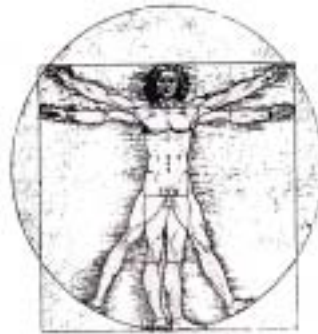


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# ***ACTA MORPHOLOGICA***

*ПУБЛИКАЦИЈА НА ЗДРУЖЕНИЕТО НА АНАТОМИ И МОРФОЛОЗИ НА МАКЕДОНИЈА*

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## SIGNIFICANCE OF SPINAL ACCESSORY NERVE PRESERVATION IN MODIFIED NECK DISSECTIONS

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### Abstract

**Introduction:** The spinal accessory nerve or eleventh cranial nerve is frequently encountered during neck surgery, and as such is at risk of iatrogenic injury, resulting in the “shoulder syndrome”. The surgical anatomy of the spinal accessory nerve within the neck is very variable, without entirely reliable landmark for its identification. Neck dissections are without doubt the most challenging form of head and neck surgery because of the difficulty of avoiding damage to the numerous vital structures. The modified neck dissection with preservation of the spinal accessory nerve is based on desire to minimize the functional deformity associated with section of the eleventh nerve.

**Material and methods:** The comparative study was created through the medical records retrospectively of 165 consecutive patients who underwent neck dissections at our institution in the past five years with attention to ultrasound and MRI preoperative findings, type of neck dissection, type of identification and dissection of spinal accessory nerve and postoperative morbidity and survival rate. Clinical and electrophysiological evaluations of the trapezius and sternocleidomastoid muscles and neurologic evaluations were performed at 3 to 6 months postoperatively.

**Results:** The safest identification of spinal accessory nerve is in posterior neck triangle where may be recognized exiting from the posterior border of the sternocleidomastoid muscle at Erb’s point. For exact preoperative planning ultrasound and MRI are superior for determining the positioning of the eleventh nerve. The mean distance between the greater auricular point and the spinal accessory nerve was 0.90 cm. Average length of the trunk from Erb’s point until the penetration in the trapezius muscle was around 5.1 cm, ranging from 4.8 to 5.4 cm. The diversity in the course from the posterior border of the sternocleidomastoid muscle and posterior neck triangle were confirmed in 9 cases (15%), predominantly at the level of entering the posterior neck triangle. The frequency of postoperative morbidity of accessory nerve was (46.7%) for radical neck dissections, (28.3%) for selective neck dissections and (8.3%) for modified neck dissections. For every separate type of dissection different subtypes were included.

**Conclusion:** Endorsed is the concept that the spinal accessory nerve injury is potentially preventable in most cases of neck surgery. Identification of spinal accessory nerve over established landmarks is unconditionally reliant on the exact preoperative mapping of the nerve with imaging diagnostics. Modified neck dissection has similar regional control rates to more comprehensive operations in appropriately selected patients and significantly reduces the risk of functional disability.

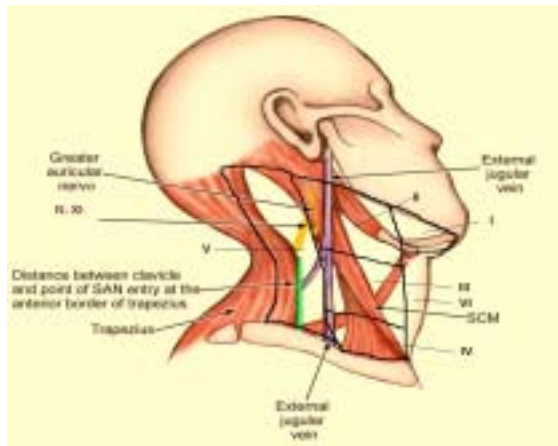
**Key words:** posterior neck triangle, spinal accessory nerve, ultrasound, neck metastases, modified neck dissection.

### Introduction

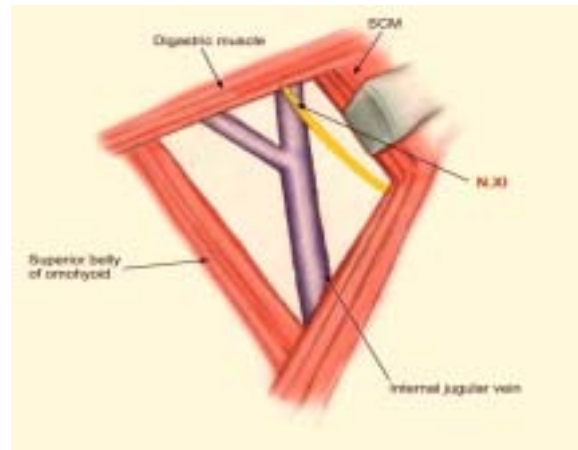
Identification of spinal accessory nerve even in the correct anatomical position is not always easy during different types of neck dissections. The surgical anatomy of the spinal accessory nerve has been properly acknowledged in the literature<sup>1,2,3,4</sup>, with evidence of significant variations. Eleventh cranial nerve topography consists of two parts. The lesser cranial part arises from cells in the nucleus ambiguus and in due course is distributed with the vagus nerve. This portion innervates the pharyngeal muscles. The main part of eleventh cranial nerve, the spinal portion, arises from a long column of nuclei situated in the ventral part of the medulla and extending to the fifth cervical segment or lower. The nerve descends in the neck through jugular foramen and near the jugular vein exits in the posterior neck triangle and supplies the sternocleidomastoid and trapezius muscles. These muscles have the following functions: elevation of the shoulder by the trapezius, rotation and tilting of head toward and away from the side of the contracting sternocleidomastoid muscle and flexion of the neck by both sternocleidomastoid muscles. This cervical part is

associated by motor or sensory contributions from the upper cervical nerves. With weakness or paralysis these functions are decreased or absent. When the lesion is nuclear or infranuclear, there is associated muscle atrophy and fasciculations<sup>5,6,7,8</sup>.

On the other hand neck dissection is an principal technique for the diagnosis (staging) and the treatment of cervical lymph node metastasis in patients with head and neck cancer. In modified neck dissections when indications are raised a very important part is preservation of the spinal accessory nerve, together with internal jugular vein and sternocleidomastoid muscle<sup>9,10,11</sup>. The pain and dysfunction associated with a loss of innervation by spinal accessory nerve has motivated surgeons to modify the classic neck dissection. The modified neck dissection with preservation of the spinal accessory nerve is based on desire to minimize the functional deformity associated with section of that nerve, combined with the recognition that in many situations the nerve intimately involved in the neck disease and its preservation does not compromise the oncological effectiveness of the more limited procedure<sup>4,12,13,14,15</sup>.



**Fig. 1.** Spinal accessory nerve and neck triangles for dissection



**Fig. 2.** Spinal accessory nerve relationship at posterior neck triangle

Since the introduction of functional neck dissection, various modifications have been made to reduce the adverse effects of radical neck dissection and have contributed to improve the quality of life and to prevent permanent sequelae and medico-legal actions following neck dissection<sup>4</sup>. Proficient knowledge of posterior neck anatomy is crucial to avoid its accidental injury during selective or modified neck dissection in almost any extensive surgery of the posterior neck. Reasonable speed and safety in identifying and preserving important anatomical structures are of vital importance and for this type of neck dissection, special attention must be paid to refined identification of the spinal accessory nerve.

**Material and methods**

Surgical study of operative alteration of the spinal accessory nerve, and trapezius muscle function of patients who underwent distinctive neck surgery was performed. This study was done not only to document the

indispensability of the trapezius muscle to shoulder-strap stability, but also to clarify the role of the eleventh cranial nerve preservation. The cross-sectional retrospective analyze was created on own clinical material from the University Clinic for Maxillofacial Surgery in Skopje, where in the last five year period, neck dissections were performed in 165 patients, of whom 59 were treated with radical neck dissection, 20 with modified neck dissection and in 83 cases with adequate type of selective neck dissection. Neck dissection with a curative intent was done in 80 patients with squamous cell carcinoma of the upper aerodigestive tract (naso- and oro-pharynx), in 26 patients with skin squamous cell carcinoma, 17 with salivary gland carcinoma, 11 with malignant melanoma and 31 with hidden primary.

**Table 1.** Type of neck dissection

Type of Neck Dissections	2007	2008	2009	2010	2011	Total
Classical Radical Neck Dissection	12	11	18	11	7	59
Modified Neck Dissection	2	2	3	5	8	20
Selective Neck Dissection	11	7	18	12	21	69
Billateral: Radical + Selective ND	2	3	5	4	4	18
<b>Total</b>	<b>27</b>	<b>23</b>	<b>44</b>	<b>32</b>	<b>40</b>	<b>165</b>

**Table 2.** Distribution of patients according to primary site disease

Timing of neck dissection	Primary site	Number of patients
Primary treatment	Oropharynx (lower lip, tongue, sublingual, jaws, pharynx)	69
Secondary treatment	Nasopharynx	11
	Skin cancer	26
	Hidden primary	31
	Salivary carcinoma	17
	Malignant melanoma	11
	Total	165

Entirely records for this study we have evaluated in 20 with modified neck dissection and 40 with selective neck dissections, comparing preoperative diagnostic work-up, intraoperative findings and relationship of the dissected and preserved eleventh nerve with tumor masses and postoperative complications. Ultrasonography, computed tomography (CT) and magnetic resonance images (MRI) were compared and analyzed about different sensitivity in the preoperative assessment. In the selected cases, the intraoperative mapping study was done to obtain the exact anatomical data by drawing the exposed accessory nerve in life size during the modified neck dissection. Measurements were made at the end of the procedure by using a caliper. After the completeness of lymph node and metastases removal from the posterior neck triangle, evaluation was performed over extensive exposure of the spinal accessory nerve. Towards the end of the dissection, the correct location of the nerve was corroborated by enlarging the exposure to confirm the position and integrity of the preserved nerve.

The dissections and clinical observations corroborate that the trapezius is a key part of a “muscle continuum” that stabilizes the shoulder. Clinical and electrophysiological evaluations of the trapezius and sternocleidomastoid muscles and neurologic evaluations were performed at 3 to 6 months postoperatively.

**Results**

Intraoperatively we found significant variances in the positioning of the spinal accessory nerve. Particular attention was created about identification of spinal accessory nerve positioning and relationship with the internal jugular vein. Our findings confirmed that the spinal accessory nerve almost always crosses the internal jugular vein anteriorly in the upper neck, with exclusion in 4 cases (6.7%) with posterior crossing at the level of posterior belly of the digastric muscle and one case with rare anomaly where accessory nerve passing through the fenestrated internal jugular vein was observed. In 20 cases we have measured the distance from jugular foramen (skull base) to crossing of internal jugular vein with mean value of 2.34 cm.



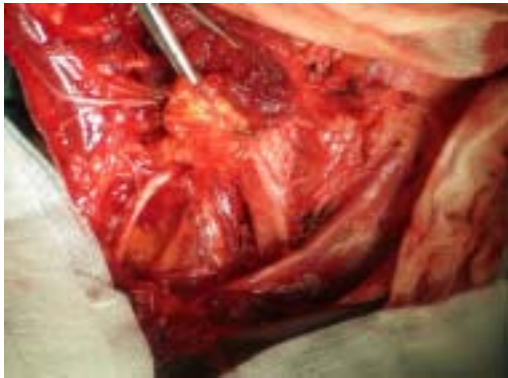
**Fig. 3.** Identification of spinal accessory nerve in radical neck dissection

The diversity in the course from the posterior border of the sternocleidomastoid muscle and posterior neck triangle were confirmed in 9 cases (15%), predominantly at the level of entering the posterior neck triangle. Hypoplastic nerve was apparent in 8.3% (5 cases), generally after removal of neck masses with greater proportions. Hyperplastic nerve was evident in only 3.3% (2 cases). In almost every case we found that spinal accessory nerve was with typical coiled appearance in its course through the posterior cervical triangle of the neck

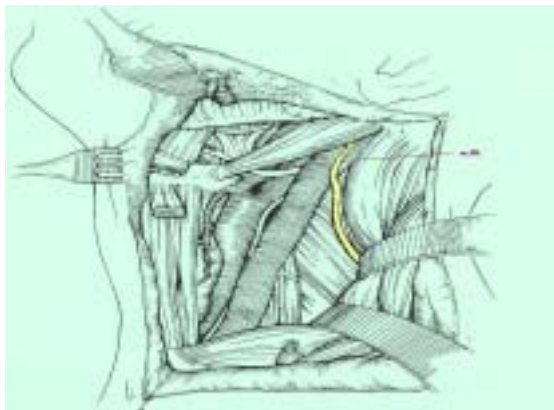


**Fig. 4.** Modified neck dissection with preserved spinal accessory nerve

The mean distance between the greater auricular point and the spinal accessory nerve was 0.90 cm. Average length of the trunk from Erb's point until the penetration in the trapezius muscle was around 5.1 cm, ranging from 4.8 to 5.4 cm. The most significant elongation was found in cases after meticulous preparation of spinal accessory nerve – usually after complete removal of neck metastases at level III - V. There were 4-8 lymph nodes in the spinal accessory nerve chain.



**Fig. 5.** Schematic appearance in modified neck dissection



**Fig. 6.** Facial nerve branching variations-schematic statistic

Measurements of abduction and the electromyographic measurements of the study group at 3 to 6 months postoperatively were found to be superior to those of the control group, although the difference between the groups was not significant. The mean number of dissected lymph nodes was significantly higher in the study group than in the control group. The frequency of postoperative morbidity of the spinal accessory nerve was highest in radical neck dissections (46.7%) in 28 cases, There was a reduced dysfunction in 17 cases with selective neck dissection (28.3%) comparing to preoperative values, while shoulder drop and scapular winging was confirmed in only 5 cases (8.3%) of modified neck dissection. The most significant was correlation after

radical neck dissection and modified neck dissection including levels IIb and V.



**Fig. 7.** Trapezius disability – shoulder drop after RND

Patients who underwent supraomohyoid selective neck dissection that involved minimal dissection of the spinal accessory nerve had minimal loss of shoulder function and usually, normal electromyograms at 16 weeks that documented less injury to the spinal accessory nerve. Again, these patients had improvement with time.

### Discussion

The main therapeutic dilemma for the therapy of metastatic carcinoma from the head and neck malignancy remains a choice of the type of neck dissection. The probability of metastases to the neck from various sites in the upper aerodigestive tract has been outlined<sup>16,17</sup>, so there are data from the basis of much of the literature on the technique and indications for functional modifications of the classical radical neck dissection. Conley and Schuller, confirmed a large percentage of metastases (42%) in close proximity to the spinal accessory nerve where it comes to lie near the internal jugular vein<sup>11</sup>. These and other investigations have designated to the introduction of modified neck dissections.

Beneficial surgical intervention in case of posterior neck metastases depends upon suitable exposure and preservation of spinal accessory nerve. This induces a consistent understanding of the anatomy of the posterior neck triangle for doing surgery safely, including the sufficient knowledge of extra cranial anatomy of spinal accessory nerve variations<sup>12,17,18</sup>. Our findings support results in previous literature in that the spinal accessory nerve is located anterior to the internal jugular vein in the majority of the cases, however, it is imperative for the surgeon to be mindful to the anatomic variability and possible posterior crossing of the internal jugular vein by the spinal accessory nerve in the neck to avoid injury to the internal jugular vein during the dissection of the nerve. Since the great auricular nerve (Erb's point) represents a constantly identifiable landmark, it allows simple and reliable identification of the course of the spinal accessory nerve. Across the posterior triangle the nerve was running superficially with either straight (78%) or coiled (22%) pathway. The accessory nerve and the phrenic nerve were similar in the anatomic evidences and



the number of motor nerve fibers. On ultrasound evaluation the accessory nerve exited the posterior border of sternocleidomastoid at a mean of 6.5 (5.0-8.5) cm below the mastoid process and penetrated the anterior border of trapezius 5.5 (3.0-7.0) cm above the clavicle with mean caliber of  $0.75 \pm 0.10$  mm.

The most common complication of neck dissection is shoulder pain and dysfunction due to manipulation of spinal accessory nerve, resulting in trapezius muscle atrophy mainly in procedures involving the posterior neck triangle<sup>18</sup>. Our study indicates that there may be a functional disability associated with any type of neck dissection in which the spinal accessory is dissected out and placed in some degree of traction. Significantly lower risk of functional disability in the modified neck dissections and confirmation that the modified neck dissection is as effective as the radical neck dissection for controlling neck disease, extend the indications for modified or selective neck dissections as more logical approach to surgical treatment of cervical neck disease. If there is no functional advantage, all other arguments for modified neck dissections become inappropriate.

The results of this study show that, on the average, neck dissection patients with their spinal accessory nerve preserved have less pain in their shoulders, less functional disability, and stronger results on their physical examination than did those with their spinal accessory nerve sacrificed without any difference in their local control and survival rate. This is of pronounced importance because any inadvertent injury of the spinal accessory nerve during surgical procedures is a cause of significant morbidity with medicolegal repercussions. The findings in this work are consistent to those in the most previous reported studies regarding the spinal accessory nerve preservation<sup>1,3,10</sup>. This study has the advantage that it originated from existing operative findings rather than cadaver dissections and, as a result, incorporated functional information and the postoperative significance of damage to some of the muscle functions.

In conclusion we can validate that the spinal accessory nerve injury is potentially preventable in most cases of neck surgery. Surface anatomical landmarks are not always reliable guide to the position and course of the nerve in the posterior triangle. Within modified neck dissections, identification of spinal accessory nerve over established landmarks is unconditionally reliant on the exact preoperative mapping of the nerve with imaging diagnostics but the sophisticated further eleventh nerve dissection and preservation is dependent to the inclusive surgical knowledge. Modified neck dissection has similar regional control rates to more comprehensive operations in appropriately selected patients and significantly reduces the risk of functional disability.

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**ANATOMY AND CLINICAL IMPORTANCE OF THE TRIANGLE OF KOCH**

Zhivadnikov Julija, Lazarova - Tosovska D, Popovski V<sup>1</sup>, Papazova M, Matveeva N, Dodevski A  
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**Abstract**

The triangle of Koch occupies the atrial component of the muscular AV septum. The tissue of AV node and the “slow” and “fast” pathway of the AVNRT are incorporated in the triangle, which makes this area clinically important.

The aim of this study was to present the morphology and clinical importance of the triangle of Koch. The study consists of two parts: basic and clinical. In the basic part, 100 human hearts fixed in formaldehyde were examined using common anatomical and histological methods. The numerical features of the triangle of Koch were measured in two different ways. In the clinical part of the study, the analysis was made on 100 patients who were tested and treated in the Electrophysiological laboratory of the Institute for Heart Diseases in Skopje. Using the data of patients weight and height, the numerical features of the triangle were calculated. The results obtained were statistically analysed.

The first type of measuring, in the basic part of the study, gave the following mean values of the length of the sides of the triangle of Koch: side a (a1)  $26.1 \pm 3.1$  mm, side b (b1)  $20.8 \pm 3.6$  mm and side c (c1)  $24.5 \pm 2.5$  mm. The mean value of the area of the triangle (P1) was  $256.2 \pm 67.6$  mm<sup>2</sup>. According to the second type of measuring the following numerical features of the triangle were obtained: side a (a2)  $20.8 \pm 2.5$  mm, side b (b2)  $13.9 \pm 2.8$  mm and side c (c2)  $19.8 \pm 2.4$  mm. The mean value of the area of the triangle (P2) was  $139.47 \pm 37.28$  mm<sup>2</sup>. In the clinical part of the study, mean value of the length of the side a (a3) was  $28.5 \pm 2.7$  mm, side b (b3)  $12.9 \pm 1.2$  mm and side c (c3)  $21.1 \pm 2.7$  mm. The mean value of the area of the triangle (P3) was  $116.6 \pm 12.3$  mm<sup>2</sup>.

Knowledge of the variations of numerical features of the triangle of Koch is fundamental for successful catheter placement in electrophysiological studies and radiofrequent catheter ablations.

**Key words:** triangle of Koch, anatomy, atrioventricular node, numerical data, statistical data, electrophysiology

**Introduction**

The application of contemporary ablation techniques for treatment of atrioventricular nodal reentry tachycardia (AVNRT) renews the interest for the morphological features of the triangle of Koch. This triangular area occupies the atrial part of the muscular septum, a sloping area that attains its AV location because of the major differences in the levels of attachment of the leaflets of the tricuspid and mitral valves on either side of the septum<sup>(1, 2)</sup>. For the first time it was described by Koch in 1909, with the heart being seen within body in the anatomic position. The sides of the triangle are the tendon of Todaro (side a) and the attachment of the septal leaflet of the tricuspid valve (side c) that converge at its apex, and the base (side b) is formed by the orifice of the coronary sinus. The tissue of AV node and the “slow” and “fast” pathway of the AVNRT are incorporated in the triangle, which dimensions vary among the patients<sup>(3, 4)</sup>.

The aim of this study was to present the morphology and clinical importance of the triangle of Koch, and to compare the data obtained directly by postmortal measurements and indirectly using mathematical formulas.

**Material and methods**

The study consisted of two parts (basic and clinical) and it included two independent groups.

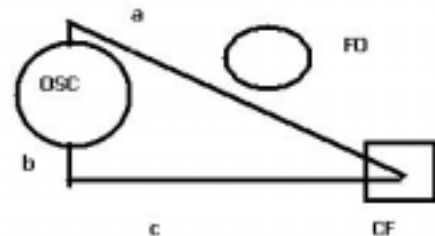
The first group comprised 100 human hearts got after autopsies of patients older than 18 years, died from noncardiac reasons. The hearts were removed intact and fixed in 10% formaldehyde, for at least 72 hours. The right atrium was opened through an incision between the superior and inferior caval venous orificies, and then by

extending an incision perpendicular to the first, along the lateral wall of the atrium into the right appendage.

The lengths of the sides of the triangle of Koch (marked as a, b and c) were measured with caliper, using two different types of measuring, which are schematically presented in Figs. 1 and 2.

*First type of measurement:*

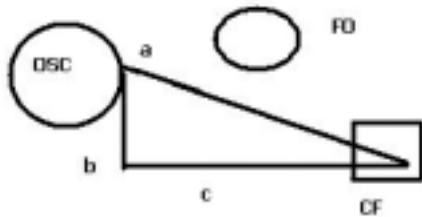
Side a (a1) is the length of the tendon of Todaro; side b (b1) - the base of the triangle- is the distance from the tendon of Todaro to the septal leaflet of the tricuspid valve (at the right angle to the leaflet) passing through the coronary sinus ostium (OSC); side c (c1) is the distance from the insertion of side b to the central fibrous body (CF), along the septal leaflet of the valve.



**Fig. 1.** First type of measurement

**Second type of measurement:**

Side a (a<sub>2</sub>) is the length of the Todaro from the central fibrous body to the nearest point of the valve of the coronary sinus ostium (OSC); side b (b<sub>2</sub>) - the base- is the distance from the anulus of the septal leaflet of the tricuspid valve to the nearest point of the valve of the coronary sinus ostium; side c (c<sub>2</sub>) is the distance between the attachment of side b to the central fibrous body (CF).



**Fig. 2.** Second type of measurement

The second group consisted of 100 patients, tested, examined and treated in the Electrophysiological laboratory of the Institute for Heart Diseases of Medical Faculty in Skopje. Numerical values of the triangle of Koch were calculated from the data for the height and weight of the patients, using the following formulas:  $a = 3.1 \pm 13.7$  (BSA);  $b = 1.8 \pm 6$  (BSA);  $c = 1.7 \pm 13.7$  (BSA);  $p = 2.56 \pm 61.5$  (BSA) <sup>(5)</sup>. The surface area of the body (BSA) was calculated according to Mosteller's formula <sup>(6)</sup>:

$$BSA (m^2) = \frac{[\text{height (cm)} \times \text{weight (kg)}]}{3600}$$

Values were expressed as mean  $\pm$  standard deviation, minimum and maximum value and difference between two means (p). The following statistical methods were used: t-test, Mann-Whitney U test and Pearson's coefficient of correlation (r).

**Results**

The first type of post mortal measurements, in the basic part of the study, gave the following mean values of the length of the sides of the triangle of Koch: side a (a<sub>1</sub>)  $26.1 \pm 3.1$  mm, side b (b<sub>1</sub>)  $20.8 \pm 3.6$  mm and side c (c<sub>1</sub>)  $24.5 \pm 2.5$  mm. The mean value of the area of the triangle (P1) was  $256.2 \pm 67.6$  mm<sup>2</sup>. The second type of measurements, the following numerical features of the triangle were obtained: side a (a<sub>2</sub>)  $20.8 \pm 2.5$  mm, side b (b<sub>2</sub>)  $13.9 \pm 2.8$  mm and side c (c<sub>2</sub>)  $19.8 \pm 2.4$  mm. The mean value of the area of the triangle (P2) was  $139.47 \pm 37.28$  mm<sup>2</sup>. Testing of the differences between the mean values obtained by using these two different types of measuring showed statistically significant differences ( $p < 0.01$ ).

In the clinical part of the study, mean value of the length of the side a (a<sub>3</sub>) was  $28.5 \pm 2.7$  mm, side b (b<sub>3</sub>)  $12.9 \pm 1.2$  mm and side c (c<sub>3</sub>)  $21.1 \pm 2.7$  mm. The mean value of the area of the triangle (P3) was  $116.6 \pm 12.3$  mm<sup>2</sup>.

Testing of the correlations between the analyzed parameters showed a direct positive correlation between the height ( $r = 0.55$ ), weight ( $r = 0.98$ ) and body area ( $r = 0.99$ ) of the patients with the length of the sides of the triangle and its area.

Testing of the significance of differences of parameters analyzed in both parts of the study showed a statistically significant difference ( $p < 0.01$ ) between the mean values of the lengths and the area of the triangle of Koch, obtained by the two types of measuring in the basic part of the study, and those in the clinical part of the study.

Significance of differences of the values between the parameters analyzed with the second type of measuring in the basic part of the study and those in the clinical part of the study was clinically insignificant.

**Discussion**

The clinical importance of the triangle of Koch is due to its functional connection with AV node <sup>(7, 8, 9)</sup>. The anatomical borders of the triangle are easy perceptible, because they are formed by well defined elements, although some authors still make questions about the permanence of the tendon of Todaro <sup>(10)</sup>.

According to our measurements the area of the triangle of Koch greatly varies among the patients. These results are in agreement with those obtained by most authors that analyzed this area <sup>(11)</sup>. Contrary to these findings, studies published by Mc Guire et al described the triangle of Koch as an area with uniform size. The distance between the tricuspid anulus and the nearest edge of the coronary sinus (mean height) was  $13 \pm 3$  mm and the distance from the central fibrous body to the nearest edge of the coronary sinus (mean length) was  $17 \pm 3$  mm <sup>(3, 11)</sup>. The mean height of the triangle measured in postmortem hearts was slightly greater than measured at arrhythmia surgery ( $15 \pm 4$  mm). In the clinical part of our study, the dimensions of the triangle of Koch were determined indirectly using the data for the height and weight of the patients. Considering the fact that AV node tissue is located at the apex of the triangle, approximately 1 cm anterior of the coronary sinus valve <sup>(3)</sup>, clinicians are interested in a part of the triangle that is smaller than the anatomic one. The sides of the triangle can be determined by measuring of distances as we did in the second type of measurements in the basic part of the study. The dimensions obtained in this way correspond with those obtained by Mc Guire <sup>(11)</sup>.

We measured the dimensions in the postmortem hearts in two different ways. As we expected, the values obtained with the measurements of the anatomical boundaries of the triangle of Koch were significantly different from those obtained with the other types of measurements, but very similar to that published by other authors <sup>(1, 3, 7, 11, 12)</sup>. However, the difference between the results of the second type of measurements and the values determined by using the formula were not significant (the mean value of the area of the triangle (P2) was  $139.47 \pm 37.28$  mm<sup>2</sup> vs  $116.6 \pm 12.3$  mm<sup>2</sup>;  $p > 0.05$ ). We concluded that the formula used for determination of the area of the

triangle of Koch refers to the part of the triangle that is smaller than the expected anatomic borders and corresponds with our second type of measurements.

In conclusion, the dimensions of the triangle of Koch vary among patients. The knowledge of the variations of its numerical features is fundamental for successful catheter placement in electrophysiological studies and radiofrequent catheter ablations.

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## EVALUATION OF SEX-SPECIFIC DIFFERENCES OF ANTHROPOMETRIC PARAMETERS OF GROWTH IN CHILDREN AGED 6

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### Abstract

**Aim:** Evaluation of sex-specific differences of anthropometric parameters that were used as indicators for growth in children aged 6.

**Examinees and methods:** The study included 217 healthy children aged 6 from Macedonian nationality. Fourteen anthropometric parameters were measured which define longitudinal, circular and transversal measures of skeleton using standard equipment and measurement technique. The following indicators were calculated: weight-for-age (BW), height-for-age (BH) and BMI.

**Results:** The majority of anthropometric parameters have shown significant sex-specific differences in favour of boys, with exception of leg length and circumferences of head, upper-arm, forearm, thigh and calf that were no significant differences. Values of the 50th percentile in boys were as follows: 24 kg for BW, 119.8cm for BH and 16.8 kg/m<sup>2</sup> for BMI. The values of these parameters in girls were: 22kg for BW, 118.7 cm for BH and 16.12 kg/m<sup>2</sup> for BMI.

**Conclusions:** These results can be used as criteria for the assessment of the morphological characteristics and detection of deviations in the growth and nutritional status in children aged 6.

**Key words:** growth, children, anthropometry

### Introduction

Childhood is one of the most vulnerable periods in human growth during which both growth and development lead to unique changes in the organism [1]. Nutrition is also an important ecological characteristic which significantly influences all phases of growth and development. That is why it is especially important to follow and assess how nutrition reflects on growth, since growth is sensitive to nutritional deficit or surplus [2]. Anthropometric examinations are non-invasive, simple and adaptable to following the speed and dynamics of children's physical growth by measuring certain dimensions that define longitudinal, transversal, circular dimensionality of the skeleton, as well as body mass and body volume [3]. They also point to sexual dimorphism and other disorders related to growth and nutritional status during that period [4].

### Aim

Evaluation of sex-specific differences of anthropometric parameters used as indicators of growth in children aged 6.

### Examinees and method

The study included 6-year-old healthy children of both sexes and of Macedonian nationality, selected randomly from different urban areas of Macedonia. The total number of examinees (n=217) was divided into two groups based on the sex criterion: (n=110 male and n=107 female).

### Anthropometry

14 anthropometric variables were selected and measured according to the International Biological Programme (IBP). For assessment of longitudinal skeleton dimensionality: body height, arm length, leg length; for assessment of

transversal skeleton dimensionality: elbow diameter, wrist diameter, knee diameter, ankle diameter; for assessment of body mass and circular dimensionality, i.e. body volume: body weight, chest circumference, head circumference, mid upper arm circumference, forearm circumference, thigh circumference, calf circumference. The following standard anthropometric equipment was used for that purpose: anthropometer by "Martin", with 1mm reading accuracy; calliper square which can read values from 1mm, as well as elastic plastic band, also with 1mm reading accuracy. The following anthropometric indexes were calculated: BMI (as a ratio of body weight and height squared) weight-for-age, height-for-age.

### Definition

According to several authors, we can define the values of these anthropometric indicators with the following percentile ranks with cut off points: [4-8]

- Normal distribution (average values) usually from 15<sup>th</sup>-85<sup>th</sup> percentile.
- The percentile rank from 5<sup>th</sup>-15<sup>th</sup> percentile points to under average values
- Values under the 5<sup>th</sup> percentile point to underweight, as well as to retarded growth, if we analyse the parameter height-for-age.
- Children whose weight-for-age, height-for-age and BMI indexes are between the 85<sup>th</sup> and 95<sup>th</sup> percentile are defined as children with above average growth with a risk of becoming overweight.
- Values over the 95<sup>th</sup> percentile point to the category of children with extremely high growth, obese children.

### Statistics

The gathered data for the relevant variables were analyzed with a descriptive statistics represented by: measures of central tendency and its deviations (arithmetical mean  $\pm$  standard deviation) and ranges (percentiles). Testing the significance of the differences between two arithmetical

**Table 1.** Body weight, body height, BMI, lengths and diameters of the extremities in 6 year-old -children from R Macedonia (mean and standard deviation)

Sex	n	Body weight (kg)	Body height (cm)	BMI(kg/m <sup>2</sup> )	Lengths (cm)			Diameters (cm)		
					Arm	Leg	Elbow	Wrist	Knee	Ankle
<b>Male</b>	110	25.22±5.34 <sup>a</sup>	120.2±5.66 <sup>a</sup>	17.31±2.67 <sup>a</sup>	53.22±2.31 <sup>a</sup>	68.28±4.55	5.52±0.82 <sup>a</sup>	4.01±0.49 <sup>a</sup>	7.99±0.84 <sup>a</sup>	5.58±0.45 <sup>a</sup>
<b>Female</b>	107	23.04±4.56	118.4±5.91	16.38±2.17	51.36±2.38	67.39±3.73	5.24±0.44	3.86±0.44	7.5±0.89	5.28±0.5

<sup>a</sup>p<0.05 vs female children (ANOVA)

**Table 2.** Circumferences of 6 year-old children from R. Macedonia (mean and standard deviation)

Sex	n	Circumferences (cm)					Calf
		Head	Chest	Mid upper	Forearm	Thigh	
<b>Male</b>	110	50.97±1.9	59.94±4.43 <sup>a</sup>	17.85±2.42	15.8±3.0	34.05±4.23	24.08±3.28
<b>Female</b>	107	50.73±1.35	58.71±4.7	18.07±2.21	15.48±1.86	33.15±3.86	23.51±2.77

**Table 3.** Sex-specific percentiles of the indexes: weight-for-age, height-for-age and Body Mass Index in 6 year-old-children from R Macedonia

	PERCENTILES									
	5	10	15	25	50	75	85	90	95	
<b>MALE</b>										
Weight-for-age	18	20	20	21	24	30	32	33	35	
Height-for-age	112	113.2	114.53	116	119.8	123.6	125.96	128	130	
BMI	13.59	14.28	14.56	15.4	16.8	18.9	20.3	20.98	22.2	
<b>FEMALE</b>										
Weight-for-age	16.95	18	18	20	22	25	28.1	30	31	
Height-for-age	109.9	111.16	111.7	113.4	118.7	122	124.41	126.2	129	
BMI	12.96	13.68	14.11	14.5	16.12	18.1	18.9	19.26	19.68	

series was done with ANOVA. The values for  $p < 0.05$  were taken as significant differences.

## Results

The mean values and the standard deviations of the examined anthropometric parameters in children aged 6, as well as their sex-specific differences are presented in tables 1 and 2.

Table 1 shows mean values and standard deviation of weight, height, BMI, upper and lower limb length, as well as the diameters of the elbow, wrist, knee and ankle.

The value of body mass in male children aged 6 is  $25.22 \text{ kg} \pm 5.34$ , the height is  $120.21 \text{ cm} \pm 5.66$  and BMI is  $17.31 \text{ kg/m}^2 \pm 2.67$ . Girls at the same age have the following values for the same parameters:  $23.04 \text{ kg} \pm 4.56$  for weight,  $118.4 \text{ cm} \pm 5.91$  height and  $16.31 \text{ kg/m}^2 \pm 2.17$  for BMI. The results of the comparative examinations of these parameters show the existence of sex-specific differences in favour of male children.

Sex-specific difference was also detected in the transversal parameters, i.e. all the diameters, in favour of the boys. In reference to the longitudinal parameters -arm and leg length- there was a sex-specific difference in arm length, whereas in leg length the difference was insignificant despite the fact that its mean value was slightly higher in boys.

Table 2 shows mean values and standard deviations of circumferences (head, chest, mid upper arm, forearm, thigh and calf). Of the circular parameters, only chest circumference showed sex-specific differences in favour of boys. The mean of the other circular parameters were slightly higher in boys, but the sex-specific difference was insignificant. Only the average value of upper arm circumference ( $18.07 \text{ cm} \pm 2.21$ ) in girls was slightly higher than the values in boys ( $17.85 \text{ cm} \pm 2.42$ ) but the sex-specific difference for this parameter was also insignificant.

The sex-specific percentiles for the indexes weight-for-age (TM), height-for-age (TV) and body mass index (BMI) for children aged 6 are given in table 3.

Limit values (5<sup>th</sup> and 95<sup>th</sup> percentile) in boys aged 6 are from 112cm on the 5<sup>th</sup> percentile and 125.96cm on the 85<sup>th</sup> percentile for height-for-age, 18 and 30 kg for weight-for-age, 13.59 and 20.3kg/m<sup>2</sup> for BMI. The corresponding values in girls are: 109.93 and 124.41 cm for height-for-age, 16.95 and 28.1 kg for weight-for-age, i.e. 12.96 and 18.9 kg/m<sup>2</sup> for BMI.

## Discussion

Several anthropometric parameters were examined in our study, parameters which define the longitudinal, circular and transversal dimensionality of the skeleton, the body mass and volume and which are used to assess the growth and degree of nutrition in children. It was concluded that the mean values for almost all examined anthropometric parameters (except for upper arm circumference) are higher in boys. There were also sex-specific differences in certain anthropometric parameters in favour of the boys. Only the differences between the circumference of the limbs and the parameter

leg length were statistically insignificant. Certain percentile ranks of the anthropometric indexes in children aged 6 were also calculated.

The values we obtained allow us to compare our values to the corresponding anthropometric examinations done on children from other areas and populations.

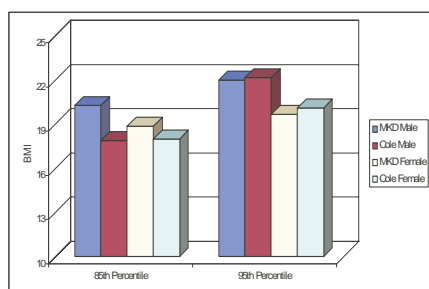
Longitudinal parameters are considered to be some of the most reliable indicators of physical growth. Transversal parameters are good indicators of bone maturity, circular parameters along with body mass point to the volume of the body, i.e. its mass. Knowing the significance of body weight and height as basic somatic characteristics is important because it can be used as index for more accurate interpretation of the growth and nutritional status of children. The index height-for-age shows the linear growth, and the deviation in its values which is detected on the 5<sup>th</sup> percentile is used to recognize children with obstacles in reaching the potential of the linear growth as a result of long-term cumulatively misbalanced nutrition or health [9]. The values of the indexes weight for age and height for age on the 50<sup>th</sup> percentile for boys aged 6 were 24kg and 119.8cm, i.e. 22kg and 118.7cm for girls. These values are slightly higher than the values of NCHS referent population [8]. Values over the 95<sup>th</sup> percentile detect children with a risk of endocrine diseases, tumours which produce growth hormones and other similar conditions. The children from our study are insignificantly heavier and higher than children in Belarus and India, and weigh less and are shorter than the children from Arlene's, Potvin's and Lindgren's study [10-14]. The index body mass, known as BMI and the index weight-for-age are parameters for monitoring the degree of nutrition during childhood [15]. Figure 1 shows the values of BMI on the 50<sup>th</sup> percentile in children from our country (MKD) and children from different areas and populations: Verona-Italy (I), Saragossa-Spain (E), Mexico (MEX) America (USA) and the referent NCHS [8,4-18].

**Fig. 1.** The values of the 50 th percentile of BMI in 6 – years-old-children from Macedonian (Mkd) and different areas and population

Figure 2 shows limit values, i.e. cut off points for BMI on the 85<sup>th</sup> and 95<sup>th</sup> percentile in male and female examinees as well as the corresponding values obtained from the



representative international referent sample of Cole et al [19, 20].



**Fig. 2.** The cut-off points for BMI of 85<sup>th</sup> for overweight and 95<sup>th</sup> for obesity by sex for 6 years old MKD children and Cole's cut off points from international survey

With this information which is used to identify persons with a risk of overweight and obese we can see that the cut off points for our male examinees are slightly higher than the international sample published by Cole. On the other hand, our female examinees had slightly lower values of BMI on the cut off points compared to the values published by Cole et al [19, 20].

The differences among the values obtained in children from this study and those from other studies, as well as the standard values, are another confirmation of the existence of population differences in the anthropometric characteristics which depend on a series of internal (genetic) and external (exogenous) factors [21]. The results of our study confirm the recommendation of WHO that every country should have its own anthropometric standards necessary for accurate classification and detection of deviations in the growth and the nutritional status of children of all ages.

### Conclusion

Based on the results of this study, we can conclude the following:

- 6-year-old male children of Macedonian nationality and from urban areas have slightly higher values for their longitudinal, transversal and circular parameters (with the exception of upper arm circumference) compared to female children.

- There were statistically significant sex-specific differences for weight, height, BMI, arm length, all 4 diameters and chest circumference in favour of the boys. In terms of the other examined parameters (leg length and 5 circumferences: head, mid upper arm, forearm, thigh and calf) there were no statistically significant sex-specific differences.

- The sex-specific percentile ranks on the 5<sup>th</sup> and 95<sup>th</sup> percentile, i.e. cut off points for the anthropometric indexes of children aged 6 were also determined.

It is recommended to use the results we obtained from this study in everyday routine practice as

anthropometric criteria for assessment and evaluation of the growth and the nutritional status. These results can also point to certain misbalances that can be used as criteria for selecting individuals for further clinical research. By defining the cut off points we can identify children who need intervention. Undoubtedly, this has huge practical importance for planning preventive measures and activities in the field of child nutrition.

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**SOCIOECONOMIC STATUS IN RELATION TO BMI IN MACEDONIAN ADOLESCENTS**

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**Abstract**

The objective of this study was to evaluate the nutritional status in Macedonian high school students in relation to their socioeconomic status (SES), education and employment of their parents. In this study 117 adolescent students (48 males and 69 females) at the age of 17 and 18 were included. We measured weight and height using standard procedures while BMI was calculated. The examinees completed the questionnaire including data for SES, parents' education and employment. Male students had significantly higher standard deviation scores for weight, height and BMI compared with females. The level of education and employment of students' mother had no significant influence on BMI but the level of education and employment of the father had a significant influence ( $p < 0,05$ ). A student whose fathers' was with a level of lower education had significantly higher scores for BMI opposite to those whose fathers have middle or higher level of education. Examinees with lower SES had significantly higher rates of BMI compared with those which SES was higher.

**Key words:** adolescents, socioeconomic status, nutritional anthropometry, obesity

**Introduction**

Obesity in childhood is a result of unhealthy sedentary lifestyle, fast food consumption and low level of physical activity. It's consequences in adulthood make this problem very important and attracts interest of researchers for early detection of health risk in child and adolescent population (1).

WHO declared an increasing obesity in 15-20% of people all over Europe. The organization stressed the seriousness of the problem especially in late puberty and adolescence, which is a very vulnerable period of life when teenagers make the eating habits and get the perception of their physical bodies (2). Besides genetic factors which are predictors for physical constitution and tendency for obesity, other factors that influence the dynamic of physical growth and development and biologic characteristics of child's body and system are: food, sociocultural or environmental factors and physical activity. There is a connection between SES and eating habits, especially in developed countries where nutritional stress is a major factor, and where influence of mass media and advertisement related to eating habits among adolescents is much higher than in developing countries (2, 3, 4, 5).

There are some anthropometrical indicators for evaluating nutritional status in children, adolescents and adults. The most used and validated is body mass index (Quetelet index =  $\text{weight}/\text{height}^2$ ) (3). It can be used as alternative indicator for direct measuring of body fat. It is an easy and simple method for early detection of nutritional status in children and adolescents. BMI is sex and age specific and it is referred as BMI - for - age (6).

The focus of this study was anthropometrically assessed nutritional status in Macedonian adolescents in relation to their socioeconomic differences.

**Material and methods****Subjects**

Data were obtained from a cross-sectional sample of students of two highschools in Skopje. The sample

included 117 healthy students (48 males and 69 females) at the age of 17 and 18, from selected schools and classes, which gave their consent for participation in the research. In order to avoid mistake in the selection of sample, volunteer students were not included. Subjects were grouped according to sex and age. The University Human Research Ethics Committee approved the experimental protocols.

**Anthropometry**

Anthropometric measurements were made during school hours, not interrupting the lessons. Body height (BH) was measured with Martin stadiometer. Subjects were standing facing ahead, and body height was measured as maximum distance from the floor to the highest point on the head. Shoes were off, both feet together, and arms at the sides. Heels, buttocks and upper back were in contact with the wall. Body height measurement can vary throughout the day, usually being higher in the morning, so to ensure reliability we measured height at the same time of the day. We measured body weight (BW) with scale, the persons standing with minimal arm movement at their side. These values were used to assess weight status, BMI ( $\text{weight (kg)}/\text{height (sm)}^2$ ). The cut-off points suggested by the WHO were used (WHO, 1995). Subject having BMI less than 18.5kg/m<sup>2</sup> were categorized as underweight, BMI from 18.5-24.9 normal weight, BMI from 25.0 – 29.9 overweight and BMI more than 30.0 obese. Every adolescent has his/her own anthropometric file with the following data: date of birth, date of examination and sex.

**Socioeconomic status (SES)**

To assess SES students completed the validated questionnaire. It contained a set of questions on household's financial situation, including car ownership, bedroom occupancy standards, holidays and home computers. Students were classified according to the summed score of the items, with the overall score being recorded to give values of FAS 1 (0-3) low SES, FAS 2 (4,5) middle and FAS 3 (6,7) high SES. They also gave

information about the level of education and employment of their parents.

### Statistics

The data were analyzed with descriptive statistics represented by measures of central tendency and its deviation (arithmetic mean value  $\pm$  and standard deviation). Testing the significance of differences between two arithmetic series was done by analysis of variance – ANOVA, and values of  $p < 0.05$  were considered to be statistically significant.

### Results

The study included 117 adolescents (69 females and 48 males) at the age of 17 and 18. Values of BMI for males and females are presented in Table 1. The values of underweight, healthy weight, overweight and obese female students were 4.53%, 79.7%, 15.9%, respectively 2.08%, 60.4%, 33.3% and 4.1% male students.

Male students have statistically higher values

for body weight, body height and BMI compared to females (Table 2).

Of all students 58,9% females and 77% males have mother and father with middle level of education, 11% females and 5,12% males have mother and father with low level of education, only 29,9% females and 25,6% males have mother and father with high level of education and only 3,4% males have father with no education (Table 3). Level of mother's education has no significant influence on mean value of BMI of the examinees, while the fathers educational level has significant influence ( $p < 0,05$ ). The examinees whose fathers were with low level of education had higher values for BMI compared to BMI of those whose fathers had middle or higher level of education (Table 4.).

Ninety students (76.9%) answered that their mothers were employed and 88.03% that their fathers were employed, contrary to those who have unemployed mothers (23.07%) and unemployed father (11.96%). (Table 5).

**Table 1.** Nutritional status of adolescents

BMI	underweight	healty weight	overweight	obese
female (69)	4.53%	79.7%	15.9%	
male (48)	2.08%	60.4%	33.3%	4.1%

**Table 2.** Body height, weight and BMI by sex (mean value (x) and standard deviation (SD))

	BH X $\pm$ SD	BW X $\pm$ SD	BMI X $\pm$ SD
Female	162.71 $\pm$ 6.23	59.44 $\pm$ 7.01	22.48 $\pm$ 2.56
Male	174.04 $\pm$ 4.37	72.39 $\pm$ 10.69	23.87 $\pm$ 3.19

**Table 3.** Descriptive statistics of level of parents' education

education	No education	Low level	Middle level	High level
<b>Mother n=117</b>		13 (11.1%)	69 (58.9%)	35 (29.9%)
<b>Father n=117</b>	4 (3.4%)	6 (5.12%)	77 (65.8%)	30 (25.6%)

Analysis of Variance  $F=2.78$   $p=0.044$

**Table 4.** Influence of level of parents' education to subjects' BMI

Father education	BMI (X $\pm$ SD)
No education (n=4)	25.55 $\pm$ 2.91
Low (n=6)	25.52 $\pm$ 2.95
Middle (n=77)	22.81 $\pm$ 3.04
High (n=30)	22.84 $\pm$ 2.21

**Table 5.** Descriptive statistics of parents' employment

Parent employment	employed	Not employed
<b>Mother (n=117)</b>	90 (76.9%)	27 (23.07%)
<b>Father (n=117)</b>	103 (88.03%)	14 (11.96%)

**Table 6.** Socioeconomic differences according to questionnaire

<b>FAS1 (Does your family has a car or van)</b>		
No	15	12.82%
Yes(one)	62	52.99%
Yes (two or more)	40	34.1%
<b>FAS2 (Do you have your own bedroom)</b>		
Ne	5	4.27%
Da	112	95.72%
<b>FAS3 (During the past year, did you go away on holiday (vacation) with your family?)</b>		
I haven't been	8	6.83%
Once	46	39.31%
Twice	40	34.18%
More than two times	23	19.65%
<b>FAS4 (How many computers does your family own)</b>		
None	4	3.41%
One	60	51.28%
Two	37	31.62%
More than two	16	13.67%

**Table 7.** Distribution of examinees according to their SES

SES	(N)	%
low	(19)	16.2%
middle	(52)	44.4%
high	(46)	39.3%

**Table 8.** Correlation of SES with BMI

SES		BMI (X±SD)
Low	(n=19)	24.24±3.37
Middle	(n=52)	22.27±2.39
High	(n=46)	23.43±3.05

To determine the SES, students described their family affluence on the basis of 4 variables which constitute the three objective indicators of Family affluence scale (FAS): Does your family has a car or van; Do you have your own bedroom; During the past year did you go away on holiday (vacation) with your family?; How many computers does your family own? The answered questions are shown in Table 6. According to FAS score 16.2 % of all the examinees lives in low SES, 44.4% middle and 39.3% high SES (Table 7). Adolescents with low SES had significantly higher values for BMI compared to those with middle SES ( $p < 0.05$ ) (Table 8).

### Discussion

Today unhealthy sedentary lifestyle, fast-food consumption, low level of physical activity cause obesity in childhood, which is a serious problem. It attracts the interest of researchers for early detection of health risk in child and adolescent population (1). Overweight and obesity carry serious health consequences that can last into adulthood such as emotional and social difficulties

and health problems. Symptoms began to appear in the early school years: high blood pressure, high cholesterol level, respiratory abnormalities, and insulin resistance, and they are predictions of other serious diseases as hearth disease, type 2 diabetes, gallbladder disease and sleep and digestive disorders (5). Besides genetic factors which are predictors for physical constitution and tendency for obesity, other important factors that influence dynamic of physical growth and development and biologic characteristics of child body and systems are: food, sociocultural or environmental factors and physical activity. The values of BMI showed that overweight and obesity was found in 31.4% of children in the fifth grade and 20 % in the first year of high school. This condition can be an alarm to undertake more preventive measures with proper education about benefits of healthy eating habits and more physical activity in children and adolescents (7). According to our results majority of the Macedonian adolescents at the age of 17 and 18 have normal weight. There were 79.9% females with normal weight and 15.9% overweight, and there were 60.4% males with normal weight, 33, and 3% with overweight and 4.1% obese. Males had statistically higher values for body weight, body height and BMI compared to females which we expected.

There is a connection between SES and eating habits, especially in developed countries where the influence of mass media and advertisement related to eating habits among adolescents is much bigger than in developing countries (2, 3, 4, 5). Macedonia belongs to developing countries with a high percent of unemployed residents (34.9% in 2007) and only 8% of people with high education (7). When family income is enough, children can have more quality food and proper nutrition intake so they can assess their potential of growth and have normal weight and healthy body. Although Macedonia has no problem with undernutrition, the world bank reports 7% of residents with low level of income, hence they cannot receive minimal calories intake (8). The influence of socioeconomic status of adolescents' and the level of education and employment of adolescents' parents have definitely influence on the morphological characteristics of adolescent body and it is different among different populations. A large number of studies have shown that children from families with low socioeconomic status have bigger BMI than those living in families with middle or high SES. The reason could be that low socioeconomic status restricts families' opportunities to adopt healthy behaviors such as eating fruit and vegetables and practicing physical activity (5). In the study of Morgenstern et al the relationship between SES and BMI was partially mediated through the higher television exposure (4). According to Turkish scientists Can Pelin et al. who made a research with healthy adolescents at age of 19, there were no statistically significant differences between those with low, middle and high SES and BMI but there was a positive correlation between educational level of adolescents' parents and BMI (9). Adolescents whose mother was with a higher level of education had significantly higher values of body weight and BMI

compared with those whose mother had middle and low level of education. Similar were the results for the father education. According to multi indicators cluster research from 2005/2006 children in Macedonia whose mothers were with middle education had more normal weight than those whose mother had no education, and the were underweight (8). We found that level of education of adolescents' mothers had no significant influence on mean values of BMI in examinees, while the educational level of fathers had significant influence ( $p < 0,05$ ). The examinees whose fathers were with low level of education had higher values for BMI compared to BMI of those whose father had middle or higher level of education. Also the adolescents with low SES had significantly higher values for BMI compared with those with middle SES.

According to Maruf PT et al. there were no differences in the influence of SES on body weight and BMI in examinees that visited private or public schools, but those who visited private schools had an average higher body height (10). Jennifer A. has found that children with family with low SES had higher BMI values, which was similar to our findings. According to her opinion those children and families had difficulty in receiving advices for healthy diets and control of body weight. It is also very important that children and adolescents with higher values of BMI have lower confidence about their physical appearance (11).

### Conclusion

The present study showed that majority of Macedonian adolescents, both male and female have normal weight and that SES has influence to nutritional status. Those who live in low SES have higher values for BMI. In our opinion it is very important to implement more educational programs in primary and secondary school about consequences of sedentary lifestyle and eating fast food and to present benefits of more physical activity and its positive effects on body and mind.

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**IMMUNOHISTOCHEMICAL ANALYSIS OF FETAL KIDNEY STEM CELLS**

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**Abstract**

Tissue specific stem cells reside both in fetal and adult tissues and represent an excellent source of replacement therapy. Studies have confirmed the existence of stem cells in human fetal kidney, though the dilemma of their location and their origin remains.

We have performed immunohistochemical analysis on fetal kidney tissues with selected stem cell markers: CD133, CD34, CD117, CD44 and the proliferation marker Ki-67.

The analyses have shown the same distribution of the positive signal in stainings with CD117 and Ki-67, located in S-shaped bodies and solitary cells in the distal tubules in the cortex, as well as solitary cells in the collecting ducts and urothelium in the medulla, which makes them candidates for progenitor cells. CD133, widely used in the search for adult stem cells, was also positive in cells other than those positive for CD117 and Ki-67. CD34 and CD44 were found to be of no value for this matter.

Broader panel of antibodies and functional studies for the clonogenic potential of the putative stem cells are needed in further characterization of fetal kidney stem cells.

**Key words:** fetal kidney, stem cells, immunohistochemistry

**Introduction**

Stem cells identified in embryos and fetuses are pluripotent cells, i.e. under certain conditions they can differentiate into any type of human cell. Their therapeutic potential has led to establishment of a new field of medical research, called Regenerative Medicine.

The organogenesis involves the formation of tissue-specific stem cells that sustain cell renewal of their own tissue for the lifetime of the organism [1]. Tissue specific stem cells reside both in fetal and adult tissues and represent excellent source of replacement therapy.

The kidney has a complex histomorphology, thus representing a great challenge for regenerative studies. At least 14 different cell types comprise the kidney [2] and need to be orchestrated. Furthermore, the varieties of renal epithelia arise from two distinct sources: the collecting duct system develops from its epithelial precursor – ureteric bud, whereas the nephron arises from the metanephric mesenchyme via the process named mesenchymal/epithelial transition [3]. This is a unique mechanism composed of highly regulated subtle processes of proliferation, apoptosis and differentiation.

In recent years, the search for organ residing stem cells, both in fetal and mature tissues and organs, has provided a lot of information, sometimes confusing and contradictory.

Studies have confirmed the existence of stem cells in human fetal kidney [2, 4, 5, 6], yet the dilemma of their location and their origin remains. Which of the above

mentioned two populations of cells can best represent nephrogenic stem cell? Are they organ specific or do they originate from the bone marrow?

The aim of this study is to analyze the expression of the putative stem cells markers in the fetal kidney tissue and to determine their location within the constitutive renal structures.

**Material**

We used 10 tissue samples from fetal kidneys obtained during post mortem analysis performed at the Institute of Pathology, Faculty of Medicine, Skopje, Macedonia. The average gestational age of the fetuses was 18 gestational weeks (ranging from 14-23). Only tissue samples from kidneys, where both macroscopic and microscopic analysis confirmed absence of any pathological process, were included in the study.

**Methods**

The tissue has been processed through standard fixation methods. Briefly, after dissection, the tissue is immersed in 10% buffered formalin solution for 18-24 hours, followed by a series of alcohols with different concentration and afterwards embedded in paraffin. The paraffin embedded tissue blocks are then cut into 4-5 μm thick sections, applied on silanized slides and stained.

Initially, the tissue sections were stained with H&E staining, after which immunohistochemical stainings with antibodies depicted in Table 1 were performed. The

**Table 1.** Antibodies used for phenotypisation of the fetal kidney stem cells

Antibody	Producer	Clone	Dilution	Localisation of the signal	Positive control
<b>CD133</b>	Miltenyi, Germany	AC133	1:11	Cytoplasmic and/or membranous	Bone marrow
<b>CD34</b>	Dako, Denmark	QBEnd-10	1:50	Cytoplasmic	Internal control (blood vessels)
<b>CD117</b>	Dako, Denmark	Polyclonal	1:60	Cytoplasmic	GIST
<b>CD44</b>	Dako, Denmark	DF1485	1:50	Membranous and/or cytoplasmic	Tonsil
<b>Ki67</b>	Dako, Denmark	MIB-1	1:100	Nuclear	Tonsil

EnVision Flex (DAKO, Denmark) visualization system was used, after pretreatment of the tissue in DAKO PT Link.

As positive control, we used tissues suggested by the producers of the antibodies (also stated in Table 1). The negative controls were performed by omitting application of the primary antibody on a control tissue section.

All microscopic analyses were performed on NIKON Eclipse 1000 microscope.

## Results

### *Morphology*

The microscopic analyses of the fetal kidney tissue showed that both cortical and medullar tissue is consistent to the gestational age of the fetuses.

The cortex consisted of undifferentiated mesenchymal tissue of closely packed spindle cells with eosinophilic cytoplasm and dark nuclei, which was getting thinner with the gestational age of the fetuses. Beneath this mesenchymal tissue, there were S- and comma- shaped bodies lined by single layer of cells, between which the structures of the ureteric bud were interposed. Precursors and well developed proximal and distal tubules were also observed.

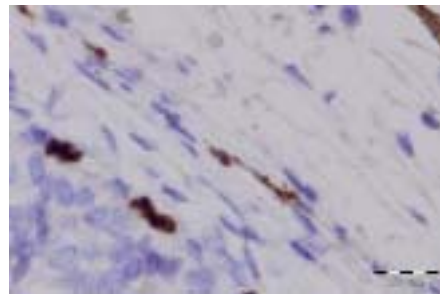
The medulla consisted of precursors of collecting tubules lined by cylindrical cells with clear cytoplasm, precursors of thick segment of the Henle loop lined with cuboidal cells with eosinophilic cytoplasm and round nuclei and precursors of thin segment of the Henle loop lined with flattened cells. Between the tubules, there was loose undifferentiated mesenchymal tissue of spindle cells with pale cytoplasm and oval nuclei, progressively decreasing with the gestational age of the fetuses.

The renal pelvis was lined by transitional epithelium of multilayered cells with clear cytoplasm and oval nuclei.

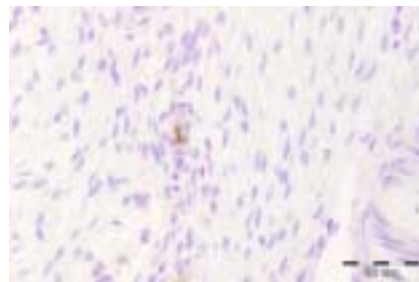
### **Immunohistochemistry**

The immunohistochemical analyses showed presence of positive cells in four of the five above mentioned markers in both compartments. All samples were negative for the CD44 staining.

**CD34.** There were many positive cells both in cortical and medullar compartment of the kidneys, but most of them were, as expected, of endothelial origin. The intensive positive signal for this staining in the rich vascular network of the renal tissue corrupted the analyses of its potential presence in other cells than endothelial. However, single positive cells were observed in the medullar mesenchymal tissue (Figure 1).



**Fig. 1.** Single mesenchymal cells in the medulla positive for CD34, x40

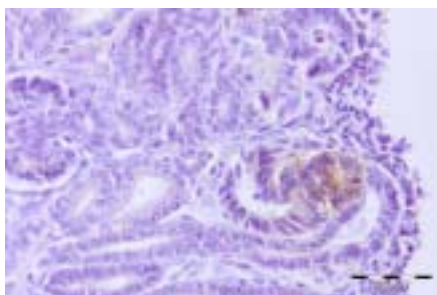


**Fig. 2.** Single mesenchymal cells in the medulla positive for CD133, x40

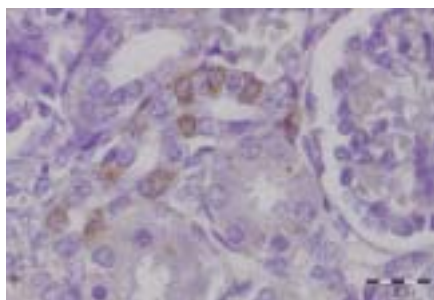


**CD133.** The pattern of staining for CD133 was equal in all analyzed specimens. Rare tubules in the cortex showed positive apical membranous signal, whereas more tubules in the medulla, mainly distal tubules and occasionally collecting ducts, showed apico-lateral membranous positivity. Single cells from the medullar mesenchymal tissue were also positive (Figure 2).

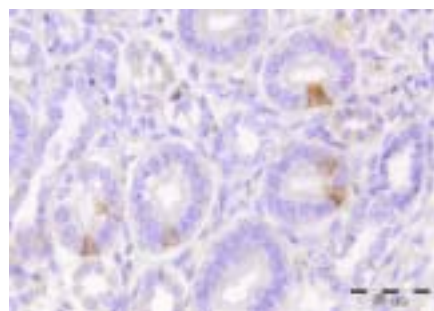
**CD117.** In the cortex, cells of the S-shaped bodies and single cells in the distal tubules between the glomeruli showed cytoplasmic positivity (Figure 3 and 4). In the medulla, 1-2 cells in each collecting duct section (Figure 5) and single cells in the loose mesenchymal tissue were positive. Single positive cells were also found among urothelial cells in the renal papillae (Figure 6).



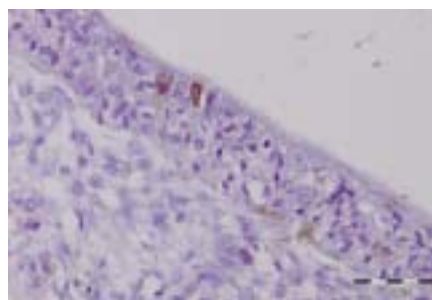
**Fig. 3.** S-shaped body in the renal cortex positive for CD117, x20



**Fig. 4.** Single cells in the distal tubules in the renal cortex positive for CD117, x40



**Fig. 5.** Single cells in the collecting ducts in the renal medulla positive for CD117, x20



**Fig. 6.** Single urothelial cells in the renal papilla positive for CD117, x20

**Ki-67.** Nuclear positivity in solitary cells with the same distribution as cells positive for CD117 was observed in the proliferation marker Ki-67. Still, the highest proliferative activity has been noticed among the cells in the undifferentiated mesenchymal tissue in the cortex.

### Discussion

Our study confirmed the expression of the putative stem cell markers in fetal kidneys, with different distribution.

CD133 is considered to be a stem cell marker by many researchers [8, 9, 10, 11], but it is also reported to be present in normal renal epithelia, too [12, 13]. Lazzeri et al. (2007), relying on previous reports of CD133 being used for marking and isolation of putative adult kidney stem cells, and Kim et al (2011) have reported its expression in the mesenchymal tissue of the cortex and some S-shaped bodies of fetal kidneys, which during organogenesis remains restricted to the parietal epithelial cells in Bowman's capsule. Our observations are not consistent to these reports due to the different distribution of the signal for CD133. Namely, in our study this protein was expressed on apical membranes of some proximal tubules in the cortex. These facts may suggest the possibility that the adult and fetal stem cells have different immunophenotype [16].

The most striking observation in our study was the expression of CD117 (c-kit) in the cortical and in the medullar structures. CD117 is also known as mast/stem cell growth factor receptor, or proto-oncogene c-kit, and represents transmembrane receptor belonging to the class III receptor tyrosine kinase family. Its natural ligand is stem cell factor (SCF), and the c-kit/SCF signaling pathway is considered to be most important in the development of both haematopoietic and non-haematopoietic stem cells. It is involved in promotion of the cell survival, proliferation, differentiation, adhesion and functional activation [17]. C-kit is normally expressed in many mature tissues: mast cells, breast epithelium, interstitial cells of Cajal, melanocytes [18, 19]; it has also been found in neoplastic

tissues with different histogenesis: myeloproliferative disorders, gastrointestinal stromal tumors (GIST), melanoma and seminoma [20, 21, 22].

C-kit has been used for selection of multipotent stem cells from the amniotic fluid [23, 24]. In the normal and developing kidney, c-kit has been reported to be present in the epithelial cells mainly of the proximal tubules and in the loops of Henle [4], which is in complete contrast to our findings.

Our observation of positive S-shaped bodies and single cells in the distal tubules in the cortex, as well as solitary positive cells in the collecting ducts and urothelium in the medulla, and the corresponding distribution of the positive signal for the proliferation marker Ki-67, makes them candidates for progenitor cells. However, studies performed with a broader panel of putative stem cell antibodies [25] have failed to identify the exact location of the stem cell sub-population in the fetal kidneys. On the other hand, the papilla has been suggested as a niche for kidney stem cells [26], which corresponds to our observation of cells positive both for CD117 and Ki-67 in this renal segment.

The fact that there is a different distribution of CD133 and CD117 in the fetal renal cells makes them inappropriate for paired use during the search for stem cells in the fetal kidneys. Another possibility, suggested by some authors [27], is that there are several types of cells with stem/progenitor cell properties: epithelial [14, 15], mesenchymal [2, 28] and those of unknown origin [29, 30], which, accordingly, would express different markers. There are only a few reports for the use of CD117 in the search for stem cells in fetal human kidneys, all of them in favor of non-specific signal for this antibody [4, 25]. Apart from that, our study found CD34 and CD44 to be useless for progenitor/stem cells identification, the first antibody due to its wide expression in the endothelial cells, which are abundant in the renal tissue, and the second one due to the lack of its expression in any cell population.

However, the fact that CD117 antibody has widely been used for selection of embryonic stem cells from amniotic fluid and other fetal tissues makes it a potential marker for this cell population. Furthermore, the specific distribution of the signal in our study, as described previously in this paper, and its correspondence to the proliferation marker Ki-67 make these cells potential candidates for progenitor/stem cells.

The above described results and review of literature point to the lack of specific surface marker(s) for progenitor/stem cells in the fetal kidney tissue, to the necessity of broadening of their panel and to the need of further functional studies, which would prove the clonogenic potential of the putative stem cells.

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## SERUM ANALYSES OF TISSUE INHIBITORS OF MATRIX METALLOPROTEINASE'S (TIMP-1, TIMP-2) IN PATIENTS WITH COLORECTAL CANCER

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### Abstract

The aim of this study was to analyze TIMP-1 and TIMP-2 serum levels in patients with CRC and to correlate the results with the pathological stage of the disease and outcome in order to evaluate the role of TIMP-1 and TIMP-2 serum levels as prognostic markers.

The investigation has been made on 82 patients with operable CRC without distant metastases, who had undergone blood tests in order to determine the TIMP-1 and TIMP-2 serum levels in the following points of time: preoperatively, as well as 3, 6, 9 and 12 months postoperatively. Pathologic staging of the disease was determined in each patient according to the pTNM classification of AJCC 2010.

Significant differences were found between serum levels of TIMP-1 and TIMP-2 obtained preoperatively and postoperatively, as well as significant association of serum TIMP-1 levels obtained preoperatively in CRC patients in stage I and III, in the 3<sup>th</sup> and in the 6<sup>th</sup> month ( $p < 0.001$ ) postoperatively as defined points of time with the outcome of CRC patients. Serum TIMP-2 levels obtained preoperatively was significantly associated with the outcome of the CRC patients. Analysis of the obtained TIMP-1 and TIMP-2 serum levels in CRC patients showed statistically significant differences with: disease progression, occurrence of liver metastasis, prior to and post chemotherapy treatment.

The results derived a conclusion that the serum levels of TIMP-1 and TIMP-2 could be indicators for occurrence and progression of CRC, as well as valuable and useful markers for following the effects of chemotherapy treatment.

**Key words:** colorectal cancer, TIMP-1, TIMP-2, prognosis, chemotherapy

### Introduction

Colorectal cancer (CRC) is the third most common malignant disease CRC accounts for an estimated 570000 new cases per year and it is the second most common cause of death in the Western-European countries and eight in the developing countries [1].

CRC prognosis predominantly depends on the disease stage, but new prognostic factors are being investigated in order to determine disease progression and outcome in patients as well as postsurgical pharmacology treatment [2].

The correct staging of each CRC patient is crucial in order to plan an optimal treatment regimen. It is widely recognised that prognostic information based on clinical and histopathological investigation may be insufficient, although tumour stage and lymph node involvement are the main prognostic tools in evaluating cancer specific survival. It is questionable to expose a large number of patients to adjuvant treatment with considerable side effects without indications that they will benefit from such treatment. Finding prognostic markers to better identify patients with higher risk for poor survival would be valuable in order to customise pre- and postoperative

treatment as well as enabling closer follow-up for these patients [3-5].

MMPs are a family of extracellular structurally related zinc-dependent endoproteases capable of degrading all the ECM components. MMPs play an important role in the physiologic degradation of ECM and are associated with tumor progression including invasion, migration, angiogenesis and metastasis [6].

Tissue inhibitors of matrix metalloproteinases (TIMPs) are a family of natural MMPs inhibitors that appear to affect many aspects of cancer biology. TIMPs role is not restricted only to MMPs inhibition but can modulate many cellular processes as cell growth, invasion, migration, metastasis, and angiogenesis and can be both anti- and protumorigenic [7, 8]. TIMPs have similar structure in about 30% to 40% and inhibit MMPs proteolytic activity building 1:1 noncovalent stoichiometric complexes. Inhibiting MMPs TIMPs have a role of remodeling of extracellular matrix (ECM). There are 4 types of TIMPs; 1, 2, 3 and 4; TIMP-1, -2, and -4 are present in soluble form and TIMP-3 is present in ECM [9, 10].

Several previous studies of TIMPs role in various cancer diseases have shown contradictory results, and

some of them have shown that serum levels of TIMPs may play important role as an indicator of occurring of CRC and its progression [11-15].

In the present study we have measured TIMP-1 and TIMP-2 serum levels in patients with CRC and we correlated the results with the pathological stage of the disease and outcome in order to evaluate the role of TIMP-1 and TIMP-2 serum levels as a prognostic markers and markers that may indicate the changes in cancer disease progression.

### Material and methods

The study included a total of 82 previously untreated CRC patients, 30 females and 53 males (age range from 43 to 75 years, averaged 67.85) with operable CRC, without detectable distant metastases, who respected the medical instructions and were available for follow-up. All the patients underwent surgical resection of the primary neoplasm at the University Clinic for Abdominal Surgery in Skopje in the period of 2 years (2007-2009).

Blood samples from all the patients were drawn before surgical treatment, as well as 3, 6, 9, and 12 months postoperatively, in order to examine the TIMP-1 and TIMP-2 serum levels. None of the CRC patients had received chemotherapy before blood sample collection. To standardize clotting conditions, all sera were separated within 1 h after blood collection, aliquoted and stored at  $-80^{\circ}\text{C}$  until assayed.

Serum levels of TIMP-1 and TIMP-2 were determined using an quantitative solid phase sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA) according to the manufacturer's instructions. High concentrations of TIMP-1 and TIMP-2 were diluted with calibrator, to produce samples with values within the dynamic range of the assay.

The histopathological analysis of surgically removed operative material was made at the Institute of Pathology at the Faculty of Medicine, Skopje, where the pathological stage was defined for every patient according to the International Union Against Cancer (UICC-pTNM) and American Joint Committee on Cancer (AJCC) 2010.

Forty three patients with Stage II B and III (A,B,C) received adjuvant chemotherapy, postoperatively at the Institute for Radiotherapy and Oncology in Skopje.

Correlations were made between the MMPs serum levels and the pathological parameters.

### Statistical analysis

Descriptive statistics (mean) are given according to normality of the distribution. Normality of the distribution was determined by Kolmogorov-Smirnov's test. Analysis of variance with Kruskal-Wallis test was first used in the analysis of different sample types. In the case of significant

results, the analyses were continued by pairing the variables and analyzing them with Mann-Whitney's U-test. Fisher's exact probability test and Pearson's Chi-Square test ( $r$ ) were used for testing the association (linearity of the correlation of serum concentrations) between TIMPs and major prognostic variables in CRC, such as grade and stage. P-values less than 0.05 ( $p < 0.05$ ) were considered as statistically significant.

### Results

There have been 17 (20.73%) patients in stage I, 40 (48.78%) patients in stage II and 25 (30.48%) patients in stage III of the CRC. Lymph node metastases were substantiated in 25 (30.48%) patients and were not found in 57 (69.51%) patients with different pT category (Table 1).

The majority of patients were with pT3N0M0 (26.82%), i.e. patients in stage II A of the disease, and the smallest number of patients were with pT4aN1M0 (4.87%), i.e. patients in stage III B of the CRC.

The mean TIMP-1 and TIMP-2 serum levels in terms of disease staging and defined points of time are shown in Table 2, Figure 1 and 1A.

The mean TIMP-1 levels in patients in all stages of the disease, decreased after tumor resection, whereas the curve of mean serum values in stage III demonstrated most intensive changes, unlike the curve of mean serum values in CRC patients in stage I and II which showed mild changes. All three curves showed decreasing values postoperatively, where the decline is most evident in 3<sup>rd</sup> month postoperatively in all CRC patients at all determined stages.

Curve of mean serum levels of TIMP-1 in CRC patients in stage II remains approximately at the same level in further defined points of time, with a slightly increase in 9<sup>th</sup> and 12<sup>th</sup> month postoperatively.

Curve of mean TIMP-1 serum levels of CRC patients in stage I showed approximately the same movement, while the curve mean TIMP-1 serum levels of CRC patients in stage III showed a plateau values from 3<sup>rd</sup> to 6<sup>th</sup> month postoperatively, then abruptly increases at 9<sup>th</sup> month and again achieves plateau up to 12 months postoperatively.

Most evident decline in mean serum levels of CRC patients was observed at 6 month postoperatively in all determined stages, and the most pronounced increase in mean serum levels was observed in CRC patients in stage III, especially in the defined points from 6<sup>th</sup> to 9<sup>th</sup> months postoperatively.

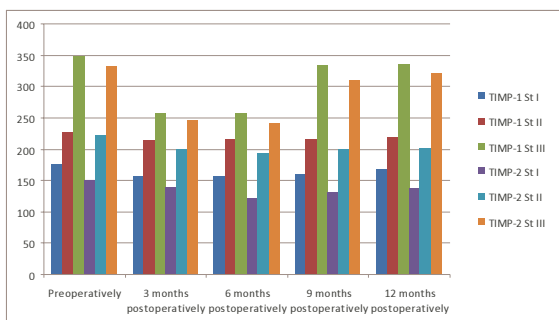
The explanation of this condition would be identical as in previously displayed results of the other investigated parameters, i.e. due to the change of condition of the disease in patients most of the stage III, in which is shown the largest percentage of deaths.

**Table 1.** Staging of patients with CRC according to AJCC

Stage	pTNM	N=(82)	(%)
I	pT1N0 M0	8	20,73
	pT2 N0 M0	9	
II	pT3 N0 M0	22	48,78
	pT4a N0 M0	18	
III	pT3 N1b M0	7	30,48
	pT3 N2a M0	9	
	pT4a N1b M0	4	
	pT4a N2b M0	5	

**Table 2.** Mean serum TIMPs (ng/mL) values in terms of stage and defined points of time

Defined points in time	TIMP-1	TIMP-1	TIMP-1	TIMP-2	TIMP-2	TIMP-2
	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
Pre-operatively	176,2	228,66	348,62	150,01	222,56	333,29
3 months postoperatively	157,74	215,05	257,98	240,91	201,49	245,99
6 months postoperatively	156,16	215,51	257,74	121,78	194,69	240,91
9 months postoperatively	161,11	216,43	334,18	132,05	201,27	310,5
12 months postoperatively	169,23	219,82	336,69	138,17	203,36	321,45

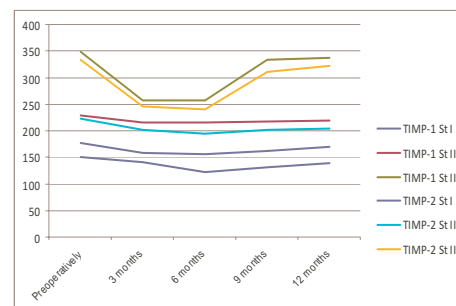


**Fig. 1.** Mean serum TIMP-1 (ng/mL) and TIMP-2 (ng/mL) levels in terms of stage and time

There were a significant differences between the mean serum levels of TIMP-1 before tumor resection and 3<sup>th</sup> and 6<sup>th</sup> month ( $p < 0,001$ ) postoperatively in CRC patients in stage I and III, between 3<sup>th</sup> and 6<sup>th</sup> month and 9<sup>th</sup> and 12<sup>th</sup> month postoperatively in CRC patients in stage III ( $p < 0,001$  among all) and between preoperative and postoperative levels during defined control points of time in CRC patients in stage II.

Regarding mean serum levels of TIMP-2 obtained prior to surgical intervention and 6<sup>th</sup> month in all CRC patients stages postoperatively we found statistically significant differences between mentioned features ( $p < 0,001$ ), as well as between 3<sup>th</sup> and 6<sup>th</sup> month and 9<sup>th</sup> and 12<sup>th</sup> month postoperatively in CRC patients in stage III ( $p < 0,01$  among all).

In table 3 are presented significant associations in TIMP-1 and TIMP-2 and outcome of the CRC patients, where it is shown that serum levels of TIMP-1 and TIMP-



**Fig. 1 A.** Mean serum TIMP-1(ng/mL) and TIMP-2 (ng/mL) levels of in terms of stage and time (preoperatively and 3, 6, 9 and 12 months postoperatively)

**Table 3.** Significant correlations of serum levels of TIMPs (ng/mL) and CRC patient's outcome

Parameter	p	R
Stage	<0,001	0,635
pT	<0,005	0,331
pN	<0,005	0,618
TIMP-1 preoperatively	<0,001	4,0279
TIMP-1; 3 months postop.	<0,001	4,0595
TIMP-1; 6 months postop.	<0,001	4,6553
TIMP-1; 9 months postop.	NS	/
TIMP-1; 12 months postop.	NS	/
TIMP-2 preoperatively	<0,001	4,5372
TIMP-2; 3 months postop.	NS	/
TIMP-2; 6 months postop.	NS	5,1124
TIMP-2; 9 months postop.	NS	/
TIMP-2; 12 months postop.	NS	/

NS – not significant

**Table 4.** Distribution of the CRC patients according received and non-received chemotherapy treatment and outcome

Stadiumn=82	With chemo-therapy	%	Without chemo-therapy	%	Poor outcome	%
Stage I	/	/	17	20,73	6	7,31
Stage IIA	/	/	22	26,82	8	9,75
Stage IIB	18	21,95	/	/	11	13,41
Stage IIIB	20	24,39	/	/	15	18,29
Stage IIIC	5	6,09	/	/	3	3,65
Total	43	52,43	39	47,56	43	52,43

**Table 5.** Mean serum levels of TIMP-1 and TIMP-2 in 20 CRC patients prior to and post chemotherapy treatment

Time	TIMP-1 (ng/mL) N=	TIMP-2 (ng/mL) N=
Prior to chemotherapy	329,03	386,19
Post chemotherapy	164,62	271,24

2 preoperatively, as well as the TIMP-1 serum levels in 3<sup>th</sup> and 6<sup>th</sup> month postoperatively, are in a significant correlation with the outcome of the CRC patients.

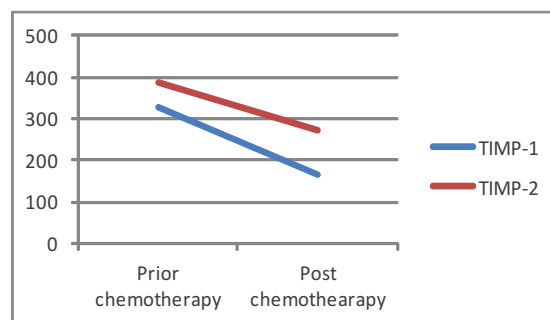
The CRC patients with different stages who received and who didn't receive chemotherapy treatment, and their outcome are shown in Table 4.

In twenty patients that were particularly monitored to the appearance of liver metastasis and subsequently were administered first-line chemotherapy treatment (in patients with stage I and II A) and additional second-line chemotherapy treatment (in patients with stage II B and III).

The liver metastasis was substantiated by imaging techniques and liver biopsy with histological confirmation with the following distribution: in 2 patients of stage I, 3 patients of stage II A, 4 patients of stage II B, 8 patients of stage III B and in 3 patients of Stage III C.

In these patients blood samples were drawn additionally for quantifying of TIMPs serum levels prior to and post chemotherapy treatment in order to determine variability of serum levels for their role as markers, which could indicate a change in the condition of the disease in CRC patients, that indicate possible progression and occurrence of metastases.

The mean serum levels of TIMP-1 and TIMP-2 in CRC patients obtained prior to chemotherapy treatment reaches approximately the levels of the mean values of the group of CRC patients in stage III C in 12<sup>th</sup> month separately, while the mean serum levels abruptly decreased ( $p < 0,001$ ) post chemotherapy treatment, i.e. similar values were obtained after resection of the primary neoplasm.

**Fig. 2.** Mean serum TIMP-1 (ng/mL) and TIMP-2 (ng/mL) levels prior to and post chemotherapy treatment

### Discussion

TIMPs are also linked with a wide variety of other functions of cell growth and survival, and at least some of these functions seem to be independent of MMP inhibition. TIMP-1 and TIMP-2 were first identified as proteins potentiating the epo-effect on proliferation and differentiation of erythroid progenitor cell [16, 17]. Additionally, the cell-growth promoting effects of TIMP-1 and -2 have been shown in various normal and malignant cell lines [18, 19]. There is some evidence supporting the hypothesis that TIMPs' role as growth stimulators and MMP inhibitors is functionally as well as possibly structurally separated [20].

The role of TIMPs in cancer is very complex, and acknowledging the fact that angiogenesis and ECM degradation are very crucial in tumor cell spreading and metastasis formation, it is obvious that TIMPs have a role of some importance in cancer. Previously the TIMPs' role as MMP inhibitors seemed most important, and there is clear evidence that downregulation of TIMP-1 and TIMP-2 expression is associated with increased invasiveness of tumors, while overexpression leads to reduced tumor growth and metastasis formation in tumors of various origins [21].

TIMP-1 (tissue inhibitor of metalloproteinases-1, human collagenase inhibitor, fibroblast collagenase inhibitor, EPA-erythroid potentiating activity) was first detected in the culture of fibroblasts and represents 28.5

KD secretory protein whose mRNA is found in macrophages, keratinocytes, endothelial cells and fibroblasts [22, 23, 24]. TIMP-1 inhibits activity of MMP-7, -14, -16, -19 and -24, but also has a role in the promotion and stimulation of cell growth by inhibiting apoptosis [25, 26]. Elevated TIMP-1 levels in blood circulation was expected to be a useful prognostic indicator for cancer patients due to inhibitory effect on MMPs, but in a number of published studies claim otherwise because TIMP-1 inhibits the apoptosis.

In meta-analysis for determining plasma and serum levels TIMP-1 as predictors of the outcome of CRC patients, Lee, Cho and Kim (2011) reported that the elevated TIMP-1 values obtained in blood samples, as well as expression of TIMP-1, are associated with shorter survival time [22].

Contemporary researches have been made in order to determine the significance of the serum TIMP-1 levels in patients of pre-invasive to invasive CRC, in order to diagnose the malignancies and to determine TIMP-1 influence on the disease's outcome [27, 28].

TIMP-2 (tissue inhibitor of metalloproteinases-2, CHIAMP-chondrocyte-derived inhibitor of angiogenesis and metalloproteinase activity, CSC-21K) could either promote the activity of MMP-2 through interaction with Mo1-MMP or to inhibit through direct action [29]. TIMP-2 can reduce proliferation of endothelial cells, fibroblasts and some tumor cell lines [32-34]. In a mouse model, TIMP-2 could protect from CRC and reduce the growth of the existing metastasis [34].

Giaginis, Nikiteas, Margeli et al. investigated TIMP-1 and -2 serum levels in 97 CRC patients and discovered a significant correlation between elevated TIMP-1 serum levels and shorter survival time, which led them to the conclusion that the elevated TIMP-1 serum levels are an independent prognostic factor for survival in patients with CRC [14].

In the examination conducted by Oberg, Hoyhtya, Tavelin, et al. (2000) on 158 CRC patients was confirmed that the elevated serum TIMP-1 levels are significantly higher in patients with an advanced stage of the disease, and that the increased values of TIMP-2 were in correlation with worse prognosis [15].

### Conclusion

In the present study significant differences were found between serum levels of TIMP-1 and TIMP-2 obtained preoperatively and postoperatively, as well as significant association of serum TIMP-1 levels obtained preoperatively, in the 3<sup>th</sup> and in the 6<sup>th</sup> month as defined points of time with the outcome of CRC patients. Serum

TIMP-2 levels obtained preoperatively was significantly associated with the outcome of the CRC patients.

Analysis of the obtained TIMP-1 and TIMP-2 serum levels in CRC patients showed statistically significant differences with: disease progression, occurrence of liver metastasis, prior to and post chemotherapy treatment.

The results derived a conclusion that the serum levels of TIMP-1 and TIMP-2 could be indicators for occurrence and progression of CRC, as well as valuable and useful markers for following the effects of chemotherapy treatment.

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**SENTINEL LYMPH NODE MAPPING IN PATIENTS WITH MELANOMA**Noveski Lazo<sup>1</sup>, Dzonov B<sup>1</sup>, Stojmenski S<sup>2</sup>, Angelevska M<sup>3</sup>, Stojanovski S<sup>3</sup>University Clinic for Plastic, Reconstructive and Aesthetic Surgery<sup>1</sup>, University Clinic for Traumatology<sup>2</sup>,  
Institute for Pathophysiology and Nuclear Medicine<sup>3</sup>, Skopje, R. Macedonia**Abstract**

Sentinel lymph node (SLN) mapping allows the surgeon to identify the draining lymphatic basin, to remove the first draining or “sentinel” lymph node(s), and to provide the pathologist with one or more nodes for meticulous examination. In this way, the patient is accurately staged with a minimally invasive procedure.

The **aim** of this study was to present two techniques for sentinel lymph node mapping used at Clinic of Plastic and Reconstructive Surgery at Clinical Center in Skopje.

A total of 60 patients with malignant melanoma that had undergone surgery at our Clinic were analyzed.

The mapping of the lymph nodes was made using 1% solution of methylen blue (first group of 30 patients) and using hand-held  $\gamma$  probe after intradermal injection of a [Tc]-99m sulfur colloid. Removed lymph nodes were sent to patohistological analysis. The analysis was made on 2 mm interval sections, after hematoxylin-eosin staining.

In all 60 patients, after the successful identification, the nodes were carefully removed and sent to histological examination. The results obtained have shown that in the first group, 16 (12 patients) from 76 removed lymph nodes were positive to metastasis, and in the second group, 11 (5 patients) from 52 removed lymph nodes were positive to metastasis.

**Key words:** sentinel lymph node, mapping, melanoma

**Introduction**

Although Cabanas used the term ‘sentinel node’ (SN) in 1977 to indicate the primary site of metastases from penile carcinoma <sup>(1, 2)</sup> the current use of this term must be attributed to Morton and his colleagues, who defined SN as the first lymph node in a regional basin that receives lymph flow from a primary tumor and thus the first lymph node encountered by tumor cells metastasizing through lymphatic<sup>(3)</sup>. The histological status of this node should therefore predict the status of at-risk regional lymphatic basins: if the SN is negative, it is unlikely that other lymph nodes in the same basin contain micrometastatic disease, while if it is positive, the risk of additional tumor in the basin is significant <sup>(4, 5)</sup>. Thus, if SN is positive a complete regional lymphadenectomy is indicated, while if SN is negative, no further surgery is required.

Sentinel lymph node (SLN) mapping allows the surgeon to identify the draining lymphatic basin, to remove the first draining or “sentinel” lymph node(s), and to provide the pathologist with one or more nodes for meticulous examination. In this way, the patient is accurately staged with a minimally invasive procedure.

The **aim** of this study was to present two techniques for sentinel lymph node mapping used at our Clinic.

**Material and methods**

A total of 60 patients with malignant melanoma that had undergone surgery at the University Clinic of Plastic and Reconstructive Surgery in Skopje were analyzed.

All patients included in the study were informed about the planned surgical procedure and they gave a written informed consent.

Patients were divided into two groups according to the technique used for sentinel lymph node mapping.

Patients met the following inclusion criteria: primary malignant skin melanoma on the extremities or trunk, without clinically present signs for metastases in the regional lymph nodes or distant metastasis. Exclusion criteria were: presence of any type of metastasis, patients after any surgical procedure that could disrupt lymphatic drainage patterns from the primary site and pregnancy.

All patients underwent routine preoperative examinations.

Patients from the first group, forty minutes before the surgery, were intradermally injected with 1% solution of methylen blue, at four places around the primary lesion. Small skin incision above the lymph group in which the region is drained was used to identify the blue colored afferent lymph vessel, that was followed to the first blue colored lymph node. After the identification, the node was carefully removed and histologically examined. The radical lymphadenectomy was performed as a separate procedure in the patients from the group with SLN positive for metastasis (SLN+). The patients with negative SLN (SLN-) did not undergo lymphadenectomy.

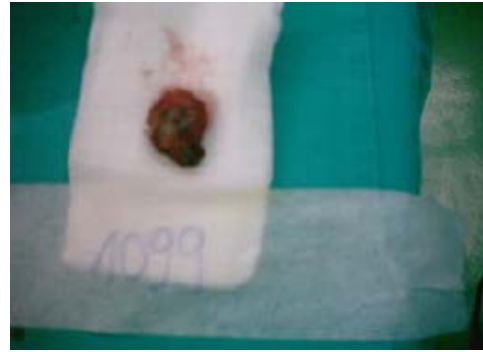
Patients from the second group underwent an intradermal injection of a radiopharmaceutical (Technetium [Tc]-99m sulfur colloid) at the site of the primary melanoma. After the injection, the patients were imaged using a large field of view g camera until enough radioactivity had accumulated in the SLN to allow localization. This generally takes between 30 and 60 minutes. Dynamic as well as static images were obtained to differentiate the first from the second echelon nodes. Anterior, posterior, and oblique images serve to localize the node in three dimensions. Once the SLN was visualized, the patient was taken to the operating room. The skin over the SLN was localized with

the aid of a hand-held  $\gamma$  probe, and general or local anesthetic was administered. In order to facilitate identification of SLN, blue dye was used in conjunction with radiocolloid. Approximately 1ml of isosulfan blue dye was injected into the skin at the site of the primary melanoma. A small incision was made over the radioactive SLN, and all blue-stained and/or radioactive lymph nodes were removed. After removal of the last SLN, the  $\gamma$  probe was used to determine that bed counts were less than 10% of the ex vivo counts of the least radioactive SLN. This ensures that all radioactive SLNs have been removed.

Removed lymph nodes were sent to pathological analysis. The analysis was made on 2 mm interval sections, after hematoxylin-eosin staining.

### Results

All 30 patients from the first group were intradermally injected with 1% solution of methylen blue. Then, a small skin incision above the lymph group that drained the region was made, and the colored afferent lymph vessels were followed to the first blue colored lymph node. After the identification, the nodes were carefully removed and sent to histological examination (Figs. 1, 2, 3). The wound was closed with direct suture and passive drainage. The median number of SLNs removed from each group was 2. No serious complications were observed. In 3 patients there were prolonged lymphorhoe that spontaneously ended in a period of three weeks after the surgery.



**Fig. 3.** Removed sentinel node

After the histological examination we found out that 16 (taken from 12 patients) from 76 removed lymph nodes were positive to metastasis.

In all 30 patients from the second group, successful radiosciintigraphic identification of the sentinel nodes, using intraoperatively  $\gamma$  hand held probe, was made (Figs. 5, 6). During the procedure 52 lymph nodes were removed. The wound was closed with direct suture and passive drainage. In six of the patients there were prolonged lymphorhoe that spontaneously ended after two weeks. The median number of SLNs removed from each group was 2.



**Fig 1.** Methylen blue injection



**Fig. 4.** Sentinel node



**Fig. 2.** Sentinel node



**Fig. 5.**  $\gamma$  hand held probe

The results of pathohistological analysis obtained that 11 (taken from 5 patients) from 52 removed lymph nodes were positive to metastasis.

### Discussion

A successful program in SLN mapping for patients with melanoma requires cooperation, communication, and commitment by physicians in nuclear medicine, surgery, and pathology.

In our study we used 1% solution of methylen blue and Technetium [Tc]-99m sulfur colloid for the visualization of the sentinel lymph nodes,.

Tc-sulfur colloid is the most commonly used agent and is usually filtered through a 50- to 100-nm or 50- to 200-nm filter before use. Other radiopharmaceuticals that have been used successfully include unfiltered technetium sulfur colloid (Tc-99 m-SC) or human serum albumin injected 24 hours before the procedure. A same-day injection of filtered Tc-99 m-SC colloid maybe an optimal method since it has been reported to provide the greatest differential counts between the SLN and the remaining basin as well as concordance between blue-stained and radioactive nodes <sup>(9)</sup>.

In our experience the median number of SLNs removed is 2, but it can range from one to five or more.

According to our experience complete lymphadenectomy is being performed in those patients that have positive SLNs (harboring metastatic melanoma). The status of the SLN has been shown to be a strong independent predictor of prognosis in clinical stage I and stage II melanoma patients <sup>(10)</sup>. Information obtained from SLN mapping is critical to accurate staging using the new AJCC staging system for patients with melanoma <sup>(11)</sup>.

The regional nodal basin is the first site of recurrence in 60% to 70% of melanoma patients with clinically localized disease who develop metastases <sup>(12)</sup>. Indeed, the pathologic status of the regional lymph nodes is the most accurate predictor of prognosis in melanoma patients <sup>(13)</sup>. Despite the intuitive appeal of removing the draining lymph nodes in patients with clinically localized disease, several prospective, randomized trials failed to demonstrate a survival advantage <sup>(1, 2, and 3)</sup>. Retrospective studies, however, suggest a survival benefit in patients undergoing elective lymph node dissection (ELND) compared with those undergoing wide excision alone <sup>(4, 5)</sup>. Once regional nodal metastases are palpable, a patient's opportunity for a long-term survival is reduced by 20% to 50% over those found to have microscopically positive lymph nodes at ELND. The 5- year survival of patients with clinical apparent regional nodal metastasis is approximately 20%, compared with approximately 50% of 5-year survival in those with clinically negative but pathologically positive regional nodes. The development of lymphatic mapping with vital blue dye and, more recently, with radio labeled colloid has provided an appealing alternative to routine ELND or wide excision alone.

The morbidity of SLN mapping using blue dye and radio colloid is small. The most common complication

of the procedure is seroma formation. Anaphylaxis after injection of blue dye has also been reported but is exceedingly uncommon. Lymph edema has also been reported after SLN biopsy. Wrone et al. reported an incidence of 1.7% in their experience with 235 procedures <sup>(14)</sup>. In general, most SLN biopsies require sedation or general anesthesia, particularly in the setting of axillary basin drainage. This must be considered when evaluating older patients with other significant medical problems who might otherwise be treated with a 1-cm margin of excision in an office setting under local anesthesia.

There have been scattered published reports of allergic reactions to isosulfan blue dye ranging from mild local reactions to anaphylactic shock. The company manufacturing isosulfan blue reported a 1.5% incidence of allergic reactions <sup>(15)</sup>. Komenaka et al published an article with a review of the literature in which they reported a total number of 1663 patients with an overall percentage of more than 1% (18/1663) in patients experiencing systemic allergic reactions. Of these, 72% (13/18) were anaphylactic shock-type reactions and 28% (5/18) were blue urticaria reactions <sup>(16)</sup>.

However, precaution is necessary in certain clinical situations. Some patients will undergo lymphatic mapping after a wide excision has been performed. Injection of blue dye in a patient who is not going to have a wide excision may lead to prolonged or permanent tattooing and is inadvisable. In addition, care must be taken in patients with facial melanoma because blue dye may extend into the dermis beyond the borders of eventual excision, and prolonged or permanent tattooing may occur.

It is important to recognize the limitations of SLN mapping as a diagnostic and prognostic procedure, since recurrence may appear in at least a third of patients but without involvement of the regional nodal basin.

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## LIMITED REAMED INTRAMEDULLARY INTERLOCKING FIXATION OF GRADE I AND II OPEN TIBIAL SHAFT FRACTURES

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### Abstract

**Introduction:** Limited reamed intramedullary nailing is a relatively new method for operative treatment of open tibial shaft fractures. This operative technique has the purpose to minimize the negative effect of intramedullary reaming and to achieve biomechanical strength and stability of the fixation.

**Objective:** The aim of the study was to evaluate the preliminary clinical results using this method of operative treatment of open tibial shaft fractures.

**Methods:** Sixty eight patients with type I and II open fractures of tibial shaft according to Gustilo classification were treated. Time of union, problems with union, infection and other complications, as well as functional results were examined.

**Results:** The results from the study showed a mean time of union of 24 weeks in group A patients, delayed union in 1 (4.5 %), non-union in 1 (4.5 %), superficial infection in 2 (9.1%). In group B, the mean time of union was 28 weeks, delayed union in 2 (4.3%) and non-union in 3 (6.5%) patients. Male union was noticed in 1 (2.2%) patient. Superficial infection in 4 (8.7%), and deep in 2 (4.3%) patients. DVT was noticed in 4 (5.8%) patients. Compartment syndrome developed in 3 (4.4%) patients.

**Conclusions:** Functional outcome showed a high percent of excellent results in the examined groups. Limited reamed intramedullary fixation is safe and effective method for operative treatment of open tibial shaft fractures, with relatively small percent of complications and excellent functional outcome.

**Key words:** limited reamed intramedullary fixation, open fractures, tibial shaft

### Introduction

Open tibial shaft fractures are relatively common injuries in everyday surgical practice as a result of the anatomy of the tibia and the susceptibility of the tibial shaft to injuries. High-energy trauma is the main cause and is frequently associated with soft tissue and bone damage. Open tibial shaft fractures treatment represents a complex problem that requires soft tissue repair and bone stabilisation through the use of established protocols. Management of open tibial shaft fractures includes an initial inspection and evaluation of the fracture, intravenous antibiotic administration, wound debridement and wound irrigation, bone stabilisation, soft-tissue coverage and the use of a bone graft, if necessary.

In regard to fracture stabilization, there are two most common techniques of fracture management, external fixation and intramedullary fixation, both with their advantages and limitations. Intramedullary fixation has an excellent fracture stabilisation, good end-functional results after rehabilitation and is generally more acceptable for patients<sup>1-7</sup>. Main disadvantage is the risk of medullary canal contamination and late development of osteomyelitis. The only way to minimise the infection risk is through initial surgical debridement and soft tissue decontamination before intramedullary nail placement. It is relatively easy achievable in Gustilo<sup>8</sup> open fractures type I-III A, while III B injuries require additional surgical debridement after the initial repair.

In regard to infection rate, the dilemma remains if reamed or non-reamed intramedullary nail placement is more susceptible to infection, for II and III Gustilo type fractures, in which there is more extensive soft tissue and

bone damage<sup>9,10</sup>. On the other hand, endosteal circulation has already suffered damage as a result of the injury<sup>11</sup>. The use of non-reamed intramedullary fixation has the goal of limiting the already damaged cortical bone circulation. This method has advantages and limitations, such as problems in bone healing and implant failure as a result of intramedullary nail fixation with a smaller-diameter nail. The technique of limited reaming has emerged in recent years as an alternative to the standard technique for reamed and non-reamed intramedullary fixation.

This technique reams the intramedullary canal with one to two-diameters less than a normal procedure, allowing bone canal widening for easier, less traumatic nail placement. This allows for the placement of a nail with a larger diameter compared to the standard non-reamed nail, thus avoiding potential complications from non-reamed fixation caused by the small diameter of the nail and also avoiding possible complications as a result of extensive reaming. The limited reaming technique "reamed-to-fix", at the same time unifies the advantages of present surgical techniques for intramedullary fixation and minimising possible complications<sup>12</sup>.

The aim of this study was to evaluate the clinical results of limited reamed intramedullary fixation in open tibial shaft fractures Gustilo types I and II with regards to complications and end-functional treatment results.

### Material and methods

During the period between 2007 and 2010, 68 patients with open tibial shaft fractures were operated with limited reaming intramedullary fixation technique at the Clinic for Traumatology in Skopje. They were divided

into two groups according to the type of open fractures: Group A, 22 patients with type I fractures and Group B, 46 patients with type II fractures according to the modified Gustilo classification from 1984 (Table 1). Gustilo type III fractures were excluded from the research. Fractures were also divided according to the AO Classification (30 type A, 28 type B, 10 type C).

Mean age of the patients was 36.4 years, of whom 40 (58.8%) were male, and 28 (41.2%) were female. High-energy trauma was the most common mechanism of injury in 52 (76.4%) patients. The research included only isolated tibial shaft fractures in adult patients. All patients were operated in the first 12 hours after the onset of the injury and all patients were treated according to established protocols. Initial clinical evaluation consisted of evaluation of the type of fracture and soft-tissue. Sterile dressing and immobilisation were initially applied. Antitetanus prophylaxis was administrated with the use of human tetabulin (250 IE). Third generation cephalosporin (2.0 g) was used for antibiotic prophylaxis, depending on the injury degree. Standard RTG investigations (AP and profile RTG) of the leg with proximal and distal joints were made.

All patients received routine preoperative preparation. Operative protocol included surgical treatment of soft-tissue damage, debridement of devitalized soft-tissues and bony fragments and wound irrigation with normal saline under low pressure. Fixation of the fracture was included in the second stage of the surgical treatment. The patient was positioned on a fracture table. Fluoroscopy enhanced imaging was used. The operative technique is identical as the classic reamed intramedullary fixation, the only difference being that reaming is done to the diameter of the nail, eventually one diameter more in the proximal end of the bone. Interlocking of the nail was done in line with the standard operative technique (proximal screws with positioning guide, distal using "free hand" technique).

In all patients wounds were closed per primam. Antibiotic prophylaxis was administered using third generation cephalosporin; clindamycin (3 x 600 mg) was additionally used for open fractures type II at least 7 days postoperatively. Antithrombotic prophylaxis was administered using low molecular heparin (standard dose of 40mg) during hospitalisation and additional 30 days after hospitalisation. Patient follow-up period was at least 12 months.

Main parameters for investigation included healing time, problems during healing (malunion, delayed union or non-union), infections and other surgical complications that could have an effect on the final functional outcome and results. Union was achieved in fractures where RTG evidence of callus formation was present and full weight-bearing was possible without pain. Mean and average time healing was determined. Delayed union was considered in fractures whose healing took longer than 6 months in Group A, 8 in group B, and fracture non-union was considered longer than 9 months in Group A, or 12 months in Group B. Malunion was determined by the occurrence of angulations greater than 15 degrees in antero-posterior or lateral RTG projections.

The effects of the type of fracture according to the AO classification on bone healing and healing difficulties were not the subject of this study. A functional evaluation of the final results was undertaken using the *Karlström-Olerud* scoring system<sup>13</sup>. In this system, the clinical data are evaluated, including pain, impairment in walking, climbing stairs, or previous sports activity, work limitation, skin condition, deformity, muscle atrophy, length discrepancy, loss of knee movement, loss ankle movement and loss of pronation/supination. There are three grades (1, 2 and 3 points) in each item, and the maximum score is 36 points.

## Results

Our results showed a mean bone fracture healing time of 24 weeks in group A and 28 weeks in group B. Non-union was present in 1 (4.5%) patient and delayed union in 2 (9.1%) patients in Group A. In group B, delayed union was present in 2 (4.3%) patients, non-union in 3 (6.5%) patients, and malunion in 1 (2.2%) patient. In 32 (47%) patients, from both groups, a nail dynamization was undertaken, according to the evaluation of the surgeon. In 4 (5.9%) patients (one from Group A and 3 from Group B) a break in the screws used for interlocking was reported in the period of fracture healing or the occurrence of selfdynamization was reported (Table 2).

There were superficial wound infections with swelling, pain and erythema in 2 (9.1%) patients in the immediate postoperative period. In Group B a superficial wound infection occurred in 4 (8.7%) patients, and deep wound infection in 2 (4.3%) patients. A wound leakage was reported in one case of deep wound infection; we administered antibiogram suitable antibiotic therapy after which the wound leakage had stopped. In one other case of a deep wound infection additional surgical wound treatment was necessary, antibiotic therapy and regular wound dressings. There was no indication for nail removal.

Deep venous thrombosis was evident in 4 patients from both groups; in one patient (4.5%) from Group A and in 3 (6.5%) from Group B. Compartment syndrome occurred in 3 cases, 1 (4.5%) in Group A and 2 (4.3%) in Group B. Mean hospitalisation period was 10 days for both groups. In 56 patients (82.35%) from both groups a pre-established physical therapy protocol was implemented. Functional results evaluation, according to *Karlström-Olerud* scoring system, was excellent in 42 (61.7%) patients, good in 18 (26.5%) patients and unsatisfactory in 8 (11.8%) patients for both groups (Table 3).

## Discussion

Open tibial shaft fractures surgical treatment is still controversial<sup>14-18</sup>. Soft tissue surgical care is very important and its adequate management is crucial for good functional. Regarding to fracture stabilisation, intramedullary fixation is widely use in modern surgical practice. Stabilisation of the fracture offers a number of advantages. It restores limb-length, decreases dead space, improves access for wound care and stops ongoing tissue damage<sup>19</sup>. There are two commonly-used options for

**Table 1.** Patient characteristics

	No. of patients	%
Gustilo type fracture:		
Group A (type I fracture)	22	32.3
Group B (type II fracture)	46	67.7
Sex:		
Male	40	58.8
Female	28	41.2
High energy trauma injury	52	76.4

**Table 2.** Postoperative results

	Group A	Group B
Bone fracture union (mean time)	24 weeks	28 weeks
Delayed union	2 (9.1%)	2 (4.3%)
Non-union	1 (4.5%)	3 (6.5%)
Superficial wound infection	2 (9.1%)	4 (8.7%)
DVT	1 (4.5%)	3 (6.5%)
Compartment syndrome	1 (4.5%)	2 (4.3%)
Mean hospitalisation period	10 days	10 days
Screw breakage	1 (4.5%)	3 (6.5%)

**Table 3.** Karlström - Olerud functional outcome score

	No. of patients	%
Excellent	42	61.7
Good	18	26.5
Unsatisfactory	8	11.8

stabilising the fracture, external fixation and intramedullary fixation<sup>20-22</sup>, both of which have advantages and pitfalls<sup>4</sup>.

External fixation is ideal for patients with severe injuries to the soft tissue because it avoids additional surgical trauma. However, external fixation does present problems. It is inconvenient to the patient, needs maintenance of hygiene, can require frequent corrections, and has a higher incidence of pin track infection (5% to 50%)<sup>4</sup>. The risk of local sepsis is also increased when the external fixator is exchanged for an intramedullary nail. Intramedullary nailing offers excellent stability and is convenient for the patient. Non-reamed intramedullary fixation has proven as an adequate way for stabilization of open fractures, even those in type IIIA and IIIB according to Gustilo, although this technique is followed by a certain percent of various complications, mostly in fracture healing and implant failure<sup>23-28</sup>.

Reamed intramedullary nailing is linked to the increased risk of infection and additional devascularisation of the already damaged bone circulation. However, vascular damage is linked to different factors, and blood flow measurement and influence of bone-healing period is difficult to be assessed in clinical conditions. Optimal management of bone stabilisation in open tibial shaft fractures is the subject of extensive

research, directed towards decrease of the occurrence of infections, less bone-healing time and lowered complication rates. The concept of limited reaming or “reamed-to-fix” of the intramedullary canal should minimise the possible disadvantages of the reamed fixation but at the same time allowing biomechanical advantage for fracture stabilisation through the use of a larger-diameter nail. At the same time, it lowers the possibility for the occurrence of complications as malunion, or implant problems which is the basic disadvantage of non-reamed intramedullary fixation.

Reaming frequently causes cortex devascularisation, additional thermal necrosis (of the inner 50-70% of the cortex<sup>29,30</sup>) and delayed healing. However, it can also fix the fracture with a larger-diameter nail thus achieving better stability<sup>31-33</sup>. There is a lower risk of fat embolism using unreamed nailing and several studies show better preservation and faster recovery of intraosseous blood supply after using a smaller-diameter nail without reaming<sup>34-39</sup>. Studies done by Court-Brown<sup>40</sup> and O'Brien<sup>9</sup> indicate that non-union is more frequent using unreamed nail. Some studies indicate higher incidence of screw breakage using unreamed nailing<sup>41</sup>.

Usage of a nail is recommended only if debridement is thorough and ‘safe’ and an external fixator



should be chosen if there are any doubts about debridement and tissue contamination. In general, a nail may be used for most grade II injuries, a careful decision taken with grade III-A injuries and a very cautious approach with grade III-B injuries<sup>42</sup>.

The results of the study showed that limited reaming technique gives similar results of bone healing and infection rates in reamed and non-reamed fixation. The results point out that open tibial shaft fractures type I should be treated as closed fractures. Using a protocol for surgical therapy and osteosynthesis in fractures type II with the limited reaming interlocking technique allows for lower surgical complications; faster fracture healing and good end functional results.

### Conclusions

Open tibial shaft fractures represent a serious surgical problem that is still the subject of many debates. An appropriate surgical treatment of soft-tissue damage is very important for successful treatment of tibial shaft open fractures. Limited reamed interlocking fixation can be applied for fixation and stabilisation of open tibial shaft fractures types I and II, according to the Gustilo classification. Our results point out that the limited reamed technique is a safe method for operative fracture stabilisation with excellent functional results, followed with only a minor rate of complications.

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**REHABILITATION OF A PATIENT WITH PERONEAL MUSCULAR ATROPHY – A CASE REPORT**

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**Abstract**

**Background:** The peroneal muscular atrophy (Charcot-Marie-Tooth disease) is a hereditary disorder characterized by signs of chronic progressive neuropathy. The treatment procedures of such patients include rehabilitation.

The purpose of the study: It is to show the rehabilitation methods in patients suffering from peroneal muscular atrophy.

**Material and methods:** The patient Lj. M. age 50, with weakness in both legs, walking difficulties with typical peroneal gait of both legs and low back pain was admitted for rehabilitation treatment. Previously, the diagnosis of Charcot-Marie-Tooth disease on the Clinic for Neurology was confirmed. The patient was assessed with a history, clinical examinations, and functional assessment with Functional Independence Measure (FIM) test and with Visual Analogues Scale (VAS) for measurement of the low back pain.

The purpose of rehabilitation was to improve the muscular trophicity, to overcome partially the contracture of the ankles, to provide the patient with orthopedic devices, to improve his gait and to help him in his social life because of his disability to perform his occupational tasks. The rehabilitation treatment included galvanic current, manual massage, exercise therapy, occupational therapy, plastic ankle-foot orthoses for both feet and for a pair of below-elbow crutches. A social worker was included during the rehabilitation process. At his first discharge, the patient was feeling better, there was no a significant change in the neurological finding, but his gait was improved, more stable and faster with the use of two plastic ankle-foot orthoses and below-elbow crutches. Later on, the patient received a disability pension.

**Conclusion:** Patients with peroneal muscular atrophy need multi-disciplinary rehabilitation program to improve their functional status and walking stability.

**Key words:** peroneal muscular atrophy, rehabilitation, orthosis, exercise, physical therapy

**Introduction**

The peroneal muscular atrophy (Charcot-Marie-Tooth disease) is a hereditary disorder characterized by signs of chronic progressive neuropathy. It is one of the most frequent genetic neuromuscular disorders with prevalence of approx. 1:2500 [1]. It occurs in both sexes, more commonly in men, begins in the second decade of life and is characterized by symmetric muscular atrophies and weakness in distal segments of the lower extremities, and less often of the upper extremities [2, 3].

The patoanatomical picture of peripheral nerves shows modifications of the myelin sheath and axon degeneration, whereas in the spinal roots it shows proliferation of connective tissue and decay of ganglion cells in the anterior horns of the spinal cord. There are degenerative changes in the muscles too, along with fibrosis [2].

The peroneal muscular atrophy is manifested with symmetric weakness and atrophy of the feet muscles, especially the ones innervated by the peroneal nerve. In the further progression of the disease, the weakness

affects the muscles of the calf muscles of the lower legs, and the lower part of the upper legs [2].

The clinical picture shows muscular atrophy of the affected muscles, pain and numbness, impairment of sensibility, and rarely even fasciculations. The Achilles tendon reflex is extinguished, and various deformities of the feet develop, as well. The disease gradually progresses causing smaller or greater disability [2]. The gait becomes more difficult [3].

The treatment procedures of such patients include rehabilitation, as well. The rehabilitation processes are directed toward the prevention of contractures, maintenance of circulation, strengthening of the remaining unaffected muscles, and improvement of walking abilities [2].

**Purpose of the study**

The purpose of this study is to present the rehabilitation methods in patients suffering from peroneal muscular atrophy.

### Material and method

The patient Lj. M. age 50, a disability pensioner, a former baker with 26 years of service; married and father of two children. The disease began in 1988 when the patient felt pain and weakness in both legs, particularly in the right one, for the first time. The disease was understood as the right-side sciatica and was treated conservatively with medical and physical therapy. Neurosurgical consultations were also made. The disease was getting worse in the following years, and the weakness began to appear in the left lower leg, as well. Few years ago, a diagnosis of peroneal muscular atrophy (Charcot-Marie-Tooth) was set up after tests (EMG, SEP, muscular biopsy, MRI of LS spine) performed at the Clinic of Neurology.

EMG: the motor nerve conduction velocity in the legs was decreased. SEP of n.tibialis with normal finding. Muscular biopsy: advance process of chronic denervation.

MRI on lumbosacral spine: disc space reduction T 11-T 12 with dorsocentral herniation of the disc. Polydiscopathy from L3 to S1 without compression of the dural sac.

Rehabilitation treatments at the Institute for Physical Medicine and Rehabilitation repeatedly followed. The patient was assessed with a history, clinical exams, and functional assessment with Functional Independence Measure (FIM) test and with Visual Analogues Scale (VAS) for measurement of the low back pain.

The patient complained of weakness, occasional stiffness, numbness, burning and coldness in both legs, particularly in the right one, walking problems, occasional pain in the left hip and lower back during long standing or walking, difficulties in walking with typical peroneal gait of both legs.

Clinically, at first admission, the patient's lower extremities were with a very pronounced weakness and hypotony of the lower leg muscles, bilaterally, especially the peroneal one, as well as weakness in the upper leg muscles. He held his feet in a slight equinus. There were trophic changes on his feet skin; the skin was slightly blue and cold at palpation. Active movements of the hips and knees were possible in good range of motion; in the both ankles the active dorsal flexion was impossible (-5 degree), and the passive one to 0 degree due to contracture. The rough muscle strength of the feet muscles was reduced. The superficial sensibility was normal. The patellar reflex was symmetric. The Achilles' tendon reflex was extinguished bilaterally. The patient could not stand on his toes and heels. He walked without aids with typical bilateral peroneal walk, on a wide base (Fig. 1.).



**Fig. 1.** Patient with Charcot-Marie-Tooth disease

The FIM score was 110 and the VAS for the low back pain was 50 mm.

The purpose of rehabilitation was to improve the muscular trophicity, to overcome partially the contracture of the ankles, to provide the patient with orthopedic devices, to improve his gait and to help him in his social life because of his disability to perform his occupational tasks.

The rehabilitation treatment included longitudinal application of galvanic current for the lower extremities, manual massage, exercise therapy (a range of motion exercises that improve the ankles function, exercises that improve muscular strength), and occupational therapy. He was given prescriptions for plastic ankle-foot orthoses (AFO) for both feet and for a pair of under-elbow crutches. For low back pain, the patient received a conventional TENS (Transcutaneous Electrical Nerve Stimulation). A social worker was included during the rehabilitation process, which made social assessment, and gave recommendations for disability pension. The rehabilitation program lasted 20 days.

At his first discharge, the patient was feeling better, there was no a significant change in the neurological finding, but his gait was improved, more stable and faster with the use of two plastic ankle-foot orthoses and below-elbow crutches. FIM test was 118 points, and VAS for low

back pain was 10 mm. Later on, the patient received a disability pension (**Fig. 2.**).

The patient received further physical treatments (once a year) in order to maintain the results achieved and to reduce his low back pain. He was adopted on his disability and satisfied with rehabilitation outcome and



**Fig. 2.** CMT patient with AFO orthoses and a below-elbow crutch.

getting disability pension. He has not visited a neurologist for several years.

The patient has been an alcoholic for many years and treated for alcohol abuse in the Hospital for Psychiatry in Kisela Voda, Skopje. The patient occasionally receives diazepam a 5 mg.

### Discussion

There are no completely precise methods of rehabilitation of patients with Charcot-Marie-Tooth (CMT) disease. However, there are a number of significant treatments accessible to CMT patients that could improve their quality of life and assist them in maintaining their independence. The rehabilitation treatment is part of the therapies applied, whereas pain treatment and surgical interventions are occasionally included.

The physical therapy is considered limited within the secondary prophylaxis, in the prevention of deformities in the muscular-skeleton system and in the progressive reduction of the capacity of movement [3].

Some authors recommend application of general ultraviolet radiation in below erythema doses to strengthen

the immune system, as well as middle-frequency currents to improve the trophic processes [3].

Another authors recommend iontophoresis with galantamin, electric stimulation (indirect and direct), diadynamic currents (rhythm syncope), middle-frequency currents with alternative or constant regime with frequency from 100 to 150 Hz, depth of 100%, as well as exercises, exercises in water, four-cell bath, paraffin therapy, healing mud therapy and manual massage [4].

Foot extension exercises are recommended to prevent shortening of the Achilles tendon. Special shoes with good ankles support may be necessary. Good evaluation of movements, application of ankle foot orthosis and training for its application is indispensable. Patients frequently need AFO orthosis for foot equines correction and gait improvement [5]. Treatment methods may use plaster splints, plastic corrective orthosis for lower extremities to correct feet deformities. Special attention should be paid to patients with impairment of the sensation for pain and heat [2].

To improve his gait, our patient received exercises, manual massage, therapy with galvanic currents, including ankle foot orthoses (AFO) and below-elbow crutches.

Some patients need under-elbow crutches or a stick to improve their gait stability, and only less than 5% of the patients need a wheel chair. It is desirable to educate the patients in relation to weight control, because obesity makes difficulties in movement. Exercises should support the capacity of each individual patient. The majority of the Charcot-Marie-Tooth patients remain physically active [6].

Adapted aids may be used occasionally to improve the activities of daily living and self-care. Wrist and hand orthoses are occasionally used. Professional occupational therapy important for employment and career may also be needed [6].

In a randomized study of 14 persons aged 7 to 30 suffering from Charcot-Marie-Tooth Type 1 A with less than 15 degrees dorsal flexion tests were made with splints to a foot setting in a maximum dorsal flexion. Splints were carried on one leg during the night for a period of 6 weeks, and on the other leg in the following 6 weeks. The results showed that the night splints increased neither the range of motion in the ankle nor the muscular strength [7].

In another randomized study that included 30 children and young adults with Charcot-Marie-Tooth and limited dorsal flexion in the ankles, the test group received serial night plasters for 4 weeks followed by a 4-week weight bearing stretches, whereas the control group was not got any intervention. The results obtained showed that the 4-week night plasters increased the dorsal flexion

of the test group, but 8 weeks later, there was no significant difference between the two groups [8].

In a clinical study testing sensitivity of various scales in rehabilitation and lungs condition with 8 CMT patients compared to healthy persons, it was determined that all the rehabilitation measures were much worse with CMT patients than was the case with the healthy persons. Treatments with walking belts, extension exercises, respiratory exercises and proprioceptive exercises were carried out twice a week for a period of 8 weeks. After the treatment, there was a significant improvement in the movement range of the ankles and in the walking time at a distance of 6 meters. The authors concluded that patients should be treated at least 2 times a year, because regression of outcome measures was determined after a 6-month period without exercises [9].

Another clinical study tested the effects of a resistance exercises program applied at home during 12 weeks and activities of daily living (ADL) between men and women with Charcot-Marie-Tooth disease in order to design a resistance exercise program that would be recommendable in respect to the activities of daily living. The 20 patients involved were doing exercises at home, and tested at a university clinic. The time required for standing up from a chair to evaluate the lower part of the body was also measured, as well as the time required for lifting the upper part of the body from lying on the back to evaluate the upper part of the body. The exercise compliance was 87%. At the beginning, the women had 80% normal strength in 4 of 10 measurements, whereas the men did not achieve 80% of the normal strength in any measurement. After training, the women had 80% of the normal strength in 8 of 10 measurements, whereas the men had 80% of the normal strength only in 1 measurement. The strength change scores did not show any difference between the sexes. After the training, the ADL was improved; however, there was no difference between the sexes [10].

A study on 8 CMT patients conducted a test to prove whether a 24-week-training interval of bicycle riding can substantially improve the psychological, neuromuscular and functional capacity and increase the resistance to fatigue in these patients. Assessments were made of the cardiovascular fitness, muscular strength, fatigue and functional capacity before and after 12 weeks in hospital training under the supervision, and again after 12 weeks training at home with no supervision. It was concluded that the training was well tolerated. The cardiovascular capacity, the isokinetic concentric strength and the measures of functional possibilities were significantly improved. All patients had an improvement in the self-assessment Visual Analogue Scale of fatigue

and pain during the exercises. The patients had an improvement in the exercise tolerance presumably because of the deconditioning effects of their sedentary lifestyle, so that the patients benefited from the functional possibilities and subjective perception of pain and fatigue. Improvement was noticed at the end of the first period of home training under supervision and was maintained after the second home training without supervision, although there was no further improvement in the performance opportunities and tolerance [11].

Our patient has been an alcoholic for many years and previously treated for alcohol abuse in the Hospital for Psychiatry. Because of long lasting disability with CMT disease, he was adopted with it. Sometimes, he took diazepam. However, CMT disease is considered not to affect the psychological status of patients.

A study with 53 patients suffering from CMT disease determined that these patients are capable of coping with the problems caused by their disease without developing an additional psychological distress when compared to persons who do not suffer from such disease. It is probably because of adaptation due to long duration of the disease, relatively mild symptoms, good cognitive functions and possibilities for rehabilitation treatment [12].

Carter G.T. et al. believe that patients with Charcot-Marie-Tooth disease should be treated in a comprehensive multi-disciplinary ward in which a neurologist, physiatrist, orthopedic surgeon, physiotherapist, occupational therapist and an orthotist should be involved. Treatment should be aimed at achieving maximum independence and quality of life [13].

### Conclusion

Patients with peroneal muscular atrophy need multi-disciplinary rehabilitation program to improve their functional status and walking stability.

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## FISTULAS IN CROHN'S DISEASE

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### Abstract

Despite the progress in treatment of patients with Crohn's disease that improves or release the symptoms, there is still no cure for the disease. Complications such as fistulas occur in patients with Crohn's disease. Fistulas arise in 35% of patients and 20% of these patients develop perianal fistulas.

**Material and methods:** 23 patients (11 females and 12 males) with fistulas due to diagnosed Crohn's disease, who attended their routine medical exams during the last two years, were included in our retrospective study of 10 years from 1995 to 2005. The analysis was made based on the following information that we gathered from each patient: Clinical history, endoscopy, histopathology, radiography and management.

**Results:** Sixteen of 23 patients got diagnose of Crohn's disease after the first occurrence of symptoms. The other 7 patients got delayed diagnosis. There were 7 patients with ileal involvement; 5 of them with perianal disease (4 fistulas, 1 abscess) and 2 with enteroenteral fistulas. Sixteen patients had Crohn colitis, 10 of them with perianal disease (7 perianal fistulas, 3 abscesses), 3 with enterocutaneous and 3 patients with enteroenteral fistulas. Eleven patients underwent surgery due to complications and symptoms refractory to medical therapy. Two patients with perianal fistulas treated only with medicamentous treatment did not achieved closure and had persistent fistulas. The perianal fistulas of four patients were resolved after fistulectomy and the fistulas of two patients after treatment consisting of antibiotic, 5ASA drugs, glucocorticoids and local dressing. One patient underwent a second fistulotomy and still had persistent perianal fistula. Four patients with enteroenteric fistulas achieved closure of their fistulas after the first operation and one of the patient who had recurrence, achieved closure after the second operation. Two patients with perianal abscesses that underwent incision converted into persistent perianal fistulas despite the type of treatment and two patients healed after the incision.

**Conclusion:** The combined therapy consisted of surgical and medical treatment was effective in management of our patients and we strongly recommend team work with gastroenterologist and surgeon involved in treating fistulas in Crohn's disease.

**Key words:** Crohn's disease, fistulas, perianal disease, management, therapy

### Introduction

Crohn's disease (CD) is described first by Crohn, Ginsberg and Oppenheimer in 1932 as a chronic inflammatory disease of the gastrointestinal tract. Despite the progress in treatment of patients with Crohn's disease that improves or release the symptoms there is still no cure for the disease [1].

Complicattions such as fistulas, abscess, perforation, strictures, hemorrhage, toxic dilatation and malignancy occur in patients with Crohn's disease and often require surgical treatment. Approximately 80% of all patients with Crohn's disease will undergo operative intervention in some point of their life and 20%-70% of them will undergo second surgical procedure [1, 2, 3].

Fistulas are common complication of Crohn's disease, appearing as enteroenteric, enterocolic, enterocutaneous, enterovaginal, enterovesical and perianal [4]. Perianal fistulas in Chron's disease occur within the range from 17% to 43% of the patients and many of them

are present at the time of diagnosis [5, 6]. The frequency of enteroenteric fistulas range from 30% to 50% of all patients with fistulas [7, 8]. The perianal fistula symptoms severity depend on the severity of inflammation and the symptoms of internal fistulas depend on their location [6, 8]. The symptoms of perianal fistulas include pain, pus fluid or fecal discharge, anal pruritus, itching and skin maceration and decresed quality of life [6, 9]. The occurrence of perianal abscess is different manifestation of the same clinical disease [6]. The internal fistulas may usually stay silent and may be difficult to diagnose [8]. The management of fistulas requires a competent care and includes medications, surgery or combination of both [4, 5, 8, 10, 11, 12, 13, 14, 15, 16].

**Aim:** In our study we made an analysis of 23 patients with Crohn's disease and fistulas as a complication of the primary disease. We observe clinical, endoscopycal and radiological data and we compared the efficacy of the different ways of treatment of the patients.



We also discuss the current aspects of medical and surgical treatment of the disease.

### Patients and Methods

A retrospective study of 10 years period from 1995 to 2005 was made using archive materials from the University Clinic of Gastroenterohepatology and University Clinic of Abdominal Surgery, Faculty of Medicine in Skopje. Twenty three patients (11 females and 12 males) with fistulas, due to diagnosed Crohn's disease who attended their routine medical exams during the last two years, were included in the study. Two patients were excluded due to lack of data. The youngest patient had 25 and the oldest 67 years and average age was 47.9 year. The analysis was made based on the following information that we gathered from each patient: Clinical history, endoscopy, histopathology, radiography and management.

Symptoms, intestinal involvement, fistula occurrence, the medicamentous and surgical treatment of Crohn's disease and complications (perianal disease, fistulas), radiological findings and endoscopic findings were analyzed. All the patients underwent barium enema, endoscopy and histopathology examination for diagnosis of the primary disease. Patients with perianal and enterocutaneous fistula underwent fistulography for visualization of fistulous channel. The patients with enteroenteral fistula were examined by irrigography and enteroclysis.

The continuous medicamentous therapy for perianal disease in all patients included: oral glucocorticoids 40-60 mg/day with gradual tapering until achieving reduction of symptoms and maintenance dose of 5 to 10 mg per day, 5ASA 500 mg three times a day and

Data were analyzed using SPSS software and mean value; frequencies, percents and Spearman's coefficient of correlation were done.

### Results

Sixteen of 23 patients were diagnosed with CD after the first occurrence of symptoms; 7 patients got delayed diagnosis with 4 of them after a year and 3 patients 2 years later. Those 7 patients with delayed diagnose had mild symptoms and their first medical examination after the above mentioned period.

All patients had variable feeling of pain, distension and nausea. Diarrhea was the main symptom in 12, bloody stool in 4, vomiting in 2 and perianal fistula was main symptom and presentation at the time of diagnose in 2 patients. One of the patients had significant loss of weight, one patient was diagnosed after operation due to ileus and one was diagnosed after appendectomy. The symptoms of perianal disease included pain, swelling, tenderness, discomfort during walking and sitting in all patients. Patients with perianal fistulas had additional skin maceration, itching and variable degree of discharge. Well known radiological features of the intestine in variable degree were present in all patients with CD: narrowed lumen, string sign, proximal dilatation, wall thickening, ulcerations, edematous mucosa, and cobblestone pattern with nodular filling. On endoscopy examination 7 patients had characteristic findings only in terminal ileum and the other 16 had colonic lesions in different parts of the large bowel. All patients had positive histological findings for Crohn's disease. Eleven patients underwent surgery due to complications and symptoms refractory to medical therapy (Table 1).

**Table 1.** Localization of the disease for the patients who underwent surgery

Patient No.	localization of disease	operation
3	terminal ileum	resection of ileum
4	colon ascendens	right colectomy
1	colon descendens	resection of c. descendens
1	colon sigmoideum	sigmoid resection
1	terminal ileum	resection of ileum and cecum
1	pan colitis	resection of terminal ileum+ subtotal colectomy

metronidazole 800-1000 mg/day until remission. All patients received maintenance dose of Azothioprine 50-100 per day. The remission was achieved in 4 to 8 weeks in all patients.

Concerning the type of the fistula as a complication of the primary disease there were 11 patients with perianal, 5 patients with enteroenteral and 3 patients with enterocutaneous fistulas. The group of patients with

perianal disease includes: 4 with perianal abscesses and the previous mentioned 11 patients with perianal fistulas (Figure 1, 2).



**Fig. 1.** Enterocutaneous fistula



**Fig. 2.** Perianal fistula

There were 3 patients with postoperative recurrence of perianal fistulas and 6 with long time persistent perianal fistulas despite different ways of treatment. Two patients with perianal fistulas treated only with medicaments did not achieved closure and had persistent fistulas. The perianal fistulas of four patients were resolved after fistulectomy and the fistulas of two patients after treatment with antibiotic, 5ASA drugs, glucocorticoids and local care. One patient underwent a second fistulotomy and still had persistent perianal fistula. One patient with perianal fistula developed enterocutaneous fistula 2 years later and one patient developed gastro colonic fistula 10 years later (Patient No 1 and No 6).

Four patients with enterenteric fistulas achieved closure of their fistulas after the first operation and one of the patient who had recurrence achieved closure after the second operation.

Two out of three patients with enterocutaneous fistulas had postoperative recurrences that resolved after the second operation.

Two patients with perianal abscesses that underwent incision converted into persistent perianal fistulas despite the type of treatment and two patients healed after the incision.

The statistical analysis showed that there was a correlation between:

a) The gender and the site of intestinal involvement (Spearman's  $\rho = 0.44$ ,  $p < 0.05$ ), colon involvement was more frequent in women;

b) The gender and the complications (Spearman's  $\rho = 0.51$ ,  $p < 0.05$ ), perianal fistulas were more frequent in women;

c) Concerning the type of treatment on recurrence and outcome (Spearman's  $\rho = 0.79$ ,  $p < 0.05$ ), medical treatment was in positive correlation with persistent fistulas.

d) There was negative correlation between the disease recurrence and healing of fistulas (Spearman's  $\rho = -0.46$ ,  $p < 0.05$ ); meaning that in patients with no recurrence, healing was more frequent than persistence. There was no correlation between site of intestinal involvement and complication.

During the treatment of complications of Crohn's disease the glucocorticoid therapy was stopped and metronidazole and other antibiotics according to antibiogram were applied.

### Discussion

Fistulas arise from ulcers progressing in transmural fissures that may penetrate surrounding soft tissue or another organ and then open into another organ or skin [8, 18]. According to their termination fistulas are classified as internal and external. Internal fistulas terminate in abdominal cavity or open in another hollow organ or abscess cavity unlike external fistulas that open on the skin. Internal fistulas may be enteroenteric, gastro colic, enterovaginal and enterovesical. In addition, external fistulas may be enterocutaneous, colocutaneous and perianal [8]. Fistulas occur in 35% of patients with Crohn's disease and 20% of them develop perianal fistulas [8, 10, 18]. Perianal fistulas are a common manifestation and may be initial presentation of Crohn's disease [10, 11]. They are usually classified as simple fistulas with or without proctitis or complex fistulas as intersphincteric, transsphincteric, extrasphincteric and submucosal fistulas [6, 10, 23]. According to some authors, the frequency of perianal fistulas in patients with Crohn's disease ranges from 17% to 43% and the risk of their occurrence increase when the disease involves the distal bowel [5, 19, 20, 21].

**Table 2.** Clinical data of the patients

Patient	Gender	Crohn's localisation	Crohn's disease complication	Treatment	No of recurrence	Treatment	Outcome
<b>Patients with perianal fistula</b>							
1	f	Colon	Perianal fistula	Fistulotomia	1 Perianal 1 New Enterocut.	Fistulotom Operation	Persistent Sanation
2	f	Colon	Perianal fistula	Fistulectomia	None	None	Sanation
3	f	Colon	Perianal fistula	Medicam.	Persistent	Medicam.	Persistent
4	f	Colon	Perianal fistula	Fistulectomia	None	None	Sanation
5	f	Colon	Perianal fistula	Medicam.	Persistent	Medicam.	Persistent
6	f	Colon	Perianal fistula	Medicam.	None 1 New Persistent	None Medicam.	Sanation Gastro colic
7	f	Colon	Perianal fistula	Fistulotomia	None	None	Sanation
8	m	Ileum	Perianal fistula	Fistulectomia	None	None	Sanation
9	m	Ileum	Perianal fistula	Medicam.	None	None	Sanation
10	m	Ileum	Perianal fistula	Fistulotomia	2 Perianal	Medicam.	Persistent
11	f	Ileum	Perianal fistula	Fistulotomia Curettage	Persistent	Medicam.	Persistent
<b>Patients with enteroenteric fistula</b>							
12	m	Colon	Enteroenteric	Operation	None	None	Sanation
13	m	Colon	Enteroenteric	Operation	None	None	Sanation
14	m	Colon	Enteroenteric	Operation	None 1 New Enterocut.	None Operation	Sanation Sanation
15	m	Ileum	Enteroenteric	Operation	None 1 Perianal	None Medicam.	Sanation Persistent
16	m	Ileum	Enteroenteric	Medicam.	None	None	Persistent
<b>Patients with enterocutaneous fistula</b>							
17	f	Colon	Enterocutaneous	Operation	None	None	Sanation
18	f	Colon	Enterocutaneous	Operation	1 Enterocut	Operation	Sanation
19	m	Colon	Enterocutaneous	Operation	1 Enterocut	Operation	Sanation
<b>Patients with abscess</b>							
20	f	Colon	Abscess	Incision	1 Perianal fistula	Medicam.	Persistent
21	m	Colon	Abscess	Incision	None	None	Sanation
22	m	Colon	Abscess	Incision	1 Abscess	Incision	Sanation
23	m	Ileum	Abscess	Incision	2 Abscess	Incision	Persistent

\*All the patients are treated with medicamentous therapy for the CD continuously. The data in the table refer to the fistula treatment.

In our study 5 patients (71,4%) out of 7 with ileal involvement and 10 (62,5%) patients out of 16 with colonic involvement developed perianal fistulas, that is not consistent with the data from the literature. According to our opinion this is result of small number of patients in our group.

The frequency of enteroenteric fistulas ranges from 30% to 50% of all patients with fistulas [7, 8]. Enteroenteric fistulas may be asymptomatic and may be difficult to diagnose. They are usually found incidentally and their presence do not always require surgery, but this approach is the most common and satisfactory method of their treatment [8]. Intra abdominal abscesses should be drained and complicated fistulas accompanied by intestinal obstruction or present symptoms should undergo surgery. It is reported that antibiotics and 6 mercaptopurine/azathioprine may be effective in closing internal fistula [10, 22].

There were 5 enteroenteric fistulas (30,4%) in our study. Four of them were treated surgically and closed after the intervention. Afterwards one of the postoperative fistulas which had recurrence as well as another medically treated fistula did not achieve closure but only reduction.

Enterocutaneous and perianal fistulas are easy to recognize [7, 10]. Perianal Disease Activity Index (PDAI) may be used to measure activity of perianal Crohn's disease. PDAI was reported by Irvine et al 1995 year [9, 24] and is a score that includes 5 parameters: discharge, pain, restriction of sexual activity, type of perianal disease and degree of induration. We find PDAI a useful tool for evaluating perianal Crohn's disease. We have recently brought it into use at our Clinics for another study of Crohn's disease. Score systems are used in order to make distinction between remission and active disease and to evaluate the severity of the disease (24). PDAI may be a useful tool in clinical trials in order to validate new treatments of perianal Crohn's disease.

The new treatment of perianal Crohn's disease prefers combined medical and surgical approach, after a correct diagnoses using endoscopy, ultrasound or MRI [11]. Many authors support this view and recommend it in treatment of Crohn's disease [8, 10, 11, 12, 13, 14, 15, 16, 26].

Daniel H. present in his article has made a review of treatment of fistulas in Crohn's disease [10]. According to author's findings, sulfasalazine or mesalazine has been used predominantly for the management of patients with fistulas and active bowel disease. Glucocorticoids should not be used in patients with fistulas, either internal or external. Metranidazol may cause initial clinical response in patients with fistulas or complete healing if used as maintaining therapy. If the antibiotic is excluded from the

therapy, relapse frequently occurs. The agent has high toxicity profile. Ciprofloxacin has also been used in treatment of fistulas with similar efficacy. According his opinion antibiotics are effective in perianal disease, usually without complete healing, but improving quality of life for long period. 6 Mercaptopurine/Azathioprine is effective in closing and maintaining closure of fistulas. The agent has high toxicity profile. Methotrexate may cause partial healing or complete closure of fistulas. Methotrexate also has high toxicity profile. Cyclosporin A is an effective in treatment of fistula, as well as active Crohn's disease. The agent may have significant toxicity. Tacrolimus (FK 506) has been used in several studies and has showed a good efficacy but high toxicity also. Infliximab, a chimeric monoclonal antibody which binds to tumor necrosis factor  $\alpha$ , has shown efficacy in the treatment of chronically active Crohn's disease and perianal fistulas in dose of 5mg/kg. It's adverse effects are less toxic, causing infusion reactions, delayed hypersensitive reactions, formation of anti-double-stranded DNA, human antichimeric antibodies, lupus like reaction and reactivation of latent tuberculosis. Other therapies as total parenteral nutrition, bowel rest, thalidomide, and hyperbaric oxygenation have also been used and shown some efficacy. The author recommends surgical intervention for complicated fistulas and large abscesses and combination of therapies in treatment of Crohn's disease and fistulas [10].

As it can be seen in table 2 we achieved medicamentous closure of 2 perianal fistulas, and only reduction of the symptoms without closure in another 2. The patients received oral glucocorticoids 40-60mg/day with gradual tapering until achieving reduction of symptoms and maintenance dose of 5 to 10 mg per day, 5ASA 500 mg three times a day, metronidazole 800-1000 mg/day until remission and azothioprine according body weight and disease activity. The mortality rate of our patients was zero in a 10 years period.

### Conclusion

In our study perianal fistulas were the most common type of fistula in patients with Crohn's disease compared to other types (enteroenteric and enterocutaneous). Regardless of the type of fistula the combination of surgical and medical treatment was effective in management and we strongly recommend team work with gastroenterologist and surgeon involved in treating fistulas in Crohn's disease.

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**IMMUNOGLOBULIN A NEPHROPATHY IN CHILDHOOD**Naunova Timovska Silvana<sup>1</sup>, Kuzmanovska D<sup>1</sup>, Petrusevska G<sup>2</sup>University Childrens Hospital, Skopje, Republic of Macedonia<sup>1</sup>Institute of Pathology, Medical Faculty, "St Cyril&Methodius University", Skopje<sup>2</sup>**Abstract**

**Objectives:** Immunoglobulin A nephropathy is a common primary glomerular disease in childhood. The most frequent clinical presentation is hematuria with/or without proteinuria, usually with good prognosis. The characteristics of IgA nephropathy in Macedonian children are unknown. The aim of the study was to describe the characteristics of immunoglobulin A nephropathy in children in the Republic of Macedonia.

**Methods:** This study was performed at the University Pediatric Clinic in Skopje as the only pediatric nephrology centre in the country. For the period of 14 years we analyzed 82 children at the age of 1-15 years who had diagnosis of glomerular disease documented by renal biopsy, with special attention to immunoglobulin A nephropathy. The data from the history and the renal biopsy findings were analyzed.

**Results:** Immunoglobulin A nephropathy was registered in 14% of cases with primary glomerular disease. The mean age of the patients was  $8.55 \pm 3.53$ , with predominance of male gender. The most common histopathological finding was focal mesangial proliferation. By immunofluorescence different patterns of IgA deposits were found in 100%. The most common clinical presentation was recurrent hematuria.

**Conclusion:** Data presented in this study reflect the specifics of *immunoglobulin A nephropathy* in Macedonian children. Its distribution is similar to that described in other countries with some differences, probably as a result of different selection of patients for renal biopsy.

**Key words:** immunoglobulin A nephritis, renal biopsy, children

**Introduction:**

Immunoglobulin A nephropathy constitutes about 10% of all glomerular disease in childhood [1-13]. The prevalence has been estimated to be about 5-25 cases per 100.000 children and is generally associated with isolated hematuria [14]. It is deemed to be monosymptomatic form of Henoch-Schoönlein disease, which is indicated by similar clinical symptoms as well as pathohistological and immunohistological findings of immunoglobulin A deposits in mesangium [15-17].

Immunoglobulin A nephropathy is a chronic disease manifested in recurring microhematuria episodes, and if proteinuria appears it is less than 1g/24h. Following an infection (respiratory, abdominal or urinary) might recur macrohematuria occasionally associated with nephritic syndrome. Today immunoglobulin A nephropathy is not considered a benign disease whereby with a number of patients in kidney failure progression and increased proteinuria is a sign of forthcoming progression [17-19].

The most common pathohistological finding is the focal mesangioproliferative glomerulonephritis. The aim of this study was to determine the characteristics of immunoglobulin A nephropathy in Macedonian children.

**Methods**

This retrospective study was performed at the University Pediatric Clinic during the period of January 1996 to December 2008 year. We analyzed a total of 82 patients at the age of up to 15 years suffering from glomerular disease. All native renal biopsies were reviewed but only glomerular diseases were analyzed with special attention to immunoglobulin A nephropathy. The diagnosis of each case was based on histological, immunopathological and clinical features. We used data

from the medical history of the patients and renal biopsy findings. The renal biopsy was done at the Clinic of Nephrology and controlled by ultrasound. The biopsy material was examined at the Institute of Pathology using the method of light microscopy and immunofluorescence. The statistical analysis of data was made with methods of descriptive statistics, presented in figures.

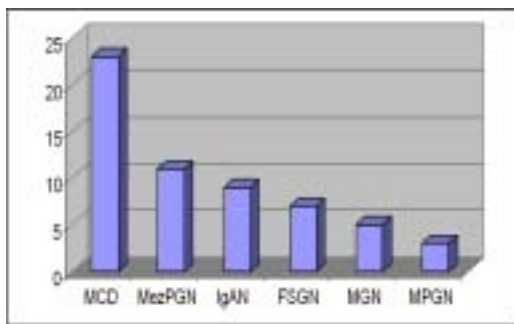
**Results**

The study included a total of 82 patients with glomerular disease in duration of 15 years. Primary glomerular disease was diagnosed in 65 patients (79%) and secondary in 17 (21%). Immunoglobulin A nephropathy was registered in 9/65 (14%) of patients with primary glomerular disease.

Fig. 1 shows the distribution of the histopathological finding in primary glomerulonephritis. Immunoglobulin A nephropathy was in third place, immediately after the minimal change nephritis (35%) and mesangioproliferative glomerulonephritis (17%).

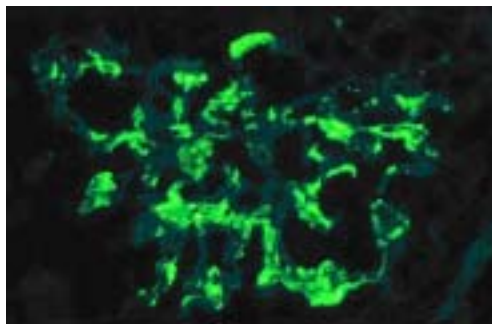
Fig 2. shows distribution of new patients with immunoglobulin A nephropathy for the period 1996 - 2008. There is an increasing trend of newly diagnosed patients with IgA nephropathy over last years.

The most common renal biopsy finding was focal mesangioproliferation in 80% of the causes, while diffuse mesangioproliferation was found in 20%. Immunohistological finding showed presence of fine granular deposits of immunoglobulin A (IgA), complement (C3) and fibrinogen in intracapillary space among the mesangial cells and the basement membrane. In only one patient there was obstruction in tubules with numerous erythrocytes and a finding of tubular degenerative changes in absence of significant pathological changes



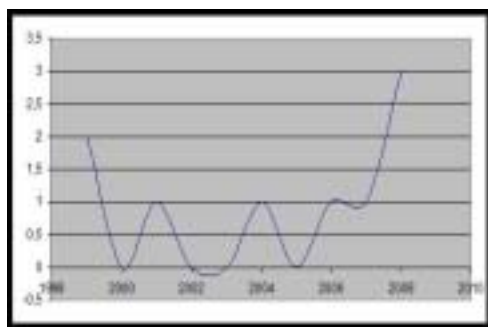
**Fig. 1.** Distribution of the histopathological finding in primary glomerulonephritis

- \* MCD - minimal change disease
- \* MezPGN - mesangioproliferative glomerulonephritis
- \* IgAN - Immunoglobulin A nephropathy
- \* FSGS - focal and segmental glomerulosclerosis
- \* MGN - membranous glomerulosclerosis
- \* MPGN - membranoproliferative glomerulonephritis

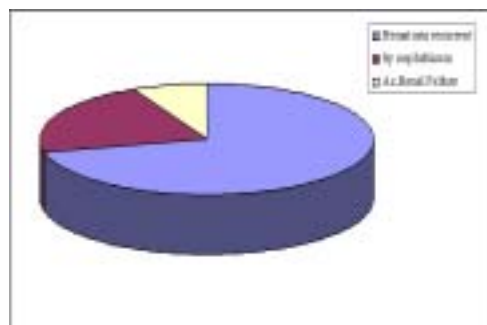


**Fig. 4.** Immunofluorescence, enlarged 1:400. Presentation of fine granular deposit of immunoglobulin IgA and complement – C3 in mesangium.

The most common clinical presentation of immunoglobulin A nephropathy was recurrent hematuria registered in 80% of patients, while 20% of registered patients were nephritic syndrome. Acute renal failure was registered only in one patient. These results are shown in Fig 5.

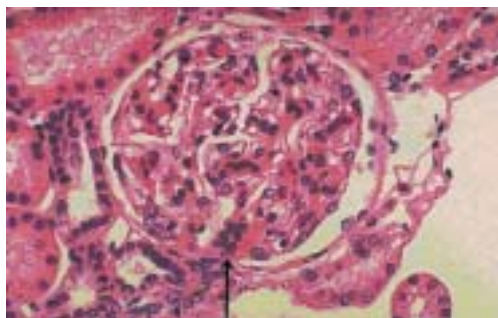


**Fig. 2.** Distribution of new patients with immunoglobulin A nephropathy for the period 1996 - 2008



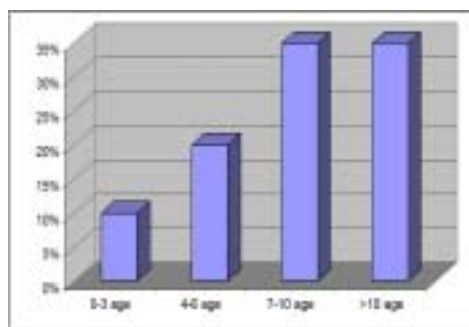
**Fig 5.** Distribution of patients with immunoglobulin A nephropathy according to clinical presentation

in glomeruli (*minimal change glomerulonephritis*). This patient showed clinical signs of acute kidney failure.

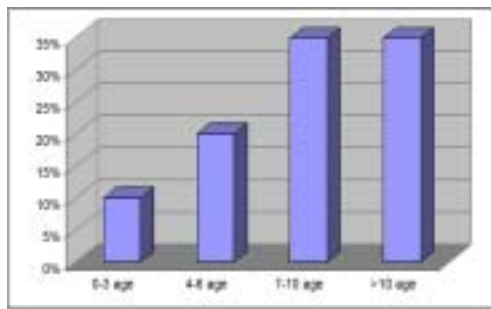


**Fig. 3.** Light microscopy, dyeing with eosinophil, enlarged 1:1000. Presentation of increased cellularity – hypercellularity in mesangium

The mean age of patients was  $8.55 \pm 3.53$  with predominance of male gender (in 56% of cases) while female gender (in 44%).



**Fig 6.** Distribution of patients with immunoglobulin A nephropathy according to age



**Fig 7.** Distribution of patients with immunoglobulin A nephropathy according to sex

Fig. 6. shows distribution of patients with immunoglobulin A nephropathy according to age. 35% of patients were aged between 7 and 10 years and older, 20% of the patients were aged between 4 and 6 years and 10% of the patients were aged up to 3 years.

### Discussion

During the period of 14 years, this study analyzed 82 children suffering from glomerular diseases and treated at the Pediatric Clinic- the only pediatric nephrology centre in the country. Therefore, the results of this study reflect the situation in the whole country.

Immunoglobulin A nephropathy was registered in 14% of patients with primary glomerular disease, being the third on the list of incidence within the group of primary glomerulonephritis, immediately after the minimal change nephritis (35%) and mesangioproliferative glomerulonephritis (17%) [14].

Similar findings were published of Covic A, Schiller A, Volovat C and Gluhouschi G in 2006. They placed immunoglobulin A nephropathy in the third place in the group of primary glomerulonephritis, immediately after membranous glomerulosclerosis (40%) and mesangioproliferative glomerulonephritis (29%). On the other hand, Schena FP in 1997 and Brazina M, Glavina-Durdov M and Scukanec-Spoljar N in 2007 published somewhat different findings in their studies where immunoglobulin A nephropathy, with incidence of 20% was the most common pathohistological finding in all glomerulonephritis types (primary and secondary). Rychlik I, Jancova E and Tesar V in 2004, also reported predominance of immunoglobulin A nephropathy (34%) in primary glomerulonephritis.

These diverse reports most probably result from differences in selecting patients when setting indication for renal biopsy. In our centre, we indicated renal biopsy in children clinically presented with syndrome of recurrent hematuria, only if there was a simultaneous presence of proteinuria with over 1g. In other centers, especially in developed tertiary nephrology units, the indication for renal biopsy is less strict, comprising all children with syndrome of recurrent hematuria [1, 3, 7, 9, 10].

As a result of broadening the indications for renal biopsy in patients with recurrent hematuria in our center, we found an increasing trend of newly diagnosed patients with IgA nephropathy over the last few years.

Regarding pathohistological findings, 80% of patients had focal mesangioproliferative glomerulonephritis. We observed diffuse mesangioproliferative glomerulonephritis in 20% of patients. In only one patient we found minimal changes and finding of tubular degenerative changes with obstructed tubule and numerous erythrocytes. This patient clinically presented with acute kidney insufficiency. In this particular case we presume that the kidney insufficiency occurred secondary, because of obstruction of the tubule with erythrocyte (Er) cylinders / or immediate (direct) effect of the erythrocytes (Er) over the tubular epithelium, as a result of emphatic macroscopic hematuria. Such findings are also described in medical literature.

In our study, 80% of patients with immunoglobulin A nephropathy had clinical signs of recurrent hematuria. This finding is also in agreement with findings in another studies [1, 3, 7].

### Conclusion

Data presented in this study reflect the specifics of immunoglobulin A nephropathy in children in our country. The identified differences with the other published studies can be explained by the differences in the selection of patients for renal biopsy.

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## PULPAL RESPONSES AFTER CARIES TREATMENT IN HUMAN TEETH: AN IMMUNOHISTOCHEMICAL STUDY

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### Abstract

The class II major histocompatibility complex (MHC) molecule expressing cells, termed dendritic cells and lymphocytes in human dental pulp are highly sensitive to exogenous antigenic stimuli. Their drastic changes in number and localization are induced by dental caries. This study investigated the responses of the immune system in 3 different clinical conditions: shallow and deep cavities and treated caries. Cells were identified immunohistochemically by using the following monoclonal antibodies: HLA-DR, CD45RO and CD20. Initial pulpal response was characterized by a localized accumulation of HLA-DR antibody-positive cells in the pulp tissue beneath the dentinal tubules communicating with the caries lesion. In the pulp of progressed caries, a large number of HLA-DR-positive cells was observed with a marked increase of other kinds of immunocompetent cells. This might indicate the occurrence of antigen presentation locally in the pulp tissue, which is very important for the immune response. In treated carious teeth, clusters consisting of HLA-DR-positive cells and CD45-positive T lymphocytes were recognized locally in the pulp tissue, regardless of cavity depth. CD20-positive B cells were seen only under the deeper cavities.

The results of this study demonstrated that dental pulps respond to cavity preparation and restoration, and that antigen presentation and cellular or humoral immunoresponses persist for many months, even after caries treatment.

**Key words:** human dental pulp, MHC class II molecule-expressing cells, lymphocytes, dental caries, caries treatment, adhesive system, immunohistochemistry

### Introduction

Caries removal, cavity preparation, and restoration with adhesive systems are generally conducted in dental practices. There has been no shortage of papers published on the subject of histological evaluations of pulpal responses to cavity preparation in animals, but very little attempts to evaluate those in human teeth.

Dental pulp is equipped with major histocompatibility complex (MHC) class II molecule-expressing cells for initiating immune responses to exogenous antigenic stimuli. In intact teeth, they are distributed mainly in and around the layer of odontoblasts and are called pulpal dendritic cells. Drastic changes in their localization are induced by human dental caries [1, 2, 3, 4, 5], and after cavity preparation in rats [6, 7, 8, 9]. Analysis of these data suggests that class II molecule-expressing cells are highly sensitive to antigenic stimuli penetrating dentinal tubules [10, 11].

Caries attack also induces changes in the distribution of lymphocytes; they become concentrated beneath the carious lesions [12, 13]. Following the exogenous invasion of microorganisms, host defence reactions, such as inflammatory and immunological reactions, take place in the pulp in order to eliminate the foreign pathogens and to maintain the local homeostasis in the pulp. Interactions between lymphocytes and MHC class II molecule-expressing cells have been shown in pulpal inflammation [14].

The focus of this paper is the influence of an operative procedure upon the distribution of MHC class II molecule-expressing cells and lymphocytes. We have investigated pulpal responses in untreated carious teeth compared with carious lesions treated with an adhesive system. We postulated that pulpal responses for cavities with caries and that with treated carious teeth would no longer have their responses after 6 months.

### Material and method

We have examined 30 human teeth from patients at the age of 9 to 14 years. Teeth were extracted from various therapeutic reasons (mostly from orthodontic reason), and immediately cut longitudinally; pulp tissue was extirpated and fixed in formalin for 24 hours at 4°C. The specimens were embedded in paraffin, according to standardized laboratory procedure. Sections were cut at 5 µm thickness and stained by the streptavidin-biotin complex immunoperoxidase method. Cells were identified immunohistochemically by using the following monoclonal antibodies: HLA-DR (for dendritic cells), CD45RO (for memory T - lymphocytes) and CD20 (for B -lymphocytes).

To verify our hypothesis, we analyzed pulpal responses in 3 different clinical conditions: shallow (n=10, pulp with caries in dentin, about 2-3 mm from the pulp chamber), deep cavities (n=10, pulp with caries deep into the dentin, 0.5-1.5 mm from the pulp chamber) and treated caries (n=10). Treatment of caries lesions was carried out on occlusal surfaces. The distance between cavity floors and pulpal walls varied from 0.5 to 3 mm. The Uni Fil Bond

dental adhesive system and GC Gradia composite resin were applied to the prepared teeth.

The depth of the carious lesion was determined by the pigmentation of hard tissues.

The main numbers of dendritic cells, T-cells and B-cells in each group were statistically analysed with ANOVA.

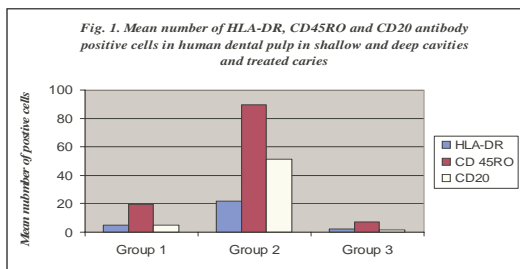
**Results**

The number of antigen-presenting and immunocompetent cells in each group is shown in Table 1 and Fig.1.

**Table 1.** Number of HLA-DR, CD45RO and CD20 antibody-positive cells in dental pulp in shallow, deep cavities and treated caries

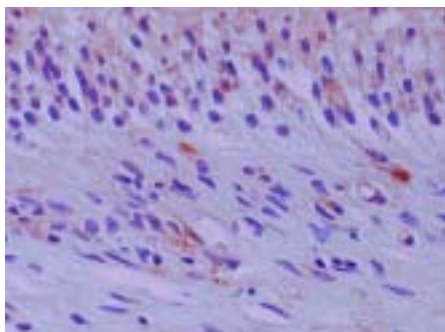
	Group 1	Group 2	Group 3
N	10	10	10
HLA-DR	5.0	22.1	2.2
CD45RO	19.5	89.6	7.1
CD20	4.7	51.2	1.5

Values are means ± SEM; N, number of samples



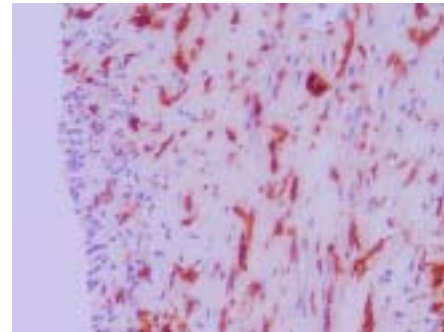
**Fig. 1.** Mean number of HLA-DR, CD45RO and CD 20 antibody positive cells in human dental pulp in shallow and deep cavities and treated caries

In shallow dentinal lesions a few HLA-DR cells were present and they were distributed mainly around an odontoblast layer and along the dentin-pulp border (Fig. 2).



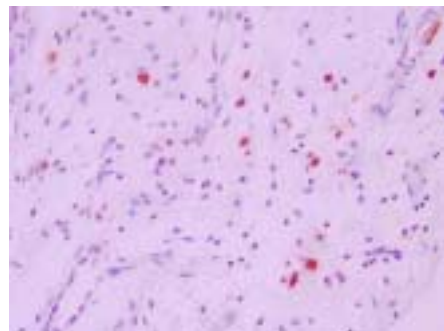
**Fig. 2.** Immunohistochemical localization of HLA-DR positive cells in the pulp with shallow cavities

As the caries lesion advanced, cells expanded toward the center of the pulp. Under deeper cavities HLA-DR-positive cells were dispersed among affected odontoblasts and they have displaced odontoblasts below the cavities. Cells were markedly increased, but not significantly (Fig.3).

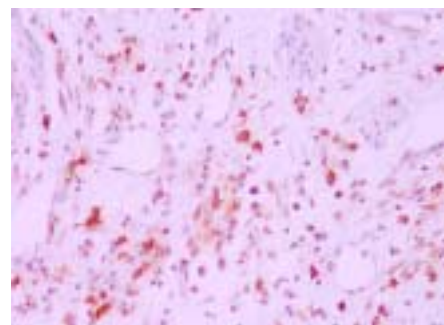


**Fig. 3.** Immunohistochemical localization of HLA-DR positive cells in the pulp with deep cavities

An increase of CD45RO-positive cells T-lymphocytes was observed in majority of specimens in teeth with moderate to deep caries. These cells were concentrated below the para-odontoblastic region, forming an aggregation. The number of T-cells was markedly increased in deep cavities and significant differences were evident between group 1 and 2 (p<0.01), (Fig. 4, Fig. 5).

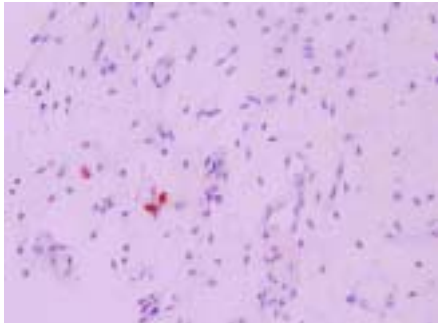


**Fig. 4.** Immunohistochemical localization of CD45RO positive cells in the pulp with shallow cavities

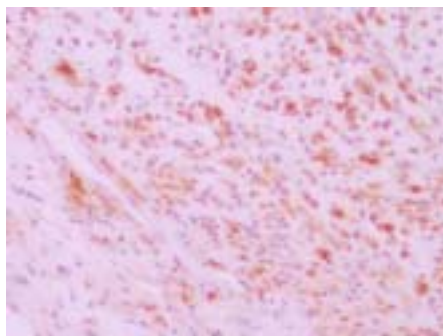


**Fig. 5.** Immunohistochemical localization of CD45RO positive cells in the pulp with deep cavities

The number of CD20-positive B-lymphocytes was much smaller than that of T-lymphocytes in most specimens. A considerable number of CD20-positive cells was detected among lymphocytes forming clusters in deeper cavities, with significant differences between group 1 and 2 ( $p<0.01$ ), (Fig. 6, Fig. 7).



**Fig. 6.** Immunohistochemical localization of CD20 positive cells in the pulp with shallow cavities

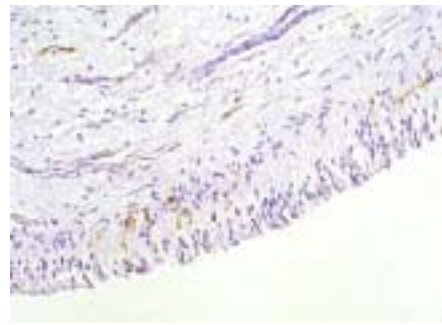


**Fig. 7.** Immunohistochemical localization of CD20 positive cells in the pulp with deep cavities

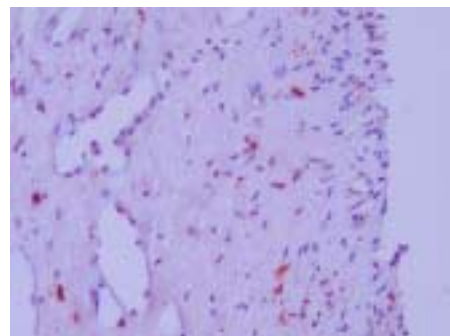
Treatment of caries lesions showed that beneath 9 of the 10 cavities samples, aggregations of HLA-DR-positive cells were recognized locally (Fig. 8), and always followed the accumulation of CD45RO-positive cells T-lymphocytes (Fig. 9). CD20-positive B-cells were seen only under deeper cavities (Fig.10). There were significant differences in the number of T-cells between group 3 and 1 ( $p<0.05$ ), and between group 3 and 2 ( $p<0.01$ ), and in the number of B cells between group 3 and 2 ( $p<0.01$ ).

### Discussion

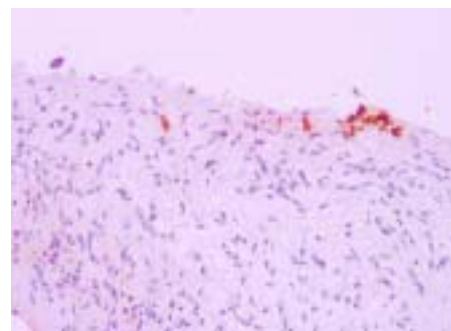
This study provides evidence that cavity depth influences the distribution of HLA-DR-positive dendritic cells and lymphocytes. Reduction in the thickness of residual dentin had an impact on the distribution of the cells. These changes are in agreement with findings from studies on the distribution of dendritic cells and lymphocytes in human teeth. Early pulpal response to bacterial diffusion of bacterial products through dentinal tubules elicits the influx of dendritic cells, T-lymphocytes



**Fig. 8.** Immunohistochemical localization of HLA-DR positive cells in the pulp of treated caries



**Fig. 9.** Immunohistochemical localization of CD45RO positive cells in the pulp of treated caries



**Fig. 10.** Immunohistochemical localization of CD20 positive cells in the pulp of treated caries

and rare B-lymphocytes. As the infection is coming closer to the pulp, the response assumes a typical mixed character, consisting of T-cells and B-cells.

On the other hand, most components of adhesive systems and composite resins are able to diffuse through the dentinal tubules and reach the pulp tissue producing noxious effects on odontoblasts [15,16] and influence the function of pulpal immunocompetent cells [17, 18, 19, 20]. In meanwhile, no aggregations of dendritic cells were recognized under prepared cavities, and no aggregations of CD45-positive lymphocytes were detected in any sample of the cavity prepared teeth. Thus, the materials

used here provided excellent sealing characteristics, and they effectively prevented the ingress of noxious substances to the dentin-pulp complex.

Decrease in the number of dendritic cells and lymphocytes were recognized under prepared cavities, with a statistical significance between caries-affected teeth and after treatment. Against our expectation, even after caries treatment, small aggregations of HLA-DR-positive dendritic cells, accompanied by CD45-positive cells T-lymphocytes, were left behind in 9 out of 10 samples. The inflamed lesions would be the result of activities by bacteria, which had existed locally deep in the dentinal tubules and survived even after the removal of caries. Presence of dendritic cells and lymphocytes after removal of the carious lesion shows that local antigen presentation and cellular and/or humoral immunoresponses persist even after careful treatment of caries.

### Conclusion

Our study demonstrated that dental pulps have different response to cavity preparation and restoration, and that antigen presentation and immunoresponses persist for many months, even after caries treatment. Further investigations are needed to ascertain how to control the bacterial activities that might have remained deep in the dentinal tubules.

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**VASCULAR PATHWAYS OF HUMAN DECIDUOUS DENTAL PULP**Georgiev Zlatko<sup>1</sup>, Petrushevska G<sup>2</sup>, Kovachevska I<sup>3</sup>, Sotirovska - Ivkowska A<sup>1</sup>, Dimova C<sup>3</sup><sup>1</sup>Faculty of Dentistry, University "Ss. Cyril and Methodius" – Skopje, R. Macedonia<sup>2</sup>Faculty of Medicine, University "Ss. Cyril and Methodius" – Skopje, R. Macedonia<sup>3</sup>Faculty of Medicine, University "Goce Delèev" – Štip, R. Macedonia**Abstract**

The transition from deciduous to permanent teeth is a unique and dynamic process in which the development and eruption of permanent teeth is coordinated with the resorption of deciduous teeth.

The vascularisation in pulp tissue of human deciduous teeth has not been as well studied as that within human permanent teeth. Such information is useful to those who diagnose and treat children's teeth.

In order to examine the reparatory pulp ability of deciduous teeth, it was our aim to determine vascular structures in contrast to the histological appearance of noncarious human primary teeth with root completion and physiological resorption, because the dental pulp is an active component in the life of the tooth.

Histological examinations of blood vessels in deciduous dental pulp were performed on a light microscope and on a Transmission Electron Microscope.

When the process of physiological root resorption in deciduous teeth is initiated, blood vessels in pulp tissue show some disturbances. Another area in which information is lacking involves changes in the blood vessels of the deciduous teeth during the period of root resorption. Endothelial cells of arterioles, venules and capillaries are cuboidal, with large pinocytotic vesicles, progressive reduction of luminal capacity, and reduction of the wall of the pulp blood vessels.

**Key words:** deciduous teeth, dental pulp, root resorption, blood vessels, ultrastructure

**Introduction**

The transition from deciduous to permanent teeth is a unique and dynamic process in which the development and eruption of permanent teeth is coordinated with the resorption of deciduous teeth. Primary teeth contrary to permanent ones, have a relatively short lifetime and functional duration, and are subordinated to an early physiological resorption of the roots.

In the past human primary teeth has received little attention compared to similar research on permanent teeth, because they are smaller and short lived, and due to the belief that the pulps are similar. Now a days, deciduous dental pulp is the origin of stem cells and is progenitor for tissues with therapeutic expectations in much disease in future<sup>1,4</sup>.

Dental pulp is a unique tissue, responsible for the tooth vitality, and when this tissue is damaged by disease; it reacts in an attempt to defend by production of protective dentine. From its non-specific and specific defensive mechanisms, depends the survival of the tissue in pathological conditions. This tissue passes three phases: phase of root formation, phase of functional duration and phase of physiological resorption of the root.

The vascularisation in pulp tissue of human deciduous teeth has not been as well studied as that within human permanent teeth. Such information is useful to those who diagnose and treat children's teeth.

Another area in which information is lacking involves changes in the blood vessels of the deciduous teeth during the period of root resorption.

**Aim:** In order to examine the reparatory pulp ability of deciduous teeth, it was our aim to determine vascular structures in contrast to the histological appearance of noncarious human primary teeth with root completion and physiological resorption, because the dental pulp is an active component in the life of the tooth.

**Methods**

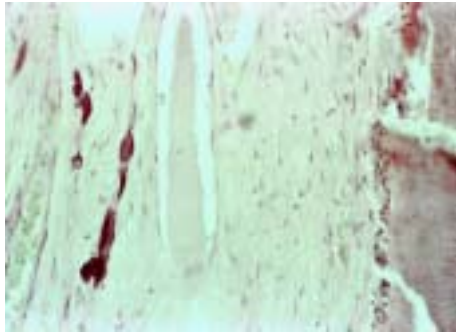
The pulps used for this research had originated from intact teeth of healthy children, aged 5 to 9 years (5 deciduous teeth without signs of physiological resorption, and 5 deciduous teeth with progressive physiological resorption).

Immediately after the extraction (performed due to orthodontic reasons, under local anesthesia), each tooth was cut perpendicularly to its long axis with a rotating carborundum disc under a water jet. The separated halves were dissected with plastic instrument, and the tooth pulp was excavated completely.

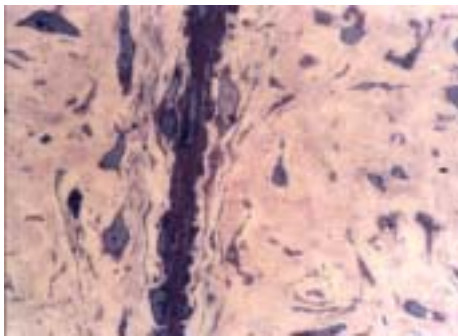
The histological examinations were performed on a light microscope Orthoplan Leitz-Wetzlar with haematoxyllin-eosin stain (HE), and on a Transmission Electron Microscope (TEM) Tesla BS 500 (60KV).

**Results**

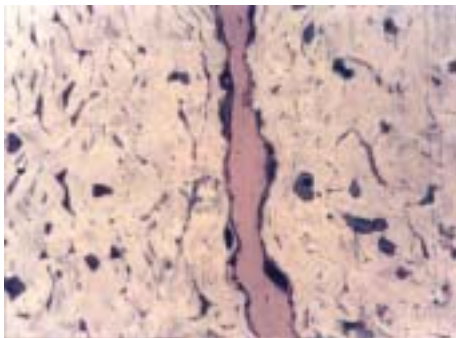
The vascular structures of deciduous dental pulp were studied in human deciduous teeth using a light microscope and a TEM (Fig. 1, 2, 3). Their fine structure corresponds with blood vessels in other tissues, and the architectural morphology is similar to that of permanent pulps, according to Rapp<sup>5</sup>.



**Fig. 1.** Deciduous teeth without progressive physiological resorption: dentin and pulp tissue with arterioles, venules and capillaries with endothelial cells and pericytes, with red blood cells, and calcifications (HE, 400x)



**Fig. 2.** Deciduous teeth without progressive physiological resorption: arterioles in pulp tissue with endothelial cells and pericytes (HE, 1000x)

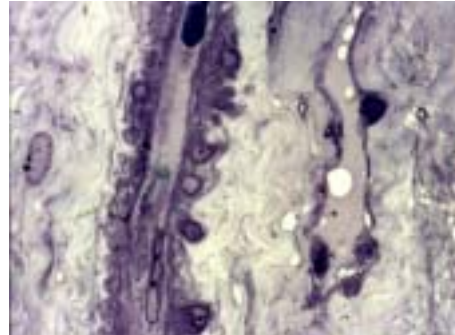


**Fig. 3.** Deciduous teeth without progressive physiological resorption: venula in pulp tissue (HE, 1000x)

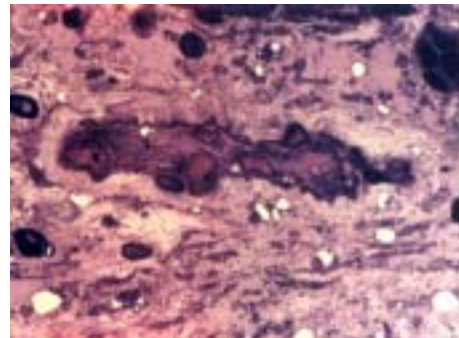
Endothelial cells are flat cells with an ovoid nucleus in the middle of the cell, which often give the luminal side of the cell a bulging contour, mostly prominent in capillaries.

Vascularisation of deciduous dental pulp in the second group of teeth, when the physiological resorption had been started, showed some disturbances. Endothelial cells were cuboidal, with large pinocytotic vesicles, like an atheromathosis with progressive reduction of luminal

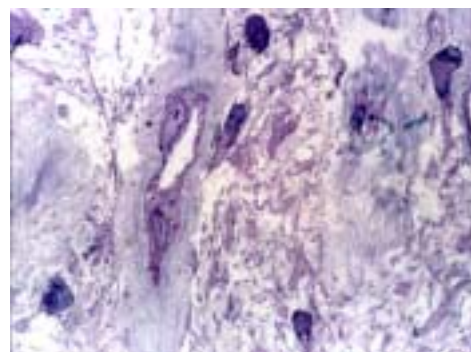
capacity, and reduction of the wall of the pulp blood vessels (Fig. 4, 5, 6, 7). In addition, development of edematous changes of the basic substance with perivascular hyalinization was observed (Fig. 6). It spoke in favor of progressive disturbance of nutrition, resulting in reduction of cells and collagen net.



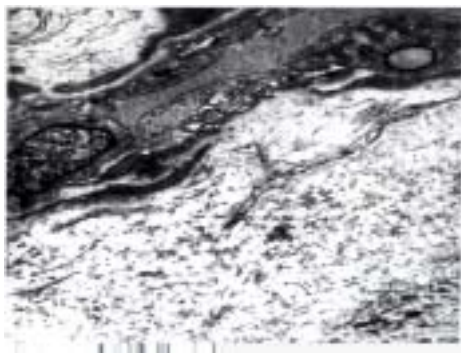
**Fig. 4.** Deciduous teeth with progressive physiological resorption: cuboidal endothelial cells with large pinocytotic vesicles (lipid material), perivascular hyalinization (HE, 1000x)



**Fig. 5.** Deciduous teeth with progressive physiological resorption: cuboidal endothelial cells (HE, 1000x)



**Fig. 6.** Deciduous teeth with progressive physiological resorption: cuboidal endothelial cells, perivascular hyalinization (HE, 1000x)



**Fig. 7.** Deciduous teeth with progressive physiological resorption: cuboidal endothelial cells with large pinocytotic vesicles (lipid material) (TEM, 17000x)

### Discussion

Physiological resorption of the root in deciduous teeth is a complex process, and unique in human organism: there is no other organ that is mineralized that after finishing its function it demineralizes and sheds. However, the increasing need of child's nutrition for growth depends on this.

When the process of physiological root resorption in deciduous teeth is initiated, the dental pulp enters in phase of involution, tending to decrease blood supply and innervations, accordingly the results of Yu & Abbott<sup>2</sup>. Blood vessels of the pulp show signs of stasis and perivascular calcifications. Under the light microscopy, regressive changes are evident in the blood vessels, as a result of edematous changes in the basic substance and perivascular hyalinization (Fig. 4, 5, 6, 7).

In the cytoplasm of the endothelial cells under TEM, there are too many pinocytotic vacuoles incorporated inside the cells, full of lipid material, because of their strong micropinocytotic activity - ingestion of saturated fats.

Because of the aforementioned changes in the endothelial cells, pulp blood vessels supply is compromised. These unavoidable circumstances lead to a decrease in all pulp functions.

With age, the pulp tissue reduces in size<sup>3</sup>. In our opinion, physiological resorption of the root produces consequences to deciduous dental pulp similar to aging - with age, nerve and blood supply to the pulp tend to decrease, and the pulp becomes more fibrous and less cellular (Yu & Abbott<sup>2</sup>).

### Conclusions

Physiological resorption of the root produces consequences to deciduous dental pulp similar to aging - with age, nerve and blood supply to the pulp tends to decrease.

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**PREVENTION OF WHITE SPOT LESIONS DURING ORTHODONTIC TREATMENT**Zabokova - Bilbilova Efka<sup>1</sup>, Sotirovska - Ivkovska A<sup>1</sup>, Kanurkova L<sup>2</sup>, Kovachevska I<sup>3</sup><sup>1</sup>Department of Pedodontic Dentistry, University Dental Clinical Centre "St. Pantelejmon", Faculty of Dentistry, Skopje, R. Macedonia<sup>2</sup>Department of Orthodontics, University Dental Clinical Centre "St. Pantelejmon", Faculty of Dentistry, Skopje, R. Macedonia<sup>3</sup>Faculty of Medicine, University "Goce Delcev", Shtip, R. Macedonia**Abstract**

The purpose of this *in vitro* study was to evaluate the effect of a paste containing CPP-ACP, GC Tooth Mousse in preventing white spot lesions (WSL) during orthodontic treatment with fixed appliances.

A total of 30 extracted human lower and upper premolars with no restorations, cracks, caries, hypoplastic areas or pliers impressions were collected for this study and used within one and six months. Enamel of buccal surface of the teeth was polished with pumice and water, rinsed and air dried. After etching the enamel surface with a 37% phosphoric acid solution for 15 seconds and rinsing for 10 seconds, teeth were dried. In this study *Ricketts Universal Ultratrim (Dentaurum, Germany)* stainless steel brackets for premolars were used. Each bracket was positioned over the mid point of the clinical crown on buccal surfaces of the prepared premolar. *Con Tec LC (Dentaurum, Germany)* was used as adhesives for bonding brackets in this study. The teeth were divided randomly into three groups, each one consisting of 10 teeth. The first group - control group included samples without any prevention; the second group included samples which enamel surface was treated with dental cream (GC Tooth Mousse) each day for 5 minutes in a period of 1 month and the third group included samples which enamel surface was treated with dental cream (GC Tooth Mousse) each day for 5 minutes in a period of 3 months.

Teeth were stored in artificial saliva for one or 3 months. After that, the samples were prepared for SEM analysis (JEOL JSM 5300), using sputter technique in a vacuum evaporator. The appropriate area of enamel surface was analyzed in order to determine micro morphology changes in the structure of the enamel, on the place of previous brackets fixation.

The application of GC Tooth Mousse dental cream after bonding appears to be beneficial in reducing the incidence of white spot lesions.

**Key words:** white spot lesions, brackets, prevention

**Introduction**

Enamel demineralization or white spot lesions around orthodontic fixed appliances is a common side effect of orthodontic treatment [1]. These orthodontic appliances tend to cause a shift of the lesion from posterior to anterior teeth and from interproximal to vestibular and lingual sites. Considering the mechanical difficulties of removing plaque with orthodontic brackets in place, proper oral hygiene is crucial. Unfortunately, patient compliance is a commodity that is unpredictable and decreasing. Consequently, the incidence of enamel decalcification and caries during orthodontic care is increasing [2]. For example, Gorelick et al. [3] found white spot lesions for nearly 50% of patients that underwent orthodontic treatment. In addition, Øgaard et al. [4] reported that these lesions can develop within 4 weeks or the average time between orthodontic visits. For a specialty whose objectives are to improve facial and dental esthetics, the presence of unsightly white spot lesions may detract from the beneficial effects of orthodontic treatment.

Many products have been developed to prevent demineralization of enamel surface. One such product is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP). CPP-ACP can be found in multiple products [5, 6].

Recaldent™ is a unique complex containing amorphous calcium phosphate (ACP) and casein phosphopeptide (CPP), obtained from milk casein. The preparation is recommended in hard tissue remineralization. The manufacturer compares the material to "liquid enamel". CPP-ACP complex makes a strong binding with dental biofilm and form calcium and phosphate reservoir. They are then incorporated into the surface of enamel and dentine [7]. The CPP-ACP complex contained in Recaldent™ is hence an ideal system for transporting free calcium and phosphate ions - and GC Tooth Mousse is the world's first product for professional use in the dental practice that contains this novel active ingredient [8]. The proposed anticariogenic mechanism of CPP-ACP involves the incorporation of the nanocomplexes into dental plaque and onto the tooth

surface, thereby acting as a calcium and phosphate reservoir. Studies have shown that CPP-ACP incorporated into dental plaque can significantly increase the levels of plaque calcium and phosphate ions. This mechanism is ideal for the prevention of enamel demineralization as there appears to be an inverse association between plaque calcium and phosphate levels and measured caries experience [9].

Some of authors [10, 11] who had evaluated the incidence of carious process influenced by input of CPP-ACP compound, demonstrated that this compound reduced the incidence of carious lesions when entered in the form of chewing gums. The chewing gums are ideal for transport of CPP-ACP compound in the mouth, because in such way it remains in the mouth long enough and shows its beneficial effect. Its presence in the plaque is confirmed after three hours later the chewing gum [12, 13].

The purpose of this *in vitro* study was to evaluate the effect of a paste containing CPP-ACP, GC Tooth Mousse in preventing white spot lesions (WSL) during orthodontic treatment with fixed appliances.

#### Material and methods

A total of 30 extracted human lower and upper premolars with no restorations, cracks, caries, hypoplastic areas or pliers impressions were collected for this study and used within one and six months. All extractions were indicated for orthodontic purposes in patients of 11-18 years of age. After being extracted, teeth were carefully inspected and only intact teeth were cleaned and stored in artificial saliva. The artificial saliva contained KCl (1.04 g/L),  $\text{NaH}_2\text{PO}_4$  (0.68 g/L),  $\text{NaHCO}_3$  (0.42 g/L),  $\text{CaCl}_2$  (0.03 g/L) and  $\text{MgCl}_2$  (0.01 g/L).

Enamel of buccal surface of the teeth was polished with pumice and water, rinsed and air dried. After etching the enamel surface with a 37% phosphoric acid solution for 15 seconds and rinsing for 10 seconds, teeth were dried. In this study *Ricketts Universal Ultratrim* (Dentaurum, Germany) stainless steel brackets for premolars were used. Each bracket was positioned over the mid point of the clinical crown on buccal surfaces of the prepared premolar. Con Tec LC (Dentaurum, Germany) was used as adhesives for bonding brackets in this study. The teeth were divided randomly into three groups, each one consisting of 10 samples:

- first group control group: without any prevention;
- second group: samples which enamel surface was treated with dental cream (GC Tooth Mousse) each day for 5 minutes in a period of 1 month;

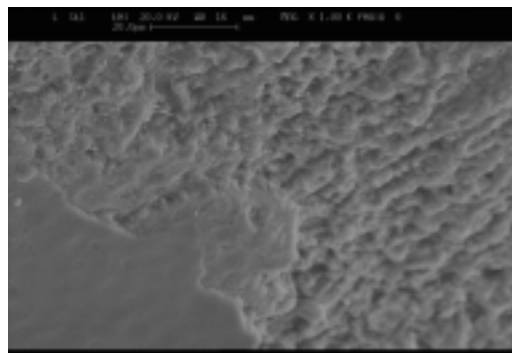
- third group: samples which enamel surface was treated with dental cream (GC Tooth Mousse) each day for 5 minutes in a period of 3 months.

Teeth were stored in artificial saliva for one or 3 months. After that, the samples were prepared for SEM analysis (JEOL JSM 5300), using sputter technique in a vacuum evaporator. The appropriate area of enamel surface was analyzed in order to determine micro morphology changes in the structure of the enamel, on the place of previous brackets fixation.

#### Results

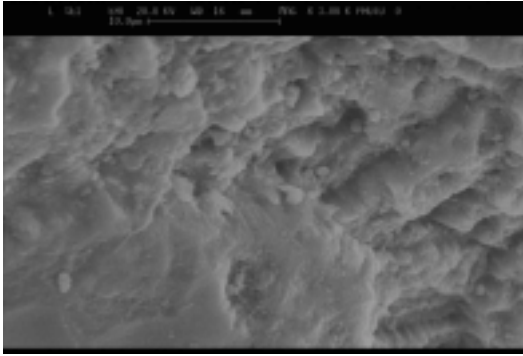
The analysis of enamel surface was performed using a scanning electronic microscopy after completion of a particular preventive treatment and bracket debonding. Comparison was made between the buccal surface of the tooth on which the brackets were fixed, and those specimens with no to prevention. In this group, micromorphology characteristics showed decomposed enamel surface with lost integrity (Fig. 1).

In the second group, comprising tooth samples that were prevented with dental cream (GC Tooth Mousse) each day for 5 minutes in a period of 1 month, inhibited demineralization of enamel in the center and on the outskirts of enamel prisms was noted (Fig. 2).



**Fig. 1.** SEM images of tooth samples from the control group: lost enamel integrity; enamel decomposition (original magnification, x 1000)

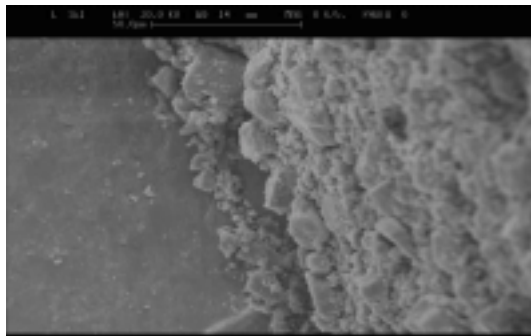
In the third group comprising tooth samples that were prevented with GC Tooth Mousse each day for 5 minutes in a period of 3 months, presence of amorphous deposits on the enamel surface was observed (Fig. 3).



**Fig. 2.** SEM images of tooth samples from the second group: inhibited demineralization of enamel in the center and on the outskirts of enamel prisms (original magnification, x 1000)

**Discussion**

The most common negative effect of orthodontic treatment with fixed appliances is the development of incipient carious lesions around brackets and bands, particularly in cases with poor oral hygiene. Caries lesions



**Fig. 3.** SEM images of tooth samples treated with GC Tooth Mousse; presence of amorphous deposits on the enamel surface

typically form around the brackets interface, usually near the gingival margin (Fig. 4 a, b). Since orthodontic appliances make plaque removal more difficult, patients are more susceptible to carious lesions. Mineral loss (demineralization) or gain (remineralization) by enamel is a dynamic physicochemical process occurring when oral bacteria form a biofilm on the enamel surface and this biofilm is exposed to fermentable dietary carbohydrates, sucrose being the most cariogenic of them [14]. Thus, every time sugar penetrates into a cariogenic biofilm and is converted to acids by bacterial metabolism, the biofilm fluid becomes undersaturated with respect to the enamel mineral, and demineralization occurs [15].

Administration of topical agents containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), maintenance of oral hygiene, and dietary control have been suggested as mechanisms to control the formation of enamel lesions during fixed orthodontic appliance treatment [16]. In contemporary orthodontic literature, fluoride and CPP-ACP applications are accepted approaches for remineralizing of the previously demineralized enamel. CPP-ACP is known to be a source of calcium and phosphate close to the sites of possible demineralization, and this is likely to inhibit demineralization, enhance remineralization or possibly both [17]. In our study the tooth treated with CPP-ACP was remineralized by calcium and phosphorus, and the resulting calcium-phosphate layer was found to be amorphous. Previous studies have demonstrated that CPP-ACP enhances the remineralization of artificially formed dentinal lesions. The suggested mechanism for this is the stabilization of calcium phosphates on the tooth surface by the casein phosphopeptides, which leads to high concentration gradients of calcium and phosphate ions, thus promoting the remineralization of hard tissues [18].



**a)**



**b)**

**Fig. 4 (a, b).** Incipient caries lesions (white spot) develop around brackets and bands due to poor oral hygiene

The role of CPP-ACP has been described as localization of ACP on the tooth surface, which buffers the free calcium and phosphate ions. This helps to maintain a state of supersaturation with respect to the enamel by suppressing demineralization and enhancing remineralization [19]. Enamel lesions, which were remineralized with topical exposure to CPP-ACP, have been shown to be more resistant to subsequent acid challenge compared with normal remineralized enamel as CPP-ACP is able to promote the remineralization of enamel subsurface lesions with hydroxyapatite. In addition, the relatively low carbonate environment of the CPP-ACP treated subsurface lesion may also exhibit both improved crystallinity and lower microstrain than might be found in normal tooth enamel [20].

### Conclusions

Within the limitations of an *in vitro* study, the results lead to conclusion that dental cream containing CPP-ACP enhances the remineralization potential of the enamel in teeth.

The application of GC Tooth Mousse dental cream after bonding appears to be beneficial in reducing the incidence of white spot lesions.

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## DETERMINING CORONARY MICROLEAKAGE OF ENDODONTICALLY TREATED TEETH RESTORED WITH TEMPORARY AND PERMANENT RESTORATIONS

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### Abstract

The aim of the study was to determine the coronary microleakage in in-vitro conditions of the endodontically treated teeth, restored with temporary and permanent fillings.

**Material:** The experimental part of the study included a total of 60 intact single root human teeth, which were endodontically treated in maximum sterile conditions. The intracanal obturation was made with the sealer AH plus and Termafill gutapercha technique. The teeth were divided in 3 groups depending on the coronary restoration: first group - composite resin, second group - Cavition and third group - dental amalgam. Next, we sterilized the teeth with water steam at temperature of 121°C and pressure of 1.5 in autoclave in duration of 15 minutes. After isolating the surface of the roots with two layers of nail polish to the enamel-dentin border, the teeth with the crowns were submerged into bacterial suspension prepared from *Proteus mirabilis*, Gram-negative motile, rod shaped bacteria, with concentration of the bacterial cells of  $10^7 - 10^9$  in milliliter of solution. They stayed in the bacterial suspension at temperature of 37°C and permanent bacterial concentration for 5 to 30 days. After the time interval of 5 and 30 days, we prepared the teeth for the procedure of histological evaluation of the coronary microleakage and the longitudinal sections were colored by Brow-Bern.

**Results:** The largest bacterial microleakage after 5 days was determined in the second test group of the teeth restored with the temporary filling material - Cavition (80%). After the period of 30 days, the bacterial microleakage was largest in the second test group and it was 70%.

**Conclusion:** Coronary restoration as a final procedure in the endodontic therapy should be realized in a period of 5 days, so that the contamination of the endodontic space can be stopped.

**Key words:** microleakage, endodontic therapy, composite resin, dental amalgam

### Introduction

The microleakage is defined as a physical movement of fluids and microorganisms from the oral cavity to the cavities through the endodontic space to the apical and periapical spaces. This discrete migration, which the endodontists studied in the sixties of the past century [1], was pointed out as a possible reason for breaking out the integrity of the coronary as well as of the intracanal filling. This can cause contamination, dissolving of the cement, creation an empty spaces and transport of infectious material out of the tooth root into the periapical zone [2, 3]. Subsequently, the success of the endodontic therapy is compromised, the patient feels discomfort; there is a need for retreatment or surgical intervention, and finally, the treated tooth is lost.

Many studies suggest that microleakage, no matter if it is coronary or apical, has effect on the success of the endodontic treatment [4, 5, 6, 7]. The researches are mostly conducted in in-vitro conditions by evaluation the microleakage of colored solutions, radioisotopes, bacterial markers etc. [1, 4, 5]. Some suggest that the apical

microleakage has a primary role in the endodontic therapy failure but also the importance of the coronary microleakage can not be neglected or forgotten. After all, tridimensional hermetically intracanal obturation means sealing of the apical and the coronary part of the tooth and it is one of the conditions for successful and prognostic positive endodontic procedure [6, 7, 8].

According to Siquera et al. [9] the most intensive bacterial leakage begins from the 2<sup>th</sup> to the 58<sup>th</sup> day, and the authors did not find any difference in the level of the salivary coronary microleakage. Although the contamination of the canal system in the coronary parts was evident, the authors thought that in-vivo conditions should be accepted with a certain reserve.

Trope, Chow and Nissan [10], alerted that endotoxin was present in the canal chamber after 24 hours, and that quality coronary restoration provided healthy periradical status.

Chailertvanitkul et al. [11] in their study of coronary microleakage, with the tracer bacteria *Fusobacterium nucleatum* and did not detect permeability

in the group where the opening was covered with modified glass ionomer cement after a period of 60 days.

The therapeutic endodontic procedure has to be realized in one session, which is not always possible, and the coronary accessory cavities are closed with temporary sealing materials [12, 13, 14]. These same materials, in the processes of mastication and loading are breaking, they fall out and the endodontic space is exposed to the conditions of the oral cavity and the possibility of contamination and infection [4, 7, 8, 15].

The contradictory literature findings about the role of the coronary microleakage in the failure of the endodontic therapy motivated us to set the aim of this research: to evaluate bacterial micropermeability of the endodontically treated teeth restored with temporary and permanent fillings.

### Material and method

For realization of the aim of this research we used 60 single root intact human teeth, extracted for orthodontic reasons. They were cleaned with a scalpel from the tissue residues and until the moment of processing they were left in saline at temperature of 37°C in order to remain wet and not modify the tissue structure. In maximum sterile conditions we did endodontic treatment of all teeth, irrigation with 2% solution of hypochlorite and canal obturation with sealer AH plus and Thermafil gutapercha technique. According to the coronary restoration, we divided the teeth in 3 groups:

- first group - 20 single root teeth endodontically treated and coronary restored with composite resin Tetric ceram and Excite adhesive system;
- second group - 20 single root teeth endodontically treated and coronary restored with temporary sealant Caviton;
- third group - 20 single root teeth restored with dental amalgam.

The teeth were left for 48 hours into saline at temperature of 37°C, in order to organize and stabilize the filling. The next step was sterilizing of the tested specimens for 15 minutes, with water steam, in autoclave, at temperature of 121°C and pressure of 1.5 at. The coating of the specimens root surface with two layers of nail polish to the enamel-cement border followed. Then we put the teeth in bacterial suspension made out of *Proteus mirabilis*, Gram-negative motile, rod-shaped bacteria, with concentration of the bacterial cells of  $10^7 - 10^9$  in milliliter of artificial saliva. Ten of the teeth stayed into the suspension for 5 days and the rest of them for 10-30 days, at temperature of 37°C, and during that time, the bacterial concentration was closely monitored for an eventual change. Next, after removing the nail polish, the teeth

were decalcified with Osteomol<sup>R</sup> (Merck), for decalcifying of the hard teeth tissues, molded into paraffine blocks from which we made longitudinal sections with thickness of 5 µm. We colored them by *Brow-Bern*, a coloring technique for histological evaluation of the bacterial presence. Verification of the microbiological microleakage of the crown to the apex of the teeth was made on bioocular microscope Eclipsa 600.

The final results were statistically analyzed with the computer program Statistica for Windows, version 6.

### Results

Determination of bacterial coronary microleakage in a period of 5 days showed that out of total 10 teeth, bacterial migration was found in 30% of the composite group, 20% in the group with dental amalgam, and the most prominent bacterial permeability was found in the temporary restored teeth (in 80% of the specimens), (Table 1).

**Table 1.** Percentage representation of the bacterial microleakage after 5 days

leakage	without		with	
	no	%	no	%
I gr.	7	70.0	3	30.0
II gr.	2	20.0	8	80.0
III gr.	8	80.0	2	20.0

In the period of 30 days, changes in the bacterial flow from the coronary level were not observed in the groups restored with composite and dental amalgam. In the teeth closed with temporary sealing Caviton, a decrease of 10% was observed on the bacterial penetration compared with the period of 5 days (Table 2).

**Table 2.** Coronary bacterial microleakage after 30 days

leakage	without		with	
	no	%	no	%
I gr.	7	70.0	3	30.0
II gr.	3	30.0	7	70.0
III gr.	8	80.0	2	20.0

Statistical analysis of the differences in the coronary bacterial microleakage according to the type of the restoration with the ANOVA test showed a statistically significant value of  $p < 0.05$ . A significant difference was

**Table 3.** Coronary bacterial microleakage according to the type of restoration

leakage 5 days	Mann-Whitney U test		U	Z	p	sig. / n.sig.
	rank sum gr 1	rank sum gr 2				
I gr / III gr	200.0	110.00	45.00	(-) 0.38	0.7	n.sig.
II gr / III gr	75.00	135.00	20.00	(-) 2.77	0.02	sig.,,
I gr / II gr	80.00	130.00	25.00	(-) 2,19	0.03	sig. ,,

found between the temporary and permanent restorative materials on the coronary level; the difference between amalgam/Cavition was significant ( $p=0.02$ ), as well as the difference between composite/Cavition ( $p=0.03$ ). The samples of the second group showed much larger bacterial microleakage (Table 3).

The F-test analysis of variance (one-way ANOVA) used for testing the differences in the bacterial permeability after 30 days showed no statistically significant difference ( $p>0.05$ ).

The Wilcoxon Matched test was used for evaluation of the bacterial microleakage from the coronary level in the groups for different time intervals (5 and 30 days). The difference between tested variables was insignificant ( $p>0.05$ ), (Table 4).

**Table 4.** Bacterial microleakage for different time intervals

leakage termafil	Wilcoxon Matched test		
	Z	p	sig. / n.sig.
5 / 30 days			
I gr.	0.0	1.0	n.sig.
II gr.	0.4	0.68	n.sig.
III gr.	0.53	0.59	n.sig.

The following images are from the longitudinal histological sections with and without bacterial microleakage.



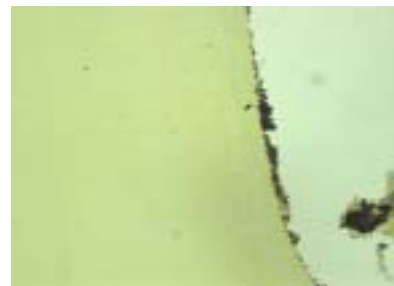
**Fig. 1.** Microleakage in group 1, after 5 days



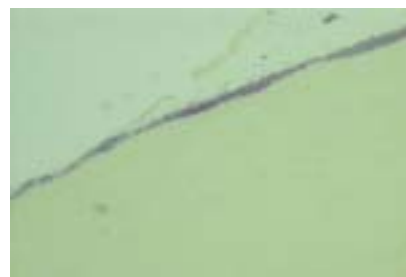
**Fig. 2a.** Bacterial penetration in group 2, after 5 days



**Fig. 2b.** Bacterial penetration in group 2, after 5 days



**Fig. 3.** Leakage in group 3, after 5 days



**Fig. 4.** Bacterial leakage after 30 days - group 1





**Fig. 5a.** Bacterial leakage after 30 days - group 2



**Fig. 5b.** Bacterial leakage after 30 days - group 2



**Fig. 6.** Bacterial leakage after 30 days - group 3

### Discussion

Microleakage, independent from the level of the crown or in the apical part, was the primary problem which endodontists had faced in their clinical practice [2, 3, 4, 5, 6, 7]. This factor, which prospectively compromises the success and the prognostic outcome of the endodontic therapy, has initiated the scientists to promote different methodologies which will, more or less, successfully determine, follow and prevent this process [1, 5, 7, 11].

Microleakage is not just a transport of fluids, but also a movement of microorganisms and their metabolic products [9, 10, 11, 16, 17]. In order to get a real picture of the coronary leakage and the possibility for penetrating into the intra-canal space we determined the flow of the bacteria *Proteus mirabilis*, classified as the most motile bacteria.

We determined different values of the coronary microleakage. The largest presence of *Proteus mirabilis* in the period of 5 days was registered in the second group where the specimens were coronary sealed with Caviton. The presented differences in the bacterial microleakage between the groups showed a statistically significant difference ( $p < 0.05$ ) as a result of the significantly higher permeability for bacteria of the temporary material for the coronary obturation in the second tested group.

Mann-Withney U test showed that the bacterial microleakage was not statistically significant between the permanent dental materials.

In the period of 30 days, there were no registered changes in the bacterial microleakage of the specimens in the first and the third group, while in the second group the values dropped for 10%, compared with the period of 5 days.

Statistical analysis of the differences in the coronary microleakage of *Proteus mirabilis* in the groups in the time interval of 30 days with the ANOVA test showed no significant difference, which was also confirmed with the Mann-Withney U test ( $p > 0.05$ ).

We evaluated the bacterial permeability from the crown to the endodontic space with the Gram-negative rod-shaped bacteria *Proteus mirabilis* with great potential for migration. The penetration of the bacteria was determined in two different periods (5 and 30 days) by a specific method of coloring and we verified the bacterial leakage in histological longitudinal sections. The permeability of Caviton for bacteria was confirmed in both tested periods in the second group. In the period of 5 days the observed microleakage was with greater intensity and resulted in higher significant difference. Similar data were also registered by Deveaux [16]. In his *in vitro* study he measured bacterial leakage of materials for temporary coronary obturation for 7 days and he suggested that the thickness of the material of 4 mm was optimal for reducing the microleakage independent of the thermocyclic procedure.

Contrary to our findings, Magura [18] on the longitudinal histological sections did not verify presence of bacteria with the coloring technique Brow-Brenn and did not find a statistically significant difference in the permeability of the temporary obturated and not sealed teeth. But, he highlighted that after 3 months the penetration of the artificial saliva statistically increased. Unlike his results, we determined coronary bacterial flow, using the same technique of coloring in the tested groups showing statistically significant values according to the material for coronary obturation and in both time intervals (period of 5 and period of 30 days).

In the period of 5 days the bacterial flow was minimal in the specimens of the first group where the occlusal cavities were obturated with composite resin. This result was in agreement with the results of Deveaux [16] and Sousa et al [19].

The dental adhesive along with the composite resin provided minimal bacterial microleakage in both tested periods. In our opinion, this is a result of its quality characteristics. Newer generations of dental adhesives like Excite, also have antimicrobial potential which allows reduction of the permeability of the bacteria. The correct layered application of the composite resin and the hybrid texture in combination with the adhesive did not allow bacterial micro-flow in 70% of the tested teeth in both tested periods. These results coincide with the findings of Chailertvanitkul [11]. For preventing the coronary flow of oral fluids and microorganisms in the endodontic space they also suggested subjecting the orificium to dental adhesive.

The incidental bacterial microleakage of 30% in both first and third tested group suggests that the permanent restorative materials minimize the coronary leakage. On the other side, we confirmed that the definite coronary restoration of the endodontically treated teeth must be made in a period of 5 days if we want a successful realization of the therapeutical procedure and positive outcome. Similar findings were also presented by Zivkovic [20] who in a period of 72 hours tested the apical bacterial permeability and found presence of bacteria in the dentine tubules independent of the used intra-canal cements.

In the period of 30 days microleakage in the second group was reduced due to cohesion and water expansion of the material for temporary sealing in the suspension. In clinical conditions, during the mastication processes, durability of these fillings is limited, or they break or fall out, and rarely keep their integrity [13, 15, 16, 18]. In *in-vitro* conditions we got these findings, but *in-vivo* researches are also needed for a complete clinical implementation.

We set a period of 5 and 30 days to expose endodontically treated teeth to bacterial suspension. The first tested date was imposed as a maximum time interval in which the endodontic procedure is desirable to be finished and with quality coronary restoration. The sealing of the transitory endodontic cavities is necessary to be realized in the shortest possible period of time after endodontic permanent canal obturation [8, 13, 21, 22, 23, 24].

### Conclusion

The analysis and the evaluation of the results obtained have shown that the bacterial coronary

microleakage exists. It is statistically limited by the quality of the coronary restoration: temporary or permanent. If the integrity of the coronary restoration is not disturbed, then canal obturation does not play a role in the coronary bacterial leaking. After the period of 5 days the temporary sealed cavities are contaminated with bacteria and the penetration is on the orificium level.

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## THE INFLUENCE OF FRONTAL PORCELAIN FUSED METAL BRIDGES ON DENTAL SOUNDS ARTICULATION

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### Abstract

**Aim:** to establish the influence of frontal PFM bridges vestibular-oral diameter on dental sounds articulation.

**Materials and methods:** the investigation of Dental sounds articulation was performed by S consonant analysis on 70 subjects, using a digital spectral analyser "Bruel and Kjaer", type 2131. The subjects were divided in 2 groups: examined (1<sup>st</sup>) group with 33 subjects missing 1 upper central incisor, replaced by PFM bridge, and control (2<sup>nd</sup>) group with 37 subjects who had intact dentition.

The connection between s-consonant articulation and the fixed partial dentures was examined by analysis of the spectral phonetic analysis of the subjects and the measurements of the vestibular-oral diameters of the upper central incisors in both groups. The measurements of the vestibular-oral diameters was made in the middle third (the place with the largest vestibular-oral diameter), and 1mm above the incisal edge.

For comparison, beside s-consonant spectral analysis, a statistical data processing of the gained results was performed.

**Results:** We noticed that the highest relative value for the consonant s in both groups is in the 10000 Hz third. There is a statistically significant difference in the s consonant articulation between the subjects in the 1<sup>st</sup> group.

The measurements of the PFM bridges with the subjects showed that the mean vestibular-oral diameter in the middle third of the upper central incisor crowns is 5,2mm, whereas 5,1mm for the pontics. The vestibular-oral diameter in the area 1mm above the incisor edge is 1,8mm, both in crowns and pontics.

In the control group, where the subjects had natural teeth, the upper central incisor vestibular-oral diameter in the middle third was 3,6mm, and 1,5mm measured 1mm above the incisor edge.

**Conclusions:** S-consonant spectral analysis confirmed that an improvement of the articulation occurred after the incorporation of the PFM frontal bridges with the subjects, missing upper central incisors.

Because of significant variations of the vestibular-oral diameter in the middle third in the frontal bridges which are fabricated, we recommend higher attention both during tooth preparation and design of this part of the crowns and the bridges as well.

**Key words:** upper central incisor, frontal PFM bridges, spectral analysis

### Introduction

The connection between dentistry and phonetics was first emphasized by Oakley in 1872 (cit. Popov [1]). Since then, articulation disorders, as a result of dental anomalies or tooth loss, have frequently been proofed. Very often speech disorders result from physiological (organic) anomalies, such as gothic palate, cleft palate, progenia, displacements, overbite, hypodontia, tongue anomalies etc [2, 3].

Articulation defects, resulting from tooth and jaw anomalies were asserted by Gavrilovic and Grkovic [4], and according to them, the prosthodontis is the one that should be eliminated or at least diminish these problems.

Intercanine upper and lower jaw teeth and their positions are most important during dental sounds (S, Z, C, dz) forming. Long, short or thick teeth, their vestibular-oral position, as well as reduced tongue space and its relation to surrounding teeth are all important factors, which lead to speech disorders.

Schonekerl [5] performed his research based on follow-ups over articulation of the sound s. He pointed out that tooth loss, as well as the prosthodontic constructions are very important in articulation of the sound s. He found out that edentulous patients are not

able to pronounce s correctly. But immediately after getting their first new dentures, they also have pronunciation problems.

Runte examined the articulation of the sound s in relation to maxillary incisor position, and stated that when they are more labial positioned, distortions in s-articulation are bigger than in palatal positioned incisors [6].

Any relocation in the volume of the individual gap, essential in forming dental sounds, may cause acoustic distortions of these sounds. Thus, inadequate prosthodontic restoration of the lost frontal teeth may cause disturbance in dental sounds articulation. If frontal crowns or bridges are fabricated with dimensions, dissimilar to natural teeth, they also can effect acoustical distortions.

In this paper an analysis of s-sound articulation was performed with patients, who received frontal PFM bridges, previously measured vestibular-oral dimension.

### Aim

The aim of this paper is to establish the influence of frontal PFM bridges vestibular-oral diameter on dental sounds articulation.

## Material and methods

The influence of frontal bridges on Dental sounds articulation, depending on their vestibular-oral diameter, was performed by S consonant analysis, the most frequent phonetic sound. The recordings of sound articulation was made at the institute for hearing, speech and voice rehabilitation in Skopje, in an adequate acoustic room, using a digital spectral analysator "Bruel and Kjaer", type 2131. The analysator readings are in Hertz and Decibel, and may graphically present the spectrum of the s-sound [7, 8].

The examination was performed on 70 subjects, divided in 2 groups. The first or examined group consisted of 33 subjects missing 1 upper central incisor, replaced by PFM bridge, whereas the second was a control group with 37 subjects, who had intact dentition, without orthodontic anomalies. All examinees were eugnathic patients (Angle 1<sup>st</sup> class).

In the first group, frontal PFM bridges were fabricated. The bridge retainer copings were made with palatal metal edges with champher edges. Crown modelation, i.e. porcelain firing was made according to the adequate natural tooth morphology.

Bridge pontic's gingival surfaces were shaped with total ridge lap design and adequate anatomy of the missing teeth, i.e. Upper central incisors.

The gingival-incisal dimension of the retainer crowns and pontics were shaped in accordance with the previously measured length of the natural crowns and homologous.

A spectral analysis of the sound s was made by the examinees group with missing upper central incisor in 3 conditions: without bridge, after cementation of the bridge, and 6 months after cementation. A comparison of the gathered sound-s spectrums in these 3 conditions was compared with the results of the control group.

In order to comprehend the influence of the vestibular-oral diameter of the bridges (crowns and pontics) on s-sound articulation we made measurements on upper central incisors in both groups.

The measurements of the vestibular-oral diameters was made in the middle third (the place with the largest vestibular-oral diameter), and 1mm above the incisal edge, using a caliper (Martin Solingen 1594), which had graduations up to 10<sup>th</sup> parts of 1mm.

After completing the measurements on the frontal bridges, a statistical analysis was performed, determining whether there is a significant difference in the s-sound articulation between the 3 above mentioned conditions and the control group. All t-test comments are in relation to a significance threshold of  $p=0.05$ .

## Results

A spectral s-sound analysis was performed with all examinees from both groups, after voice normalization, to avoid changes in intensity. For normalization a terz level of 1000Hz was adopted, where 1000 Hz = 0 dB.

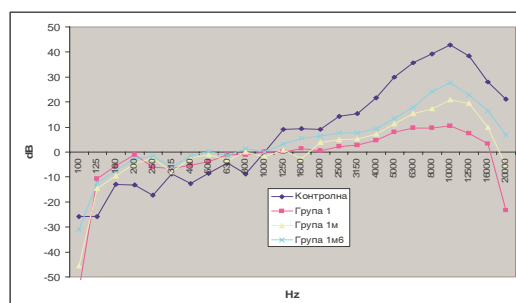
The control group's s-sound spectrum shows that energy dominates in the terz, which central frequency is 10000 Hz, i.e. Energy maximum can be determined

between the 8000 Hz and 12500 Hz terz. The highest relative value of the s-sound level in the 10000 Hertz terz is 42 dB in the control group. The analysis of the s-sound spectrum in the intact eugnathic control group presents a reference value.

The s-sound spectrum in the study group, missing 1 upper central incisor shows highest energy between 6300-12500 Hz terz. The highest relative value of the s-sound level in the terz is 10dB, whereas after bridge cementation it is in the 22 dB terz. Highest energy was noticed in the terz with central frequencies between 8000 and 12500 Hz.

After 6 months adaptation period, the highest energy is again in the terz between 8000 and 12500 Hz, but the highest relative value of the level in the 10000 Hertz terz is getting closer to the control group and is 28 dB.

Comparison of the s-sound spectrums between the control group and the study group in all 3 conditions (without bridge, after bridge cementation and after 6 months adaptation) is presented in Fig. 1.



**Fig. 1.** s-sound spectrum in the study group patients in all 3 conditions, compared with the control group spectrum  
Group 1- condition without bridges  
Group 1m- condition with bridges after cementation  
Group 1m6- condition after 6 months

Beside s-sound spectrum analysis, a statistical data processing of the gained results was performed. The statistical analysis was done to compare the gained results of highest relative value of the terz level with central frequencies of 10000 Hz, were the highest energy for all 3 conditions was noticed. A comparison was made between all 3 conditions and separately with the control group.

In Table 1 is shown the s-sound articulation in the study group, compared in the 3 conditions, while in Table 2 is presented a comparison of s-sound articulation between the control and the study group in all 3 conditions, separately.

The measurements of the vestibular-oral diameters of the PFM frontal bridges in the study group resulted with:

- Mean v-o diameter of the upper central incisor crowns is 5.2 mm in the middle third, and 1.8 mm in the area 1mm above the incisal edge.
- Mean v-o diameter of the upper central incisor pontics is 5.1 mm in the middle third, and 1.8 mm in the area 1mm above the incisal edge.

**Table1.** s-sound articulation in the study group

Conditions	X	Std	p (t)	Significancy
<b>P 12</b>	-4.070	9,246	0,022	yes
<b>P 13</b>	-7.700	9,166	0,001	yes
<b>P 23</b>	-3.948	7.187	0,006	yes

for 10000 Hz

P 12 – comparison between condition without bridges and with bridges

P 13 – comparison between condition without bridges and after 6 months

P 23 - comparison between condition with bridges and after 6 months

**Table 2.** Comparison of s-voice articulation between the controll group examinees and examinees from the study group

Conditions	X	Std	p (t)	Significancy
<b>Without bridge</b>	64.233	8.555	0.034	yes
<b>With bridge</b>	64.213	8.545	0.916	no
<b>6 months after</b>	64.221	8.443	0.931	no

for 10000 Hz

**Table 3.** Vestibular-oral diameter of upper central incisors in examinees from both groups

Crown (middle 1/3)	Pontic (middle 1/3)	Control group (middle 1/3)
5.2 mm	5.1 mm	3.6 mm
Crown (1mm above incisal edge)	Pontic (1 mm above incisal edge)	Controll group (1 mm above incisal edge)
1.8 mm	1.8 mm	1.5 mm

In the control group, where the examinees had natural teeth, the mean v-o diameter of the upper central incisors crowns is 3.6mm in the middle third, and 1.5mm in the area 1mm above the incisal edge. The gained values are shown in Table 3.

**Discussion**

Fixed partial dentures in the upper frontal region, besides esthetic, must also meet phonetic criteria. Proper dental sounds articulation is directly dependant on speech organs anatomic. The oral cavity and its contents are important for voice articulation, which functions thru strictures, formed by it organs (teeth, alveolar ridges, tongue and palate). It is known that loss of teeth and supporting structures, as well as prosthodontic constructions in oral cavity change the articulation space, thus influencing sound articulation and speech, regardless the high adaptability of the articulation structures [9].

The connection between s-consonant articulation and the fixed partial dentures was examined by analysis of the s-voice spectrum by patients, missing 1 upper central incisor, before prosthodontic rehabilitation, after bridge cementation and after 6 months recall. A comparison of the gathered results was made in these 3 conditions, as well as with the results from the control group, which we presumed had correct s-sound articulation. The s-sound spectrum in the patient group, missing 1 upper central incisor shows highest energy between 6300-12500 Hz terz. The highest relative value of the s-sound level in the terz is 10 dB in this group, which is far below the level of the highest relative value of the s-

sound spectrum level in the control group, being 42 dB. This proofs that the absence of 1 upper central incisor causes lisping during s-sound articulation. This lisping is known as sygmatisms [10], which is often caused by teeth and their positions in the dentition.

After bridge cementation, a rising of the highest relative value of the voice level in the 22 dB terz can be noticed in the s-sound articulation spectrum, and the lisping is reduced.

After 6 months adaptation period, the highest energy is in the terz between 8000 and 12500 Hz, which is the same frequency area as in the control group, and the highest relative value of the level in the 10000 Herz terz gets similar to the control group and is 28 dB.

Maximum identity with the intact teeth examinees can not be expected, because of the new resonance circumstances, i.e. FPDs, which should be fabricated very close to the natural counterparts.

The statistical analysis of the s-sound articulations in all groups and conditions showed significant statistical differences in the study group.

There was a statistically significant difference between the examinees from the I (all 3 conditions) and II group, only when the I group examinees were without bridges. There was no statistically significant difference in s-sound articulation between the I group and the control group, immediately after cementation or after 6 months adaptation.

The position of the frontal bridges in anterior-posterior direction can influence voice articulation mechanisms [11].

In order to realize the influence of the vestibular-oral diameter of the bridges (crowns and pontics) on s-sound articulation we made measurements on upper central incisors natural crowns, crowns and pontics in both groups.

Upper central incisor's (PFM crowns and pontics) vestibular-oral diameter in the study group showed certain differences, compared to the control group, i.e. 1.6mm in the middle third and 0.3mm in the area 1mm above the incisal edge for the crowns, and 1.5mm and 0.3mm for the pontics, respectively. Obviously, the central incisor's v-o diameters, measured in the middle third show the largest differences, whereas in the incisal area they are insignificant. It is possible to make the PFM crowns thinner in the incisal area because of their resistance towards abrasion and pressure.

Certain gaps in the bridge's design, between the crowns and pontics (space for the interdental papillae) can also enable airstreams, which could interfere proper s-sound articulation. According to Radlovic [12] all upper frontal bridge pontics must be in contact with the vestibular side of the alveolar ridge because of esthetic and phonetic reasons.

Beside that, the crown's oral surfaces design should be made with great attention, because the S-sound is formed between it and the lower incisor incisal edges [13].

The spectrum analysis of s-sound articulation showed that upper frontal PFM bridges for the replacement of 1 missing upper central incisor are influencing s-sound articulation. Thanking the excellent articulation organ's adaptive mechanisms towards the presence of the prosthodontic works, s-sound articulation improves with time. Patient's neuromuscular activities enable adaptation to the new prosthodontic construction by compensatory feedback articulation mechanisms [14]. Auto correction fixes the oral cavity volume.

But, significant is the v-o diameter deviations of PFM constructions, in the middle crown third. Therefore we recommend, during designing, greater attention to this part, which will lead to even better s-sound articulation. These findings were confirmed by the patient's subjective evaluation, which is also an important indicator.

### Conclusion

Through s-sound spectrum analysis, an improvement in the articulation, after fabrication and cementation of adequate PFM frontal bridges, has been stated with patients, missing upper central incisor. This improvement is especially noticeable after the adaptation period of 6 months.

After the statistical analysis and comparison of the gained results, a statistically significant difference in articulation was noticed with the subjects before bridge fabrication and the control group.

Because of significant variations of the vestibular-oral diameter in the middle third in the frontal PFM bridges which are fabricated, we recommend higher attention during tooth preparation and designing of this part of the crowns and bridges.

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В в	У у	О о	О о
Г г	Г г	П п	П п
Д д	Д д	Р р	Р р
Ѓ ѓ	Г г	С с	С с
Е е	Е е	Т т	Т т
Ж ж	Zh zh	Ќ ќ	К к
З з	Z z	У у	У у
С с	Dz dz	Ф ф	Ф ф
И и	И и	Х х	Х х
Ј ј	Ј ј	Ц ц	Ц ц
К к	К к	Ч ч	Ч ч
Л л	Л л	Џ џ	Џ џ
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