Injury. *Transl Perioper Pain Med* 2016; 1(2): 24-32.
35. Lloyd CM. IL-33 family members and asthma - bridging innate and adaptive immune responses. *Current opinion in immunology* 2010; 22(6): 800-806.
36. Miller AM. Role of IL-33 in inflammation and disease. *Journal of inflammation* 2011; 8(1): 22.
37. Brittany M. Salter, *et al*. IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. *Respiratory Research* 2016; 17: 5.
38. Lee HY, *et al*. Blockade of IL-33/ST2 ameliorates airway inflammation in a murine model of allergic asthma. *Exp Lung Res* 2014; 40(2): 66-76.
39. Kaur D, *et al*. IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk. *Allergy* 2015; 70(5): 556-567.
40. Ma XY, Luo YL, Lai WY. IL-33 levels in peripheral blood plasma and sputum of patients with bronchial asthma and their correlation with symptoms of patients, pulmonary function, and eosinophil numbers. *J Third Mil Med Univ* 2011; 33: 1526-1529.
41. Zhang HQ, Wu LF, Sun ZL. Serum levels of IL33 and IL5 in patients with allergic rhinitis and bronchial asthma. *Journal of Zhejiang Medicine* 2013; 35: 1755-1756.
42. Li QY, Huang XX, Zhang HY. Significance of changes of serum interleuin in patient with acute attack of bronchial asthma. *China Medicine* 2013; 8: 1556-1557.
43. Gluck J, Rymarczyk B, Rogala B. Serum IL-33 but not ST2 level is elevated in intermittent allergic rhinitis and is a marker of the disease severity. *Inflamm Res* 2012; 61: 547-550.
44. Rui Li, *et al*. Interleukin-33 and receptor ST2 as indicators in patients with asthma: a meta-analysis. *Int J Clin Exp Med* 2015; 8(9): 14935-14943.
45. Azazi EA, *et al*. Serum levels of Interleukin-33 and its soluble receptor ST2 in asthmatic patients. *Egyptian Journal of Chest Diseases and Tuberculosis* 2013.
46. Prefontaine D, *et al*. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* 2009; 183: 5094-5103.
47. Prefontaine D, *et al*. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 2010; 125: 752-754.
48. Li YM, Cheng XM. Clinical significance of IL-18and IL-33 levels changes in the treatmentof bronchial asthma with acute attack stageby glucocorticoids. *Practical Pharmacy and Clinical Remedies* 2013; 16: 897-899.
49. Liu SP, Ma R, Li XA. The relationship between interleukin 33 promotes and lung inflammationin asthmatic children. *Chin J Diffic and Compl* Cas 2013; 12: 422-424.
50. Liao XM. Changes of plasma interleukin 33 inasthmatic patients and its clinical significance. *Journal of Clinical Pulmonary Medicine* 2014; 19: 33-35.
51. Hamzaoui A, *et al*. Induced sputum levels of IL-33 and soluble ST2 in young asthmatic children. *J Asthma* 2013; 50: 803-809.
52. Bartosz Stolarski, *et al*. IL-33 Exacerbates Eosinophil-Mediated Airway Inflammation. *J Immunol* 2010; 185(6): 3472-3480.
53. Pecaric-Petkovic T, *et al*. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009; 113(7): 1526-1534.
54. Keisuke Oboki, *et al*. IL-33 and Airway Inflammation Allergy. *Asthma Immunol Res* 2011; 3(2): 81-88.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 56-63

MMR

DOI:10.1515/mmr-2017-0011
Original article

DOPPLER COMPARATIVE MEASUREMENTS IN THE RECONSTRUCTION OF LIMBS WITH FLAPS AND GRAFTS

ДОПЛЕР КОМПАРАТИВНИ МЕРЕЊА КАЈ РЕКОНСТРУКЦИЈА НА ЕКСТРЕМИТЕТИ СО РЕЗАНКИ И ТРАНСПЛАНТАТИ

Boro Dzonov[1], Lazo Noveski[1], Suzana Nikolovska[2] and Elizabeta Zhogovska[1]

[1]University Clinic for Plastic and Reconstructive Surgery, [2]University Clinic of Dermatovenerology, Medical Faculty, Ss Cyril and Methodius University of Skopje, Republic of Macedonia

Abstract

Doppler technique was first described by the Australian physicist and mathematician Christian Doppler. Doppler effect is defined as a reflection of high frequency sound waves of different frequency when they come in contact with the movable structure in the blood vessel. Waves that go to transducers are coded red, while waves that move away from the transducer are coded blue. Doppler main types can be classified as following: continuous wave (CW) Doppler, spectral Doppler, color Doppler and Power Doppler color. The study was realizedat the University Clinic for Plastic and Reconstructive Surgery. It is a randomized prospective study. During the study two groups of 30 patientseach were formed. Each patient was required a permission for reconstructive surgery procedure and an informed consent for participation in the study. For all patients a specially designed questionnaire (non-standardized) was filled out.

1. First (I) group of patients treated with flaps. In this group a type of reconstructive technique with skin or complex flapshas been applied.
2. Second group (II) of patients treated with grafts (split thickness grafts). In this group applied reconstruction comprised application of skin grafts with partial thickness.

The study included patients with defects of the skin and soft tissues, whohad an indication for reconstructive surgery procedure. Exclusion criteria of patients for participation in the study were: children under 14 years of age, adults over 75 years, people with systemic diseases that can affect the results of reconstructive intervenetions and patients who have without periosteum bone-like surface defect as contraindication for skin grafting. The results of the reconstructive procedures according to the objectives set were investigated clinically into three time periods: preoperative, postoperative day 7 and day 30 postoperatively. The following investigations were carried out: determination of the circulation levels by means of Doppler; determination of the levels of limbs circulation is distal to the site of reconstruction inthe pre-and postoperative period (day 7 and day 30);
For the evaluation of blood flow the following parameters were used:

- PSV-Peak systolic velocity
- PI - Pulsatility index

RI - Resistance indexPI and RI were calculated using the formula:

- PI = PSV - EDV / Vmean
- RI = PSV - EDV / PSV

EDV indicates the flow velocity in late diastole and V mean, the average speed of blood flow through the artery. By assessment of arterial status before and after surgery through the analysis ofvascular waves at different locations of the vascular tree of the upper and lower extremities, we registered significant difference between the two examined groups, which speaks in favor of the use of flaps in reconstruction of the lower limbs.

Keywords: Doppler, flap, transplant (graft), blood flow

Апстракт

Доплер техниката првпат била опишана од австралискиот физичар и математичар Кристијан Доплер. Ефектот на доплерот се дефинира како рефлексија на високофреквентни звучни бранови со различна фреквенција, кога доаѓаат во контакт со подвижните структури во крвниот сад. Брановите што одат кон трансдукторот се кодирани со црвена боја, додека брановите кои се движат подалеку од трансдукторот се кодирани со сина боја.
Главните видови доплер може да се класифицираат според следните типови: континуиран бран (CW) доплер, спектрален доплер, колор доплер и power колор доплер.

Correspondence to: Dzonov Boro, University clinic for plastic and reconstructive surgery-Skopje, R. Macedonia; E-mail: bdzonov@yahoo.com

Студијата е работена на Клиниката за пластична и реконструктивна хирургија. Изработена е рандомизирана проспективна студија.

Во текот на студијата се формирани две групи испитаници од по 30 пациенти. Од секој пациент е побарана дозвола за оперативен реконструктивен зафат и информативна согласност за учество во студијата.

За сите пациенти е пополнуван специјално конструиран прашалник (нестандардизиран).

1. Прва (I) група пациенти третирани со резанки. Во оваа група е применет тип на реконструктивна техника со кожна или со сложена резанка.
2. Втора (II) група пациенти третирани со трансплантати (split thickness grafts). Во оваа група, применетата реконструкција се состои од апликација на кожни трансплантати, со делумна дебелина на кожа.

Во студијата се вклучени пациенти со дефекти на кожа и на меки ткива, кај кои има индикација за реконструктивен зафат. Екслузивни критериуми на пациенти (испитаници) од студијата се деца под 14 годишна возраст, возрасни лица над 75 години, лица со системски заболувања, кои можат да ги афектираат резултатите од реконструктивните зафати, и пациенти со депериостирана коска, како подлога на дефектот, како контраиндикација за примена на кожен трансплантат.

Резултатите од реконструктивните оперативни зафати, според поставените цели, се клинички испитувани во три временски периоди: предоперативно, 7. ден постоперативно и 30. ден постоперативно.

Спроведени се следните испитувања:

- Одредување на циркулацијата со помош на доплер
- Одредување на циркулацијата на екстремитетите, дистално од местото на реконструкција, пред и постоперативно (7. и 30. ден);

За евалуација на крвниот проток беа користени следните параметри:

• PSV-максимална брзина на протокот во систола (англ. Peak systolic velocity)

• PI – индекс на пулсативност (англ. pulsatility index)

• RI – индекс на отпор (англ. resistance index)

PI и RI се пресметуваат со формулите:

• PI= PSV - EDV / Vmean

• RI = PSV - EDV / PSV

EDV ја означува брзината на протокот на крајот од дијастолата, а Vmean, средната брзина на протокот на крвта низ артеријата.

Оцената на артерискиот статус, пред и по операцијата, преку анализа на васкуларните бранови на различни локации од васкуларното стебло на горните и на долните екстремитети, регистриравме сигнификантна значајност меѓу двете испитувани групи, кои одат во прилог на примената на резанки кај долни екстремитети.

Клучни зборови: доплер, резанка, трансплантат, крвен проток

Introduction

Doppler technique was first described by the Australian physicist and mathematician Christian Doppler. Doppler effect is defined as a reflection of high frequency sound waves of different frequency when they come in contact with the movable structure in the blood vessels. Waves thatgo to transducers are coded red, while waves that move away from the transducer are coded blue. Doppler main types can be classified as following: continuous wave (CW) Doppler, spectral Doppler, color Doppler and Power Doppler color. In the arterial Doppler ultrasonography of theextremities, the images are obtained by providing a clear evaluation of anatomical structures and atheromatous plaques. In a normal artery of the extremities, i.e. without pathological substrate and with normal flow, there is a three-phase flow model (Figure 1A). Firstly, the high velocity flow is the result of the cardiac cycle, and then an inverse flow occurs in early diastole, which is followed by a progressive flow rate in late diastole [1]. Three phase wave is characteristic for the arteries supplying muscles, which have a high peripheral resistance. During exercise or transit ischemia, there is a loss of the three-phase model. In arterial occlusive disease, flow velocity increases measured by Doppler on places where the artery lumen is narrowed.In contrast, vascular resistance decreases as a result of collateral circulation and vasodilation distal from the obstruction. As the disease progresses, the three-phase model of the flow is reduced to a two-phase flow (Figure 1B).

This is due to the initial loss of elasticity of the artery walls. If the disease progresses, the flow loses its pulse nature and turns into a single-phase signal with increased diastolic flow as a result of regional vasodilation (Figure 1C). By enclosure measurements using Doppler, the degree of arterial patency in limbs is classified into 4 categories: 1) normal (0% stenosis), 2) 1-49% stenosis, 3) 50-99% stenosis, and 4) complete occlusion (100% stenosis) [2]. The criteria for evaluation of arterial stenosis of the lower limbs and limbs in general, according to the flow speed arebased onpeak systolic velocity-PSV and velocity ratio-VR, when the speed of the flow is normal PSV is less than 1.5 and VR is 1.5: 1. [3]. In the case of 0-49% stenosis PSV is between 1.5 and 2 and VR e 1.5-2: 1. In stenosis between 50-99%, PSV is >2.0 and VR is calculated as> 4: 1. According to the diagnostic criteria for hemodynamic important and influential stenosis of 50-99% PSV is doubled on the site of the lesion, compared tothe more proximal segment of vessel (PSV greater than 200 cm/s, with evidence of turbulence).

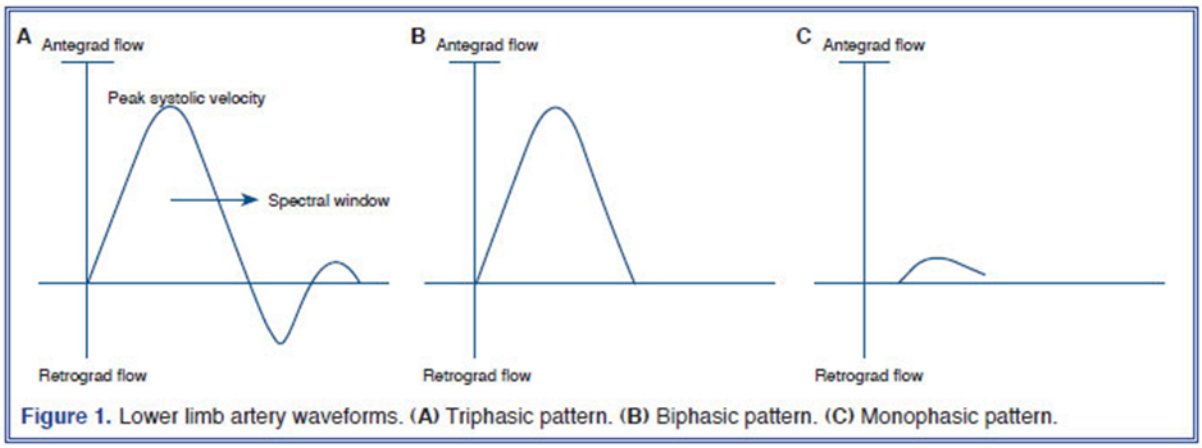


Fig. 1. Lover limb artery waveforms

The most important first test, following physical examination, which helps to determine arterial disease of the limbs is ankle-brachial index (ABI). To perform this test a device for blood pressure and Doppler ultrasound with continuous waves are required.This test compares the blood pressure obtained with a Doppler on a. dorsalis pedis or a. tibialis posterior (which is higher) with blood pressure of the two compared to brachial pressures. Generally, 0.9 ABI is considered normal, from 0.4 to 0.9 reflects mild to moderate peripheral arterial disease and 0.4 ABI suggests severe arterial disease of the lower extremities. ABI as a test has emerged as one of the most applicable in the diagnosis of diffuse atherosclerosis, cardiovascular risk and overall survival in many patient populations.For example, in a study of 2,023 middle-aged men who were screened with ABI, the relative risk of death from cardiovascular causes and coronary causes were significantly higher in patients with ABI <0.90 than in patients with normal ABI [4]. In a population of 1,492 women aged over 65 years, the relative risk of death fromheart diseases and cardiovasculars disease was significantly higherwhen baseline ABI was 0.90 [5].
About 30 years ago, initial clinical studies concluded that Doppler ultrasonography can be used in the diagnosis of arterial occlusion of the extremities [6]. In the following years, many authors have presented data suggesting that angiography can be successfully replaced by Doppler [7,8]. As a result of rapid technological progress and cost reductions, Doppler ultrasonography is used in many hospitals and clinics. In an early well-designed study of 40 patients, it was found that Doppler ultrasonography had a sensitivity of 92% and specificity of 98% in the aorto-iliac artery occlusion [9]. For femoro-popliteal stenosis, these values are calculated to be with 88% and 98% accuracy. The success of Doppler ultrasonography for diagnosis of stenosis was because the sensitivity and specificity reached 100% accuracy in the aorto-iliac arteries and up to 90% and 100% accuracy in the femoro-popliteal arteries. It has been discovered that diagnostic accuracy of Doppler decreases in the distal parts of the limbs, in a study that evaluated 24 patients and 213 arterial segments for calculating the Doppler sensitivity in detecting stenosis of the arteries of lower leg [10]. However, the author concludes that ultrasonography had quite a high success in uncovering the stenosis in the lower limbs and arteries.
If the entire lower limb is taken into consideration, contrast-MRA has a high diagnostic value for diagnosis of stenosis, with a sensitivity of 95% (92 to 99.5%) and specificity of 97% (64-99%) [11]. In one newer study 668 segments in atotal of 249 patients were evaluated, and the sensitivity and specificity were excavated to be statistically different for the Doppler ultrasonography (76% and 93%) and MRA (84% and 97%) compared with the two techniques [12]. Newer studies have less methodologycal deficiencies due to the development of technological level, as well as the experience gained in the time to recognize the pathological or non-pathological results.
Doppler ultrasonography is comparable in sensitivity with angiography, especially in femoro-popliteal artery stenosis or occlusion. However, its sensitivity in lower leg area is relatively smaller. Distal arteries are often difficult to be visualized because of their small size. Moreover, age-related or accelerated calcification of the wall of the arteries can easily disrupt the implementation of the Doppler signals [13]. However, with respect to the latter Doppler has the advantage of detecting less calcified areas suitable for anastomosis in patients wheresurgical approach is planned [14].

Material and Methods

The study was realizedat the University Clinic for Plastic and Reconstructive Surgery. It is a randomized prospective study. During the study two groups of 30 patients each were formed. Each patient was required a permission for reconstructive surgery and an informed consent for participation in the study. A specially

designed questionnarire (non-standardized) was willed out for each patient.

1. First (I) group of patients treated with flaps

In this group a type of reconstructive technique with skin or complex flapshas been applied.

2. Second group (II) of patients treated with grafts (split thickness grafts)

In this group the applied reconstruction consisted of application of skin grafts with partial thickness.

The study included patients with defects of the skin and soft tissues, whohave an indication for reconstructive surgery procedure. Exclusion criteria of patients for participation in the studywere: children under 14 years of age, adults over 75 years, people with systemic diseases that can affect the results of reconstructive interventions and patients with bone-like surface defect without periosteum as contraindication for skin grafting).

The results of the reconstructive procedures according to the objectives set were investigated clinically into three time periods: preoperative, postoperative day 7 and day 30 post-operatively. The following investigations were carried out.

Determination of the circulation levels by means of Doppler- determination of the levels of limbs circulation distal to the site of reconstruction pre-and postoperatively (day 7 and 30).

With Doppler ultra-sonography it was possible to assess arterial status before and after the surgery, through the analysis of the vascular waves at various locations of vascular stem from the upper and lower extremities. Examination of the upper and lower extremities were performed in the lying position of the patient after10 minutes rest in bed in rooms with t = 22-24 C^0. For the evaluation of the radial and ulnar artery, hands were placed in a small flexion and relaxed position with the probe atthe levelof radio-carpal wrist. In evaluation of a. tibialis posterior, patients were in right or left decubital position and for a. tibialis anterior the flexion of the knee was performed. All measurements of the arteries of the lower extremities were performed in the malleolar line. The angle of the ultrasound during the analysis of vascular waves was $\leq 60^0$. Doppler spectral quantitative examinations of vascular waves were performed with Medacord PVL, Medasonics, USA and Dopplex Assist Huntleigh -Healthcare, UK with a 7.5- MHz linear probe. For the evaluation of blood flow the following parameters [15, 16] were used:

• PSV-Peak systolic velocity
• PI - Pulsatility index
• RI - Resistance index

PI and RI were calculated using the formula:

• PI = PSV - EDV / Vmean
• RI = PSV - EDV / PSV

EDV indicates the flow velocity in late diastole and V mean, the average speed of blood flow through the artery.

Results

The average value of PULSATILITY INDEX of the radial artery on day 0 was 7.2±1.9, the seventh day after surgery dropped to 7.0±1.9, to rise after the 30th day to 7.1±1.9. The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of RESISTENCE INDEX ofthe radial artery on day 0 was 0.8±0.09, the seventh day after surgery dropped to 0.76±0.08, to increase after day 30 to 0.8 ±0.08. The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of PEAK SYSTOLIC VELOCITY (cm/s) ofthe radial artery on day 0 was 64.4±9.5, the seventh day after surgery dropped to 62.5±9.4, to increase after day 30 to64.1 ± 9.8.

The difference between registeredvalues on a day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of PULSATILITY INDEXof the ulnar artery on day 0 was 7.2±1.9, the seventh day after surgery dropped to 7.0±1.9, to increase after day 30 to 7.1±1.9.

The difference between registered values on a day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p>0.05).

The average value of RESISTENCE INDEXof the ulnar artery on day 0 was 0.8±0.09, the seventh day after Surgery dropped to 0.76±0.08, to increase after day 30 to 0.8±0.08. The difference between registered values on a day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of PEAK SYSTOLIC VELOCITY (cm/s) ofthe ulnar artery on a day 0 was 62.8±8.85, the seventh day after surgery decreased to 61.6±8.6, to increase after day 30 to62.8±8.4. The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of PULSATILITY INDEX of the radial artery on a day 0 was 7.4±0.9, the seventh day after surgery dropped to 7.1±0.9, to increase after day 30 to 7.3±0.8.

The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of RESISTENCE INDEX of the radial artery on a day 0 was 0.8±0.1, the seventh day after surgery droppedto 0.76±0.08, to increase after day 30 to 0.8±0.08. The difference between registered values on a day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).

The average value of PEAK SYSTOLIC VELOCITY (cm/s) ofthe radial artery on day 0 was 65.2±9.3, the seventh day after surgery declined of 62.1±10.1, to increase after day 30 to 63.5±9.8.

The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of PULSATILITY INDEX of the ulnar artery on day 0 was 7.1±0.8, the seventh day after surgery dropped to 6.9±0.9, to reduce after 30 days to6.6 ±1.7. The difference between registered values on a day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of RESISTENCE INDEX of the ulnar artery on day 0 was 0.08±0.08, the seventh day after surgery it dropped to 0.7±0.07, to increase after day 30 to 0.75±0.07. The difference between registerred values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p>0.05).
The average value of PEAK SYSTOLIC VELOCITY (cm/s) ofthe ulnar artery on day 0 it was 62.8±9.4, the seventh day after surgery decreased to 60.8±9.4, to increase after day 30 to61.2±9.6.
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The difference of the average values of PULSATILITY INDEX of the radial artery between patients with flaps reconstruction and reconstruction with transplant on hand onday 0, the seventh and day 30was statistically insignificant (p> 0.05).
The difference of the average values of PULSATILITY INDEXof the ulnar artery between patients with flaps reconstruction and transplant reconstruction on hand on day 0, the seventh and day 30 was statistically insignificant (p> 0.05).
The difference of the average values of RESISTENCE INDEX of the radial artery of patients with flaps reconstruction and transplant reconstruction on hand on day 0, the seventh and day 30 was statistically insignificant (p> 0.05).
The difference of the average values of RESISTENCE INDEX of the ulnar artery between patients withflaps reconstruction and transplant reconstruction on hand on day 0, the seventh and day 30 was statistically insignificant (p> 0.05).
The difference of the average values of PEAK SYSTOLIC VELOCITY in the radial artery between flaps reconstruction and transplant reconstruction on hand on day 0, the seventh and day 30 was statistically insignificant (p> 0.05).
The difference of the average values of PEAK SYSTOLIC VELOCITY of the ulnar artery between patients with flaps reconstruction and transplant reconstruction on hand onday 0, the seventh and day 30 was statistically insignificant (p> 0.05).
The average value of PULSATILITY INDEX of posterior tibial artery on day 0 was 12.4±2.5, the seventh day after surgery decreased to 12.1±2.3, to increase after 30 days to 12.5±2.5.
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of RESISTENCE INDEX of the posteriortibial artery on day 0 was 1.23±0.085, the seventh day after surgery dropped to 1.20±0.09, to increase after day 30 to 1.23±0.08.
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statisticcally insignificant (p> 0.05).
The average value of PEAK SYSTOLIC VELOCITY (cm/s) of the posterior tibial artery onday 0 was 58.0± 12.3, the seventh day after surgery declined to 55.7±11.4, to increase after day 30 to57.5±11.7. The difference between registered values on day 0, day 7 and day 30 according to ANOVA test wasstatistically insignificant (p> 0.05).
The average value of PULSATILITY INDEX of the anteriortibial artery on day 0 was 12.5±2.6, the seventh day after surgery decreased to 12.4±2.6, to increase after day 30 to 12.5±2.6.
The difference between registered values on day 0, day 7 and day 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of RESISTENCE INDEX of the anterior tibial artery on day 0 was 1.2±0.09, the seventh day after surgery it dropped to 1.15±0.09, to increase after day 30 to 1.2±0.09.

Table 1. Average values of the three indexes-pulsatility index, resistence index and peak systolic velocity in patients with transplant reconstruction on lower extremity on day 0, day 7 and day 30 on a. tibialis posterior

	number	**average**	**minimum**	**maximum**	**Std.Dev.**
PTA *– posterior tibial artery* ***PI*** *– PULSATILITY INDEX*					
PTA 0 PI	14	12.60143	9.02	16.23	1.94060
PTA 7 PI	14	11.35571	8.1	13.26	1.52737
PTA 30 PI	14	11.77714	8.87	13.49	1.48047
PTA *– posterior tibial artery* ***RI****-RESISTENCE INDEX*					
PTA 0 RI	14	1.21071	1.02	1.31	0.07956
PTA 7 RI	14	1.11143	0.97	1.24	0.07794
PTA 30 RI	14	1.15714	0.98	1.27	0.08695
PTA *– posterior tibial artery* ***PSV*** *– PEAK SYSTOLIC VELOCITY (cm/s)*					
PTA 0 PSV	14	53.84786	39.37	72.03	10.27836
PTA 7 PSV	14	50.98786	38.57	70.38	10.31863
PTA 30 PSV	14	52.44643	39.24	71.68	9.97724

The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of PEAK SYSTOLIC VELOCITY (cm/s) of the anterior tibial artery on day) was 58.5± 12.9, the seventh day after surgery declined to 57.3±13.2, to increase after day 30 to 58.2±13.03.
The difference between registered values on day 0, day 7 and day 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of PULSATILITY INDEX of the posteriortibial artery onday 0 was 12.6±1.9, the seventh day after surgery decreased to 11.4±1.5, to increase after day 30 to 11.8±1.5 (Table 1).
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of RESISTENCE INDEX of the posterior tibial artery on day 0 was 1.2±0.08, the seventh day after surgery it dropped to 1.1±0.08, to increase after day 30 to 1.2±0.09 (Table 1).
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically significant for p<0.05 (p=0.009995).
Tukey HSD-test, test for multiple comparison, aims to detect which difference (among many) is "credited" to the overall significant result; in this case the difference is statistically significant between zero day and seventh day for p <0.05 (p=0.007241).
The average value of PEAK SYSTOLIC VELOCITY (cm/s) of the posterior tibial artery on day 0 was 53.8± 10.3, the seventh day after surgery declined to 51.0±10.3, to increase after day 30 to 52.4±10.0 (Table 1). The difference between registered values on day 0, day 7 andday 30 according to ANOVA test was statistically insignificant (p> 0.05).
The average value of PULSATILITY INDEX of the anteriortibial artery on day 0 was 7.1±0.8 seventh day after surgery it dropped to 6.9±0.9, to reduce after 30 days to 6.6 ± 1.7.
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p<0.05).
The average value of RESISTENCE INDEX of the anterior tibial artery onday 0 was 0.08±0.08, the seventh day after surgery it dropped to 0.7±0.07, to increase after day 30 to 0.75±0.07.
The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically significant for p <0.05 (p=0.020537).
Tukey HSD-test, test for multiple comparison, aims to detect which difference (among many) is "credited" to the overall significant result; in this case the difference is statistically significant between zero day and seventh day for p <0.05 (p=0.015254).
The average value of PEAK SYSTOLIC VELOCITY (cm/s) of the anteriortibial artery on day 0 was 62.8±9.4, the seventh day after surgery it decreased to 60.8±9.4, to increase after day 30 to 61.2±9.6. The difference between registered values on day 0, day 7 and 30 according to ANOVA test was statistically insignificant (p> 0.05).
The difference between the average values of PULSATILITY INDEX of the posteriortibial artery between patients with a flap reconstruction and transplant reconstruction on leg on day 0, day 7 and day 30 was statistically insignificant (p> 0.05).
The difference between the average values of PULSATILITY INDEX of the anteriortibial artery between patients with a flap reconstruction and transplant reconstruction on leg on day 0, day 7 and day 30 was statistically insignificant (p> 0.05).
The difference of the average values of RESISTENCE INDEX of theposterior tibial artery between patients with flap reconstruction and transplant reconstruction on leg on day 0 wasstatistically insignificant, but the difference wasstatistically significant in theaverage values between 7 and 30 day for p <0.05 (p=0.011364; p=0.040244).
The difference between the average values of RESISTENCE INDEX of theanteriortibial artery between patients with flap reconstruction and transplant reconstruction on leg on day 0, day 7 and day 30was statistically insignificant (p>0.05).
The difference between the average values of PEAK SYSTOLIC VELOCITY of theposteriortibial artery between patients with flap reconstruction and transplant reconstruction on leg on day 0, day 7 and day 30 was statistically insignificant (p>0.05).
The difference between the average values of PEAK SYSTOLIC VELOCITY of theanteriortibial artery between patients with flap reconstruction and transplant reconstruction on leg on day 0, day 7 and day 30 was statistically insignificant for p>0.05.

Discussion

The values that are registered with Doppler ultrasonography in comparisonwithtwo surgical techniques were in favor of the reconstruction with flaps, but without statistical significance. In comparative results with similar studies by other authors, especially in the postoperative period, the reference values were similar. Due to the uniqueness of the test, comparison was made with valuesfrom other studies in their control groups or in groups with low occlusion of arterial blood vessels of the limbs. The conclusion is that the values we have obtained are very close to normal.
Thus, in the study by Williams *et al*. RI index in the group without stenosis was 0.89±0.20 [17]. According to Suzuki *et al*. RI index was 0.90±0.20 in the normal control group and in the group with minimal stenosis [18].
In the postoperative period the reference values were similar as in the study of Taylor *et al*. [19], which shows that the circulation depends onthe type of flap used for

reconstruction. Ono *et al.* [20] and Sananpanich *et al.* [21] have shown the dependence of circulation in the postoperative period on the flaps applied and the involvement of major blood vessels of the limbs in reconstructive procedure.

Therefore, recommendable reconstruction techniques are thoseinvolvingsuperficial and perforator blood vessels. This is confirmed in the study of Innocenti *et al.* [22]. Of course, when using larger perforators or magistralblood vessels as basis for the flaps we have significantly worse outcomes on circulation distal to the reconstruction, which has been demonstratedbyPanse*et al.* [23]. In the study of Pollock *et al.* PSV of upper limb arteries in control group ranged from 40-53 sm/s, while in the group of RSD 44-95 sm/s [24]. In the study of Rashad *et al.* in which measurements were made for PSV in groups of patients with diabetic ulcers the values in the group with normalized circulation of PSV were 49-53 sm/s, while in the group without improvement of circulation the PSV values were 19 sm/s [25]. In Takahsai *et al.* study PI values of the arteries of the lower extremity, foot and lower leg ranged from 7.97±2.29 in diabetic patients without neuropathy, which is equal with the normal findings, to 3.0 ±0.69 in patients with diabetic neuropathy [26].

Moore *et al*. presented PI index values in the ulnar artery depending on the position of the limb, from 3:06 to 3.36 [27].

The transplant reconstructions leadto lower values obtained by measurements distal to the application. This is especially true for the later postoperative period, when transplantation was made circumferential on limbs, which causesfibrosis and constriction of soft tissues [28].

Conclusion

By assessment of arterial status before and after surgery through analysis of vascular waves at different locations of the vascular tree of the upper and lower extremities, we have registered significant/difference between the two examinedgroups, whichspeaks in favor of the use of flaps in reconstruction of the lower limbs

Conflict of interest statement. None declared.

Reference

1. Hatsukami TS, Primozich J, Zierler RE, Strandness DE Jr. Color Doppler characteristics in normal lower extremity arteries. *Ultrasound Med Biol* 1992; 18: 167-171.
2. Lau JF, Weinberg MD, Olin JW. Peripheral artery disease. Part 1: clinical evaluation and noninvasive diagnosis. *Nat Rev Cardiol* 2011; 8: 405-418.
3. Allen PL, Gallagher K. The peripheral arteries. In: Allen PL, Dubbins PA, Pozniak MA, McDicken N, editors. Clinical Doppler ultrasound. 2nd ed. London, England: *Churchill Livingstone* 2006; 73-99.
4. Kornitzer M, Dramaix M, Sobolski J, *et al.* Ankle/ arm pressure index in asymptomatic middle-aged males: an independent predictor of ten-year coronary heart disease mortality. *Angiology* 1995; 46: 211-219.
5. Vogt MT, Cauley JA, Newman AB, *et al.* Decrease dankle/arm blood pressure index and mortality inelderly women. *JAMA* 1993; 270: 465-469.
6. Demorais D, Johnston KW. Assessment of aorto-iliac disease by non-invasive quantitative Doppler waveform analysis. *Br J Surg* 1981; 68: 789-792.
7. Kohler TR, Nance DR, Cramer MM, *et al*. Duplex scanning for diagnosis of aortoiliac and femoropopliteal disease: a prospective study. *Circulation* 1987; 76: 1074-1080.
8. Legemate DA, Teeuwen C, Hoeneveld H, *et al.* Spectral analysis criteria in duplex scanning of aortoiliac and femoropopliteal arterial disease. *Ultrasound Med Biol* 1991; 17: 769-776.
9. Legemate DA, Teeuwen C, Hoeneveld H, Eikelboom BC. Value of duplex scanning compared with angiography and pressure measurement in the assessment of aortoiliac arterial lesions. *Br J Surg* 1991; 78: 1003-1008.
10. Hatsukami TS, Primozich JF, Zierler RE, *et al.* Color Doppler imaging of infrainguinal arterial occlusive disease. *J Vasc Surg* 1992; 16: 527-531.
11. Collins R, Burch J, Cranny G, *et al*. Duplex ultrasonography, magnetic resonanc angiography, and computed tomography angiography for diagnosis and assessment of symptomatic, lower limb peripheral arterial disease: systematic review. *BMJ* 2007; 334: 1257.
12. Leiner T, Kessels AG, Nelemans PJ, *et al*. Peripheral arterial disease: comparison of color duplex US and contrast-enhanced MR angiography for diagnosis. *Radiology* 2005; 235: 699-708.
13. Hingorani AP, Ascher E, Marks N, *et al*. Limitations of and lessons learned from clinical experience of 1,020 duplex arteriography. *Vascular* 2008; 16: 147-153.
14. Hingorani A, Ascher E, Marks N. Preprocedural imaging: new options to reduce need for contrast angiography. *Semin Vasc Surg* 2007; 20: 15-28.
15. Abigail Thrush, Timothy Hartshorne. Peripheral Vascular Ultrasound how, why and when. *Elsevier Limited* 2005; 114-155.
16. Satish K Barghava. Textbook of Color Doppler imaging. *Ajanta Offset and Packagings Ltd. New Delhi* 2010; 25-45.
17. Williams DT, Hardinh KG, Price P. An evaluation of the efficacy of methods used in screening for lower-limb arterial disease in diabetes. *Diabetes Care* 2005; 28: 2206-2210.
18. Suzuki E, Egawa K, Nishio Y, *et al.* Prevalence and major risk factors index in Japanese patients with type-2 diabetes. of reduced flow volume in lower extremities with normal ankle-brachial. *Diabetes Care* 2003; 26: 1764-1769.
19. Taylor GI, Doyle M, McCarten G. The Doppler probe for planning flaps: Anatomical study and clinical applications. *Br J PlastSurg* 1990; 43: 1-16.
20. Ono S, Sebastin SJ, Yazaki N, *et al*. Clinical applications of perforator-based propeller flaps in upper limb soft tissue reconstruction. *J Hand Surg Am* 2011; 36: 853-863.
21. Sananpanich K, Tu YK, Kraisarin J, Chalidapong P. Reconstruction of limb soft-tissue defects: Using pedicle perforator flaps with preservation of major vessels, a report of 45 cases. *Injury* 2008; 39(4): 55-66.
22. Innocenti M, Baldrighi C, Delcroix L, Adani R. Local perforator flaps in soft tissue reconstruction of the upper limb. *Handchir Mikrochir Plast Chir* 2009; 41: 315-321.
23. Panse N, Sahasrabudhe P. The ulnar digital artery perforator flap: A new fl ap for little fi nger reconstruction: A preliminary report. *Indian J Plast Surg* 2010; 43: 190-194.

24. Pollock FE Jr, Koman LA, Smith BP, Poehling GC. Patterns of microvascular response associated withreflex sympathetic dystrophy of the hand and wrist. *J Hand Surg [Am]* 1993; 18A: 847-852.
25. Rashad A Bishara, Wassila Taha, Ihab Akladious, Muhammad A. Ankle Peak Systolic Velocity: New Parameter to Predict Nonhealing in Diabetic Foot Lesions New Parameter to Predict Nonhealing in Diabetic Foot Lesions. *Allam Vascular* 2009; 17(5): 264-268.
26. Takahsai T, Yoshiki N, Emoto M, *et al*. Sympathetic function test of vasoconstrictor changes in foot arteries in diabetic patients. *Diabetes Care* 1998; 21; 1495-1501.
27. Moore AE, Muirhead JC, Sharples K, Stringer MD. The effect of upper limb position on ultrasonographic arterial flow to the ulnar nerve. 2014 Apr; 69(4):356-61. doi: 10.1111/anae.12543.
28. Harrison CA, MacNeil S. The mechanism of skin graft contraction: An update on current research and potential future therapies. *Burns*. 2008; 34: 153-163.

DE GRUYTER
OPEN

Mac Med Review 2017; 71(1): 64-71

MMR

DOI:10.1515/mmr-2017-0012
Original article

DETECTION OF PLACENTAL CHROMOSOMAL ABERRATIONS IN EARLY SPONTANEOUS ABORTIONSIN CORRELATION WITH THE HISTOLOGIC FINDINGS

ДЕТЕКЦИЈА НА ХРОМОЗОМСКИ АБНОРМАЛНОСТИ НА ПОСТЕЛКАТА КАЈ РАНИТЕ СПОНТАНИ АБОРТУСИ ВО КОРЕЛАЦИЈА СО ХИСТОЛОШКИТЕ ПРОМЕНИ

Katerina Kubelka-Sabit[1], Gorgi Bozinovski[2], Jasar Dzengis[1], Vanja Filipovski[1], Slobodan Lazarevski[1], Mitko Ivanovski[1] and Dijana Plaseska-Karanfilska[2]

[1]Clinical Hospital Acibadem Sistina Skopje, [2]Research Center for Genetic Engineering and Biotechnology "Georgi D Efremov", Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia

Abstract

Using a variety of molecular techniques, it has been established that loss of pregnancy occurs in one to two thirds of all fertilized embryosin the first trimester. In about 50% of the cases, chromosomal abnormalities are the cause of early spontaneous abortion. Several histological characteristicsof the placenta, such as presence of villous stromal cavitations, fetal erythrocytes, umbilical cord, fetal tissue,etc. are suggested as predictive factors for aneuploidy.

Two hundred and thirty one cases were analyzed in this prospective study, 50 cases were control artificial abortions and 181 cases were early spontaneous abortionsanalyzed in the period from May 2012 to December 2014. Standard histopathological analysis and molecular techniques based on polymerase chain reaction were used to analyze the samples.

Usingmolecular techniques, aneuploidy was detected in 53.1% of the samples. The most frequently detected aneuploidy was trisomy 16, followed by trisomy 22, 21, 14 and 18. The molecular analysis also enabled distinction of maternal and paternal origin of the alleles.

In the histopathological sample analysis, binary logistic regression analysis indicated the presence of trophoblastic proliferation (p=0.008) and the absence of fetal red blood cells (p=0.001) as independent significant factors in the prediction of aneuploidy in early spontaneous abortion.

In conclusion, our results show that clinically relevant and accurate diagnosis of early spontaneous abortion which can determine its causecan only be achieved bya controlled process of selection of the material, histopathological and molecular analysis, followed bya necessary correlation of these results.

Keywords: aneuploidy, histopathological analysis, miscarriage, molecular analysis

Correspondence to: Katerina Kubelka-Sabit, Clinical Hospital AcibademSistina, Skupi 5a, 1000 Skopje, R. Macedonia; Phone: +389 70 36 53 38; Fax: +389 2 30 99 599; E-mail:catkubelka@yahoo.co.uk; katerina.kubelka@acibademsistina.mk

Апстракт

Со употреба на различни молекуларни техники е утврдено дека во првиот триместар настанува губење на дури една до две третини од сите фертилизирани ембриони. Во околу 50% од случаите, причина за ран спонтан абортус се хромозомските абнормалности. Како предвидувачки фактор за анеуплоидија кај постелката се користат неколку хистолошки карактеристики, како вилозни празнини, нуклеирани еритроцити, папочна врвка, фетално ткиво и сл.

Во проспективната студија беа анализирани 231 случај, од кои 50 случаи на намерен абортус и 181 случај на ран спонтан абортус, обработени во периодот од мај 2012 година до декември 2014 година. За анализа на примероците е употребена стандардна хистопатолошка анализа и молекуларни техники, базирани на полимераза верижна реакција.

Со употреба на молекуларните техники беше детектирана анеуплоидија кај 53,1% од примероците. Најчесто детектирана анеуплоидија беше трисомија 16, следена со трисомија 22, 21, 14 и 18. Исто така, молекуларните анализи овозможија разликување на алелите по потекло од мајката и од таткото.

При хистоморфолошката анализа на примероците, бинарната логистичка регресиска анализа го посочи присуството на трофобластна пролиферација (p=0.008) и отсуството на фетални еритроцити (p=0.001) како независни сигнификантни фактори во предикција на анеуплоидија кај раните спонтани абортуси.

Како заклучок-добиените резултати покажаа дека за точна и клинички релевантна дијагноза на спонтан абортус, односно утврдување на причината за негово настанување е потребно јасно дефинирање на процесот на селекција и обработка на материјалот, преку хистопатолошка и молекуларна анализа,

до неопходната меѓусебна корелација на резултатите од овие анализи.

Клучни зборови: анеуплоидија, молекуларна анализа, спонтан абортус, хистопатолошка анализа

Introduction

In 1977, the World Health Organization defined abortion as an extraction or expulsionof a fetus weighing 500 grams or less. Today, abortion is defined as a spontaneous loss of pregnancy before the fetus is able to survive outside the womb. The term miscarriage is used for many complications of early pregnancy. Therefore, in 2005 the European Society of Human Reproduction and Embryology (ESHRE) introduced a revised terminology that refers to early miscarriage. Thus, loss of pregnancy after a positive urine test for βHCG (β Human Chorionic Gonadotropin) or elevated serum βHCG before ultrasound or histological verification of pregnancy is defined as a biochemical abortion. Such abortions usually occur before the third or fourth, but certainly before the sixth week of gestation. It is thought that 30% of human concepts are lost in the preimplantation period, while another 30% are destroyed after implantation, but before the delayed menstrual period [1].

The clinical term miscarriage is used when there is an ultrasonic or histological confirmation of intrauterine pregnancy. Clinical miscarriage can be further divided into early clinical miscarriage, which occurs before the twelfth week of gestation or late clinical miscarriage, which occurs between the twentieth and twenty-first week of pregnancy.

The incidence of early clinical miscarriage is about 15%, with considerable variation depending on maternal age [2,3]. For women aged 20 to 24 years, the incidence of early clinical miscarriage is 10%, but it rises to 51% among women aged 40 to 44 years [4].

Late pregnancy losses between 12 and 21 weeks are less common, with an incidence rate of 4% [4].

Unlike sporadic miscarriages, recurrent miscarriages have significantly lower incidence rate and if only clinical miscarriages are considered, the prevalence is 0.8% to 1.4%. Although clear criteria for the definition of recurrent miscarriage are not yet established, ESHRE recommends that recurrent miscarriage is the one preceded by two or more consecutive pregnancy losses before the 22nd week of gestation [4]. According to this definition, one out of every three women hasexperienced recurrent miscarriages [5].

In most medical facilities in the world, products of conception from spontaneous abortions are sent for routine histopathological analysis. This analysis is intended primarily to confirm the presence of intrauterine pregnancy. It is also important torule out a possible molar pregnancy, which increases the risk of persistent trophoblastic disease or choriocarcinoma. Also, using additional molecular diagnostic techniques such as flow cytometry, *in situ* hybridization and polymerase chain reaction, it is possible to detect aneuploidy or to accurately determine the type of molar pregnancy [6].

Chromosomal abnormalities are common in early spontaneous abortions [7,8]. According to some studies, the incidence of aneuploid zygotes in general is around 40% [8]. Aneuploidy rarely occurs in successful pregnancies (0.6%), but is far more common in miscarriages in the first trimester, when it occurs in approximately 50-60% of cases [9,10]. It is thought that chromosomally abnormal embryos suffer adverse selection in the first few weeks of life, with mechanisms likely involving implantation and placental development (so-called fetal-maternal interactions). The frequency and type of chromosomal abnormalities that occur in spontaneous abortions vary with gestational age of the fetus and maternal age. Loss of pregnancy that occurs in the first weeks of pregnancy is characterized by a wide range of unusual aneuploidies, whereas loss of pregnancy later in the gestation is usually a consequence of aneuploidy typically found in live births, such as trisomy 21, 18 or 13 [11].

Fetal aneuploidies are found in 90% of cases with pregnancy loss between the first and sixth weeks, while about 50% of pregnancy losses are due to aneuploidy between the eighth and eleventh week of gestation. The proportion of abortions that are due to aneuploidy decreases with gestational age and isaround 6 to 12% after the twelfthweek of gestation [11]. When a heart rate is detected by ultrasound examination, the risk of aneuploidy is thought to be less than 5% [5]. The frequency of sporadic pregnancy loss and all fetal chromosomal abnormalities increases with maternal age [12].

The aim of the study was to detect aneuploidies in early spontaneous abortions. Also, to identify the subtle histological changes of the placenta that would eventually suggest presence of aneploidy.

Material and methods

This study wasdesigned as a prospective one. It included a total of 181 consecutive cases of early miscarriage diagnosed in the period from May 2012 to December 2014. Additional control group included 50 cases of early artificial abortions.

The study cases and the control cases were analyzedand evaluated in the Laboratory for histopathology and cytology at the "Acibadem Sistina" Hospital and the Research Centre for Genetic Engineering and Biotechnology "George D. Efremov" at the Macedonian Academy of Sciences and Arts in Skopje.

All products of conception werefirst analyzed macroscopically fresh (non-fixed) (Figure 1). Then, 500 mm^3 of placental tissue for molecular detection of chromo-

somal abnormalities and 500-1000 mm^3 of decidual tissue for comparative genetic analysis of the motherwere selected and frozen in liquid nitrogen for further molecular diagnostics.

Fig. 1. Macroscopic selection of placental and decidual tissue

In the next step, additional representative samples of placental and decidual tissue were selected and fixed for 24 hours in 10% neutral buffered formalin. After wards, the samples were processed in a tissue processor, using a standard tissue processing procedure.

The paraffin tissue blocks were cut in 4µ thin sections, which were deparaffinized, rehydrated and stained with hematoxylin and eosin.

Microscopic analysis of the placental tissue was performed according to the criteria described below.This analysis was performed on 143 samples of spontaneous abortions (two cases were excluded from this analysis due to lack of adequate quantity of placental tissue) and 34 samples from the control group. A standard light microscope Leica DM2500 equipped with 4, 10, 20, 40 and 100 times objectives was used for this analysis.

The following characteristics of the placental tissue were analyzed: villous contours, appearance of the villous stroma (mucoid or hydropic change, cavitations), presence of fetal erythrocytes, trophoblastic hyperplasia and trophoblastic stromal inclusions.

Molecular analysis of the tissue was performed in 145 samples from the study group and in 34 samples from the control group. The reasons for exclusion of the rest of the samples are given in Table 1.

A small volume of about 2-3mm3 of the frozen tissue samples was transferred in 1.5 ml sterile tube. Samples were washed with 1ml 1xPBS (phosphate buffer). Then, the samples were centrifuged at 12000 rpm at 4˚C for 5 minutes. The resulting sludge was digested in a waterbath at 56˚C in 500 µl digestion solution containing 20 µl proteinase K (10mg/ml). After 2 hours (time required for digestion), DNA (deoxyribonucleic acid) extraction was performed using a mixture of equal amounts of phenol (250µl) and chloroform (250µl). The solutions were mixed together for 5 minutes; then the mixturewas centrifuged 5 minutes at 12000 rpm. After spinning, the resulting upper liquid phase was transferred to a 1.5 ml sterile tube and the cycle was repeated. By adding 1ml of 96% ethanol, the DNA was precipitated and then centrifuged 30 minutes, at 12000 rpm at 4˚C. The samples were rinsed with 70% ethanol and centrifuged at 12000 rpm for additional 5 minutes. The excess of ethanol was removed and DNA was left for few minutes at room temperature until complete evaporation of the ethanol. Depending on the amount of DNA obtained, it was further dissolves in 50 µl sterile water.

In order to determine the possible presence of chromosomal aneuploidy in the placental tissue, QF-PCR (Quantitative Fluorescent Polymerase Chain Reaction) analysis was performed on ABI-PRISM 3100 and 3500 genetic analyzers. In this PCR method, one of the primers used for amplification is fluorescently labeled. The obtained products are fluorescent, therefore apart from the quailtative characteristics of the PCR amplification (amplicon size), the quantity of the amplicons in the amplified genome can also be determined. The analysis is performed in a multiplex PCR reaction analyzing fourshort tandem repeats (STR) on chromosomes 18 (D18S535, D18S391, D18S390 and D18S386), 21 (D21S1435, D21S1446, D21S1411 and D21S1414), 13 (D13S631, D13S305, D13S258 and D13S1817), two on the X chromosome (DXS6803 and HPRT), and amelogenin locus (AMXS) to determine the gender. These markers are chosen to be trinucleotide repeats and have higher heterozygosity to be informative. PCR reactions were performed in a mixture with a total volume of 20 µl, containing 1xPCR buffer, 1.5-2.0 mM MgCl2, 200 microns ofdNTP, 25pM of the respective primers (direct and reverse), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 0.1 0.5µg DNA. PCR reaction was carried out on Thermal Cycler (Applied Biosystems 2720) under the following conditions: initial denaturation at 95°C for 10 min with activated AmpliTaq Gold polymerase, followed by 28 cycles of denaturation at 95°C, annealing at 58°C for 1 min and elongation at 72 °C for 1 min and 30 seconds.

If chromosomal aneuploidies were not detected with the QF-PCR analysis, the subtelomeric regions on all chromosomes were further analyzed using the MLPA (Multiplex Ligation Probe Amplification) method. This method is performed using two kits: SALSA MLPA kit P036-D Human Telomere-3 and SALSA MLPA P070-D Human Telomere-5 (MRC Holland, Amsterdam). These two kits contain 45 different oligonucleotide sequences from different subtelomeric regions of the short and long arms of all autosomal and sex chromosomes. The MLPA method allows simultaneous amplification of all 45 target DNA

sequences. Each MLPAprobe contains short specific sequences (22-43 nucleotides) and auniversal direct and reverse PCR primer. In order to distinguish the amplified products, probes also contain non-hybridizing sequence of variable length (19-364 nucleotides). The hybridization reaction is performed overnight and the target DNA sequences connect with the hybridizing probes in a ligation reaction. Ligated products are further amplified in a PCR reaction. The relative quantity of each PCRproduct is proportional to the copy numbers of the target sequence.The relative amount of each PCR product is proportional to the number of copies of the target sequence. PCR products of different sizes are separated by automated capillary electrophoresis. The hybridization reactions, ligation and amplification were performed on ABI 9800 Thermal Cycler (Applied Biosystems, USA).

The statistical analysis was performed using statistical program SPSS forWindows, 17.

Results

Chromosomal aberrations were significantly more often detected in the study group of early spontaneous abortions than in the control groupof artificial abortions (p=0.000001). In fact, aneuploidy was detected in only 5.9% of the control cases (2/34%), whereas in 53.1% of the studycases (77/145). Results from the molecular analysis of both groups are shown in detail in Table 1.

The most commonly detected chromosomal abnormality was trisomy 16, which was found in 14.5% of cases.

Theageofthepatients did not influence the occurrence of any of the chromosomal abnormalities (p>0.05). Patients with trisomy 16, trisomy 18, trisomy 21, trisomy 22 and Turnersyndrome were insignificantly older than patients without these abnormalities (38.0±4.2 vs 36.16±5.7; 40.4±4.2 vs 36.26±5.6; 37.0±6.4vs36.36±5.5; 37.69±4.4 vs 36.27±5.63 and 36.67±5.7 vs 36.37±5.6, consequently).

Histological analysis was performed in 143 cases from the study group and 34 cases from the control group. These results were compared with the molecular test results.

Statistically significant association was found between the presence of trophoblastic hyperplasia on the villous surface and aneuploidy (p=0.0004). Trophoblastic hyperplasia was present in 32(47.76%) samples with normal genotype and in 58(76.32%) samples with aneuploidy. There was no significant difference in the type of trophoblastic proliferation, since both focal and diffuse trophoblastic proliferation were more often present in aneuploid cases (p=0.79).

Table 1. Chromosomal abnormalities in the study group and the control group

	Control group n=50	**Study group n=181**
Chromosomal abnormalityn (%)		
Normal	32 (94.12)	68 (46.90)
Trisomy 2	0	1 (0.68)
Trisomy 4	0	1 (0.68)
Trisomy 6	0	1 (0.68)
Trisomy 7	0	2 (1.38)
Trisomy 8	0	3 (2.07)
Trisomy 9	0	1 (0.68)
Trisomy 10	0	1 (0.68)
Trisomy 14	0	6 (4.14)
Trisomy 15	0	3 (2.07)
Trisomy 16	1 (2.94)	21 (14.48)
Trisomy 18	0	5 (3.45)
Trisomy 14/Trisomy 20	0	1 (0.68)
Trisomy 21	0	5 (3.45)
Trisomy 22	0	13 (8,97)
Triploidy	1 (2.94)	5 (3.45)
Monosomy 21	0	1 (0.68)
Turner Sy	0	6 (4.14)
3p deletion	0	1 (0.68)
Total	34 (68)	145 (80.11)
Inadequate for molecular analysisn (%)		
Identical maternal and fetal profile	3 (18.75)	22 (61.1)
Poor quality DNA	0	2 (5.56)
DNA isolation not possible	13 (81.25)	12 (33.33)
Total	16 (32)	36 (19.89)

Table 2. Multivariate analysis of predictive histologic factors associated with early spontaneous abortions

Variable	**OR 95% CI for OR**	**p value**
Presence vs absence of trophoblastic proliferation	2.956 (1.322 – 6.609)	0.008**
Presence vs absence of trophoblastic stromal inclusions	0.699 (0.325 – 1.506)	0.361
Presence vs absence of irregular villous contours	1.86 (0.874 – 3.957)	0.008
Presence vs absence of stromal cavitations	0.948 (0.192 – 4.681)	0.948
Hydropic vs mucoidvillousstromal change	2.142 (0.781 – 5.875)	0.139
Pronounced edema vs mucoid villous stromal change	2.52 (0.68 – 9.337)	0.167
Presence vs absence offetal erythrocytes	0.124 (0.035 – 0.438)	0.001**

**p<0.01

The presence of fetal erythrocytes was more often seen in aneuploid than in euploidplacentas (64.47% vs 59.7%). However, this difference was not statistically significant (p=0.65).
Many nucleated red blood cells were significantly more often detected in the villous vasculature of euploid placentas compared to aneuploid ones (8.96% vs 1.82%, p=0.042).
Binary logistic regression analysis was used to determine the independent morphologic characteristics as predictors in early spontaneous abortions. Presence of villous trophoblastic hyperplasia (p=0.008) and fetal erythrocytes (p=0.001) were found to be independent significant predicting factors (Table 2).
All other histological parameters, such as villous contours, villous vascularity or presence of trophoblastic stromal inclusions did not show any significant variations between euploid and aneuploid placentas (Figure 2).

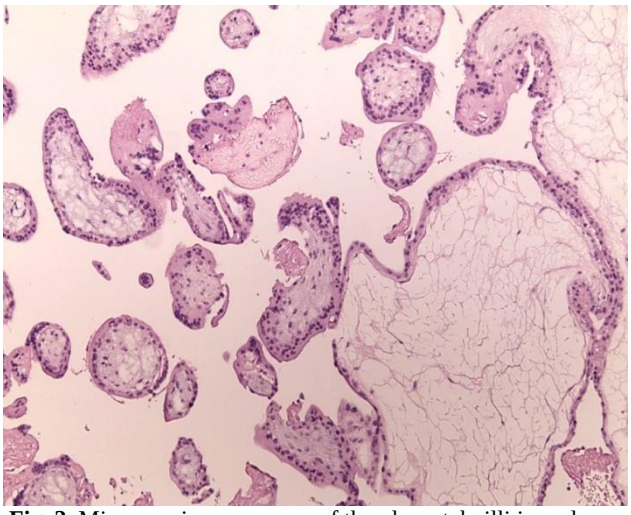


Fig. 2. Microscopic appearance of the placental villi in early spontaneous abortions (hematoxylin and eosin, x10)

Discussion

Placental role in pregnancy loss is not yet clear. Several authors suggest that aneuploidplacentas in early miscarriages have abnormal histology [13-15]. According to Genest *et al*. (1995), five histological features of the placenta can be used as predictive factors for abnormal karyotype: villous cavitation, fetal erythrocytes, abnormalities of amniotic membrane, presence of umbilical cord and presence of fetal tissue [14]. These authors suggest that villous dysmorphic features and presence of cystic cavities in the villous stroma are associated with triploidy while four characteristics (villous hydrops, absence of umbilical cord and fetal tissueand absence of fetal erythrocytes) are associated with trisomy [14].
In our study, only the presence of trophoblastic hyperplasia and paucity of fetal erythrocytes correlated with placental aneuploidy. Fetal erythrocytes in the villous vasculature in our study were more scarce in spontaneous abortions (62%) compared to artificial abortions (91%). There was also a significant difference in the presence of fetal red blood cells between euploid and aneuploid cases. In fact, according to the literature, fetal red blood cells are present mostly in normal pregnancy or partial molar pregnancy, while they are absent or present in significantly lower number in aneuploid placentas or complete molar pregnancy [14].
Regarding the findings of irregular villous contours, we did not find significant difference between euploid and aneuploid cases, nor between the control and study group. According to the literature, villous surface contours in aneuploid placentas should be irregular, jagged, unlike the smooth convex surface of euploid villi [14,16]. However, placental morphology in early pregnancy is not only influenced by karyotype, but also by gestational age and degenerative changes that are a consequence of intrauterine retention of a dead fetus. These degenerative changes are usually presented with stromal fibrosis, hypovascularization, stromal edema, attenuation of trophoblasts etc. [21]. On the other hand, the morphological changes of aneuploid placentas are very similar [22]. Other studies were also published in which authors found no significant correlation between the findings of chromosomal abnormality and histological changes in the placenta [16,21].
According to Jauniaux and Burton (2005), the main histological examination criteria for early pregnancy loss should be: villous contours, development oftrophoblasts (assessment of the degree of hyperplasia or hypoplasia), appearance of the villous stroma (degree of edema and fibrosis), development of the fetal circulation and intervillous fibrin deposition. According to these authors, so called villous type 1 tissuewith pronounced villous stromal edema is frequently associated with chromosomal abnormalities [16].
According to other authors, trophoblastic stromal inclusions are often associated with chromosomal abnormalities or genetic disorders without apparent chromosomal abnormality. However, longer periods of intrauterine death (3-8 weeks) should be taken into account,since the appearance of such artifacts can reduce the predictive value of placental histology in identifying aneuploid early spontaneous abortions [16].
We detected significantly more chromosomal abnormalities in cases of spontaneous abortions (53%) in comparison to the artificial ones (6%). These results correlate with other results reported in the literature. Namely, it is considered that chromosomal abnormalities can be found in about half of the early miscarriage cases [14]. On the other hand, when losses of pregnancy in the second or third trimester of pregnancy had been analyzed, chromosomal abnormalities werefound in only about 20% of cases [16]. The rate of aneuploidy

inartificial abortions is expected to range between 2.5 to 4.5% [17], which is in concordance with our results.

Most fetal chromosomal abnormalities are numeric (86%), while small percentage are structural abnormalities (6%) or other genetic mechanisms, including chromosomal mosaicism (8%) [17]. It not yet established whether chromosomal aberrations are more common in recurrent miscarriages, but it has been noted that around 50% of abortions in these women had chromosomal abnormalities [17]. Carp *et al.* (2001) published an incidence of 29% among women with three or more spontaneous abortions [18]. Ogasawara *et al*. (2000), however have shown that, as the number of abortions increases, the incidence of chromosomal aberrations is reduced [19]. The risk of recurrence of numerical abnormalities in subsequent pregnancies is small, so according to some authors karyotyping of fetal tissue in all cases of abortion is not considered an effective method in daily practice [17]. However, some authors routinely perform karyotyping or genotyping of fetal tissue from spontaneous abortions, since the discovery of chromosomal abnormalities in the fetus allow plausible explanation for the loss to the anxious couples.

Macroscopic analysis is acommonly used selection method in pathology laboratories. Trained and experienced medical staff can easily recognize fragments of placenta and decidual tissue. But if products of conception are scarce, mixed with large amount of blood, the selection of adequate material for analysis can be significantly impaired. In many institutions worldwide, laser microdissection isa method of choice for selection of placental fragments for further molecular analysis. However, according to our results, macroscopic tissue selection method, especially if trained pathologist is directly involved in the selection process, is simple, inexpensive and less time consuming method. Of course, experience is of utmost importance. Thus, during the initial selection of the first 50 samples, the failure rate of obtaining a good quality sample ranged up to 36%. But in the last 100 samples, in which the selection of tissue samples was carried out exclusively by an experienced pathologist, the rate of inadequate samples for molecular analysis was reduced to just 5%. This method of selection of material for genetic analysis by freezing fresh tissue is also used by other researchers such as Kokawa*et al.* (1998) [20].

There are several methods used for detection of numerical chromosomal abnormalities in spontaneous abortions. One is standard cytogenetic evaluation of tissue from spontaneous abortions. According to the literature, this method has certain limitations. Above all, vital tissue is mandatory, which may be a problem in cases with longer intrauterine retention of a dead fetus [23]. There is also a significant risk of contamination with vital decidual tissue from the mother, which can possibly give normal female karyotype originating from the mother (30-40% of cases) [24,25], not the fetus. In addition, there is a high risk of bacterial contamination [14,26]. For example, Diego-Alvarez *et al*. (2007) investigated a total of 517 samples of early miscarriage and analyzed them by a conventional cytogenetic karyotyping. Successful karyotyping was performed in only 321 cases, i.e.in about 60% of cases [27]. On the other hand, the advantage of conventional cytogenetic analysis is the ability to detect structural rearrangements, which although rare, could explain the cause of early miscarriage in 4-8% of cases [14].

Fluorescent*in situ* hybridization (FISH) allows rapid determination of aneuploidy. Several studies have shown that FISH is an effective and rapid technique for the diagnosis of numerical aberrations in amniocytes with significantly lower costs than traditional karyotyping [17,28]. Namely, all other molecular techniques such as MLPA, QF-PCR and array-CGH (Comparative Genomic Hybridization) have a definite advantage over conventional karyotyping. This refers to lower failure rate, shorter analysis time and significantly higher resolution [24,29]. Using FISH and QF-PCR, few chromosomes can be analyzed in one reaction, while MLPA and array-CGH methods can analyze copy number variations in 48 regions of the genome in a single reaction.

In this study we used two molecular methods to detect aneuploidy in placentas. TheQF-PCR method is based on amplification of chromosome-specific repetitive DNA sequences, so called STR (Short Tandem Repeats). These sequences are stable and polymorphic; they vary in length in different individuals, depending on the number of tri-, tetra- or penta-nucleotiderepetitions. The sample DNA was amplified in a PCR reaction using fluorescent primers. Thus, PCR products can be visualized and quantified as a peak of respective repetitive sequences of varying length. When DNA from normal heterozygous individual is amplified (whose DNA contains alleles of different length), two peaks in a zone are expected to be seen. When DNA from individuals who have certain trisomy isamplified, three peaks (three alleles), ortwo peaks (two alleles), one of which will be two times wider than the other, will appear in the relevant area [30].

If excessive or absent signal (or abnormal ratio) of a marker is found in an otherwise normal multiplex reaction, this could be due to fetal constitutional duplication or deletion of a chromosomal segment where the appropriate marker is located. In such cases, it is recommended to test the parents and retest the neighboring markers in order to identify the size of the alleged duplication or deletion [30].

Another very important advantage of the QF-PCR method is its ability to detectcontamination with maternal DNA. This property presents a significant advantage over FISH method for example, which can not distinguish contamination with maternal DNA in normal female karyotype. Thus, in our study maternal DNA

contamination was found in 12% of the samples in the study group. Of particular importance in the diagnosis of spontaneous abortion is the possibility of the QF-PRC method to be performed on archival material, or tissue samples from paraffin blocks. The concordance of the QF-PCR results with karyotype, according to some authors is around 95% [31].

Whenwe did not find any aneuploidy with the QF-PCR method, subtelomeric regions of all chromosomes were further analyzed using MLPA analysis. When comparing MLPA with array-CGH, MLPA method is cheaper and provides reliable and efficient detection of chromosomal numerical aberrations and subtelomeric copy number variations. The success rate ranges up to 98% and according to the literature, the method shows concordance> 90% with the karyotype [24]. However, the MLPA method has some disadvantages. Thus, the number of copies in this analysis is compared with a haploid set of chromosomes, rendering detection of polyploidy, low-grade mosaicism, balanced translocation or maternal DNA contamination impossible [24].

In this study the most common abnormality detected was trisomy 16, followed by trisomy 22, 21, 14 and 18. Other authors also refer highest incidence of trisomy 16 in early spontaneous abortions [17,28], followed by trisomy 13, 18, 21 and 22 [17]. It is considered the most common trisomy in early miscarriages with an incidence of about 1.5% of all clinically recognized pregnancies. Most fetuses with trisomy 16 end in miscarriage between 8 to 15 weeks of pregnancy [32].

Chromosomal abnormalities are the cause of pregnancy loss in 50 to 80% of cases depending on maternal and gestational age at the time of pregnancy loss [5]. Turner syndrome is the most frequent chromosomal abnormallity of sex chromosomes, found in 20 to 25% of cytogenetically abnormal fetuses by some authors [33,34].

Triploidy and tetraploidy result from abnormal fertilization and are not compatible with life. Primary pathogennic mechanism in triploidy is fertilization of a normal haploid egg by two sperm cells [35], although other pathogenetic mechanisms may lead to triploidy. In our study, triploidy was detected in 3.5% of the cases.

In conclusion, this study allowed clarification of the etiological causes of early spontaneous abortion ina large group of patients.Histopathological analysis alone could not predict aneuploidy in early spontaneous abortion. Therefore, in order to obtain a correct diagnosis valuable to the clinicians, products of conception from early spontaneous abortions should be submitted for histopathological analysis and molecular genotyping whenever possible.

Conflict of interest statement. None declared.

References

1. Jauniaux E, Kadri R, and Hustin J. Partial mole and triploidy: screening patients with first-trimester spontaneous abortion. *Obstet Gynecol* 1996; 88(4 Pt 1): 616-619.
2. Angiolucci M, *et al*. Association between different morphological types and abnormal karyotypes in early pregnancy loss. *Ultrasound Obstet Gynecol* 2011; 37(2): 219-225.
3. Coelho FF, *et al*. Detection of aneuploidies in spontaneous abortions by quantitative fluorescent PCR with short tandem repeat markers: a retrospective study. *Genet Mol Res* 2016; 15(3).
4. Larsen EC, *et al*. New insights into mechanisms behind miscarriage. *BMC Med* 2013; 11: 154.
5. Hyde KJ, and DJ. Schust, Genetic considerations in recurrent pregnancy loss. *Cold Spring Harb Perspect Med* 2015; 5(3): a023119.
6. Fram KM. Histological analysis of the products of conception following first trimester abortion at Jordan University Hospital. *Eur J Obstet Gynecol Reprod Biol* 2002; 105(2): 147-149.
7. Wu Z, *et al*. [Detection of chromosome aneuploidies in spontaneous abortion villus samples by quantitative fluorescence PCR]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi, 2016; 33(2): 227-230.
8. Qumsiyeh MB, *et al*. Cytogenetics and mechanisms of spontaneous abortions: increased apoptosis and decreased cell proliferation in chromosomally abnormal villi. *Cytogenet Cell Genet* 2000; 88(3-4): p. 230-235.
9. Warburton D, *et al*. Does the karyotype of a spontaneous abortion predict the karyotype of a subsequent abortion? Evidence from 273 women with two karyotyped spontaneous abortions. *Am J Hum Genet* 1987; 41(3): 465-483.
10. Jacobs PA, *et al*. Trisomy 13 ascertained in a survey of spontaneous abortions. *J Med Genet* 1987; 24(12): 721-724.
11. Wapner RJ and Lewis D. Genetics and metabolic causes of stillbirth. *Semin Perinatol* 2002; 26(1): 70-74.
12. Munne S, *et al*. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil Steril* 1995; 64(2): 382-391.
13. Salafia C, *et al*. Placental and decidual histology in spontaneous abortion: detailed description and correlations with chromosome number. *Obstet Gynecol* 1993; 82(2): 295-303.
14. Genest DR, *et al*. Fetoplacental histology as a predictor of karyotype: a controlled study of spontaneous first trimester abortions. *Hum Pathol* 1995; 26(2): 201-209.
15. Jindal P, *et al*. Placental pathology of recurrent spontaneous abortion: the role of histopathological examination of products of conception in routine clinical practice: a mini review. *Hum Reprod* 2007; 22(2): 313-316.
16. Jauniaux E, and Burton GJ. Pathophysiology of histological changes in early pregnancy loss. Placenta 2005; 26(2-3): 114-123.
17. Fejgin MD, *et al*. Fluorescent in situ hybridization: an effective and less costly technique for genetic evaluation of products of conception in pregnancy losses. *Acta Obstet Gynecol Scand* 2005; 84(9): 860-863.
18. Carp H, *et al*. Karyotype of the abortus in recurrent miscarriage. *Fertil Steril* 2001; 75(4): 678-682.
19. Ogasawara M, *et al*. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. *Fertil Steril* 2000; 73(2): 300-304.
20. Kokawa K, Shikone T, and Nakano R. Apoptosis in human chorionic villi and decidua during normal embryonic development and spontaneous abortion in the first trimester. *Placenta* 1998; 19(1): 21-26.

21. Fukunaga M, *et al*. Is There a Correlation Between Histology and Karyotype in Early Spontaneous Abortion? *International Journal of Surgical Pathology* 1995; 2(4): 295-300.
22. Paul M, *et al*. Early molar pregnancy: experience in a large abortion service. *Contraception* 2010; 81(2): 150-156.
23. Carp HJ, *et al*. Prognosis of subsequent pregnancies after recurrent spontaneous abortion in first trimester. *Br Med J (Clin Res Ed)* 1987; 295(6603): 925-926.
24. Saxena D, *et al*. Utility and limitations of multiplex ligation-dependent probe amplification technique in the detection of cytogenetic abnormalities in products of conception. *J Postgrad Med* 2016; 62(4): 239-241.
25. Cheng HH, *et al*. Chromosome distribution of early miscarriages with present or absent embryos: female predominance. *J Assist Reprod Genet* 2014; 31(8): 1059-1064.
26. Wu T, *et al*. Molecular cytogenetic analysis of early spontaneous abortions conceived from varying assisted reproductive technology procedures. *Mol Cytogenet* 2016; 9: 79.
27. Diego-Alvarez D, *et al*. MLPA as a screening method of aneuploidy and unbalanced chromosomal rearrangements in spontaneous miscarriages. *Prenat Diagn* 2007; 27(8): 765-771.
28. Lescoat D, *et al*. Fluorescent in situ hybridization (FISH) on paraffin-embedded placental tissues as an adjunct for understanding the etiology of early spontaneous abortion. *Prenat Diagn* 2005; 25(4): 314-317.
29. McClelland LS, *et al*. Implementation and experience of an alternative QF-PCR and MLPA diagnostic strategy to detect chromosomal abnormalities in fetal and neonatal pathology samples. *Pediatr Dev Pathol* 2011; 14(6): 460-468.
30. Hulten MA, Dhanjal S, and Pertl B. Rapid and simple prenatal diagnosis of common chromosome disorders: advantages and disadvantages of the molecular methods FISH and QF-PCR. *Reproduction* 2003; 126(3): 279-297.
31. Zou G, *et al*. Quantitative fluorescent polymerase chain reaction to detect chromosomal anomalies in spontaneous abortion. *Int J Gynaecol Obstet* 2008; 103(3): 237-240.
32. Yong PJ, *et al*. Clinical aspects, prenatal diagnosis, and pathogenesis of trisomy 16 mosaicism. *J Med Genet* 2003; 40(3): 175-182.
33. Chaithra P, MSaCS. An Overview of Genetic and Molecular Factors Responsible for Recurrent Pregnancy Loss. *Int J Hum Genet* 2011; 11(4): 217-225.
34. Nagy B, *et al*. Detection of sex chromosome aneuploidies using quantitative fluorescent PCR in the Hungarian population. *Clin Chim Acta* 2015; 445: 2-6.
35. Zaragoza MV, *et al*. Parental origin and phenotype of triploidy in spontaneous abortions: predominance of diandry and association with the partial hydatidiform mole. *Am J Hum Genet* 2000; 66(6): 1807-1820.

DE GRUYTER OPEN

Mac Med Review 2017; 71(1): 72-74

MMR

DOI:10.1515/mmr-2017-0013

Case report

SYMPLASTIC LEIOMYOMA OF THE UTERUS- CASE REPORT

СИМПЛАСТИЧЕН ЛЕЈОМИОМ НА УТЕРУС-ПРИКАЗ НА СЛУЧАЈ

Snezana Georgieva[1], Tanja Nikolova[1] and Natasha Nikolova[2]

[1]University Clinic of Obstetrics and Gynecology, Skopje, [2]Private Medical Center "Promedika", Skopje, Republic of Macedonia

Abstract

We present a case of atwenty-four-year old, nulligravid woman with suspicionof molar pregnancy and admittedat the University Clinic of Obstetrics and Gynecology in Skopje. On bimanual examination uniformly enlarged uterus was detected. Ultrasonography revealed snow storm echo signal in the uterine cavity. Dilation and curettage wereperformed and only a slight quantity of material was obtained. Serum bHCG was negative. MRI revealed a finding of the tumorous formation on the anterior wall of the uterus deforming and dislocating the uterus without any additional information. Open surgery was planned and tumorectomy was performedinour hospital. Histopathology analysis of the obtained material revealed symplastic myoma of the uterus. The patient got spontaneously pregnant later, but the pregnancy finished as a spontaneous missed abortion and instrumental revision was performed. Five months later the patient got pregnant again. She had regular pregnancy course and delivery at 37.5 gestational week.

Keywords: symplasticleiomyoma, uterus, pregnancy

Апстракт

Во овој труд презентиравме случај на дваесетичетиригодишна жена, нулигравида, која поради сомнение за моларна бременост беше примена на Универзитетската клиника за гинекологија и акушерство во Скопје. На бимануелен преглед е најден униформно зголемен утерус. Ултразвучно е добиен сигнал на "снежна бура" во утериниот кавитет. Направена е дилатација и киретажа на утериниот кавитет, но е добиен оскуден матерјал.
Наодот на бета ХЦГ во серум беше негативен. На МРИ на мала карлица еприкажана туморозна формација на предниот ѕид на утерусот, која го деформира и дислоцира, без информација за друг патолошки наод. Со отворена хирургија е направена туморектомија во нашата клиника.
Хистопатолошката анализа на добиениот оперативен материјал потврди симпластичен миом на утерусот. Подоцна пациентката спонтано забремени, но бременоста заврши со мисетирање на плодот и спонтан аборус, проследен со инструментална ревизија. Пет месеци подоцна пациентката повторно забремени, текот на бременоста уреден со завршување на истата во 37,5 гестациска недела.

Клучни зборови: симпластичен лејомиом, утерус, бременост

Introduction

Leiomyomas are the commonest smooth muscle tumors of the uterus and they are further classified according to their pathological features. Majority of the uterine leiomyomas are very easy to diagnose, but some of the subtypes, such as symplastic leiomyomas mimic malignancy with their cellular characteristics [1]. Symplastic leio-myomas are also known in the literature as atypical and bizarre leiomyomas and are very rare benign tumors of the myometrium, around 0.5% of all mesenchymal ute-rine tumors [2]. This makes them of a great interest for both pathologists and clinicians. These tumors require a precise histology analysis in order not to be misclassi-fied as leiomyosarcoma, because of their completely different therapeutic management.
They are characterized with high cellularity, numerous widely distributed bizarre myocytes with moderate to severe cytological atypia. Cellular necrosis is absent andmitotic index is less than 10 mf/hpf [3]. These tumors appear in the same age group and the size as well as location are similar to those of common leiomyoma, but clinical behavior and prognosis are different, varying in correlation with the number of the mitotic activity [2].
This case report presents a case of symplastic leiomyoma of the uterus in a young nulligravid woman.

Correspondence to: Snezana Georgieva, University Clinic of Obstetrics and Gynecology, "Vodnjanska" 17, 1000 Skopje, R. Macedonia; E-mail: gsnezana@gmail.com

Case report

We present a case of a twenty-four-year old, nulligravid woman, who has been admitted at the University Clinic of Obstetrics and Gynecology in Skopje, with suspicion of molar pregnancy. She reported anamnestic data for irregular bleeding from the uterus which was preceded by athree-month period of amenorrhea. Previous menstrual cycles were reported to be regular, 5-7/28 days with ave-rage flow associated with slight dysmenorrhea.

On bimanual gynecological examination she was found to have uniformly enlarged uterus, about 20 gestational weeks of size, but mobile, soft and non-tender.

Ultrasonography evaluation ofthe uterus was performed with a finding of a tumorous formation centrally positioned, which ranged 112x87mm in crossed diameters. It was associated with a waxen cells appearance (snow storm echo) and was stuffing the uterine cavity, leaving only a thin layer of the uterine wall.

Serum bHCG was analyzed and a negative result was obtained.

Tumor markers: Carcinoembryonic antigen (CEA), Cancer antigen 125 (CA 125), Cancer antigen 72-4 (CA 72-4) and Cancer antigen 19-9 (CA 19-9) were within the reference ranges. Conventional PAP smear was performed one month before and was negative for a presence of cervical cellular atypia.

Magnetic resonance imaging (MRI) revealed a finding of atimorous formation on the anterior wall of the uterus with the already above mentioned dimensions. On MRI the tumor was deforming and dislocating the uterus.

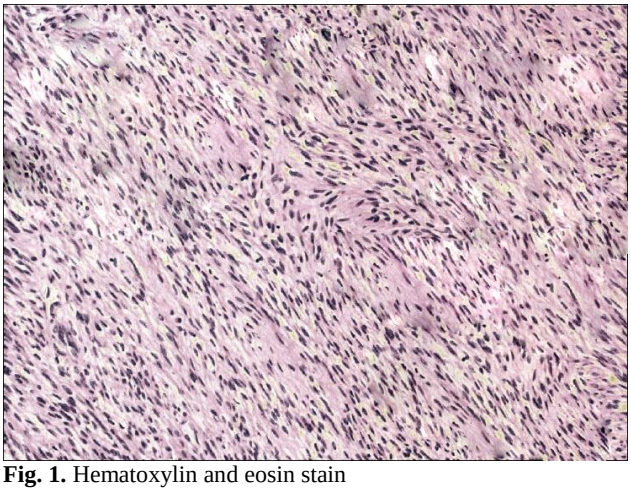


Fig. 1. Hematoxylin and eosin stain

Diagnostic and therapeutic dilatation and curettage of the uterine cavity was performed and only a slight quantity of material was obtained. Material was sent for histology verification at the Institute for Radiotherapy and Onco-logy in Skopje. The finding confirmeda regular endo-metrium in secretory phase of the menstrual cycle.

The patient was adequately prepared for open surgery andtumorectomy was performed. During the operation, because of the vast tumor dimensions, the uterine cavity was opened, but it was afterwards successfully recon-structed. Surgeryand the postoperative period were with regular course. Operative material was sent and analyzed at the Institute for Radiotherapy and Oncology in Skopje and the report revealed a presence of symplastic leio-myoma of the uterus (Figure 1).

Control hysterosalpingography was planned just to check the uterine cavity after its reconstruction three months following the operation, but in the meanwhile spontaneous pregnancy occurred. Unfortunately this pregnancy finished as early missed abortion in the eighth gestational week and instrumental revision was performed. Five months later thepatient got pregnant again. The pregnancy course was regular and uncomplicated and the patient delivered by Cesarean section in 37.5 gestational week.

Postpartum follow-upon ultrasound waswithout tumor recurrence.

Discussion

Symplasticleiomyomas belong to a mesenchymal type of uterine neoplasms. In different studies patients present with different clinical features. Our patient was with sym-ptoms of amenorrhea and a slight dysmenorrhea. She was slightly younger thanpatients reported by Downes *et al*. where their age ranged from 25 to 51 years [1].

In some cases this type of myomaisclassified as myometrial dysplasia (atypical myometrial hyperplasia). Most often symplastic leiomyomas are managed with minima-lly invasive surgical techniques. Dependent on cellular features, symplastic leiomyoma raise the question of postoperative recurrence and a possibility of distant metastases [4,5]. Since ourpatient was young and wanted to preserve fertility, conservative surgical treatment with myomectomy was performed.

In the follow-up period the patient got pregnant twice and her second pregnancy finished successfully in term, at 37.5 g.w.It suggested that symplasticmyoma in our ca-se had no negative impact on the patient's fertility. The studies published on postoperative management and fo-llow-up of patients after myomectomy have concluded that the behavior of thistype of tumors is still not clear [6].

For more precise diagnostics immunohistochemical straining methods are being used. p16, p53, Ki 67, Desminexpre-ssion and Calponin h1 are usually used [7]. In cases where there is an overexpression of p16, p53 and Ki 67, and a reduced expression of Calponin h1 and Desmin, leiomyosarcoma should be considered [7-9].

Diagnostic process in cases of symplasticmyoma may be very hard, if the clinician takes into consideration only the ultrasound finding. Enlarged uterus and irregular

ute-rine bleeding after a period of amenorrhea in this case were misleading in the direction of molar pregnancy. Additional investigations may help much in reaching the right diagnosis and planning the adequate therapy.

Conclusion

Symplastic leiomyomas are characterized with high mi-totic activity, bizarre cells, nuclear atypia, but the tumor is benign and should be well-differentiated from leio-myosarcoma. Proper evaluation of the operative material should be done and careful postoperative follow-ups are mandatory. In cases with finished reproduction, total hysterectomy should be considered.

Conflict of interest statement. None declared.

References:

1. Downes KA, Hart WR. Bizarre leiomyomas of the uterus: a comprehensive pathologic study of 24 cases with long-term follow-up. *Am J Surg Pathol* 1997; 21(11): 1261-1270.
2. Rammeh-Rommani S, Mokni M, Stita W, *et al.* Uterine smooth muscle tumors: retrospective epidemiological and pathological study of 2760 cases. *J Gynecol Obstet Biol Reprod (Paris)* 2005; 34(6): 568-571.
3. Bell SW, Kempson RL, Hendrickson MR. Problematic uterine smooth muscle neoplasms. A clinicopathologic study of 213 cases. *Am J Surg Pathol* 1994; 18(6): 535-558.
4. Seidman MA, Oduyebo T, Muto MG, *et al*. Peritoneal disse-mination complicating morcellation of uterine mesenchymal neoplasms. *PloS One* 2012; 7(11): e50058.
5. Yoon G, Kim T-J, Sung C-O, *et al*. Benign metastasizing leiomyoma with multiple lymph node metastasis: a case report. *Cancer Res Treat Off J Korean Cancer Assoc* 2011; 43(2): 131-133.
6. Sung CO, Ahn G, Song SY, *et al*. Atypical leiomyomas of the uterus with long-term follow-up after myomectomy with immunohistochemical analysis for p16INK4A, p53, Ki-67, estrogen receptors, and progesterone receptors. *Int J Gynecol Pathol Off J Int Soc Gynecol Pathol* 2009; 28(6): 529-534.
7. O'Neill CJ, McBride HA, Connolly LE, McCluggage WG. Uterine leiomyosarcomas are characterized by high p16, p53 and MIB1 expression in comparison with usual leiomyomas, leiomyoma variants and smooth muscle tumours of uncer-tain malignant potential. *Histopathology* 2007; 50(7): 851-858.
8. Horiuchi A, Nikaido T, Ito K, *et al*. Reduced expression of calponin h1 in leiomyosarcoma of the uterus. *Lab Investig J Tech Methods Pathol* 1998; 78(7): 839-846.
9. Bodner-Adler B, Bodner K, Czerwenka K, Kimberger O, Leodolter S, Mayerhofer K. Expression of p16 protein in patients with uterine smooth muscle tumors: an immunohis-tochemical analysis. *Gynecol Oncol* 2005; 96(1): 62-66.

Mac Med Review 2017; 71(1): 75-81

MMR

DOI:10.1515/mmr-2017-0014

Guidelines

HOW TO RECOGNIZE AND AVOID POTENTIAL, POSSIBLE, OR PROBABLE PREDATORY OPEN-ACCESS PUBLISHERS, STANDALONE, AND HIJACKED JOURNALS

КАКО ДА СЕ ПРЕПОЗНААТ И ИЗБЕГНАТ ПОТЕНЦИЈАЛНИТЕ, МОЖНИТЕ ИЛИ ВЕРОЈАТНИ ИЗДАВАЧИ И СПИСАНИЈА - ГРАБЛИВЦИ СО ОТВОРЕН ПРИСТАП И КИДНАПИРАНИ СПИСАНИЈА

Lenche Danevska[1], Mirko Spiroski[2], Doncho Donev[3], Nada Pop-Jordanova[4] and Momir Polenakovic[4]

[1]Central Medical Library, Medical Faculty, Ss Cyril and Methodius University of Skopje, [2]Faculty of Medicine, Ss Cyril and Methodius University of Skopje, [3]Institute of Social Medicine, Medical Faculty, Ss Cyril and Methodius University of Skopje, [4]Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia

This paper has already been published in Prilozi (Makedon Akad Nauk Umet Odd Med Nauki) 2016 Nov 1;37(2-3):5-13. doi: 10.1515/prilozi-2016-0011

Abstract

Introduction. The Internet has enabled an easy method to search through the vast majority of publications and has improved the impact of scholarly journals. However, it can also pose threats to the quality of published articles. New publishers and journals have emerged so-called open-access potential, possible, or probable predatory publishers and journals, and so-called hijacked journals. It was our aim to increase awareness and warn scholars, especially young researchers, how to recognize these journals and how to avoid submission of their papers to these journals.
Methods. Review and critical analysis of the relevant published literature, Internet sources and personal experience, thoughts, and observations of the authors.
Results. The web blog of Jeffrey Beall, University of Colorado, was greatly consulted. Jeffrey Beall is a Denver academic librarian who regularly maintains two lists: the first one, of potential, possible, or probable predatory publishers and the second one, of potential, possible, or probable predatory standalone journals. Aspects related to this topic presented by other authors have been discussed as well.
Conclusion. Academics should bear in mind how to differentiate between trustworthy and reliable journals and predatory ones, considering: publication ethics, peer-review process, international academic standards, indexing and abstracting, preservation in digital repositories, metrics, sustainability, etc.

Keywords: predatory publishers, standalone journals, hijacked journals, open access

Correspondence to: Lenche Danevska, MSc, Central Medical Library, Medical Faculty, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia, E-mail: lencedanevska@yahoo.com

Апстракт

Вовед. Интернетот овозможи лесен метод за пребарување огромен број публикации и го зголеми влијанието на научните списанија. Меѓутоа, тој исто така претставува и закана за квалитетот на објавените трудови. Се појавија нови издавачи и списанија, т.н. потенцијални, можни или веројатни издавачи и списанија-грабливци со отворен пристап и т.н. киднапирани списанија. Целта на трудот беше да се подигне свесноста и да се предупредат научниците, а особено младите истражувачи, како да ги препознаат овие списанија и како да избегнуваат да ги поднесуваат своите трудови во вакви списанија.
Методи. Преглед и критичка анализа на релевантната објавена литература, Интернет извори на информации и лично искуство, размислувања и погледи на авторите на трудот.
Резултати. Најмногу го консултиравме блогот на Џефри Бел од Универзитетот во Колорадо. Тој е библиотекар во Денверската академска библиотека и на својот блог редовно ги дополнува двете листи: првата за потенцијални, можни или веројатни издавачи-грабливци, и втората за потенцијални, можни или веројатни грабливи и киднапирани списанија со отворен пристап. Во трудот се претставени и ставовите на други автори во врска со оваа проблематика.
Заклучок. Научниците треба да прават разлика меѓу списанија кои се веќе афирмирани и кредибилни и списанијата-грабливци, притоа имајќи ги на ум следните работи: етика во публикувањето, процес на рецензирање на трудовите, интернационални академски стандарди, индексирање и вклучување

во бази на податоци, зачувување во дигитални репозиториуми, метрика, одржливост, итн.

Клучни зборови: издавачи-грабливци, списанија-грабливци, киднапирани списанија, отворен пристап

Introduction

Over the past several years there has been a debate in academic circles on the issue of how to recognize potential, possible, or probable open-access predatory scholarly publishers and how to avoid publishing in so-called hijacked journals.

Researchers, scholars, doctors and academic staff need to publish the results of their work and make them accessible to their colleagues and to the public. Also, academics tend to publish as many papers as possible in order to be promoted within their academic institutions. At this point, they have to cope with the issue of how to choose a relevant, reliable, true, peer-review journal, indexed in a reputable scientific database and then submit their manuscripts for publication. Since the emergence of these potential, possible, or probable open-access predatory publishers and journals this task has become even more daunting for scholars. Online hackers and cybercriminals have built fake or counterfeit websites for journals that actually mimic reputable journals.

Background: In 2008 Jeffrey Beall, an academic librarian and researcher at the University of Colorado, Denver, U.S.A. had received a large number of e-messages inviting him to submit articles to journals he was not familiar with. He then started extensive research on open-access publishers and coined the term "predatory scholarly open-access publishers". Such publishers use a business model where authors have to pay in order to publish their articles. In the *Chronicle of Higher Education* from 2012 Beall gives his definition of *predatory open-access publishing: "Predatory open-access publishers are those that unprofessionally exploit the gold open-access model for their own profit. That is to say, they operate as scholarly vanity presses and publish articles in exchange for the author fee. They are characterized by a various level of deception and lack of transparency in their operations. The open-access publishing model seems like a recipe for abuse: The more articles a publisher publishes, the more money it makes"* [1].

The aim of this paper is to give authors guidelines on how to recognize hijacked journals and to avoid cooperation with potential, possible, or probable predatory open-access publishers; that is, to raise awareness of dishonest publishing practices.

Methods

To accomplish the set aim, we have used information from the relevant published literature and registered websites available to the public, as well as our personal experience, thoughts, and observations.

First of all, we have consulted the blog platform and website of Jeffrey Beall (See: https://scholarlyoa.com) [2].

We have also consulted the list compiled and updated by Dr. Mehrdad Jalalian, a physician, journalist, book publisher and publication ethics researcher, who considers himself the world's leading researcher on the topics of hijacked journals (See: http://www. mehrdadjalalian. com/index.php/updates-of-hijacked-journals) [3].

To further illustrate the topic, we present the characteristics of predatory publishers and hijacked journals (Box 1).

Box 1. Characteristics of Predatory Publishers and Hijacked Journals

Large fees for articles revealed only after papers are submitted
Aggressively campaigning for academics to submit articles or serve on editorial boards
Listing academics as members of editorial boards without their permission
Appointing fake academics to editorial boards
Mimicking the name or web site style of more established journals
Improper use of ISSNs
Fake or non-existent impact factors
Accepting articles quickly with little or no peer review or quality control
Journals are not listed in standard periodical directories (such as Urlich's Periodicals Directory)

Results

Potential, possible, or probable predatory publishers

Predatory publishers use spam email to invite authors to publish their manuscripts, usually indicating large fees after papers are submitted. In fact, predatory publishing uses the open-access publishing business model, where it is very easy to set up an open-access publishing website. These websites can be created by almost anyone who has some knowledge of how to design them [4,5]. They charge publication fees to authors without providing the editorial and publishing services associated with legitimate journals. Sometimes they even negotiate a lower fee, when a potential author would comment on excessive fees. They indulge in very unethical and unscholarly practices just to collect money for publication. These publishers do not respect

any of the policies and guidelines given by the Council of Science Editors [6], International Association of Scientific, Technical & Medical Publishers (STM) Code of Conduct [7], or Committee on Publication Editors (COPE) [8]. Instead, to promote, preserve and make the published material available, these publishers exploit the author-pays model for their own profit. They also call academics to serve on the editorial boards in order to present an impression that it is a respectable journal. They have little or no peer review in most cases. Some claim to assess submission within 72 hours and digitally publish them upon acceptance and receipt of the fee. *"If the peer-review process were only that simple!"*-says Robert Bartholomew in his paper [9, 10]. Many of them have no digital preservation and they can disappear at any time, which will result in the loss of content.

Declan Butler, in his excellent paper on the explosion of open-access publishing [11], offers a checklist to identify reputable publishers. He warns authors to perform due diligence before they submit their manuscripts to a journal. He advises authors to: check whether the publisher has verifiable contact information, check whether editorial board list includes recognized experts with full affiliations, check whether the journal prominently displays its policy for author fees, be cautious when receiving e-mail invitations to submit to journals or to become an editorial board member, etc.

Beall has created *Beall's List of potential, possible, or predatory scholarly open-access publishers* (See: https://scholarlyoa.com/publishers/) [12] and he updates it regularly. He published his first list of predatory publishers in 2010 and in 2012 he posted his criteria for determining and evaluating publishers (See: https://scholarlyoa.com/2012/11/30/criteria-for-determining-predatory-open-access-publishers-2nd-edition) [13].

Here is one example of a medical publisher with some problems, which has been recently presented by Beall on his site [14]. It is InnoVison Health Media, which is a Minnesota-based publisher of six online medical journals. Beall has investigated this publisher and discovered that there were many editorial problems, including late issues, poor editing practices, etc. as well as questionable editorial boards. Therefore, Beall recommends that researchers consider publishing their papers in higher-quality journals than in InnoVision's offerings.

Potential, possible, or probable predatory open-access publishers have no transparency in publishing operations, provide insufficient information or hide information about author fees, falsely claim to have their content indexed in legitimate abstracting and indexing services, operate based in a Western country chiefly for the purpose of functioning as a vanity press for scholars in a developing country, copy "authors guidelines" from other publishers, do not use ISSN numbers, DOI numbers or use them improperly, and so forth.

Potential, possible, or probable predatory scholarly open-access journals (standalone journals)

These standalone journals do not have an official publisher behind their work. They act essentially alone, that is to say, on behalf of one or several individuals. Those who publish in predatory journals are, for the most part, young and inexperienced researchers from developing countries [15]. We believe that economic and sociocultural conditions in these developing countries have contributed to the differences found in authorship between predatory and nonpredatory journals [16]. These new journals are actually competing for authors and their money and offer little in return [17].

Jeffrey Beall has also created a list of questionable, scholarly open-access standalone journals (See: https:// scholarlyoa. com/individual-journals/) [18].

For example, one of the journals on his list is American Journal of Advances in Medical Science (ARNACA) [19]. If you visit this website, you will discover that the chief editor, associate editor, and managing editor are from India, and all the members of the Editorial Board are from Asian countries. Furthermore, all the reviewers are from India, yet the journal title is "*American* Journal of Advances in Medical Science".

Hijacked journals

Hijacked journals are those that try to defraud academics and researchers by using the name and reputation of the original journals. They usually send e-mails to attract their victims who are from certain countries (usually low and middle-income countries).

The journal falsely claims to have an impact factor and to be included in reputable databases. It lacks peer-review, or the corresponding author is asked to suggest reviewers, who are subsequently used later by the publisher. Hijacked journals are usually not listed in standard periodical directories or are not cataloged in library databases.

The number of hijacked journals has rapidly increased over the past several years. People included in this process have managed to cheat professors and Ph.D. scholars who are in urgent need of publishing their articles in journals that are found on the Web of Science Journal Citation Reports. Hijackers create a journal website and attract authors by indicating an impact factor of the journal, which means that it is a Thomson Reuters indexed journal, and by conducting the peer review process in just a couple of weeks.

Also, many require considerable manuscript processing charges for authors. Such journals are considered to be primarily interested in making quick money and paying little or no attention to peer review [16].

A study by Jalalian and Mahboobi has shown that many of the fake journals have started to imitate the features of respectable scientific journals, and not only

some relatively young journals but also such with a long tradition [20]. They even mimic the name of the journals. Among these journals are Wulfenia Journal, Jokull Journal, or Sylwan.
Authors can be easily deceived when they receive an invitation to submit their manuscript to journals whose title or logo closely resembles a highly respected publication [17].
Here is one example: The real Wulfenia journal may be found on the following website http://www.landesmuseum.ktn.gv.at/210226w_DE.htm?seite=15 [21] where there is a warning on the other website where the hijacked Wulfenia Journal is found (Box 2).

Box 2. Warning at the genuine Wulfenia journal regarding websites of the hijacked Wulfenia Journal [21]

The real Wulfenia journal website:
http://www.landesmuseum.ktn.gv.at/210226w_DE.htm?seite=15
Warning about the other website where the hijacked Wulfenia Journal is found:
Warning!
The websites
www.wulfeniajournal.at
www.wulfeniajournal.com
www.multidisciplinarywulfenia.org
are not the official websites of the journal "Wulfenia: Mitteilungen des KärntnerBotanikzentrums" published by the Regional Museum of Carinthia. These websites criminally usurp the identity of the official journal. They fraudulently use false information, a false editorial board, and false publication requirements to encourage authors to submit articles and to transfer page fees to a bank account in Yerevan (Armenia).

The list of hijacked journals created by Jeffrey Beall can be seen at https://scholarlyoa.com/other-pages/hijacked-journals/ [22]. He updates it regularly.
There is also another list created by Dr. Mehrdad Jalalian, journalist, and researcher, who is particularly concerned with the issue of hijacked journals [23]. His hijacked journal list can be found and consulted from the following website: http://www.mehrdadjalalian.com/index.php/list-of-hijacked-journals-and-fake-publishers/30-hiajcked-journal-list-2014-first-edition-june-2014 [24].
We have to emphasize the fact that this issue of hijacked journals is a great threat for medical sciences, that is, for clinical practice and health policy making. Many of the articles published in these journals will appear in the search results when retrieving literature and will be a source of new medical hypotheses that can be used to attack the reliability and validity of future clinical research [25-27].
Mehdi Dadkhah and Giorgio Bianciardi [28] in their paper discuss the possible ranking of predatory journals. First, they present criteria for detection of predatory journals, which include: editorial members' credentials, review process, and publishing, announcements, Open Access policies and publication charges. Further in their paper they present their predatory ranking metric entitled "predatory rate", based on the noted criteria.

Discussion

Open-access is a noble concept by which research is freely accessible to scholars and the public. It has brought substantial changes to higher education. Many open-access journals are legitimate and contribute to scientific knowledge, but recently a significant number of untrustworthy journals has appeared [9,29].
New terms have been coined: predatory publishers and predatory journals referring to fraudulent publication practices. In the literature much has been lately written on predatory journals, but not on hijacked journals [5,16, 30-33]. The intention of those who have dealt with the issue has been to raise awareness among scholars how to recognize and avoid submission of manuscripts to potential, possible, or probable predatory journals and hijacked journals.
This study has purposely been presented in biomedical journals published in Macedonia since we belong to this academic community. It is an imperative of the editorial board of the journals to inform scholars about this new threat on the publishing scene. Academics involved in faculty and staff promotion processes should warn and advise young scholars where to submit their papers for publication and the tenure and promotion review committees should be prepared to conduct a serious assessment of articles published in standalone or hijacked journals. Unethical scientists earn tenure and promotion at the expense of the honest [34]. The higher education sector has to employ academic rigor so as to maintain quality and integrity within publishing practice. Academics have to be more skilled in their own digital skills that will help them to identify fraud on the Internet and "low credibility", counterfeit, and predatory journals [35,36].
Scientists must be able to recognize publishing fraud. Although there is no real clue to the problem, suggestions have been offered how to combat predatory publishers and journals. There is an ongoing debate over the use of black- and white-lists of journals, as well as over the use of metrics, being identified as a problematic factor and needs further elaboration in some other study [29].

"A blacklist is easier to compile and maintain than a white list and by its nature contains more updated information than a white-list could. I often hear criticisms of my lists. Some believe that the predatory publishing problem is really a small problem, and my highlighting the problem is making it appear bigger than it really is. Others claim that we really need to give these predatory publishers a larger opportunity to succeed, that it is not fair to attack people from poor countries...."-said Jeffrey Beall in Learned Publishing [4,11,37].

Open-access associations such as Open Access Scholarly Publishers Association (OASPA) and Directory of Open Access Journals (DOAJ) should have a set of criteria to which publishers and journals must comply with in order to be considered trustworthy. In fact, OASPA was founded in 2008 after facing the challenges of OA journals. DOAJ is continually working to strengthen the journal approval process and it has already tightened up its inclusion criteria, with the purpose of serving as a white-list, as opposed to Beall's black-list. Also, regarding medical publishers and journals, the World Association of Medical Editors (WAME) has collaborated with the Committee on Publication Ethics (COPE), DOAJ and OASPA and has developed *Principles of Transparency and Best Practice in Scholarly Publishing.* Editors of peer-reviewed medical journals should adhere to these principles [38,39].

Beall said that he engaged himself in this topic partly by his sense of duty, as an academic librarian, to evaluate online resources and to help patrons to "recognize scholarly publishing scams and avoid them", and partly by the "private and very positive feedback" he receives from researchers and librarians [11]. Thus, academics may consult Beall's weblog and check the credibility of the listed journals. They cannot solely rely on his list, but should make their own evaluations as well.

Some criticize Beall's work or wonder whether it is fair to classify these publishers as "predatory" [40-42] stating that Beall is acting as prosecutor, judge and jury on who is predatory and who is not. Some say that it is an open question whether it is fair to classify these publishers and journals as "predatory" [43]. However, many state that Beall's list is widely read and consulted by librarians and researchers, and they applaud his efforts to reveal shady publishing practices. Some publishers, for example, the Academic Research Publishing Agency publish journals that cover very broad subject areas. It is difficult to image how a single journal of this publisher, *International Journal of Research and Reviews in Applied Sciences* [44] can validate papers from such a wide range of scientific fields (computer science, mathematics, economics, applied physics, nuclear engineering, chemistry, and many more) [43].

We are absolutely confident that scholars should avoid publishing their papers in hijacked journals; academics should refuse membership on Editorial Boards of such journals and they should not accept reviewing any papers submitted for publication in hijacked journals. The question on whether to accept the already published papers in these hijacked journals during the tenure and promotion processes is still under debate, since not all authors are informed about the existence and identification of these journals. Some authors suggest that already-published papers in the hijacked journals deserve a second chance, that is, these papers can be published in other legitimate journals and cannot be considered as plagiarized papers [31].

The situation is slightly different with the potential, possible, or probable open-access publishers and/or journals. These categories contain different sub-groups that are very roughly categorized in the Beall's list [41]. There is a real possibility that publishers and journals from poor, underdeveloped and developing countries be unfairly compared with those from developed

Box 3. Items to be checked or questions to be answered prior to making decision where to submit the manuscript for publication

the exact title of the journal
names of the editors
its place of publication and journal's business address
contact information
publication fees
sustainability
indexing databases
ISSN number
statement of publishing ethics, COPE membership
impact factor in the Thomson Reuters list
the quality of the already published papers, evidence of peer review
preservation that is depositing the digital content with a trusted, financially secure library (for example many publishers deposit their digital contents in the British Library)
have leading scholars in the field you are interested in, have already published articles in those journals
consult black- and white-lists of journals
think critically and don't do anything to compromise your career
resist the temptation to publish quickly
share information about fraudulent practices on scholarly social networks

countries. It means that high criteria from highly developed countries are also applied for developing countries creating comparison bias [45,46]. Therefore, recommendations given for hijacked journals cannot be entirely applied to predatory publishers and/or journals. It is crucial to raise awareness among scholars for their existence and to increase efforts for their recognition and identification as well as to advise the editorial board to improve their quality and to follow the principles of international publishing standards.

In our opinion, everyone should check the following items or look for answers to some questions prior to making his/her decision where to submit the manuscript for publication (Box 3).

Further studies are necessary in order to cover other aspects of potential, possible, or probable predatory publishers and journals, including the used metrics and financial issues.

Recently, at the meeting of the Annual Assembly of the Macedonian Association of Medical Editors (MAME), held on 13 April 2016, special attention was given to "Critical analysis of publishing in journals with Open Access", emphasizing the journals which should not be considered for submitting papers to them. At the MAME website (See: www.mame.mk) [47] separate links are available to approach "Potential, possible, or probable predatory open access publishers", "Potential, possible, or probable predatory open access journals", "Hijacked journals" and "Wrong metrics for journals". Predatory publishers and journals were recognized as the most serious problem and threat. It was proposed to inform the Faculty of Medicine and the "Ss Cyril and Methodius" University in Skopje and other universities in R. Macedonia to adjust the procedure and criteria for election in academic educational and scientific titles so as not to recognize the papers published in journals by publishers whose names can be found in Beall's list [12]. The warning was also directed to hijacked journals that are kidnapped by another publisher to earn huge sums illegally.

Since the issue on predatory publishers and hijacked journals is certainly targeting medical scholars, we decided to publish this paper in a number of Macedonian biomedical journals in order to warn not only young scholars, but also all medical professionals and academic institutions on the threat of being falsely attracted to publish their manuscripts in illegitimate journals.

Conflict of interest statement. None declared.

References

1. Elliott C. On predatory publishers: a Q&A with Jeffrey Beall. The chronicle of higher education. 2012 [Internet]. Available from: http://chronicle.com/blogs/brainstorm/on-predatory- publishers-a-qa-with-jeffrey-beall/47667.
2. Scholarly Open Access: Critical analysis of scholarly open-access publishing. Available from: https://scholarlyoa.com/page/4/.
3. Jallalian M. Academic Journalism, Publication Ethics. Hijacked Journals. Available from: http://www.mehrdadjalalian.com/index. php/updates-of-hijacked-journals.
4. Beall J. "Predatory" Open-Access Scholarly Publishers. *Charleston Advisor* 2010; 0-17.
5. Lukic T, Blesic I, Basarin B, *et al*. Predatory and fake scientific journals/publishers-a global outbreak with rising trend: A review. *Geogr Pannonica* 2014; 18(3): 69-81.
6. Council of Science Editors. CSE is a dynamic com- munity of editorial professionals dedicated to the responsible and effective communication of science. [Internet]. Available from: http://www.councilscienceeditors.org/.
7. STM. The Global Voice of Scholarly Publishing. Code of Conduct [Internet]. Available from: http://www.stm-assoc.org/membership/code-of-conduct/.
8. Committee of Publication Ethics. Promoting integrity in research publication [Internet]. Available from: http://publicationethics.org/.
9. Bartholomew RE. Science for sale: the rise of predatory journals. *J R Soc Med* [Internet]. 2014; 07(10): 384-385. Available from: http://jrs.sagepub.com/lookup/doi/10.1177/0141076814548526.
10. Bowman JD. Predatory publishing, questionable peer review, and fraudulent conferences. *Am J Pharm Educ* 2014; 78(10): 1-6.
11. Butler D. Investigating journals: The dark side of publishing. *Nature* [Internet]. 2013; 495(7442): 433-435. Available from: http://www.nature.com/news/investigating-journals-the-dark-side-of-publishing-1.12666.
12. Beall's List: Potential, possible, or probable predatory scholarly open-access publishers [Internet]. Available from: https://scholarlyoa.com/publishers/.
13. Criteria for Determining Predatory Open-Access Publishers [Internet]. Available from: https://scholarlyoa.files.wordpress.com/2015/01/crit eria-2015.pdf.
14. Scholarly Open Access. A Medical Publisher with Some Problems [Internet]. Available from: https://scholarlyoa.com/2013/12/26/a-medical-publisher-with-some-problems/.
15. Clark J, Smith R. Firm action needed on predatory journals. *BMJ* [Internet]. 2015; 350: h210. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25596387\nhttp://www.bmj.com/cgi/doi/10.1136/bmj.h210.
16. Xia J, Harmon JL, Connolly KG, *et al*. Who publishes in "predatory" journals? *J Assoc Inf Sci Technol* [Internet]. 2015; 66(7): 1406–17. Available from: http://dx.doi.org/10.1002/asi.23265.
17. Kearney MH, Thorne S, Chinn PL, *et al*. Predatory publishing: What authors need to know. *Res Nurs Heal* 2015; 38(1): 1-3.
18. Scholarly Open Access. List of Standalone Journals. Potential, possible, or probable predatory scholarly open-access journals [Internet]. Available from: https://scholarlyoa.com/individual-journals/.
19. American Journal of Advances in Medical Science [Internet]. Available from: http://www.arnaca.fopras.org/.
20. Jalalian M, Mahboobi H. Hijacked journals and predatory publishers: Is there a need to re-think how to assess the quality of academic research? *Walailak J Sci Technol* 2014; 11(5): 389-394.
21. Landes Museum Karnten. Wulfenia [Internet]. Available from: http://www.landesmuseum.ktn.gv.at/210226w_DE.h tm?seite=15.
22. Scholarly Open Access. Hijacked Journals [Internet]. Available from: https://scholarlyoa.com/other-pages/hijacked-journals/.

23. Jalalian M, Dadhkah M. The full story of 90 hijacked journals from August 2011 to June 2015. *Geogr Pannonica* 2015; 19(2): 73-87.
24. Jalalian M. Academic Journalism, Publication Ethics. Hijacked journal list 2014, first edition, June 2014 [Internet]. Available from: http://www.mehrdadjalalian.com/index.php/list-of-hijacked-journals-and-fake-publishers/30-hiajcked-journal-list-2014-first-edition-june-2014.
25. The Institutions of Engineering and Technology. Journal hijackers target science and open access [Internet]. Available from: https://www.researchinformation.info/news/analysis-opinion/journal-hijackers-target-science-and-open-access?News_id=1660.
26. Dadkhah M, Stefanutti C. Hijacked journals are emerging as a challenge for scholarly publishing. *Polskie Archiwum Medycyny Wewnetrznej* Poland 2015; 783-784.
27. Beall J. Medical Publishing Triage-Chronicling Predatory Open Access Publishers. Ann Med Surg. [Internet]. *Elsevier* 2013; 2(2): 47-49. Available from: http://dx.doi.org/10.1016/S2049-0801(13)70035-9.
28. Dadkhah M, Bianciardi G. Ranking Predatory Journals: Solve the Problem Instead of Removing It! *Tabriz Univ Med Sci* [Internet]. 2016; 6(x): 1-4. Available from: http://dx.doi.org/ 10.15171/apb.2016.001.
29. Wehrmeijer M. Exposing the predators. Methods to stop predatory journals [Master Thesis] [Internet]. 2014. Available from: http://openaccess.leidenuniv.nl/bitstream/handle/1887/28943/MA-thesis_Wehrmeijer_PredatoryJournals.pdf?sequence=1.
30. Dadkhah M, Obeidat MM, Jazi MD. How Can We Identify Hijacked Journals? *Bull Electr Eng Informatics* 2015; 4(2): 83-87.
31. Jalalian M. A second chance for authors of hijacked journals to publish in legitimate journals. *Electron Physician* 2015; 7(2): 1017-1018.
32. Jalalian M. Hijacked journals are attacking the reliability and validity of medical research. *Electron Physician* 2014; 6(4): 925-926.
33. Dadkhah M, Borchardt G. Hijacked Journals: An Emerging Challenge for Scholarly Publishing. *Aesthet Surg J* 2016; 36(6): 739-741.
34. Beall J. Predatory publishers are corrupting open access. *Nature* [Internet]. 2012; 489: 179. Available from: http://www.nature.com/news/predatory-publishers-are-corrupting-open-access-1.11385.
35. McNaught K. The Changing Publication Practices in Academia: Inherent Uses and Issues in Open Access and Online Publishing and the Rise of Fraudulent Publications. *J Electron Publ* 2015; 18(3). Available from: http://quod.lib.umich.edu/j/jep/3336451.0018.308?vi ew=text;rgn=main.
36. Willinsky J. Open access and academic reputation. *Ann Libr Inf Stud* [Internet]. 2010; 57(September): 296-302. Available from: http://nopr.niscair.res.in/handle/123456789/10242.
37. Beall J. Predatory publishing is just one of the consequences of gold open access. *Learned Publishing* 2013; 79-84.
38. World Association of Medical Editors. Principles of Transparency and Best Practice in Scholarly Publishing [Internet]. Available from: http://www.wame.org/about/principles-of-transparency-and-best-practice.
39. Bohannon J. Who's Afraid of Peer Review? Science (80-) [Internet]. 2013; 342(6154): 60-65. Available from: http://www.sciencemag.org/content/342/6154/60.full.pdf+html.
40. Crawford W. Ethics and access 1: The sad case of Jeffrey Beall. *Cites & Insights* 2014; 14(4): 1-14.
41. Crawford W. Journals, "Journals" and Wannabes: Investigating the List. *Cites & Insights* 2015; 14(7): 1-45.
42. Berger M, Cirasella J. Beyond Beall's List: Better understanding predatory publishers. *Coll Res Libr News* [Internet]. 2015; 76(3): 132-1325. Available from: http://crln.acrl.org/content/76/3/132.short.
43. Haug C. The Downside of Open-Access Publishing. *N Engl J Med* [Internet]. 2013; 368: 791-793. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMp1214750.
44. Academic Research Publishing Agency. *Int Res Rev Appl Sci* [Internet]. Available from: http://www.arpapress.com/.
45. McKerlich R, Ives C, McGreal R. Measuring use and creation of open educational resources in higher education. *Int Rev Res Open Distance Learn* 2013; 14(4): 90-103.
46. Shen C, Bjork B-C. "Predatory" open access: a longitudinal study of article volumes and market characteristics. BMC Med [Internet]. *BMC Medicine* 2015; 13(1): 230. Available from: http://bmcmedicine.biomedcentral.com/articles/10.1186/s12916-015-0469-2.
47. Macedonian Association of Medical Editors. *Ethics* [Internet]. Available from: http://www.mame.mk/wordpress/etika-2/.

УПАТСТВО ЗА ПРИЈАВА НА ТРУД ОД СОРАБОТНИЦИТЕ НА ММП

"Македонски медицински преглед"(ММП) е стручно списание на Македонското лекарско друштво, првенствено наменето на лекарите од општа практика, специјалистите од одделните медицински дисциплини и истражувачите во областа на базичните медицински и други сродни науки.
Списанието ги има следниве рубрики и категории на трудови:

1. **Изворни трудови**
2. **Соопштувања за клинички и лабораториски искуства**
3. **Прикази на случаи**
4. **Од практика за практика**
5. **Едукативни статии**
6. **Вариае (**писма од редакцијата, општествена хроника**,** прикази на книги, извештаи од конгреси, симпозиуми и други стручни собири, рубриката „Во сеќавање„ и др).

Изворните трудови имаат белези на научни трудови, додека трудовите категоризирани во рубриките 2-5 имаат белези на стручни трудови.
Во ММП се објавуваат трудови на членовите на МЛД или на членови на други стручни здруженија. Авторите се одговорни за почитувањето на етичките начела при медицинските истражувања, а изнесените ставови, изведени од анализата на сопствените резултати, не се нужно и ставови на Редакцијата на ММП.
Редакцијата ги испраќа ракописите на стручна рецензија; рецензентот (ите) и Редакцијата ја определуваат дефинитивната категоризација на ракописот кој е прифатен за печатење. Редакцијата го задржува правото ракописите да ги печати според рецензираниот приоритет.
Упатството за соработниците на ММП е во согласност со Ванкуверските правила за изедначени барања за ракописите кои се праќаат до биомедицинските списанија.

1. ТЕКСТ НА РАКОПИСОТ

Сите ракописи се испраќаат во електронска форма на електронската адреса (е-маил) на МЛД-ММП, со двоен проред и најмногу 28 редови на страница. Трудот се поднесува на англиски јазик латиничен фонт Times New Roman големина 12 и апстракт на македонски јазик. Лево, горе и долу треба да се остави слободна маргина од најмалку 3 см, а десно од 2,5 см.. Редниот број на страниците се пишува во десниот горен агол.
Ракописот на трудот треба да е придружен со писмо на првиот автор, со изјава дека истиот текст не е веќе објавен или поднесен/прифатен за печатење во друго списание или стручна публикација и со потврда дека ракописот е прегледан и одобрен од сите коавтори, односно со придружна декларација за евентуален конфликт на интереси со некој од авторите.

Насловната страна треба да има: наслов на македонски и англиски, имиња и презимиња на авторите, како и институциите на кои им припаѓаат, имињата на авторите и насловот на установата се поврзуваат со арапски бројки; автор за кореспондеција со сите детали (тел. е-маил); категорија на трудот; краток наслов (до 65 карактери заедно со празниот простор); како и информација за придонесот за трудот на секој коавтор (идеја, дизајн, собирање на податоци, статистистичка обработка, пишување на трудот).

Насловот треба концизно да ја изрази содржината на трудот. Се препорачува да се избегнува употреба на кратенки во насловот.

Изворните трудови и **соопштувањата** го имаат следниов формален редослед: насловна страна, извадок на македонски јазик (вовед, методи, резултати, заклучок) со клучни зборови, извадок на македонски јазик со клучни зборови, вовед, материјал и методи, резултати, дискусија и заклучоци, литература и прилози (табели, графици и слики) и легенди за прилозите во еден фајл.

Приказите на случаи треба да содржат вовед, детален приказ на случајот, дискусија со заклучок и литература со прилози.

Извадокот на македонски јазик треба да содржи најмногу 250 зборови и да биде структуриран со сите битни чинители изнесени во трудот: вовед со целта на трудот, методот, резултати (со нумерички податоци) и заклучоци. Заедно со извадокот, треба да се достават и до 5 клучни, индексни зборови.

Извадокот на англиски јазик мора да е со содржина идентична со содржината на извадокот на македонски јазик. Клучните зборови треба да се во согласност со MeSH (Medical Sibject Headings) листата на Index Medicus.

Воведот треба да претставува краток и јасен приказ на испитуваниот проблем и целите на истражувањето, со наведување на етичкиот комитет односно институцијата која го одобрила испитувањето (клиничка студија која се работи според принципите на Хелсиншката декларација за пациентите и нивните права).

Методите треба да бидат точно назначени, за да се овозможи повторување на прикажаното истражување. Особено е важно да се прецизираат критериумите за селекција на опсервираните случаи, воведените модификации на веќе познатите методи, како и идентификација на употребените лекови според генеричното име, дозите и начинот на администрација.

Резултатите треба да се прикажат јасно, по логичен редослед. Резултатите се изнесуваат во стандардните СИ единици. Во текстот треба да се назначи оптималното место каде ќе се вметнат табелите и илустрациите, за да се избегне непотребното повторување на изнесените податоци. Значајноста на резултатите треба да се обработи статистички, со детален опис на употребените статистички методи на крајот на делот *методи*.

Дискусијата треба да ги истакне импликациите од добиените резултати, споредени со постојните сознанија за испитуваниот проблем.

Заклучоците треба да не бидат подолги од 150 зборови.

2. ПРИЛОЗИ

Како прилог-документација на трудовите предложени за печатење, може да се достават до 5 прилога (табели, фигури,/слики - илустрации).

Табелите се доставуваат на крајот на трудот во истиот фајл. Секоја табела треба да има свој наслов и реден број кој ја поврзува со текстот. Хоризонтални и вертикални линии на табелата не се дозволени; ознаките на колоните во табелата се пишуваат скратено или со симбол, а нивното објаснување се пишува на дното на табелата, во вид на легенда.

Илустрациите се доставуваат со реден број како слика во црно-бела техника, а секоја слика треба да е придружена со легенда (опис).

Микрофотографиите може да содржат посебни ознаки во вид на стрелки или симболи. Покрај описот на сликата, мора да се наведе и зголемувањето и видот на боењето на препаратот (ако тоа веќе не е направено во секцијата *материјал и методи*).
Сите ознаки на фотографиите мора да бидат доволно големи, за да може јасно да се распознаат и по смалувањето во печатницата, при нивното вклучување во печатената страница на списанието.

3. ЛИТЕРАТУРА

Цитираната литература се пишува на крајот на трудот по заклучоците, со редни броеви според редоследот на појавувањето на цитатот на текстот на трудот ставени во средни загради и без простор меѓу нив (ако се последователни треба да се поврзани со цртичка, на пр. [3-6]).
Литературата се цитира на следниов начин (кратенките за насловите на списанијата треба да се според листата прифатени vo Index Medicus):
а) ***статија во списание*** (се наведуваат сите автори, ако ги има до 4 или помалку; ако ги има повеќе од 4 се наведуваат првите 3 автори и се додава: *и сор*.) Neglia JP Meadows AT, Robison LL *et al*. Second neoplasms after acute lymphoblastic leukemia in childhood. N Engl J Med 1991; 325:1330-6.
б) заеднички автор
GIVIO (Interdisciplinary group for cancer care evaluation). Reducing diagnostic delay in breast cancer. Possible therapeutic implications. *Cancer* 1986; 58: 1756-61.
в) ***без автор*** **-** анонимно. Breast screening: new evidence. (*Editoriall Lancet* 1984; i :1217-8).
г) ***поглавје во книга или монографија***
Weinstein L, Swartz MN. Pathogenic properties of invading microorganisms. Vo: Sodeman WA Jr, Sodeman WA, Ed. Pathogenic physiology: mechanisms of disease. Philadelphia; W B Saunders, 1974: 457-72.

Првите отпечатоци на трудовите им се праќаат на авторите за корекција: авторите се должни коригираниот отпечаток да и го вратат на Редакцијата на ММП во рок од 2 дена.

Уплата за испечатен труд во списанието ММП изнесува 3.000, 00 денари и се уплаќаат на жиро сметката на: Македонско лекарско друштво
300000000211884 – Комерцијална банка
со цел на дознака : уплата за стучен труд

Адресата на Редакцијата
Даме Груев бр. 3
Градски ѕид блок II,
1000 Скопје,
Тел.: ++ 389 02 3162 577

Електронска адреса (Е-маил): mld@unet.com.mk

Известување за рецензентите за ММП

Во склад со правилникот на УКИМ рецензентите што навремено и одговорно ќе ја одработат рецензијата ќе добијат 0.4 бода кои се собираат за унапредување во академските звања. Бодовите можат да се добијат и ретроградно преку побарување во МЛД – 3162 557.