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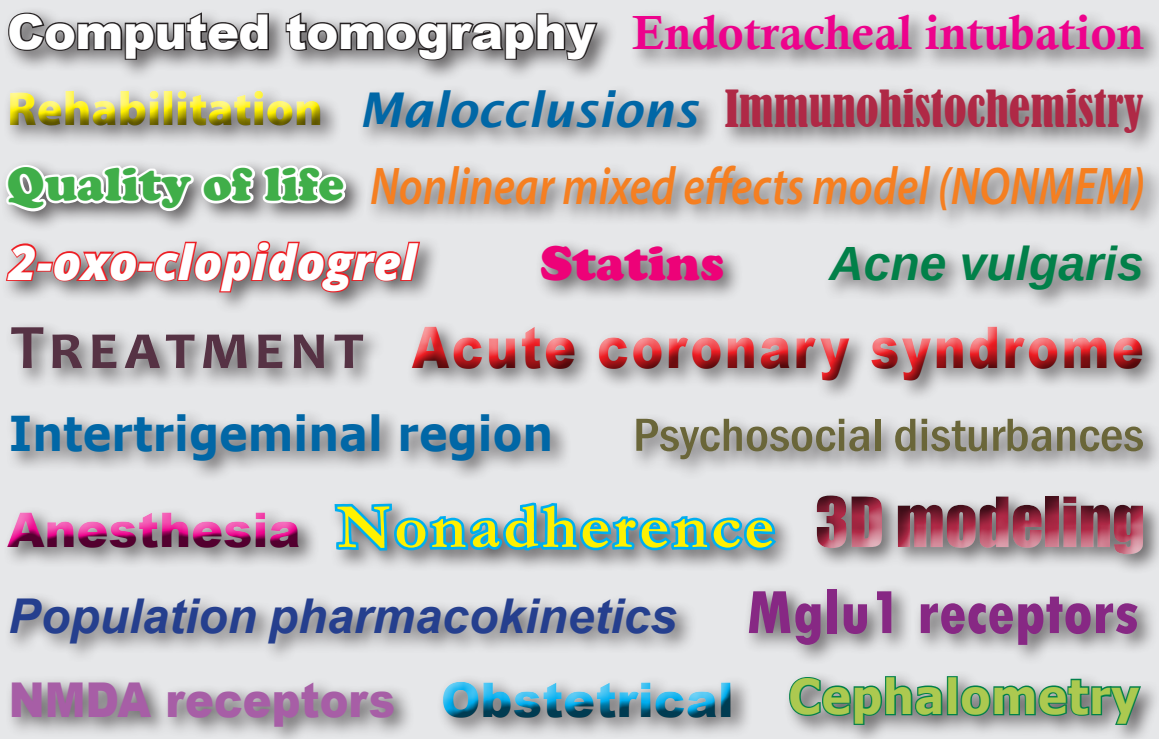

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AUTHOR GUIDELINES

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The manuscript should contain following subdivisions:

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4. Valença MM, Martins C, Andrade-Valença LPA. Trigeminal neuralgia associated with persistent primitive trigeminal artery. Migrâneas cefaléias (Brasil) 2008; 11:30–32.
5. Belenkaya RM. Structural variants of the brain base arteries. Vopr neirokhir 1974; 5:23–29. (Russian)

Abstract:

6. Tontisirin N, Muangman SL, Suz P, et al. Early childhood gender in anterior and posterior cerebral blood flow velocity and autoregulation. In Abstract of Pediatrics 2007. (doi:10.1542/peds. 2006-2110; published online February 5).

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Chapters:

9. Lie TA. Congenital malformations of the carotid and vertebral arterial systems, including the persistent anastomoses. In: Vinken PJ, Bruyn GW (eds) Handbook of clinical neurology, vol. 12. North Holland: Amsterdam, 1972; pp 289–339.

Unpublished data:

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12. Cerebrovascular embryology, in: power point; 2000. http://brainavm.oci.utoronto.ca/staff/Wallace/2000_curriculum/index.html

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Editorial

HOW TO REJECT DOGMAS AND EMBRACE REAL SCIENCE

In 1316 AD, the anatomist Mondino de Luzzi from Bologna published his Anathomia. He was the first to introduce anatomical teaching with human cadavers. Andreas Vesalius of Brussels (1514-1564), the pioneer of modern anatomy, famous for his masterpiece of Renaissance Anatomy, "*De humani corporis fabrica*" („The Fabric of the Human Body"), first published in 1543, had broken long lasting dogmas in anatomy. Educated on ancient scholars including Hippocrates, Aristotle and Galen, was able to reveal and to reject many dogmas that had been active from the 2nd century AD. Although he remained a Galenist to his dying day, he emphasized his belief that Galen had never dissected a human body. Vesalius himself showed a strong talent for dissection and became a demonstrator for one of his professors. It was probably at this point that he began to appreciate the value of direct visual inspection, and soon started to understand that some of Galen's descriptions were more closely related to animal than human anatomy [1]. From his earliest days as a professor of surgery and anatomy in Padua, or even earlier when working as a demonstrator in Paris, Vesalius discovered more gaps between Galenic descriptions and real human anatomy. The success of his book could be attributed to his more than 270 woodcut illustrations. The illustration showing his personal appearance, including an oversized head and short, stubby forearms and hands, led us to conclude that he suffered from hypochondroplasia, a condition of short-limb dwarfism [1]. Recently, both editions of the original *Fabrica* have been published by Karger - a modern edition with a total of over 1,400 pages in A3 format, with greatly enhanced illustrations, and an impressive weight of 14 kg [2].

One of interesting Galenic dogmas was that the hypothalamic infundibulum and the pituitary gland represent a draining route for mucus or phlegm (in Latin "pituita") passing from the brain ventricular parts to the nasopharynx. Perfectionism of the Renaissance genius artists Michelangelo Buonarroti and Leonardo da Vinci obligated them to dissections of human bodies. "Can you imagine there is huge and long snake in the human womb", wrote Michelangelo [3]. His painting on the ceiling of the Sistine Chapel in the Vatican in Rome uses the hypothalamic-pituitary region as a backdrop to his depiction of the „Creation of Man". Drawings by Leonardo da Vinci taken from the "Codici di Anatomia" of the Windsor's collection have three-dimensional representation of the cerebral ventricles. He assigned the third ventricle as "*Sensus communis*", the site of afference and elaboration of all external and internal signals in the human body [4]. These outstanding contributions are an example of how to reject dogmas and embrace real science and, at the same time, a confirmation of magic interactions between the anatomy and art.

Three-dimensional (3D) printing was first described by Charles W. Hull in 1986, and has been extensively used worldwide over the past 30 years. Due to its precise reconstruction of intricate anatomical structures, there is an increasing use of 3D printing in medical sciences, ranging from basic anatomy to surgical practice and innovative research application [5]. Contemporary medical students in their learning of anatomy during practical lessons use

3D images, CT scans, plastinated specimens instead of hands-on dissection. The future will show how progressive this approach is, in comparison to the *anatomia sensata.*

Acknowledgement: to Vladimir Vukovic, Institute for Biomedicine, Eurac Research, Affiliated Institute of the University of Lübeck, Bolzano, Italy, for thoughtful and useful comments.

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Editor-in-Chief

Ljiljana Šaranac

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Original Article

ANALYSIS OF THE HUMAN CEPHALOMETRIC PARAMETERS IMPORTANT FOR DENTAL PRACTICE

Stojanka Arsić[1] , Milena Trandafilović[1], Sonja Janković[2], Dragana Ilić[2], Bojan Nedović[3], Nikola Vitković[4], Miloš Stojković[4], Milica Tufegdžić[5], Jelena Mitić[4], Miroslav Trajanović[4]

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Abstract. *Cephalometry is a measurement of the head by imaging, also taking into account the layer which consists of all the soft tissues of the head. Following the introduction of computed tomography (CT), 3D reconstruction of the head and neck structures and 3D analysis of angular and linear cephalometric parameters was enabled. This study aimed to determine the characteristic cephalometric parameters, using the 2D reconstruction of the multi-slice CT (MSCT) images, which are essential for computer designing of the parametric-geometric-mathematical model (PGMM) of the human skull. We conducted the study on 20 CT scans of adult patients (12 males and 8 females), taken from the radiology archive of the Clinical Center in Niš. Measurements were done on 2D reconstruction images of preselected 3D images of the human head created using MSCT. The values of 29 linear cephalometric parameters (LCP) and 20 angular cephalometric parameters (ACP) were determined. Statistically significant differences between males and females were noted for the distance between the points Sella and Supraorbitale and for the distance between the points Subspinale and Labium superius. Mean values of cephalometric parameters obtained by measurements on 2D CT images can be used to generate normative parameters which represent values used to generate 3D PGMM of the human skull. This PGMM of the skull may allow a more accurate diagnosis, better selection of treatment methods and more accurate prognosis for healing in orthodontics, implantology, oral and maxillofacial surgery.*

Key words: *cephalometric parameters, computed tomography, 3D modeling.*

Introduction

Cephalometry means measurements on the head, taking into account the cover that builds all the soft tissue of the head, regardless of whether these measurements are performed on a live patient or a cadaveric material. The basic principles for obtaining a valid profile radiographic image were made by Pacinni in 1922 as cited by Broadbent [1]. The beginning of the application of this method is related to 1931 when Broadbent [1] introduced a cephalostat in the technique for recording the head profile, a device by which the recording can be done from the same projection with greater certainty, because the head could always be placed almost in the same position relative to anode and film.

Radiography made by the help of digital X-ray machines dramatically facilitated the clinical approach to the cephalometric analysis. The advantages of this method are reflected in the storage, transmission, improvement of image quality, reduced patient radiation, and the ability to analyze cephalometric parameters using a computer [2]. Digital radiography also provides better visualization of the soft tissue structures. However, the disadvantages of these methods, classical and digital 2D radiography, may also be the inability to identify cephalometric points due to the superposition of the bone structures as well as reporting radiographic magnification and rotation of the head in the cephalostat [3].

After the introduction of 3D reconstruction of the head and neck with the help of computerized tomography (CT), 3D analysis enabled more precise diagnostics in the field of maxillofacial surgery, orthodontics and implantology. On the same CT image, 2D reconstruction can be made at any level, and the researcher can choose which plane he will use as a plane of the cross-section and in this way simulate the profile cephalometric image (virtual cephalogram) without re-radiating the patient. Also, the position of the patient's head doesn't need to be strictly defined (eliminates the use of cephalostat) during radiography because there is the possibility of rotating a 3D image on a computer [4].

The study aimed to determine the characteristic cephalometric parameters using the 2D reconstruction of multi-slice computed tomography (MSCT) recordings that are significant for the computer construction of the parametric-geometric-mathematical model (PGMM) of the human skull.

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Material and Methods

We performed morphometry on 20 CT images of adult patients (12 male and 8 female) of the average age of 61 ± 12.76 years, obtained from the archives of the Radiology Center, Clinical Center Niš. The Institutional Review Boards at Clinical Center Niš approved all procedures according to the Declaration of Helsinki. We selected the subjects by a random method and the only criterion for selection was the absence of defects, fractures, and pathological processes on the bones of neurocranium and viscerocranium and on the surrounding soft tissues.

Measurements were performed on 2D reconstructions previously selected 3D images of the human heads performed on the 64-slice CT or MSCT (Aquillion 64, Toshiba, Japan) according to a standard recording protocol: 120 kVp, 150 mA, rotational time 0.5 s, cross-section thickness 0.5 mm. As a plane of cross sections for 2D reconstructions, a mediosagittal plane that passed through the middle of the mandibular symphysis and which is at right angles crossed the coronal plane that passed through the points of the mandibular angles was taken.

The values of 29 linear cephalometric parameters (LCP) and 20 angular cephalometric parameters (ACP) were determined by using the previously defined cephalometric points (Table 1; Figure 1). The values of these 49 parameters were used to evaluate the shape, size, and position of the three craniofacial complexes: (1) the cranial base, (2) the middle of the face, (3) the mandible (Tables 2 and 3; Figures 2 and 3). For further analysis, the values of 7 parameters were added as the cephalometric points on the soft tissues.

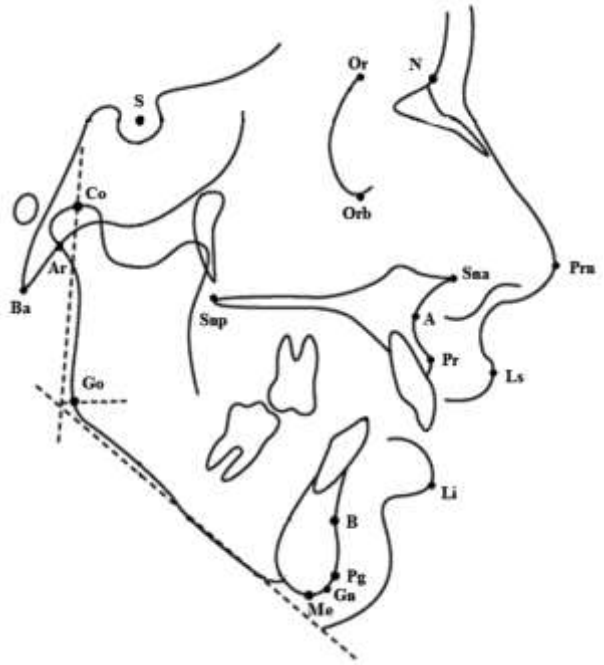

Fig. 1 Schematic representation of the topographic cephalometric points from Table 1 on the human profile image – A, Ar, B, Ba, Cd, Go, Gn, IOrb, Li, Ls, Me, N, Pg, Prn, Pr, S, Sna, Snp, Sorb.

Table 1 Topographic cephalometric points characteristic for the profile image of the human head

Cephalometric point	Definition
Articulare (Ar)	The section of the mandibular head with the shadow of the external, exocranial surface of the basilar part of the occipital bone
Basion (Ba)	The lowest point of the basilar part of the occipital bone located on the anterior margin of the foramen magnum, at the medial plane; on the profile image, it is seen as the lowest point on the profile shadow of the occipital bone
Condylion (Cd)	The highest point of the mandibular head on the condylar process
Gonion (Go)	The point where the symmetry of the angle between the tangents of the inferior margin of the mandibular body and the posterior margin of the mandibular ramus cut mandibular contour at the level of the mandibular angle
Gnathion (Gn)	The point at which the symmetric line of the angle that build the tangents at the lower edge of the mandible and the extended plane Nasion-Pogonion, cut the outer edge of the shadow of the beard; it is located between the points Pogonion and Menton
Infraorbitale (IOrb)	The most inferior point on the inferior margin of the aditus orbitae
Labrale inferius (Li)	The most prominent point of the inferior lip
Labrale superius (Ls)	The most prominent point of the superior lip
Menton (Me)	The lowest point of the shadow of the beard, in which the shadow of the beard and the shadow of the inferior margin of the mandible are joined
Nasion (N)	The connection between the internasal and nasofrontal suture; on the profile image, it is the most anterior point of the nasofrontal suture
Pogonion (Pg)	The most prominent point of the beard profile
Pronasale (Prn)	The most prominent point of the nose
Prosthion (Pr)	The lowest point on the profile of the maxillary alveolar process at the level of the incisors
Sella (S)	The middle of the contour of the sella turcica on the profile image of the head
Spina nasalis anterior (Sna)	The apex of the anterior nasal spine
Spina nasalis posterior (Snp)	The apex of the posterior nasal spine
Subspinale (A)	The point on the deepest part on the concave profile of the alveolar process of the mandible
Supramentale (B)	The deepest point of the concave profile of the chin
Supraorbitale (SOrb)	The highest point on the superior margin of the aditus orbitae

Table 2 The names and definitions of the linear cephalometric parameters

No	Name	Definition
		Cranial measurements
1	S-N	The distance between Sella and Nasion points; a cranial base in a mediosagittal plane
2	S-Ar	The distance between the points Sella and Articulare
3	N-Ba	The distance between the points Nasion and Basion represents a cranial base in a mediosagittal plane (Ricketts analysis)
4	S-Ba	The distance between the points Sella and Basion
5	S-SOrb	The distance between the points Sella and Supraorbitale
		The middle of the face
6	S-Go	The distance between the points Sella and Gonion
7	N-A	The distance between the points Sella and Subspinale; sagittal referent plane anterior-posterior position of the maxilla in the Steiner plane
8	S-Gn	The distance between the points Sella and Gnathion
9	N-Pg	The distance between the points Nasion and Pogonion
10	N-Sna	The distance between the points Nasion and Spina nasalis anterior
11	N-Pr	The distance between the points Nasion and Prosthion
12	S-Snp	The distance between the points Sella and Spina nasalis posterior
13	S-Pr	The distance between the points Sella and Prosthion
14	Snp-Pr	The distance between the points Spina nasalis posterior and Prosthion
15	S-IOrb	The distance between the points Sella and the most inferior point on the inferior margin of the aditus orbitae
		The mandible
16	Ar-Go	The distance between the points Articulare and Gonion
17	Go-Gn	The distance between the points Gonion and Gnathion; the mandibular plane in the Steiner analysis
18	Go-Me	The distance between the points Gonion and Menton; the mandibular plane
19	N-Me	The distance between the points Nasion and Menton; total face height
20	N-B	The distance between the points Nasion and Supramentale; an anterior-posterior position of the mandible in the Steiner analysis
21	Me-Sna	The distance between the points Menton and Spina nasalis posterior
22	Co-Gn	The distance between the points Condylion and Gnathion
23	Co-Go	The distance between the points Condylion and Gonion; posterior or facial height
24	Go-Pg	The distance between the points Gonion and Pogonion; the length of the mandibular body
		Soft-tissue measurements
25	Prn-(N-Pg)	The distance between the points Pronasale and line between the points Nasion and Pogonion
26	A-Ls	The distance between the points Subspinale and Labium superius
27	Li-Pg	The distance between the points Labium inferius and Pogonion
28	Ls-(N-Pg)	The distance between the points Labium superius and line between the points Nasion and Pogonion
29	Li-(N-Pg)	The distance between the points Labium inferius and the line between the points Nasion and Pogonion

Table 3 Names and definitions of angular cephalometric parameters

No	Name	Definition
		Cranial measurements
1	N-S-Ar	The angle between the points Nasion-Sella-Articulare
2	Ba-S-Or	The angle between the points Basion-Sella-Orbitale
3	Ba-S-N	The angle between the points Basion-Sella-Nasion
		The middle of the face
4	S-N-A	The angle between the points Sella-Nasion-Subspinale
5	SOrb-Pr-Snp	The angle between the points Supraorbitale-Prosthion-Spina nasalis posterior
6	S-SOrb-Pr	The angle between the points Sella-Supraorbitale-Prosthion
7	A-N-B	The angle between the points Subspinale-Nasion-Supramentale
		Mandible
8	S-N-B	The angle between the points Sella-Nasion-Supramentale
9	N-S-Gn	The angle between the points Nasion-Sella-Gnathion
10	SN<GoGn	The angle between the line Sella-Nasion and Gonion-Gnathion
11	S-Ar-Go	The angle between the points Sella-Articulare-Gonion
12	Ar-Go-Me	The angle between the points Articulare-Gonion-Menton
13	Ar-Go-N	The angle between the points Articulare-Gonion-Nasion
14	N-Go-Me	The angle between the points Nasion-Gonion-Menton
15	SN<GoM	The angle between the line Sella-Nasion and Gonion-Menton
16	S-N-Pg	The angle between the points Sella-Nasion-Pogonion
17	Co-Go-Me	The angle between the points Condylion-Gonion-Menton
18	N-A-Pg	The angle between the points Nasion-Subspinale-Pogonion
		Soft-tissue measurements
19	Li-Go-Me	The angle between the points Labium inferius-Gonion-Menton
20	Prn-A-Ls	The angle between the points Pronasale-Subspinale-Labium superius

<, angle between two lines; -, angle between the points

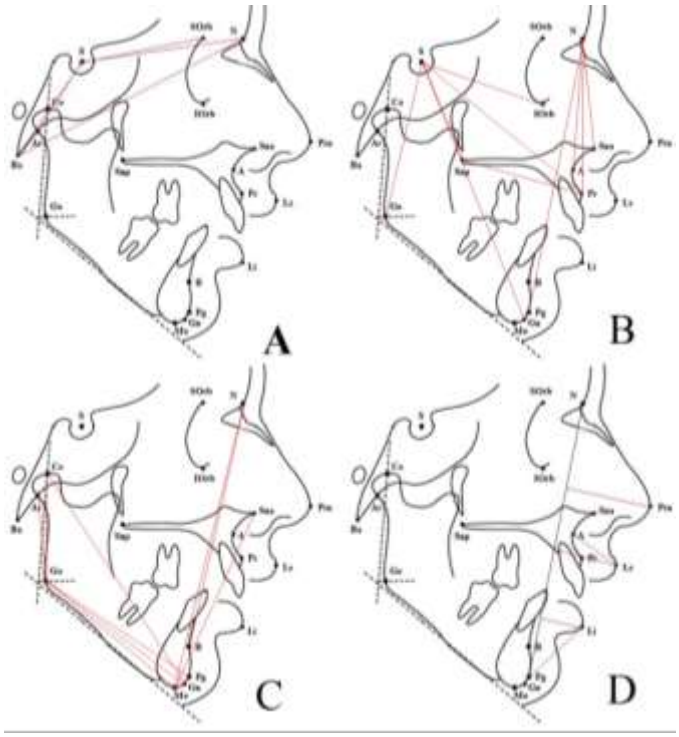

Fig. 2 Schematic representation of linear cephalometric parameters from Table 2 on the human profile image – (A) LCP cranial measurements: S-N; S-Ar; N-Ba; S-Ba; S-SOrb; (B) LCP in the middle of the face: S-Go; N-A; S-Gn; N-Pg; N-Sna; N-Pr; S-Snp; S-Pr; Snp-Pr; S-IOrb (C) LCP of the mandible: Ar-Go; Go-Gn; Go-Me; N-Me; N-B; Me-Sna; Co-Gn; Co-Go; Go-Pg; (D) LCP of the soft tissue: Prn-(N-Pg); A-Ls; Li-Pg; Ls-(N-Pg); Li-(N-Pg).

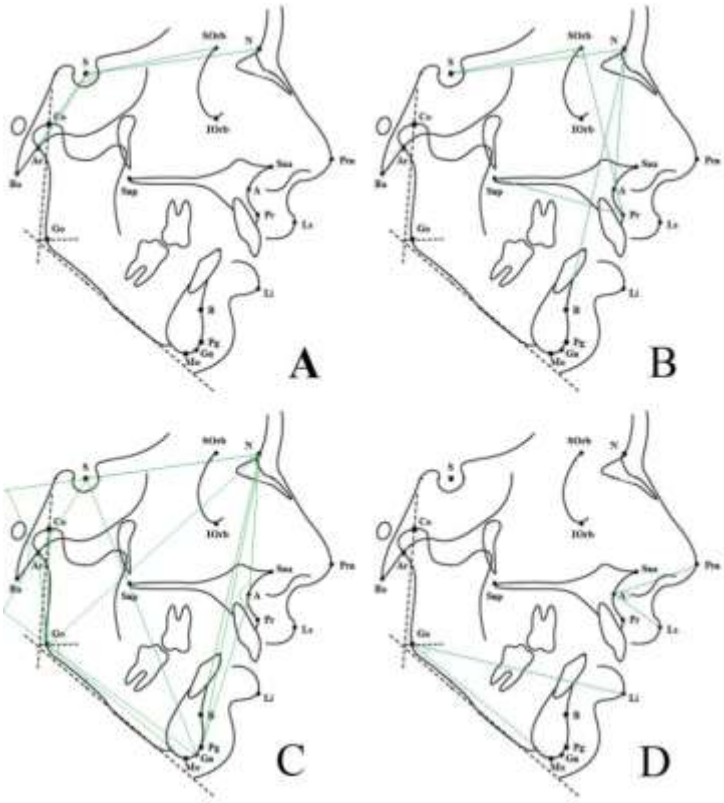

Fig. 3 Schematic representation of angular cephalometric parameters from Table 3 on a human profile image – (A) ACP cranial measurements: N-S-Ar; Ba-S-Or; Ba-S-N; (B) ACP in the middle of the face: S-N-A; SOrb-Pr-Snp; S-SOrb-Pr; A-N-B; (C) ACP of the mandible: S-N-B; N-S-Gn; SN<GoGn; S-Ar-Go; Ar-Go-Me; Ar-Go-N; N-Go-Me; SN<GoM; S-N-Pg; Co-Go-Me; N-A-Pg; (D) Soft-tissue measurements: Li-Go-Me; Prn-A-Ls.

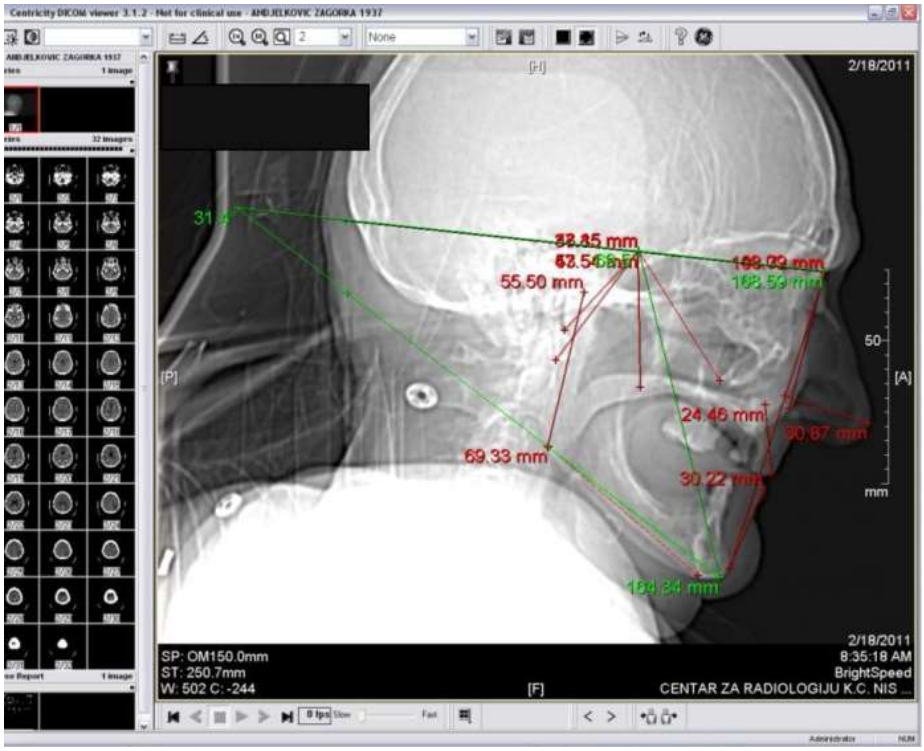

Fig. 4 Measurement of the linear and angular cephalometric parameters in Centricity DICOM viewer window

The measurement of LCP and ACP was performed by manually drawing the measuring lines between previously defined and identified cephalometric points on 2D reconstructions by three researchers, three times for each of the cephalometric parameters, at a time interval of 24 hours, by the Centricity DICOM Viewer program, version 3.12. (Figure 4).

The LCP values are in millimeters (mm) and for ACP in degrees. Statistical significance between male and female groups was defined as a two-sided p value of 0.05 for all analyses, which were carried out using the STATA software package v15.1 (Stata Corporation, College 162 Station, Texas).

Results

We included 20 subjects (12 male and 8 female) with a mean age of 61±12.76 years in this study. The sample was 100% Caucasian. There were no significant differences between males and females regarding age. The mean values and standard deviations M(SD) of the LCP and ACP are shown in Tables 4 and 5, respectively. An independent-samples t-test reported significant differences between males and females for the distance between the points Sella and Supraorbitale and for the distance between the points Subspinale and Labrale superius.

Table 4 The linear cephalometric parameters in males and females

No	Abbreviation	Male N=12 M(SD)	Female N=8 M(SD)	t	p
1	S-N	65.94 (8.81)	64.65 (2.37)	0.09	.929
2	S-Ar	44.94(10.45)	47.93 (9.07)	0.66	.518
3	N-Ba	101.09(13.69)	99.94 (6.53)	0.22	.828
4	S-Ba	58.81(21.62)	52.66 (9.05)	0.76	.459
5	S-SOrb	47.57 (7.17)	37.19(13.58)	2.24	.038
6	S-Go	79.19(16.31)	78.45 (6.30)	0.12	.905
7	N-A	54.92(10.46)	56.28 (6.31)	0.33	.746
8	S-Gn	118.75(23.17)	117.63 (4.49)	0.13	.895
9	N-Pg	111.15(19.23)	107.47(10.56)	0.49	.629
10	N-Sna	51.77(10.91)	48.98 (4.55)	0.68	.505
11	N-Pr	62.39(13.72)	66.10 (9.02)	0.67	.511
12	S-Snp	43.65 (9.53)	46.00 (3.01)	0.67	.511
13	S-Pr	83.92(16.46)	84.64 (7.34)	0.12	.909
14	Snp-Pr	50.28 (9.48)	47.76 (5.90)	0.68	.502
15	S-IOrb	50.35 (9.99)	44.52 (4.71)	1.53	.143
16	Ar-Go	37.00 (8.54)	32.51 (8.59)	1.15	.265
17	Go-Gn	71.58(16.07)	72.04 (5.34)	0.08	.939
18	Go-Me	68.53(15.54)	67.77 (4.62)	0.13	.895
19	N-Me	115.91(19.45)	111.78 (9.02)	0.56	.583
20	N-B	95.50(15.76)	91.86 (7.97)	0.60	.556
21	Me-Sna	67.60(10.21)	64.84 (6.07)	0.68	.502
22	Co-Gn	107.34(20.42)	103.57 (11.88)	0.47	.644
23	Co-Go	56.77(10.23)	56.89 (5.02)	0.03	.976
24	Go-Pg	72.21(15.53)	71.85 (4.84)	0.06	.950
25	Prn-(N-Pg)	36.24 (6.34)	31.72 (3.98)	1.79	.090
26	A-Ls	22.50 (4.94)	17.90 (4.05)	2.18	.042
27	Li-Pg	34.58(12.77)	30.08 (3.18)	0.97	.345
28	Ls-(N-Pg)	15.91 (4.59)	11.14 (6.73)	1.89	.075
29	Li-(N-Pg)	14.97 (5.63)	11.48 (5.53)	1.37	.188

Table 5 Angular cephalometric parameters in males and females

No	Abbreviation	Male N=12 M(SD)	Female N=8 M(SD)	t	p
Cranial measurements					
1	N-S-Ar	117.67 (17.01)	114.67(6.61)	0.47	.642
2	Ba-S-Or	125.50 (13.66)	121.09(7.11)	0.84	.414
3	Ba-S-N	120.15 (14.88)	116.74(5.91)	0.61	.548
The middle of the face					
4	S-N-A	77.32 (9.98)	75.19(13.27)	0.41	.686
5	Or-Pr-Snp	59.64 (3.64)	74.97(36.97)	1.45	.165
6	S-Or-Pr	92.42 (6.87)	86.17(16.95)	1.15	.263
7	A-N-B	6.27 (4.67)	4.39 (3.12)	0.99	.332
Mandible					
8	S-N-B	77.56 (6.13)	77.69 (4.73)	0.051	.960
9	N-S-Gn	74.21(15.91)	71.39 (8.25)	0.46	.652
10	SN<GoGn	31.45(12.79)	29.11 (9.19)	0.44	.662
11	S-Ar-Go	152.88(14.58)	155.01(11.37)	0.35	.732
12	Ar-Go-Me	120.50(11.05)	120.46 (8.04)	0.01	.993
13	Ar-Go-N	73.75(36.30)	56.11(23.04)	1.21	.240
14	N-Go-Me	73.88 (6.57)	66.31(16.11)	1.47	.159
15	SN<GoMe	33.55(12.73)	40.41(25.91)	0.79	.439
16	S-N-Pg	77.55 (8.54)	79.91 (4.80)	0.70	.488
17	Co-Go-Me	115.82 (8.38)	118.66 (9.83)	0.69	.497
18	N-A-Pg	170.13 (6.54)	170.07 (6.97)	0.02	.987
Soft-tissue measurements					
19.	Li-Go-Me	27.31(3.94)	28.43(3.72)	0.64	.532
20.	Prn-A-Ls	70.74(11.66)	77.34(12.65)	1.20	.250

Discussion

Creating the 3D PGMM of the skull is a complex process that is used today in dentistry, primarily in the modern diagnostics and preoperative planning. For generating of 3D models of physical objects, different computer-aided design (CAD) techniques are used. In the field of reverse engineering, various techniques are applied to the scanned models that originate from a physical model (bones or skull in the whole), with the aim to create a satisfactory CAD model. The process of creating a CAD model can be a complicated or straight forward procedure, which depends on the complexity of the physical model. The final product of this process is a valid 3D PGMM [5].

In our investigation, the cephalometric parameters that can be used for the creation of 3D PGMM of the skull were measured, and we propose a more comprehensive list of 49 cephalometric parameters. Such generated PGMM of the skull could have different applications in medicine and technology. PGMM of the skull can be used for the creation of a real 3D bone model with the purpose of studying the effects of different loads on the bone model of the skull. Also, the PGMM of the skull can also be used for the analysis of the different implants in oral and maxillofacial surgery. We point out that, this model can be used for personalized implant production using Additive Manufacturing. The measurement of the cephalometric parameters on the profile CT images provides new opportunities in the research and clinical work in dentistry.

With the help of the study of the X-ray images series, many of the cephalometric characteristics of people in different parts of the world have been found, as well as the specificity of these parameters depending on the age and gender [6−8], so measuring the cephalometric parameters in our population is an essential morphometric procedure for determining the standard values of that population.

This study measured characteristic cephalometric parameters using the 2D reconstruction of MSCT recordings that are significant for the CAD of the PGMM of the human skull. We report no significant differences between males and females for all LCP and APC except for the distance between the points Sella and Supraorbitale and for the distance between the points Subspinale and Labrale superius. The both LCP were larger in males. There is a few studies with cephalometric analysis between genders. Among Filipinos, the male group had a longer anterior cranial base, total facial height, longer lower anterior facial height, longer ramus height, longer lower posterior dentoalveolar height, and total mandibular length. Statistical significance for these differences was not noted only in the group defined by 9.5 years of age [7]. During cephalometric analysis of Slovenians in the period of the mixed dentition (6 to 14 years of age), significant differences between genders were found only for anterior and posterior face height, with boys showing larger values [8]. In these studies, the most LCP and ACP were defined as well as in our study.

Cephalometric parameters of the patient may also be compared with the established population norms to confirm the diagnosis of dentofacial malocclusion or deformities [9]. A long-lasting untreated malocclusion, such as skeletal open-bite, deep-bite and unilateral open-bite, can lead to the symptoms and signs associated with the dysfunction of the temporomandibular joint [10]. Therapy of this condition involves the use of orthodontics that can lead to unwanted effects in the form of tooth and jaw movement, which is also referred to as an increase of S-N-A angle. It shows the anteroposterior position of point A (Subspinale) to the base of the skull, as so as it speaks of the sagittal position of the viscerocranium to the neurocranium [11]. Also, the analysis of the craniofacial parameters in patients with Marfan's or fetal alcohol syndrome is the most critical characteristic of these diseases [12−14]. Postoperatively, in the surgical treatment of craniomaxillofacial disorders, deformities, and facial asymmetries, it is essential to monitor the clinical condition, facial condition and conduct the cephalometric analysis [15−18].

Many studies have focused on the reliability of digital methods so that the measurements obtained by these methods are compared with classical methods of measurement [19−22]. Studies have shown that the values of digitally measured cephalometric parameters do not differ, or are more precise, from manually measured values [4]. Also, due to the higher speed of measurement, priority is given to the measurement on the digital imaging [2, 20, 21].

Conclusion

The mean values of the cephalometric parameters obtained by measuring on 2D CT images can be used for obtaining the normative parameters which will be used as input set for the creation of a 3D PGMM of the human skull. PGMMs of the human skull generated in our study provide better conditions for a correct diagnostic, more valid choice of treatment and better prognosis of healing in orthodontics, implantology, oral and maxillofacial surgery.

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Original Article

THE INFLUENCE OF THE SAGITTAL DENTOSKELETAL PATTERN ON THE VALUE OF THE SOFT TISSUE PROFILE ANGLES - A CEPHALOMETRIC STUDY

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Abstract. *The aim of this study has been to establish the values of soft tissue profile angles in subjects with dentoskeletal Class I, Class II Division 1, Class II Division 2, and Class III pattern, in order to examine the influence of sagittal dentoskeletal relation on the value of angular profile parameters. This comparative cephalometric study included the examination and the analysis by lateral cephalograms to evaluate soft tissue profile angles for 120 adult Caucasian subjects (60 women and 60 men) from the mid Balkan region divided into four groups towards ANB angle and incisors inclination. The following angles were examined: angle of facial convexity, facial convexity angle for the lower face and the angle of total facial convexity. By investigating the influence of the sagittal dentoskeletal pattern on the value of facial convexity angles, significant differences have been established between subjects with Class I and Class II Division 1 and 2 for all examined angles ($p<0.001$; $p=0.011$), while the differences between Class I and Class III are only significant for the facial convexity angle and facial convexity angle for the lower face, while the differences in the overall facial convexity angle are not significant ($p=0.067$). There are significant differences between subjects for all examined angles except the total facial convexity angle between Class I and Class III.*

Key words: *cephalometry, face, malocclusions*.

Introduction

The human face profile represents the perspective of a person's face at an angle of 90° in relation to the "en face" projection. It can be viewed from the aspect of aesthetics and the aspect of harmony. The aesthetic aspect is very important because the human face plays a key role in basic social interactions. Therefore, it is no surprise that people with pleasant faces are considered socially more successful in life. When assessing facial beauty, psychological effects and personal attitudes are superimposed [1, 2]. Profile harmony, unlike aesthetics, is defined by numerous parameters (among them the angle ones are predominant), making it clearly defined and measurable. Angle thought that universal measurement for assessing different facial profiles harmony [3] cannot be applied, because the profile morphotype is affected by various factors such as: ethnic and geographical [4], gender [5], ecological, biological, age and nutritional factors [6].

On the other hand, the profile aesthetics is the ultimate result of some other factors that are not related only to the profile, such as hairstyle, color and shape of eyes, color and texture of complexion [7–9].

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What is the role of facial profile harmony in sagittal dentoskeletal pattern? Correlation between the facial profile harmony on the one hand and the sagittal dentoskeletal pattern and occlusal relationship on the other hand, has been the subject of research since the beginning of the last century, when Angle observed that the effect of sagittal malocclusion on facial contours produces different profile disharmonies. Angle also concluded that the profile balance quality would be proportional to the proximity with normal occlusion [3]. It has been established that Class I is more connected with a pleasant profile and Class III with the least evaluated profile aesthetics, which indicates that the sagittal position of the lower jaw affects the quality of profile aesthetics [10, 11]. However, Bittner et al [12] consider that there is no unconditional relationship between the profile harmony and the sagittal occlusal relationship, i.e. that occlusal deviations are only partially seen in the face. The occlusal relationship of Class II or III at the dental level basically does not have to be related to the skeletal disorder, while some subjects with Class I occlusion presented skeletal deviations adequately compensated by occlusion. Consequently, normal occlusion does not always indicate the profile harmony [12, 13].

The aim of this study has been to establish the values of soft tissue profile angles of facial convexity, facial convexity for the lower face and the overall angle of facial convexity in subjects with dentoskeletal Class I, Class II Division 1, Class II Division 2, and Class III

pattern, in order to, in this way, examine the influence of sagittal dentoskeletal relation on the value of angular profile parameters, as well as to examine the significance of established differences for each angle separately.

Material and Method

The study was conducted at the Dental Clinic in Nis. Before the commencement of the study, each volunteer gave an informed consent as to the purpose and nature of the study. All work was performed in accordance with the Declaration of Helsinki and was approved by the Faculty's Ethics Committee, (General project title of Clinical and Experimental Examination of the Stomatognathic System and ModernTherapeutic Procedures, Project Number 11, of March 8, 2017, Nis, Republic of Serbia).

This study included the examination and the analysis of cephalometric radiography derived lateral cephalograms to evaluate profile angles for 120 adult Caucasian subjects (60 female and 60 male) from the mid-Balkan region (Serbia), which were taken from the patient archives. Cephalometric radiography derived lateral cephalograms were recorded during routine diagnostic procedures for patients who were examined at the Department of Jaw Orthopedics at the Clinic of Dentistry in Niš, aged between 18–30 years, and who underwent orthodontic therapy for the first time. Patients were excluded from the study if they had a history of trauma, craniofacial anomalies, cleft lip and palate, and previous orthodontic, prosthetic or orthognathic surgical treatment. Cephalometric radiographs of the head were done using a cephalostat (head-holding device). All patients included in the study underwent a detailed clinical assessment and analyses of their dental and skeletal profiles, as well as soft tissue profiles on cephalometric radiography. The equipment used for the imaging analyses was the Rotograf Plus (20090 Buccinasco MI Italy) (Number and series: 00036045), and the CEI-OPX/105X-ray tube (CEI, Bologna), which had a protective filter (2.5mm a luminum - equivalent). Lateral cephalometric films were taken from a distance of 165cm from the tube, using a cephalostat to ensure rigid head fixation. The patients were placed in the cephalostat in such a way that the sagittal plane of the head was at a 90° angle to the path of the X-rays. The Frankfort horizontal plane (from the lower edge of foramen orbitale and upper rim of the external auditory canal) was parallel to the ground, the teeth were in central occlusion position, and the lips were in relaxed position. No correction for magnification factors was required, since all the radiographs were taken with the same equipment and the same proportions. Each cephalogram was fixed on the viewing box with the profile to the right, and the acetate tracing paper was fixed by tape at the top. The soft tissue and skeletal features were traced manually in a darkened room, using a 0.5 mm lead pencil. All the image tracing was done by the main investigator. Subjects were divided into four groups. The criteria for categorizing into four groups in the study was the size of the ANB angle according to Steiner and the angle inclination of the upper incisors. The cephalometric ANB angle was the parameter that defined the sagittal relationship between the upper and lower jaw as orthognathic, distal, or mesial (Fig. 1). The points that determined the ANB angle included, point (N), the nasion, located on the suture between the frontal and nasal bones; point A, the lowest point on the line between the anterior nasal spine and the prosthion (alveolar point); and point B, the lowest point from the line between the infradentale and the pogonion (midline of the chin).

The first group was with an orthognathic jaw relationship (Class I) and the ANB angle between 2–4°. The second group was with a distal jaw relationship, an ANB angle >4°, and the inclination angle of the upper incisor >22° (Class II, Division I, or Class II/1).The third group was with a distal jaw relationship, an ANB angle >4° and the inclination angle of the upper incisors inclination <22° (Class II, Division 2, or Class II/2). The fourth group consisted of a mesial jaw relationship and an ANB angle <1° (Class III). Each group consisted of 30 subjects (15 female, 15 male). Since subjects with Class I generally show a harmonic profile due to the orthognatic jaw relationship, this group is taken as a control and compared to the other three groups.

Then, on the radiograph of each patient, the following anthropometric soft tissue points were determined (Table 1, Fig. 2).

By pulling lines from these points, the following profile angles have been formed (Fig. 3):

1. Facial convexity angle, excluding the nose (G-Sn-Pg) – angle between glabella to subnasale (Sn) line and subnasale (Sn) to pogonion (Pg) line;

2. Facial convexity angle for the lower profile part (G-Sn-Pg/1) – angle between glabella to subnasale (Sn) line and subnasale (Sn) to pogonion (Pg) line (supplementary angle to the previous angle);

3. Total facial convexity angle including the nose (G-Prn-Pg) – angle between glabela (G) to pronasale (Prn) and pronasale (Prn) to pogonion (Pg) line.

Since these are angular measures, all results are expressed in degrees (°).

Statistical analysis

Statistical analysis of obtained morphometric data was performed by IBM SPSS Statistics (version 25). Results of the Kolmogorov-Smirnov test showed that majority of the morphometric parameters were not normally distributed. Consequently, significance of detected differences were evaluated by non-parametric Mann-Whitney U test. In the statistical evaluation, the following levels of significance were used: Not significant $p>0.05$; Significant $0.05 \geq p > 0.01$(*); Highly significant $0.01 \geq p > 0.001$(**); Very highly significant $p \leq 0.001$(***); p = probability value.

Results

Descriptive statistics of the average angular measurements for different parameters in all four groups with different jaw relationship (Class I, Class II/1, Class II/2, Class III) are shown in Table 2. In the Table 3, statistical differences of average values of the examined angles between the group with Class I and the other three groups are shown.

Table 1 Facial landmarks used for the determination of angular parameters.

Glabella (G)	the most anterior point of the middle line of the forehead
Subnasale (Sn)	the point where the upper lip joins the columella
Pronasale (Prn)	the most prominent point of the tip of the nose
Pogonion (Pg)	the most anterior point of the chin

Table 2 Descriptive statistics for Class I, Class II division 1, Class II division 2 and Class III (mean value, standard deviation and min-max value).

Classes	I	II/1	II/2	III
G-Sn-Pg	166.23±4.58	159.30±6.23	163.00±7.56	174.13±6.42
Min–max	158.0–174.0	139.0–167.0	151.0–182.0	165.0–189.0
G-Sn-Pg/1	13.77±4.58	20.70±6.23	17.00±7.56	5.87±6.42
Min–max	6.0–22.0	13.0–41.0	-2.00–29.00	-9.0–15.0
G-Prn-Pg	141.17±5.02	136.30±6.24	137.43±5.33	144.57±6.62
Min–max	129.0–150.0	121.0–147.0	127.0–145.0	131.0–160.0

Table 3 Statistical differences between Class I and other groups (Z value; p - probability value).

Classes		I-II/1	I-II/2	I-III
G-Sn-Pg (Z)		-4.167	-2.555	-4.564
	p	<0.001***	0.011**	<0.001***
G-Sn-Pg/1 (Z)		-4.167	-2.555	-4.564
	p	<0.001***	0.011**	<0.001***
G-Prn-Pg (Z)		-3.030	-2.681	-1.831
	p	0.002**	0.007**	0.067

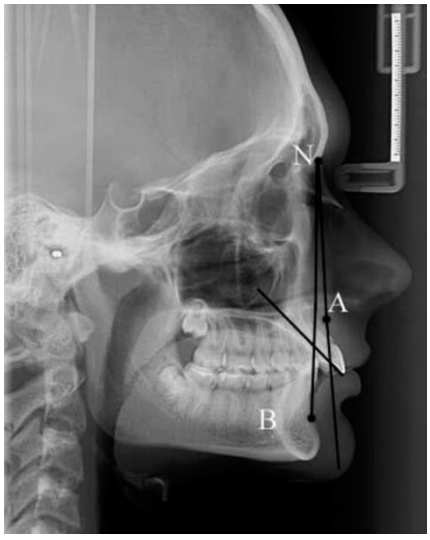

Fig. 1 The cephalometric ANB angle and the angle of inclination of upper incisors.

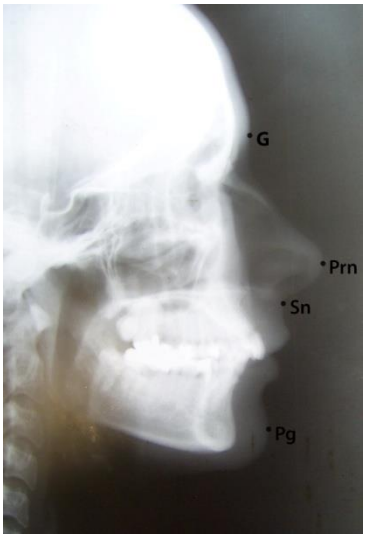

Fig. 2 The landmarks used in this investigation: glabella (G), pronasale (Prn), subnasale (Sn), pogonion (Pg).

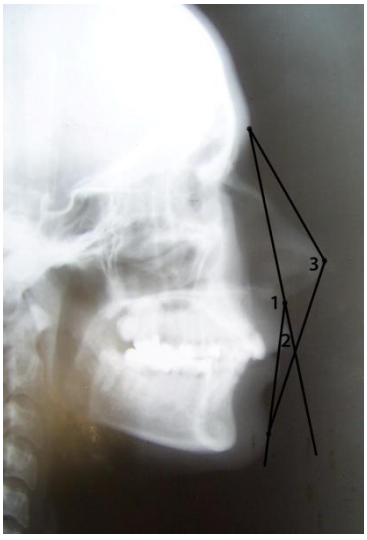

Fig. 3 Angular parameters: 1. the facial convexity angle (excluding the nose) (G–Sn–Pg); 2. the facial convexity angle for lower part of facial profile (G–Sn–Pg/1); 3. total facial convexity angle (including the nose) (G–Prn–Pg).

Discussion

The development of the facial soft tissue profile is the result of complex changes in the facial hard and soft tissue structures [14]. Altemus found a high variability in the thickness of soft tissue of some profile segments [15]. This was confirmed by subsequent researches of this topic [16–21]. Although the skeletal profile is inclined to "correction" during maturation, the soft tissue profile has remained relatively convex, with convexity decreasing with the age growth [22–24].

The established average values of the facial convexity angle for Caucasian subjects with a class amount to (according to different authors): 168.8 ± 4.96° [5], 168 ± 6° [10], 169.4± 3.2° [20], and according to the current study, 166.23 ± 4.58°, that is somewhat lower than the quoted ones. For subjects with Class II, the values are significantly lower - 159.30 ± 6.23 ($p<0.001$) for Division 1; 163.00 ± 7.56° ($p = 0.011$) for Division 2. Contrary to the previous one, for subjects with Class III, the average value of this angle is 174.13 ± 6.42 ($p<0.001$) (Table 2, 3). A similar result was obtained by Godt et al [25] with significant differences of this angle between the subjects with a different dentoskeletal pattern, as expected, because the undeveloped mandible in Class II is positioned more Pogonion posterior while overdeveloped in Class III is positioned more anterior than in Class I. Other studies of the influence of sagittal jaw relationship to the profile harmony confirm that the facial convexity angle is extremely sensitive to sagittal skeletal disorders [5, 10, 26]. Fortes et al [26] by comparing the values of this angle in the Caucasion Brazilian subjects with a pleasant and unpleasant facial profile, established an average of 169.20 ± 3.88° for a pleasant facial profile and 165.17 ± 5.81° for unpleasant facial profiles, which implicitly points out that the reduction of this angle affects the aesthetic perception in a negative way. The difference is statistically significant.

The facial convexity angle for the lower face represents the supplementary angle to the previous one and allows an estimation of the chin projection in relation to the middle part of the face.According to Reis et al [10] its normal value is 12.32 ± 3.93°, and according to Uysal et al [27] it is 14.2° for women and 12.1° for men. When comparing the value of facial convexity angle of the lower face, the significance finding is identical to the previous angle (G-Sn-Pg), with the average value for subjects with Class II/1 (20.7 ± 6.23) and Class II/2 (17 ± 7.56°) is higher while for subjects with Class III (5.87 ± 6.42°) it is lower compared to subjects with Class I (13.77 ± 4.58°) (Table 2). Similar findings were published by other authors [10, 28, 29]. According to these studies, people whose facial convex angles are above 16.25° or below 8.39° have an aesthetically disharmonic profile.

Furthermore, there is a direct connection between the profile convexity and an unpleasant aesthetic profile [10]. For a female profile, the recommended type is slightly convex, while for a male profile, the advantage is given to the straight profile. Any increase of this angle above standard deviation of the average harmonic profile was associated with a reduced result of profile aesthetics. It is believed that women with increased and men with reduced anterior chin projection are less attractive [30, 31].

For the values of total facial convexity angle (G-Prn-Pg), for the group of our subjects with dentoskeletal Class I pattern, average values of 141.17 ± 5.02° have been determined (Table 2). This is close to the values established for the Croatian population 141.55 ± 4.074° [5], but the values of this angle do not differ much with other researchers: Wen et al [4] 141.5° for men and 141.0° for women; Panadian et al [13] determined 141.54 ± 4.96 for men and 140.92 ± 4.79 ° for women.

By comparing differences of average values between groups, significant differences have been established between Class I and II/1 (136.30 ± 6.24°; $p = 0.002$), Class I and II/2 (137.43 ± 5.33°; $p = 0.007$). Differences in values between Class I and III have not shown any significance (Table 3).

Fortes et al [26] compared this angle with Caucasian Brazilian subjects with a pleasant and unpleasant facial profile and set a value of 142.67 ± 4.72° for pleasant facial profiles and 139.10 ± 4.95° for unpleasant facial profiles. The difference is statistically significant. It is interesting that the published papers examining this problem pointed to the fact that the total facial convexity angle, with nasal projection taken into account is not related to profile aesthetics. What might the explanation be? The facial convexity angle is directly related to the sagittal jaw relationship, i.e. to the sagittal pattern, and therefore any changes in its value are directly related to the sagittal deviations between jaws. The total facial convexity angle, however, gives an estimation of the nasal projection in relation to the chin and forehead. The change in this convexity type can be associated not only with skeletal disagreements but also with higher or smaller nasal projection, that does not similarly affect the aesthetic profile estimate [32].

However, some researchers think otherwise - the main factors responsible for the unpleasant profile aesthetics were the nose in 38.35% of cases and chin in 18.9% of cases [10, 33].

Conclusion

By investigating the influence of the sagittal dentoskeletal pattern on the value of facial convexity angles, significant differences have been established between subjects with Class I and Class II Division 1 and 2 for all examined angles, while the differences between Class I and Class III are only significant for the facial convexity angle and facial convexity angle for the lower face, while the differences in the total facial convexity angle are not significant.

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Original Article

ANALYSIS OF PATIENTS' NONADHERENCE TO STATIN THERAPY FROM CARDIOVASCULAR EVENT TO CARDIOVASCULAR REHABILITATION

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Abstract. *Numerous studies have pointed to low adherence to statin, which decreases as time period from acute cardiovascular event elapses. The aim was to analyze the cause of not taking statin by patients who were referred to rehabilitation after coronary event. Study population and methods. The research included the total of 573 patients, average age 60.3, while 305 (53.1%) of them were patients who experienced the first cardiovascular event. The stated research was conducted by means of a questionnaire and implied active participation of the researchers in terms of monitoring the possession and use of medication during rehabilitation. On arrival to rehabilitation, 98 (17.1%) patients did not have statin. They stated that they had never used statins before or that they stopped using them shortly after the event. This subgroup had significantly unfavorable values of lipid parameters ($p<0.001$), abdominal obesity ($p<0.01$), physical inactivity ($p<0.01$), more comorbidities ($p<0.001$), more prescribed medications on daily level ($p<0.05$), lower education degree level ($p<0.01$) and lower monthly income ($p<0.001$). Independent factors for not taking statin were: female gender, low monthly income and large number of comorbidities ($R = 0.291$, $R2 = 0.85$, adjusted $R^2 = 0.80$, std. error of the estimate = 0.36151; $p < 0.001$). The patients themselves stated that the first reason for not taking statin was lack of financial funds (45.9%), while the second reason was normalization of laboratory results (21.4%). Three months after acute coronary event, 17.1% of patients in Serbia stopped taking statin. Lower adherence to statin closely correlates with female gender, low financial income and multiple comorbidities.*

Key words: *Statins, treatment, nonadherence, cardiovascular events, rehabilitation.*

Introduction

The importance of statin in secondary prevention of coronary disease was proved in 1994 after the publication of 4S Study (Scandinavian Simvastatin Survival Study) which verified the reduction of mortality by 30% and coronary events by 34% [1]. After that, statin became a standard medication in secondary prevention therapy and was to be found in all manuals which refereed to secondary prevention of new cardiovascular events [2]. Observational study which was conducted in Europe showed that the percentage of prescribed medication at discharge after acute coronary event was very low, as well as that target values of lipid parameters were not in accordance with the recommendations, regardless of the fact that adequate medications were used [3]. The authors of another study have emphasized that statins were prescribed in 90% of hospital discharges and that they are now present in therapy of 84% of patients after 12 month follow-up [4]. When it comes to clinical practice, statins are not very welcomed by patient or even by some doctors. There are numerous prejudices which discriminate statins as "dangerous medications which destroy liver and muscles". Additionally, statin-based therapy is not always optimal and does not help in achieving targeted values of lipid parameters [5,6].

Having in mind the above stated, the objective of the study was to analyze the cause of not taking statin by patients who were referred to rehabilitation after coronary event, as well as to propose measures for increasing adherence to statin therapy.

Study Population and Methods

The research included all patients with coronary events (acute myocardial infarction with or without stent implementation, coronary revascularization) who were referred to cardiovascular rehabilitation at the Institute for Treatment and Rehabilitation "Niška Banja". The patients came from all Serbian regions, except Belgrade. The stated research started in January 2013 at one of Cardiovascular Rehabilitation departments. By the end of 2016, the research included 573 patients who consecutively came to rehabilitation, 305 (53.1%) of whom had one cardiovascular event, while others came after a recurrent cardiovascular event.

Research was carried out by means of a questionnaire and active participation of the researchers. A patient could answer the question by circling one of the offered answers. If the formulation of the answer was not satis-

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factory, the patient could give her/his own answer to the questions which referred to the reasons for not taking statins. Additionally, patients were free to write the primary reason for not taking statin. During the first examination, the researchers registered all medications that patients brought with them and monitored the use of medication during rehabilitation.

On examination, the weight, height and waist circumference of patients were measured. All patients were subjected to standard laboratory analysis with the aim of assessing risk factors - glycemia and lipid status (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides). Arterial blood pressure was measured on daily basis throughout three-week rehabilitation and the average values were calculated.

Statistical data analysis was carried out by means of SPSS 17.0 software. The results were shown either as arithmetic mean and standard deviation (X±SD) or as absolute value and percentage. Student's t-test was used for testing parameter values. The value of $p < 0.05$ was accepted as statistically significant. Non-parametric values were tested by χ^2-test. Spearman's rank correlation coefficient was used for assessing statistical significance of correlation. Multivariate regression analysis was carried out with the aim of defining independent predictors for not taking statin.

Results

In terms of gender structure, there were 68.6% of male and 31.4% of female patients (Table 1). Majority of patients completed four-year secondary education – 284 (49.6%), while 171 (29.8%) patients completed elementary education. The total of 86 (15%) patients had higher education degree and 32 (5.6%) patients had no formal education whatsoever. On average, the patients who did not use statins had lower education degree level ($p<0.01$; Table 1). The subgroup of patients who did not take statin had higher values of total cholesterol, LDL cholesterol ($p<0.001$) and triglycerides ($p<0.01$). Moreover, general obesity and abdominal obesity ($p<0.01$), physical inactivity ($p<0.01$) and presence of positive heredity for cardiovascular disease ($p<0.001$) were quite present among the members of this subgroup. Additionally, patients from this subgroup had more prescribed medications a day as compared to the subgroup which took statins (8.2±2.8 vs. 7.5±2.8; $p<0.05$).

Multivariate regression analysis included the following parameters: age, gender, education, income, number of comorbidities, recurrent cardiovascular events, time which elapsed from cardiovascular event, total number of medications taken per day. This statistical method defined female gender (coefficient $\beta = 0.152$), monthly income (coefficient $\beta = -0.162$) and comorbidities (coefficient $\beta = 0.129$) as independent factors for not taking statin in this model (for model: $R = 0.291$, $R^2 = 0.85$, adjusted R^2 0.80, std. error of the estimate 0.36151; $p < 0.001$).

Table 2 shows the distribution of previously formulated answers in terms of the reasons for not taking statin.

In terms of percentage of patients who used some kind of reminder for taking medication (medication dosette, notes, telephone, another person, etc.), the figures were not much different in group which did not take medications as compared to the group which took medications (30.6% vs. 34.9%).

When asked to state the main reason for not taking statin, majority of patients – 45 wrote that the main problem was lack of financial funds (Figure 1), 21 patients wrote that their laboratory results of lipid status were normal, 11 patients had new health problems, 10 patients were not prescribed statin until the period of rehabilitation, physician discontinued therapy in 7 patients, while 4 patients wrote down that the main reason for not taking statin was their forgetfulness and negli-

Table 1 Clinical features of all patients and differences in parameters in patients who take statin as compared to patients who do not take statin.

Parameters	All patients	Patients who take statin	Patients who do not take statin
Gender m/f	393 /180	346 / 129	47 / 51***
Age	60.3 ± 9.9	60.3 ± 9.0	60.5 ± 10.1
Education (level)*	2.8 ± 0.9	2.9 ± 0.9	2.6 ± 0.6**
Smoking (n/%)	288 (50.2%)	252 (46.9%)	36 (36.7%)
Waist (cm)	99.0 ± 11.8	98.4 ± 12.2	101.8 ± 9.4*
Abdominal obesity (n/%)	292 (50.9%)	228 (48.0%)	64 (65.3%)**
Body mass index (g/m^2)	27.5 ± 4.2	27.3 ± 4.2	28.6 ± 4.4**
Total cholesterol (mmol/L)	4.4 ± 1.1	4.4 ± 1.0	4.9 ± 1.1***
HDL cholesterol (mmol/L)	1.1 ± 0.3	1.1 ± 0.4	1.0 ± 0.2
LDL cholesterol (mmol/L)	2.5 ± 0.9	2.5 ± 0.9	2.9 ± 0.9***
Triglyceride (mmol/L)	1.8 ± 1.0	1.9 ± 0.9	2.1 ± 1.5**
Glycemia (mmol/L)	6.1 ± 2.1	6.1 ± 2.1	6.0 ± 1.7
Systolic pressure (mmHg)	124.2 ± 15.8	123.8 ± 14.4	126.4 ± 21.3
Diastolic pressure (mmHg)	77.7 ± 6.2	77.6 ± 6.1	78.2 ± 6.7
Heredity (n/%)	354 (61.8%)	273 (57.5%)	81 (82.6%)***
Physical inactivity (n/%)	148 (25.8%)	110 (23.1%)	38 (38.8%)**

m – male; f – female; HDL – high-density lipoprotein; LDL – low-density lipoprotein

Table 2 Distribution of potential reasons for not taking statin

Potential reason for not taking the medication	Number of patients (n / %)
Physician did not recommend the medication upon hospital discharge	28 (28.6)
Medication was not prescribed by primary health protection physician	41 (41.8)
Low monthly income in the family	71 (72.4)
I forgot to take the medication (always or sometimes)	39 (40.1)
Lack of information regarding medication benefits	32 (32.6)
Medication was discontinued when lipid status was within normal limits	36 (36.7)
I am cured (by-pass or stent), I do not need medication	12 (12.2)
I am afraid that "medication does not go well with other medications"	82 (83.7)
I am afraid that I may damage other organs	38 (38.8)
Medication caused new health issues	14 (14.3)
I was worried (frightened) when I read medication instruction	0 (0.0)
I cannot purchase the medication (no specific reason)	58 (59.1)
I decided to stop taking the medication on my own	89 (90.8)

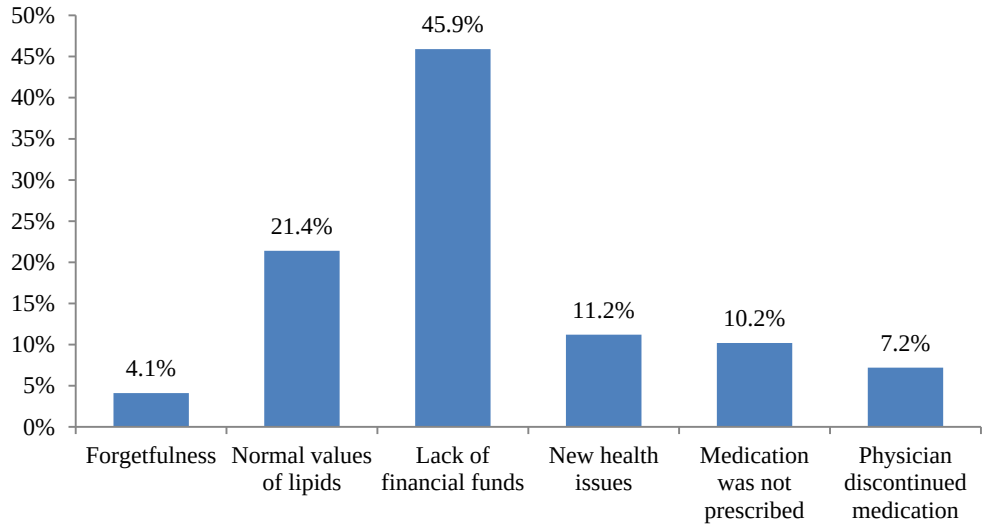

Fig. 1 Distribution of main reasons for not taking statin, according to patient's statements

gence. In terms of health issues that the patients connected with the use of statin, 32 (6.7%) patients reported minor health issues (as compared to subgroup that did not take statins $p<0.05$), but they still took the prescribed therapy.

Discussion

Nowadays, there is a tendency for standardizing the definition of adherence in medical therapy with the aim of using and comparing data in electronic database, which would make an exceptional base for conducting future meta-analysis [7]. This study could not determine Proportion of Days Covered (PDC) and/or Medication Possession Ratio (MPR), having in mind that the patients have been referred to rehabilitation from various regions in Serbia. However, we could detect the patients who do not take statins by simple physical inspection of the medications which they have brought at the first examination. We have found that 17.1% of the patients stopped taking statins after 3.2 months from the last acute cardiovascular event. The patients have filled out a questionnaire which contained the reasons for not taking statins and could write down the main reason for not taking the medication.

It is well known that high blood cholesterol levels are associated with an increased risk of CVD events and deaths, and the use of statins is associated with a significant reduction in that risk [8]. In the modelling-based study of Yang et al., under the 2013 Guidelines for primary prevention of atherosclerotic cardiovascular disease using statin therapy, up to 12.6% of total annual atherosclerotic cardiovascular disease deaths could be prevented among adults aged 40–75 who are eligible for statin treatment [9,10]. However, these prevented deaths could be accompanied by additional cases of diabetes or myopathy. The study by Xie et al. pointed out the importance of adherence to statin therapy in prevention of major adverse cardiac events (MACE) and thus clinicians should aim to achieve higher dosage, if tolerable [11]. Therefore, nonadherence represented an important therapy problem in clinical medicine, especially in terms of implementation of guideline for good clinical practice. The study by Kumbhani et al. proved low ad-

herence to medications for secondary prevention (48.2%) after one-year follow-up of arteriosclerosis (37154 patients), as well as high frequency of unwanted cardiovascular events and mortality in nonadherent group [12]. Bansilas et al. demonstrated that frequency of large unwanted cardiovascular events was significantly lower in cases of total adherence (statins and ACE inhibitors), i.e. ≥80% of days covered by therapy, as compared to partial adherence and nonadherence in patients after myocardial infarction [13]. They found that adherence had to be minimum 40% for longer period of time so that the difference in disease outcome would be noticeable. Furthermore, after examining a group of patients with diabetes, Ruokoniemi et al. stated that reduced MACE incidence was observed in patients without any documented cardiovascular disease at statin initiation odds ratio (OR) 0.87 (95% CI 0.78–0.96) overall and OR 0.80 (95% CI 0.66–0.97) for those who were subjected to 5-year or longer follow-up [14]. The authors concluded that good adherence to statins (the proportion of days covered ≥ 80%) predicted reduced incidence of MACEs, irrespective of the presence of coronary heart diseases at statin initiation.

In order to increase the level of adherence, which is based on forgetfulness and/or negligence, the literature has offered various types of reminders for taking medications. For instance, daily alarms combined with individual or partner feedback improved statin medication adherence [15]. The total of 40% of the members of our group stated that they occasionally forgot to take the medication. However, this was not a crucial reason for not taking statin, as forgetfulness was equally present in both subgroups.

Based on research results, Latry et al. concluded that adherence to statins was poor, but better for those patients with higher number of associated cardiovascular risk factors [16]. The results confirmed that long-term drug treatments were a difficult challenge, particularly for patients who could not see the benefit or felt that they were at risk. Patients at high risk for cardiovascular events were suboptimally dosed with statins, had high rates of discontinuation and low rates of adherence. Despite the use of statin therapy, atherosclerotic cardiovascular disease-related inpatient visit rates were high, particularly among those patients at highest risk because of a recent acute coronary syndrome hospitalization [17]. In terms of our study, an independent factor for not taking statins was large number of comorbidities. Additionally, larger number of prescribed medications that patients had to take significantly reduced adherence to statins. Larger number of comorbidities and prescribed medications required significant financial funds in home budget, which reduced adherence to statins. Our study has proved that one of independent factors for not taking statins is low family income, which has a logical correlation with two previously stated reasons. We have not noticed differences in answer distribution in tested subgroups when it comes to statin significance, prolongation of life by the use of statins, normalization of lipid parameter level, organ damage due to statins or fear from medication interaction. Therefore, our research has demonstrated that reasons for not taking statins are not subjective, but are objective, i.e. they correlate with education degree, financial conditions, the fact that physician has not prescribed medication or discontinued medication. This points to the significance of the problem of adherence to statin in therapy. Unfortunately, this problem goes way beyond medical profession.

Conclusion

On average, 17.1% of patients in Serbia have stopped taking statins 3.2 months after acute coronary event. Low adherence to statins was closely correlated with female gender, low home budget and larger number of comorbidities.

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Original Article

POPULATION PHARMACOKINETICS OF 2-OXO-CLOPIDOGREL IN PATIENTS WITH ACUTE CORONARY SYNDROME

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Abstract. *The aim of the study was to develop a population pharmacokinetic (PK) model for clearance of 2-oxo-clopidogrel in patients with acute coronary syndrome (ACS). Population pharmacokinetic analysis was performed by using 72 plasma concentrations from the same number of patients (mean age of 60.82±10.76 years; total body weight (TBW) of 73.63±9.67 kg) with ACS using non-linear mixed-effect modeling (NONMEM). Validation of the final PPK model was carried out through the bootstrap analysis with 200 runs and it was used to estimate the predictive performance of the pharmacokinetic model. The typical mean value for 2-oxo-clopidogrel clearance (CL), estimated by the base model (without covariates), in our population was 39.2 l h−1.The value of aspartate transaminase and co-medication with digoxin were determinants of a derived population model. The final regression model for the clearance of 2-oxo-clopidogrel was the following: CL (lh^{-1}) = 1.7 + 1.31*AST + 115*DIGOXIN. The derived PK model describes the clearance of 2-oxo-clopidogrel in patients with ACS, showing that the value of aspartate transaminase and co-medication with digoxin are the most important covariate. This finding will provide the basis for future PK studies.*

Key words: *2-oxo-clopidogrel, acute coronary syndrome, population pharmacokinetics, clearance, Nonlinear mixed effects model (NONMEM).*

Introduction

Clopidogrel, a second generation thienopyridine, is P2Y12 subtype of adenosine diphosphate (ADP) receptor antagonist. To date, a large number of conducted studies have shown clinical benefit of treatment with clopidogrel in addition to aspirin as dual antiplatelet therapy for acute coronary syndrome (ACS) and/or undergoing percutaneous coronary intervention (PCI) [1–3]. It is still cornerstone antiplatelet therapy for coronary heart disease despite some advantages of new P2Y12 receptor antagonists such as prasugrel and ticagrelor [4, 5]. The higher treatment cost of novel antiplatelet drugs, as well as its higher risk of major bleeding, maintained clopidogrel as first –line therapy option and extensively prescribed drug worldwide.

Clopidogrel is a prodrug, and its biotransformation by a 2-step, cytohrome P_{450} - dependent process, is requested for conversion to its active metabolite. Introduction of one oxygen atom into clopidogrel, and its conversion to 2-oxo-clopidogrel is the first step of its hepatic metabolism with contribution of CYP1A2, CYP2B6, and CYP2C19 by 35.8, 19.4 and 44%, respectively [6]. In the next oxidation process, 2-oxo-clopidogrel is converted into the pharmacologically active metabolite R-130964 by CYP2B6 (32.9%), CYP2C9 (6.76%), CYP2C19 (20.6%), and CYP3A4 (39.8%) enzymes [7]. The genes encoding CYP3A4/5 and CYP2C19 are polymorphic and accountable in variability of clopidogrel pharmacokinetics. Based on the available information regarding a correlation between both pharmacokinetic parameters of clopidogrel and H4, active metabolite of clopidogrel, as well as the Cmax of the H4 isomer and platelet aggregation, the intestinal absorption of clopidogrel may be considered as rate-limiting process [6, 8]. Additionally, the latter observations allowed the prediction of pharmacodynamics response to clopidogrel using pharmacokinetic data on clopidogrel or 2-oxo-cxopidogrel. Furthermore, previous publications have suggested both active metabolite's instability and existance in the plasm for a short period [6, 9]. In accordance with the previously mentioned, in this study we evaluated the clinical and demographic factors that influence 2-oxo-clopidogrel clearance, supposing this is relevant for vicagrel, novel analog of clopidogrel and its further development in phase II/III studies [10].

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The focus of our study was the evaluation PK variability of 2-oxo-clopidogrel in patients with acute coronary syndrome (ACS) using the population PK approach.

Methods

Patient Data

The investigation was performed at the Clinic of Cardiology, Clinical Center Niš, Serbia during three months (from February to May 2016) after obtaining permission of the Ethics Committee of the University of Nis, Faculty of Medicine. All patients were informed about the details related to the study protocol and were included after their written consent. The including criteria were: patients of both sexes older than 18 years, with diagnosis of acute coronary syndrome (ACS) with/without ST-elevation as confirmed by the cardiologists, using the electrocardiogram and the biochemical tests. The exclusion criteria for our study were: pregnant and lactating women, presence of mental disorders and patients' refusal to participate in the study.

Our study population consisted of 72 patients treated with clopidogrel in accordance with Institutional Review Board/Human Subjects Research Committee requirements. The dual antiplatelet therapy (acetylsalicylic acid and clopidogrel) was administered in all patients. Clopidogrel loading dose was 300mg or 600 mg and it varied depending on the revascularization procedure (PCI or fibrinolytics) and patients' status. After that, clopidogrel was administered once daily, at the maintenance dose of 75mg or 150mg. Besides that, all patients also received angiotensin-converting enzyme *(*ACE*)* inhibitors and other drugs presented in Table 1. This data and other clinical and demographic data of patients (ejection fraction (ER), their concomitant diseases, total body weight (TBW), age of patients and sex), were obtained from medical records, while data of life habits were recorded in a conversation with patients.

Blood sampling and laboratory analysis

The study protocol included taking four blood samples from all patients: two for the routine laboratory tests immediately after the admission of patients to hospital, and two blood samples after three days of starting therapy with clopidogrel for the patients' genotyping and measuring of the drug concentrations, respectively.

Oxoclopidogrel concentrations were measured from the serum samples at the steady–state concentrations using the ultrahigh-performance liquid chromatography with diode array detector-mass spectrometry analysis (UHPLC-DAD-MS). The UHPLC was carried out on a Dionex Ultimate 3000 UHPLC system equipped with a DAD-detector and also connected to LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, USA). The process of separations was performed on a Poroshell 120 EC-C18 column (4.6×50mm, 2.7μm; Agilent technology, USA) at room temperature (25°C). The absorption was recorded on DAD-detector (with total spectral range between 200nm and 800nm), set at three detection wavelengths of 240, 220 and 300nm, simultaneously. Mass spectrometric analysis was performed using an LCQ 3D-ion trap mass spectrometer with electrospray ionization (ESI) in positive ion mode. MS-spectra were acquired by full range acquisition of m/z 300–500. For fragmentation study (MS/MS), a data dependent scan was performed by deploying the collision–induced dissociation at 25eV. The range of the linearity for oxoclopidogrel was …with the lowest the limit of detection and quantification of serum concentration of oxoclopidogrel was 0.5ng/mL and 50.0 ng/mL respectively. All the serum samples for this type of the analysis were obtained in Cmax concentrations, two hours after oral administration of morning dose of clopidogrel.

The patients' genotyping analysis was included before isolating manually the genomic DNA from the whole blood leukocytes and the following small nuclear polymorphisms (SNPs) which was determined by using the PCR (polymerase chain reaction) method: ABCB1 C34335T (rs1045642), CYP2C19*2 (rs4244285) and *17 (rs12248560), as well as CYP2C9*2 (rs1799853). The commercial mix KAPA2G Readymix (KAPA2G Ready-Mix FastHotStart; Kappa Biosystems, Boston, MA, USA), the reaction mixture with specific primers were used for SNPs detections. For detection of gene polymorphism of ***ABCB1 C3435T*** were used primers *C (5′−GGTGTCA CAGGAAGAGATC−3′),* (5′−CAGCCGGGTATAGTCA CAGGAAGATATT−3′) and the reverse *(5′−GGCCAGA GAGGCTGCCACAT−3′),* for the detection of gene polymorphism of ***CYP2C19*2*** primers were used the forward *(5′−AATTACAACCAGAGCTTGGC−3′),* the reverse *(5′−TATCACTTTCCATAAAAGCAAG−3′),* for the recorded of gene polymorphism of ***CYP2C19*17*** used the forward *(5′−GCCCTTAGCACCAAATTCTC−3′)* and the reverse *(5′−ATTTAACCCCCTAAAAAAACACG−3′)* and the end for the detection of ***CYP2C9*2*** gene polymorphism was used the forward *(5′−GTATTTTGGCCTGA AACCCATA−3′)* and the reverse *(5′−GGCCTTGGTT TTTCTCAACTC−3′).* The reaction condition consisted of the initiation at 95°C for 2 minutes followed by different number of cycles. In the case of ABCB1 no restriction enzyme was used, since the primers were allele specific. SNPs determination was performed after vertical electrophoresis in 8% polyacrylamide gel (ABCB1 C3435T and CYP2C19*2) or horizontal electrophoresis in 2% agarose gel (CYP2C19*17 and CYP2C9*2).

Pharmacokinetic analysis

For population pharmacokinetics (PPK) analysis of oxoclopidogrel and the estimates of its pharmacokinetics parameters (PK) of the target population, we applied the NONMEM software (version 7.3.0.) (Icon Development Solutions, USA) with ADVAN 1 subroutine which well describes a one-compartment model without absorption [11]. This model has given the best estimate of the main PK parameters of oxoclopidogrel, the apparent oral clearance (CL/F) and its inter-individual and residual (intra-individual) variability. The choice of appropriate subroutine was based on the literature data of clopidogrel and

oxoclopidogrel pharmacokinetics and testing of different PPK models (using the lowest value of the minimum of objective function (MOF) as the mean statistical criteria between the tested models). In this step, with the aim to form the base PPK model of investigated drug, we also analyzed various models errors for the estimation of variability, using an exponential, additive, proportional and combined model errors. The parent drug, clopidogrel was administered orally in all patients and the influence of oral bioavailability (F) was not considered in the analysis. The base PPK model of oxoclopidogrel was built using the collected data of 72 patients with ADVAN1 subroutine from the software library (without tested covariate).

Covariate of interest for the PPK analysis of oxoclopidogrel were the following: total body weight, age, sex, total daily dose of clopidogrel, ejection fraction, cholesterol and triglyceride levels, red blood cells (RBC) count, creatinine clearance (*calculated for each patient using the* Cockcroft-Gault *equation)*, aspartate transaminase (AST) and alanine transaminase (ALT), ABCB1 and CYP2C9 genotypes, CYP2C19 phenotype, concomitant disease as the presence of diabetes mellitus (DM) type 2, life habits such as smoking status of patient and co-medication with other drugs: beta-blockers, diuretics, spironolactone, amlodipine, amiodarone, digoxin, pantoprazole, sulphonylureas and statins (atorvastatine, rosuvastatine and simvastatine). The influence of 25 covariates on the pharmacokinetic disposition of oxoclopidogrel, were analyzed through the univariate regression models. This process consisted of adding only one of tested covariate in the base model (in a linear or nonlinear way), and then, its statistical significance was assessed on the reduction in the MOF value between this model and the base PPK model. The MOF is defined as -2loglikelihood and, therefore, its required reduction was at least 6.63 ($p<0.01$, df=1). As the results of this complex process, we recorded all covariate with the significant influences on the PK parameters of oxoclopidogrel, and after that, were added in the full model, simultaneously. Further, the analysis was performed in the opposite manner, removing each individual covariate from the full model and noting the differences in the MOF value. In this process of backward removal, the main statistical criteria was stronger, with increasing of the MOF values by $\geq$10.83 ($p<0.001$, df=1) [12]. Thus, the final PPK model of oxoclopidogrel consisted of only those covariate which satisfied both approach selection processed (forward addition and backward removal), suggesting their particular influence on pharmacokinetics of the investigated drug. Integral part of this analysis was a graphic inspection of data fitting between predicted, population values of concentration versus obtained, measured concentrations of oxoclopidogrel, which was obligatory conducted during all phases.

Validation of the final PPK model was carried out through the bootstrap analysis with 200 runs. This type of the internal validation was recommended for a small number of samples per patient by the Guidance for Industry Population Pharmacokinetics, FDA, for the estimation of the stability and predictive performances of the final PPK model [13]. Values of PK parameters and its variability from the bootsrap analysis were compared with the values from NONMEM analysis and if similar, that indicated a good predictive performance of the derived PPK model.

Results

The study population consisted of 72 patients of both sexes (28 female and 44 male) in order to assess the mean population value of the clearance of oxoclopidogrel, as the main metabolite of clopidogrel. The range of patients age was 37 to 85 years, including middle-age to elderly patients, with the average value of

Table 1 Respondent's characteristics (demographic, biochemical, genetic and clinical data)

Characteristics of population	Investigated set (mean values ± SD*)	Range of investigated set
Number of patients	72	
Number of observations	72	
Gender (male/female)	44/28	
TBW (kg)	73.63 ± 9.67	55–106
Age (years)	60.82 ± 10.76	37–85
Clopidogrel daily dose (mg/day)	84.38 ± 24.98	75–150
Oxoclopidogrel plasma concentration (mg/l)	0.29. ± 0.39	0.0008–1.7309
ABCB1 genotype:	72	
- CC	- 19	
- CT	- 34	
- TT	- 19	
CYP2C19 phenotype:	72	
- PM	- 4	
- IM	- 19	
- EM	- 25	
- URM	- 24	
CYP2C9 genotype:	72	
-CC	- 56	
-CT	- 15	
-TT	- 1	
Ejection fractions (%)	51.32 ± 12.27	28–79
Cholesterol levels (mmol/l)	5.52 ± 1.35	2.95–10.87
Triglyceride levels (mmol/l)	2.19 ± 3.85	0.14–33
RBC counts (10^{12}/l)	4.66 ± 0.68	3. 7–6.12
AST (U/l)	117.21 ±169.81	10.2–930
ALT (U/l)	34.20 ± 26.57	1.27–146.7
Creatinine clearance (l h^{-1})	4.38 ± 1.47	1.28–7.23
Diabetes mellitus	19	
Smokers	24	
Co-medications with:		
- Pantoprazole	- 58	
- Beta blockers	- 49	
- Diuretics	- 21	
- Spironolactone	-14	
- Amlodipine	-12	
- Amiodarone	- 8	
- Digoxin	- 8	
- Sulphonylureas	- 4	
- Statins:	- 69	
- Atorvastatine	- 60	
- Rosuvastatine	- 2	
- Simvastatine	- 7	

*SD- standard deviation

body weight of 74kg. Clopidogrel tablets were routinely administered orally, once daily at the dose of 75mg (n=63, 87.5%) or 150mg (n=9, 12.5%). The presence of DM type 2 was showed as the most common concomitant disease (n=19, 26.39%), while almost a third of the population were active smokers (n=24, 33.33%). In terms of co-medications with other drugs, three groups were administered most frequently: statins (95.83%) as the drugs that reduce cholesterol levels (atorvastatine (83.33%), rosuvastatine (2.78%) and simvastatine (9.72%)), the gastroprotective therapy with the proton pump inhibitors (PPIs) (pantoprasole (80.56%)) and beta blockers (BBs) (68.06%). The other co-administered drugs were: diuretics (29.17%), spironolactone (19.44%), amlodipine (16.67%), amiodarone (11.11%), digoxin (11.11%) and sulfonylurea derivatives (5.56%). The average serum concentration of oxoclopidogrel was 0.29±0.39 followed with a wide range from 0.0008 to 1.730mg per liter in the population. Overall, demographic data, biochemical, genetic and clinical data of the respondents are presented in Table 1.

The mean population value of oxoclopidogrel clearance without examining the effects of different covariate was 39.2 liters per hour in the base model. This analysis was carried out using one compartmental model with no absorption from NONMEM software, since the active metabolite was the objective of our investigation. Further, the process of forward selection was conducted in order to assess the influence of each individual factor on pharmacokinetics disposition of the drug. The values of MOF, evaluated by univariate models which examined the effects of separate factors on oxoclopidogrel clearance, are shown

Table 2 Values of MOF for the base model and univariate regression models of examined covariates with their statistical significance in the process of building of the full PPK model

Clearance models	Minimum of objective function	p-value **
BASE MODEL		
CL= θ_1 *EXP(ETA(1))	1047.867	
UNIVARIATE REGRESSION MODELS		
CL= θ_1*EXP(ETA(1)) + θ_2 *AGE	1030.711	**<0.01**
CL= θ_1*EXP(ETA(1)) + θ_3 * TBW	1046.725	>0.01
CL= θ_1*EXP(ETA(1)) + θ_4* SEX	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_5* DD	1047.108	>0.01
CL= θ_1*EXP(ETA(1)) + θ_6* ABCB1	1046.007	>0.01
CL= θ_1*EXP(ETA(1)) + θ_7* CYP2C19 genotype	1046.103	>0.01
CL= θ_1*EXP(ETA(1)) + θ_8* CYP2C9 genotype	1046.477	>0.01
CL= θ_1*EXP(ETA(1)) + θ_9* EF	1047.001	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{10}* CHOL	1031.930	**<0.01**
CL= θ_1*EXP(ETA(1)) + θ_{11}* TGL	1047.449	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{12}* RBC	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{13}* AST	990.892	**<0.01**
CL= θ_1*EXP(ETA(1)) + θ_{14}* ALT	1047.866	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{15}* CLcr	1047.138	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{16}* DM	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{17}* TOB	1047.524	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{18}* PANT	1047.424	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{19}* BB	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{20}* DIU	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{21}* SPI	1047.860	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{22}* AML	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{23}* AMI	1040.078	**<0.01**
CL= θ_1*EXP(ETA(1)) + θ_{24}* STAT	1047.867	>0.01
CL= θ_1*EXP(ETA(1)) + θ_{25}* DIG	1035.918	**<0.01**
CL= θ_1*EXP(ETA(1)) + θ_{26}* SUL	1047.867	>0.01
FULL MODEL		
CL= θ_1 *EXP(ETA(1)) + 0.0000000845 * AGE +0.000000717*CHOL+1.31*AST+0.00000101*AMI+ 115*DIG	985.943	

CL clearance (l/h); θ_1 typical value of CL; ETA (1) interindividual variability in CL; θ_2 to θ_{26} slopes of the covariate effects; TBW patient´s body weight (kg); SEX takes the value 1 for male and 0 for female; DD daily dose of clopidogrel (mg/day); ABCB1 genotype (C=wild type, T=increased P-glycoprotein activity); CYP2C19 phenotype (PM=poor metabolizer, IM=intermediate metabolizer, EM=extensive metabolizer, URM=ultrarapid metabolizer); CYP2C9 genotype (C=wild type, T=decreased activity); EF ejection fractions; CHOL cholesterol; TGL triglyceride; RBC red blood cells; AST aspartate transaminase; ALT alanine transaminase; CLcr creatinine clearance (l h^{-1}); DM presence of diabetes mellitus; TOB takes the value 1 for smokers and 0 for non-smokers; co-medication with PANT pantoprazole, BB beta blockers, DIU diuretics, SPI spironolactone, AML amlodipine, AMI amiodarone, STAT statine, DIG digoxin, SUL sulphonylureas takes the value 1 if the patient received co-medication and 0 otherwise.

** p-value for the MOF difference between the base and tested models

in Table 2. Required statistical significance (for $p<0.001$) in this step was the minimal difference in the MOF values of 6.63 between the base and a single regression model.

Thus, the full model consisted of the following covariates: age, cholesterol level, aspartate transaminase and co-medications with amiodarone and digoxin. Its effects on clearance of oxoclopidogrel had to be confirmed by the backward removal process with the strong statistical requirements (difference in MOF ≥10.83 for $p<0.001$). In the next table, marked as Table 3, we presented this process which resulted in the confirmation of two covariates with significant influence on the apparent clearance of oxoclopidogrel and its variability. These were the value of aspartate transaminase and co-medication with digoxin in the target population, described in the form of the equation as:

$$CL\ (lh^{-1}) = 1.7 + 1.31*\underline{AST + 115*DIGOXIN}$$

Table 3 The process of backward removal of covariate from a full PPK model of the clearance of oxoclopidogrel

Covariates	Difference in minimum objective function	p-value **
Age	1.798	>0.001
Cholesterol	1.557	>0.001
AST	50.084	**<0.001**
Amiodarone	0.969	>0.001
Digoxin	17.162	**<0.001**

** p-value for the MOF difference between the base and tested models

During the PPK analysis, the value of MOF was reduced by 61.924 units from the base to the final model. Furthermore, both inter-individual and residual variability (expressed as coefficient of variation) were 31.18% and 59.72% in the base model and were reduced to 16.01% and 41.51% in the final model, respectively. Much better correlation between measured oxoclopidogrel concentrations versus its population, predicted concentrations (ng/ml) in the final model is shown in Figure 1. The estimates of the apparent clearance of oxoclopidogrel, both investigated variability, and also the effects of aspartate transaminase and co-medication with digoxin, are confirmed by the bootstrap analysis indicating a good precision and the stability of the final model (Table 4).

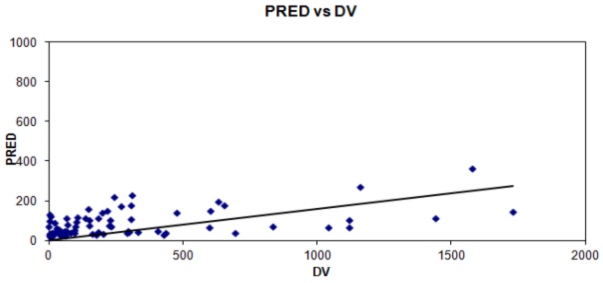

Fig. 1 Scatter-plot of predicted serum oxoclopidogrel concentrations (PRED) versus its measured serum concentrations (DV) expressed as ng/ml in the final model.

Discussion

In the present study we developed and used a population pharmacokinetic model for the clearance of 2-oxo-clopidogrel in routinely treated Serbian patients with ACS. We observed that the typical mean value for 2-oxo-clopidogrel clearance, estimated by the base model (without covariates) in our population was 39.2 l h^{-1}. Also, we noted large interindividual and residual variabilities in this phase.

Another potential source of pharmacokinetics variance of 2-oxo-clopidogrel is metabolic activity of CYP enzyme, especially the activity of CYP2C19 and CYP3A4 isoforms. The hepatic metabolism of 2-oxo-clopidogrel involves dominantly CYP219 and 3A4 isoforms, with 20.6 and 39,8% of contribution, respectively [7]. The interindividual variability in the enzyme activity is partially genetically determined. Among genetic factors, the impact of the following potential covariates was evaluated: CYP2C19*2, CYP2C19*17, and ABCB1 3435 TT genotype. Our findings showed that CYP2C19 genotypes did not influence the clearance of 2-oxo-clopidogrel. Additionally, clearance of 2-oxo-clopidogrel is not altered by ABCB1 3435 TT genotype. Future studies are required to verify these results, especially due to small sample size. The latter fact is an obvious limitation of this study, as well as only one sample per patient. Another PK analyses available in the literature are focused on the kinetics of inactive carboxylic acid metabolite [14, 15] or the H4 isomer which has the antiplatelet activity [16] and to the best of our knowledge no studies are available dealing with 2 oxo-clopidogrel kinetics.

Table 4 Parameter estimates in the final model of oxoclopidogrel

Parameter	NONMEM		Bootstrap Analysis	
	Estimate	95% CI*	Estimate	95% CI‡
CL/F (l/h)	1.7	1.17 – 2.23	1.66	1.05 – 2.27
AST	1.31	0.902 – 1.718	1.40	0.907 – 1.893
DIGOXIN	115.00	86.46 – 143.54	119.00	88.65 – 149.35
Interindividual variance of CL - ω^2_{CL}	0.0253	0.0154 – 0.0352	0.0261	0.0141 – 0.0381
Residual variance - σ^2 (exponential)	0.159	0.055 – 0.263	0.162	0.065 – 0.259

* (Estimate) ± 1.96 x (standard error of the estimate)
‡ 2.5th and 97.5th percentile of the ranked bootstrap parameter estimates

Supposing the possibility that concomitant administration of drugs which share the same CYP450 metabolizing isoenzyme may alter the systemic exposure of 2-oxo-clopidogrel, it was expected that any of them would have some influence on 2-oxo-clopidogrel. However, none of the drugs that are at least partially metabolized by CYP2C19 and 3A4 isoenzymes, influenced the clearance of 2-oxo-clopidogrel significantly. This could be explained by the relative contribution of additional CYP enzymes on 2-oxo-clopidogrel kinetics.

We tested the influence of 25 covariates on the pharmacokinetic disposition of 2-oxo-clopidogrel. In our final model, the only important factors influencing 2-oxo-clopidogrel clearance, from among the covariates tested, were the value of aspartate transaminase and co-medication with digoxin. Previous publications have suggested that both drugs (clopidogrel and digoxin) are substrates of P-glycoprotein (P-gp), but the coadministration of clopidogrel with digoxin did not impact the pharmacokinetics of clopidogrel [17]. However, our results clearly show that 2-oxo-clopidogrel CL is notably increased when digoxin is added to therapy. This was unexpected and the mechanism by which digoxin increases CL of 2-oxo-clopidogrel remains obscure.

The results of our study show that the value of aspartate transaminase significantly increases clearance of 2-oxo-clopidogrel. None of our patients had liver damage and their ALT values were in the normal range, or slightly above it. Its variability may be attributed to subtle myocardial damage in ACS. Additionally, the available information regarding correlation of liver test and the hemodynamic status is very scarce [18]. Therefore, future studies are required to verify these results.

Conclusion

Our PPK model for the clearance of 2-oxo-clopidogrel in routinely treated adult patients with ACS showed that the the value of aspartate transaminase and concomitant therapy with digoxin were the main subjects of 2-oxo-clopidogrel pharmacokinetic variability.

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Original Article

IMMUNOHISTOCHEMICAL IDENTIFICATION AND DISTRIBUTION OF GLUTAMATERGIC NMDA AND mGlu1 RECEPTORS IN THE PONTINE INTERTRIGEMINAL REGION IN RATS

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Abstract. *Local glutamate simulation of intertrigeminal region (ITR) in the lateral pons evoked immediate cardiovascular and respiratory effects proposing its role in central cardiorespiratory control. Since pharmacological studies provided only functional evidence for the existence of glutamate receptors in the ITR and thereby specifying putative neurochemical substrate involved in this control, here we employed immunohistochemistry to examine expression and distribution of NMDA and mGlu1 receptors in this structure. Thirty adult male Sprague-Dawley rats were perfuse-fixed, their brains frozen and cut into sequential series of 20 µm thick sections through the ITR. Immunohistochemistry was performed using polyclonal antibodies against NMDA-NR1, NMDA-NR2A and mGlu1 receptors. Labeled neurons in the ITR were analyzed using light microscope and computerized image analysis system for quantification of relative immunoreactivity as the mean of integrated optical density (IOD), and counting the immunopositive cells. Light microscopic analyses demonstrated NMDA-NR1-immunoreactivity mainly localized in the neuronal cell bodies with sparse distribution on primary dendrites, while NMDA-NR2A-immunoreactivity was basically somatically distributed. The mGlu1-immunoreactivity was moderate and observed both in neuronal bodies and primary dendrites or extracellular matrix suggesting somatodendritic localization. Quantitative analyses of IOD showed very strong expression of NMDA-NR1, weak of NMDA-NR2A and strong-to-moderate expression of mGluR1, with differences in immunostaining signal distribution over rostro-caudal span of the ITR. Counting of immunopositive cells followed similar expression profile. Our data directly confirm the presence of glutamatergic NMDA and mGlu1 receptors in the ITR apparently involved in signaling pathways by which this region modulates cardiorespiratory functions such as blood pressure, heart rate and breathing.*

Key words: *intertrigeminal region, NMDA receptors, mGlu1 receptors, immunohistochemistry, rats*.

Introduction

Dorsolateral pontine neurons located in the intertrigeminal region (ITR), parabrachial complex (PB) and Kölliker-Fuse nucleus (KF) are important components involved in cardiorespiratory coupling, a dynamic property of homeostasis involved in control of blood pressure, heart rate and breathing [1, 2]. This coupling depends mainly on local circuitries and direct anatomical connectivity within these neurons and their inputs to forebrain structures involved in regulation of respiratory and cardiovascular functions [3, 4].

Previous studies posited particular role of glutamatergic neurotransmission for proper synchronization between breathing and cardiovascular dynamics as glutamate is shown to be essentially involved in both ascending and descending pathways of sympathetic respiratory and cardiovascular inputs [5]. In addition, evidence based on detection of mRNA by in situ hybridization suggests that a large proportion of the brainstem neurons that contribute to respiratory and cardiovascular functions and presumably in their coupling are glutamatergic i.e. positive to vesicular glutamate transporter-2 [6, 7]. Indeed, it has been shown that local microinjections of glutamate into the PB, KF or ITR neurons elicit transitory cessation of breathing and increase in arterial blood pressure in anesthetized rats [1, 8–13]. While further neuroanatomical investigations of PB/KF nuclei confirmed glutamate receptor subtypes involved in these effects [10, 14–18], no morphological evidence exist to support presence of specific glutamate receptors in the ITR. In the set of previous pharmacological studies of ITR using subtype-selective antagonists of glutamate receptors only functional evidence for their existence therein was provided [12, 19–21]. Therefore, in the present study immunohistochemistry was performed using specific antibodies against NMDA (-NR1 and -NR2A) and mGlu1 glutamate receptors in order to determine distribution and expression patterns of these receptors within the ITR.

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Methods

All experimental procedures were reviewed and approved by the local bioethics committee and conducted in compliance with the principles outlined in the EU and USA guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU and NIH Publications No. 80-23, revised 1996). Thirty adult male Sprague-Dawley rats (270-300 g) were kept individually in standard cages in a temperature and humidity-controlled environment under a 12:12 h light/dark cycle and with unlimited access to food and water. All efforts were made to minimize the number of animals used and their suffering.

To obtain brain tissue, animals were deeply anesthetized with the combination of ketamine 80 mg/kg and xylazine 5 mg/kg given intraperitoneally and transcardially perfused with 150 ml of cold 0.9% saline containing 1 U/ml heparin, followed by 200 ml of 4% freshly prepared paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4) as a fixative. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Immediately following perfusion, brains were removed from the skull, post-fixed in the same fixative for 2 hours, and then cryoprotected by immersion in 30% sucrose in 0.1 M PBS at 4°C for several days. For tissue processing the brainstem blocks were frozen and cut into an sequential series of 20 µm-thick coronal sections using a cryostat microtome (Leica CM 1850, Nussloch, Germany) and collected consecutively into three serial screen-bottom trays immersed in cold 0.01 M PBS (pH 7.4). Thus, for each rat, approximately 25 sections were collected and equally spaced in three series (about 60 µm apart) for the entire pontine ITR. This allowed systematic examination of distribution of glutamate receptors' immunoreactivity in the rostro-caudal extent of the structure (-9.16 mm to -9.80 mm from bregma), according to rat brain atlas [22]. One section from every group of serial sections was used for immunostaining against NMDA-NR1, -NR2A, or mGlu1a receptors, while the others serve as a specificity control of labeling or processed for cresyl violet Nissl staining to examine the morphological features of the ITR. All sections from each alternate series were processed under identical conditions (i.e. time, temperature, and concentration of reagents).

Immunohistochemistry was carried out using polyclonal rabbit anti-NMDA-NR1, polyclonal rabbit anti-NMDA-NR2A and polyclonal goat anti-mGlu1a antibodies as well as corresponding secondary biotinylated antibodies, all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Briefly, the free-floating sections were treated with 3% H_2O_2 for 10 min to suppress endogenous peroxide activity, followed by 2% normal serum for 30 min at 37 ºC to block nonspecific binding sites. Then the sections were incubated with polyclonal anti-NMDA-NR1 (sc-9058), anti-NMDA-NR2A (sc-9056) or anti-mGlu1a (sc-47130) antibodies at 4ºC overnight. The primary antibodies were diluted 1:100 in a carrier containing 2% normal serum in 50 mM Tris-buffered saline (TBS, pH 7.4). Thereafter, appropriate biotinylated secondary antibodies diluted 1:300 in the same carrier as primary antibodies, and avidin-biotin-peroxidase complex solution (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) diluted 1:100 in TBS, were applied to the sections for 30 min each at room temperature. Between each of the steps, 50 mM TBS (pH 7.4) with 0.05 % Triton X-100 was used to thoroughly rinse the sections three times for 10 min by swaying. Immunoreactivity was detected by processing sections first with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) in TBS-Triton (pH 7.7) for 5 min, and then with addition of equal volume of DAB containing 0.01% H_2O_2 for the next 5 min at room temperature. The reaction was stopped by transferring the sections in ice-cold TBS and rinsing. Finally, the sections were mounted onto gelatin-coated slides, dehydrated, cleared, and sealed. Labeled sections were analyzed using Axio Observer Z1 microscope (Carl Zeiss, Göttingen Germany), linked to camera. Captured images were analyzed by computerized image analysis system (Image J, NIH, Bethesda, USA) for quantification of relative protein levels as the mean of integrated optical density (IOD) and for counting the immunopositive cells.

All antibodies used in the study (anti-NMDA-NR1, anti-NMDA-NR2A and anti-mGlu1a) were well characterized and their specificities established by the manufacturer. The rabbit polyclonal anti-NMDA-NR1 and anti-NMDA-NR2A antibodies are purified immunoglobulins raised against amino acids 19-318 or 23-76 mapping within an extracellular domain of human NMDA-NR1 or NMDA-NR2A, respectively. By Western blot analysis, anti-NMDA-NR1 antibody specifically yielded single bands between 100 and 150 kDa in mouse brain extract. Specificity of anti-NMDA-NR2A antibody was confirmed by a single band at the expected molecular size (200 kDa) in Western blots of H4 whole cell lysate. The goat polyclonal anti-mGlu1a antibody is an affinity purified immunoglobulin raised against a peptide mapping within an extracellular domain of mGluR1 of human origin. These antibodies have also been successfully used by several groups (for NMDA receptors see: [23, 24]; for mGlu1a receptors see: [25, 26].

Set sections adjacent to those processed for immunohistochemistry were used to verify the specificity of the labeling. This was achieved by running some slides in parallel through the entire procedure with the omission of the primary antibodies. No staining was observed in these control sections. As an additional control, the staining pattern obtained in this study was compared with previously published data on the distribution of NMDA-NR1 and mGluR1a immunoreactivity in the distinct brain regions. For instance, our NR1-stained sections shown strong immunoreactivity in the hippocampus and hypothalamus as found by Petralia et al. [27], whereas strong mGluR1a-immunoreactivity was exhibited in the cerebellar cortex (molecular and Purkinje

cell layers), as reported by Baude et al. [28]. The third series of the sections were processed with Nissl cresyl violet method in order to assess the cytoarchitectonic boundaries of the ITR using rat brain atlas [22].

For quantitative analysis of NMDA-NR1, NMDA-NR2A and mGlu1a receptor-like immunoreactivity in the ITR, the comparative sections were digitally photographed under the same exposure condition and analyzed using microscope-based image-analysis system ImageJ. Low power images (5 x objective) were used to outline the ITR and order sections from rostral to caudal level relative to bregma. For each rat, an average of ten immunostained sections were sampled at a high magnification (40 x objective) and converted to binary images. To calculate the number of immunoreactive cells in each section and to measure the intensity of the immunoreaction by optical densitometry an unbiased counting frame of 100 µm x 100 µm was used. This allowed assessment of changes in the number of expressing cells and the relative amount of the peptides. Approximately the same level of the ITR was chosen for every antibody and receptor-like immunoreactivity was consider positive for NMDA-NR1, -NR2A or mGlu1a if punctate staining was observed within the cell body or along the dendritic processes. To prevent multiple counting, neurons from every third evenly spaced section were included in the analysis and only immunopuncta inside the counting frame or touching its upper or right edge were counted. The counting was repeated at least twice for each section analyzed, which ensured that the number of profiles obtained was similar. Since light intensity can directly affect optical densitometric values for all of the measurements, lighting conditions were held constant all the time. Details regarding methodology for image processing and calibration were taken from publication by Jovanovic et al. [29].

Statistical analyses were done using one-way analysis of variance and Student's t-test. Data are expressed as mean ± SEM, and difference consider significant if $p < 0.05$.

Results

Light microscopic analyses demonstrated a very strong homogenous positive staining in all sections investigated through different levels of ITR. Immunoreaction product was generally present in both cell bodies and proximal dendrites of labeled neurons as well as in the neuropil, mainly in dendritic processes. Immunoreactivity of NMDA-NR1 was predominantly located somatically since plasma membrane of many labeled neurons showed clearly visible immunoreaction product (Fig. 1). Fewer neurons also displayed NMDA-NR1

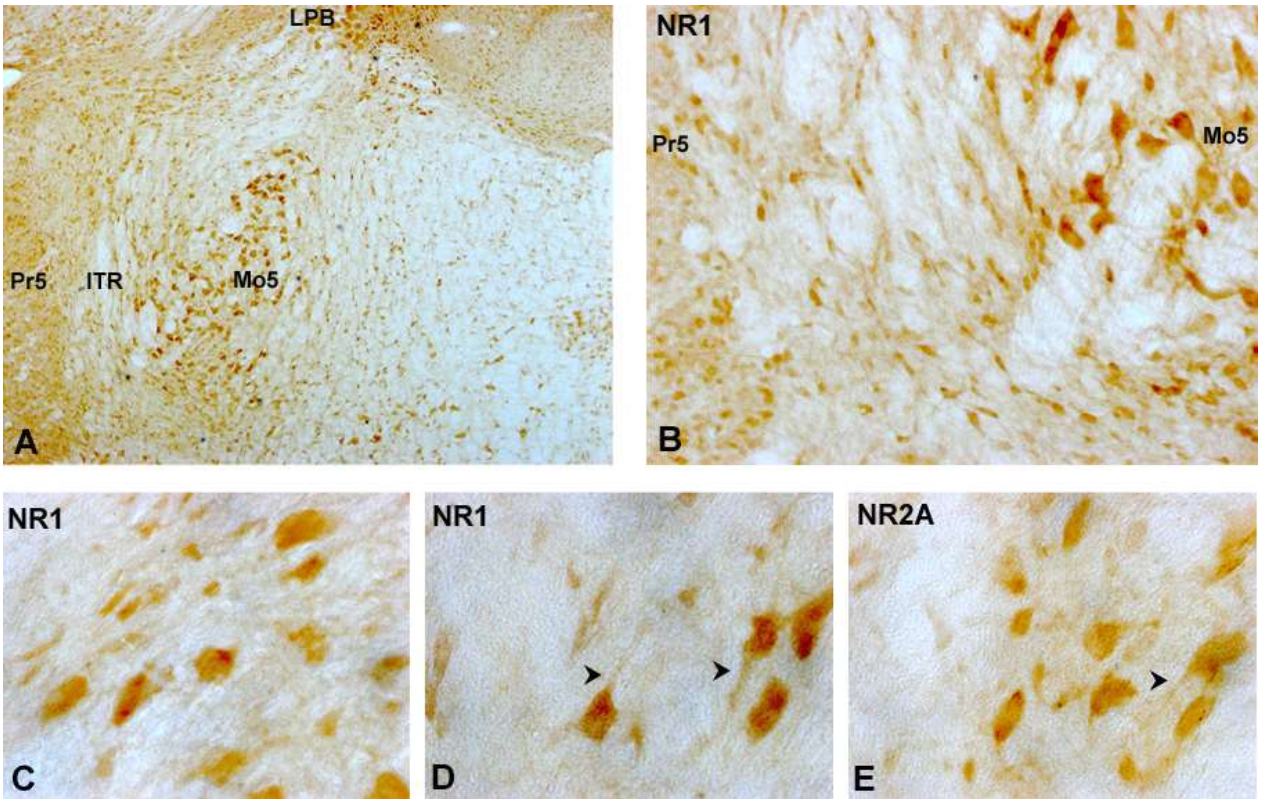

Fig. 1 Coronal sections of rat brain at the level of pontine ITR processed using specific antibodies against NMDA-NR1 and -NR2A receptors. Immunopositive reaction was detected in the cytoplasm of neurons and sparcely in the primary dendrites (arrowheads). Magnification (by objective): A (x 5), B (x 20), C-E (x 63). Abbreviations: ITR (intertrigenimal region), Pr5 (principal sensory trigeminal nucleus), Mo5 (motor trigeminal nucleus), LPB (lateral parabrachial complex nuclei).

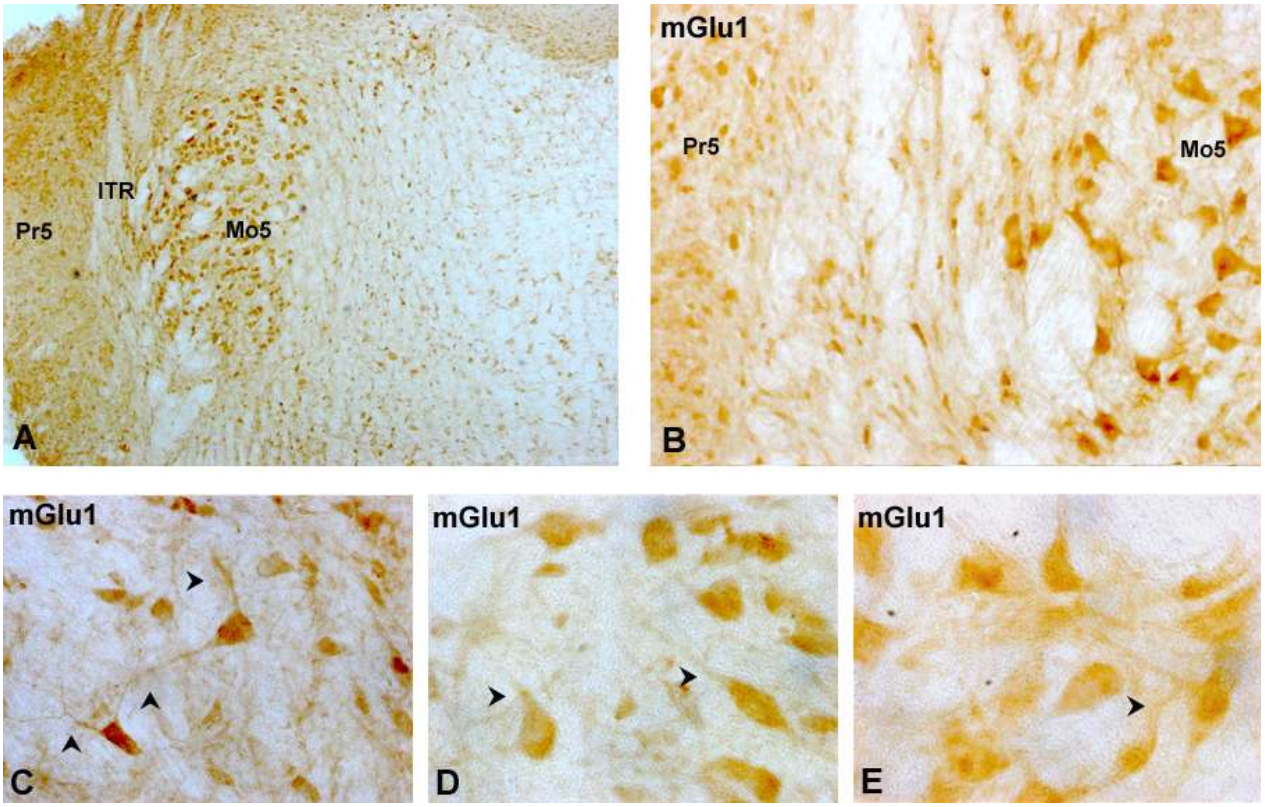

Fig. 2 Coronal sections of rat brain at the level of pontine ITR processed using specific antibodies against mGlu1 receptors. Immunopositive reaction was detected both in the cytoplasm of neurons and in primary dendrites (arrowheads). Magnification (by objective): A (x 5), B (x 20), C (x 40) D-E (x 63). Abbreviations: ITR (intertrigenimal region), Pr5 (principal sensory trigeminal nucleus), Mo5 (motor trigeminal nucleus).

positive immunoreaction on long thin profiles, which are presumably primary dendrites (Fig. 1C, D). At high magnification, NMDA-NR2A immunoreactivity consists of discrete products predominantly distributed in the soma of neurons scattered in the ITR (Fig. 1E). In mGlu1a labeled neurons immunoreactivity was rather homogeneously present both on primary dendrites and in the neuronal cell bodies (Fig. 2). In these sections, moderate fiber immunoreactivity with no apparent cellular staining was also noticed. Overall, both somatic and dendritic staining was observed (Fig. 2C, D, E). Control sections had no specific immunoreactivity above background (data not shown).

Quantitative analyses of immunostained sections at the same level of ITR from each rat revealed differences in NMDA-NR1, -NR2A and mGlu1a with respect to labeling intensity and numbers of labeled neurons. The most prominent staining intensity is for NMDA-NR1, moderate for mGlu1a and weak for -NR2A as measured by integrated optical density (Fig. 3). For both NMDA-NR1 and mGlu1, densest immunoreaction was distributed in medial part of the ITR, i.e. in sections extending from -9.30 to -9.68 mm relative to bregma. In the most caudal sections, i.e. -9.80 mm relative to bregma, apparently smaller numbers of neurons were stained for mGlu1 comparing to NMDA-NR1. Moreover, expression of the -NR2A immunoreactivity was significantly lower ($p < 0.05$) than for the other two receptor subtypes throughout the ITR. Similar results were obtained for counting immunoreactive cells for each receptor subunits analyzed at the same rostro-caudal level of the structure as summarized in Table 1.

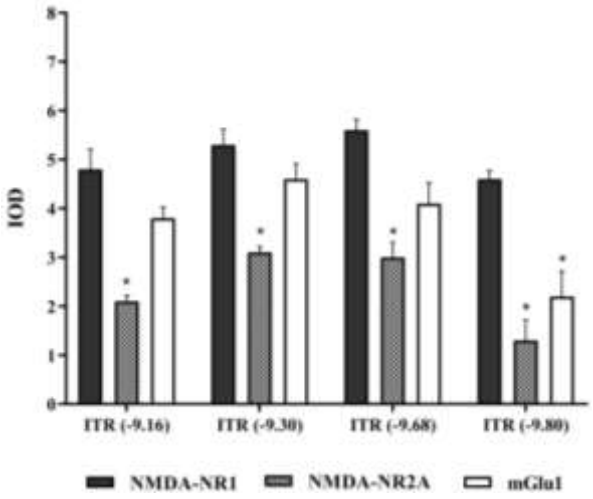

Fig. 3 Quantification of expression of NMDA-NR1, NMDA-NR2A and mGlu1 specific proteins using integrated optical density (IOD) at the level of ITR. Results are expressed as mean ± SEM for IOD at every rostrocaudal level of every analyzed ITR sections (*$p < 0.05$).

Table 1 Number of immunopositive neurons for glutamatergic NMDA-NR1, NMDA-NR2A and mGlu1 receptors in the whole rostro-caudal dimension of the ITR.

Rostro-caudal dimension of ITR	NMDA-NR1	NMDA-NR2A	mGlu1
-9.16 mm	86 ± 18	21 ± 17	43 ± 25
-9.30 mm	111 ± 21	35 ± 18	73 ± 28
-9.68 mm	91 ± 23	26 ± 12	77 ± 36
-9.80 mm	53 ± 14	28 ± 14	54 ± 18

Results are expressed as mean ± SEM immunopositive neurons in the rostro-caudal span of the ITR. Caunting sections were determined relative to bregma using rat brain stereotaxic atlas [22]. Overall lower number of NR2A immunopuncta was found in the ITR structure comparing to those for NR1 or mGlu1 receptors.

Discussion

Using light microscopic evaluation and IOD quantitative analyses of NMDA-NR1, -NR2A and mGlu1a immunopositive puncta, we showed here that glutamatergic NMDA and mGlu1 receptors are clearly expressed in the ITR. This is of particular importance since it represents the first direct confirmation of these receptors in the ITR, whose functional existence was previously suggested in a set of pharmacological studies with specific antagonists [12, 20, 21]. Moreover, our study revealed distribution of these receptors within the whole extent of ITR structure, as well as their specific localization at neuronal level that is necessary to delineate synaptic mechanisms involved in its cardiorespiratory control.

Previous immunohistochemical studies [23, 30] have shown that expression of -NR1 subunit could serve as a reliable marker of NMDA receptor presence in the brain since it is core component of the functional NMDA receptor complex. Our analysis of the ITR at the light microscopic level, showed that NMDA-NR1 and -NR2A immunopositive puncta are predominantly located in neural cell bodies, with sparse neuropilar (at the primary dendrites) and extracellular matrix distribution within the ITR. On the other hand, mGlu1 immunopositivity was present on neural-dendritic sites since it is equally distributed both in neuronal cytoplasm and primary dendrites. Overall, the distribution of NMDA-NR1 subunit was quite similar to that of mGlu1a, however, the pericellular labeling that characterized some of the immunoreactive neuropil structures outlining the soma and proximal dendrites of ITR neurons was not encountered in the NR1-immunostained material. Furthermore, using quantitative IOD analysis of immunolabeled neurons with subtype-specific antibodies, we detected different degree of expression of these neurons within the ITR. More specifically, we found intense expression of -NR1, intense-to-moderate of mGlu1, and quite low expression for -NR2A proteins. These results further confirmed our light microscopic findings, and also suggest relatively higher density of NMDA comparing to mGlu1 receptors in the structure. Thus, ITR functional NMDA receptor complexes are most likely composed of NR1/NR2A subunits given their similar neuronal distribution. This aligns with the previous findings of similar NMDA complexes in the neighboring pontine structures, as well as with the evidence of their predominant expression in the pontomedullary region during postnatal development [31-33]. Based on histological and ultrastructural colocalization for -NR1 and -NR2A subunits in various neuronal populations, Petralia et al. [27] concluded that this type of NMDA receptor complex is mainly postsynaptically localized. Accordingly, NMDA receptors identified in our study are presumably postsynaptic receptors. Presence of mGlu1 immunopositivity in the ITR is also in correlation with previous study where their expression in lateral pontine PB and KF neurons was described [17]. However, exact synaptic localization of these receptors is difficult to reveal using conventional light microscopic examination, particularly when immunopositive puncta are visible on postsynaptic sites touching certain presynaptic elements, or when they are present on presynaptic sites which terminate on postsynaptic neuronal elements [34].

Importantly, these immunohistochemical findings corroborate our previous observations from pharmacological experiments, and additionally provide evidence for presence of NMDA and mGlu1 receptors in the ITR, which are apparently involved in its signaling pathways for neuromodulation of respiratory and cardiovascular functions. In these studies it has been shown that glutamate injected into the ITR can elicit immediate apnea, prolong vagal reflex apnea induced by serotonin injection [12, 20, 21], and increase systolic blood pressure [13]. Furthermore, local non-selective blockade of ITR glutamatergic receptors with kynurenic acid was able to suppress glutamate-induced central apnea and to increase reflex apnea evoked by systemic serotonin injection [12]. On the other hand, selective blockade of NMDA receptors in the ITR was enough to abolish glutamate central apnea [20], while local selective antagonism of mGlu1 prolonged reflex apnea without changing ITR response to glutamate stimulation [21]. Given these facts, presumed role of NMDA receptors, likely localized on somatic, postsynaptic sites in the ITR, is in mechanisms of dampening acute transitory perturbations in pontine cardiorespiratory centers, while mGlu1 receptors, distributed on somato-dendritic sites of its neurons, are involved in modulation of cardiorespiratory reflexes induced by excitations of vagal afferents.

In summary, this study provides direct evidence for the presence of glutamatergic NMDA and mGlu1 receptors in the ITR, and thereby add in defining synaptic mechanisms involved in its regulation of respiratory and cardiovascular homeostasis together with other pontine and ventrolateral medullar structures.

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Original Article

THE EFFECT OF REMIFENTANIL ON INTUBATION CONDITIONS IN PATIENTS UNDERGOING CESAREAN DELIVERY UNDER GENERAL ANESTHESIA: COMPARISON OF TWO DOSING REGIMENS

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Abstract. *The objective of our study was to compare the effects of two remifentanil dosing regimens, used during induction-delivery period of cesarean section, and of remifentanil-free control on maternal intubating conditions and hemodynamic response to endotracheal intubation as well as on neonatal outcome. Seventy seven ASA physical status I–II women with singleton term pregnancy, who were scheduled for elective cesarean section in general anesthesia and have given written informed consent, were enrolled in this prospective, randomized controlled study and divided in three groups: A – 31 patient received 1 μg/kg remifentanil bolus before the induction of anesthesia, followed by 0.15 μg/kg/min remifentanil infusion that was stopped after the skin incision; B – 27 patients received only 1 μg/kg remifentanil bolus; C – 19 patients did not receive remifentanil until the delivery of the baby. Intubating conditions were qualified as excellent, good or poor. Group A had significantly higher number of patients with excellent intubating conditions ($p = 0.011$); majority of patients with good intubating conditions were in group C ($p = 0.017$). Systolic, diastolic, main arterial pressure and heart rate raised significantly in group C compared to A and B ($p < 0.001$). Neonatal outcome did not differ between groups – all neonates were vital with first minute Apgar scores ≥ 8. In conclusion, our dosing regimen of remifentanil 1μg/kg bolus given immediately before the induction followed by 0.15 μg/kg/min interrupted after skin incision provided the best compromise between the achievement of excellent intubating conditions, attenuation of maternal hemodynamic stress response to endotracheal intubation and avoidance of neonatal respiratory depression.*

Key words: *anesthesia, obstetrical, endotracheal intubation, remifentanil*.

Introduction

The time interval from induction to anesthesia to the delivery of the baby (induction-delivery, I-D interval) during caesarean section performed under general anesthesia (GA) represents very vulnerable period concerning both maternal and fetal/neonatal wellbeing. All medications that the mother receives (except muscle relaxants) will cross uteroplacental membrane and affect the fetus directly (hearth and respiratory rate, muscle tone) and indirectly (by influencing maternal hemodynamics, uteroplacental perfusion, uterine tone) [1–3]. This is the reason why the doses of anesthetics are traditionally reduced as much as possible, which could lead to light anesthesia with increased risk of maternal intraoperative awareness (reported incidence of 0.2-0.9%) [4, 5] and exaggerated neuroendocrine stress response to laryngoscopy, endotracheal intubation and surgical stimuli. Mechanical stimulation of pharyngeal and laryngeal proprioceptors during direct laryngoscopy, endotracheal intubation and cuff inflation activates hypothalamo-pituitary-adrenal axis, with subsequent increase in hearth rate (up to 20%), blood pressure (40–50%), capillary wedge, intracranial and intraocular pressure, possibly leading to severe cardio- and cerebrovascular complications [6–9]. Increasing number of vulnerable patients in obstetrics population nowadays makes this problem more and more serious. We can expect much more parturients with high-risk pregnancies, advanced age, morbid obesity and complex comorbidities and, consequently, much more need for some drug that could help us blunting the unwanted effects of endotracheal intubation [9, 10].

Among different pharmacological options used to attenuate hemodynamic response to endotracheal intubation and surgical incision (direct vasodilatators, β-blockers, calcium channel blockers, α_2 agonists, anticonvulsant drugs such as gabapentin, magnesium, local anesthetics) opioids are still the most extensively used

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[11, 12]. It seems that remifentanil, ultra-short acting synthetic opioid, due to its specific pharmacokinetics, could be the appropriate drug to use during I-D interval, where a brief but intense analgesia without prolonged effect is desirable [13–18].

Remifentanil has a rapid onset of action (1–1.5 min), rapid redistribution and context sensitive half time 3-5 min; its metabolism depends on nonspecific tissue and plasma esterases [19]. Remifentanil does cross the placenta, but, unlike other opioids, appears to be rapidly metabolized and redistributed in the fetus leaving the smaller possibility of unwanted consequences (mainly neonatal respiratory depression, muscle rigidity, low Apgar and neurobehavioral scores) [19–21].

In the present study we investigated the effects of two remifentanil dosing regimens on intubating conditions and maternal blood pressure and heart rate response to the intubation in attempt to find the most effective remifentanil dose that would not adversely affect neonatal outcome.

Material and Methods

The study was approved by the local ethics committee. Seventy-seven ASA physical status I-II women with singleton term pregnancy, who were scheduled for elective caesarean section in general anesthesia and have given written informed consent, were enrolled in this prospective, randomized controlled study. Exclusion criteria were known cardiac, respiratory, neurologic, renal, endocrine, psychiatric disorders, history of drug or alcohol abuse, morbid obesity, preeclampsia, predicted difficult airway management (Mallampati score > 2a), active labor, known fetal congenital abnormalities or signs of fetal compromise. All patients refused regional anesthesia, or had absolute/relative medical contraindications to regional anesthesia.

In the operating room patients were placed supine with left uterine displacement, standard monitoring (noninvasive blood pressure, electrocardiography, pulse oximetry, capnography – using bedside monitor, model BSM-2301k, Nihon Kohden Corporation, Tokyo, Japan and bispectral index – BIS electroencephalogram, using BIS-Vista monitoring system Norwood, Massachusetts, USA) was initiated and two intravenous lines established, one for remifentanil infusion (using Perfusor fm B/Brown , Melsungen AG, Germany), the other for the administration of other medications and fluids.

Patients were randomly allocated (using envelope method) to one of the following groups:

1. group (A) – 31 patient received 1 μg/kg remifentanil bolus, given over 30 s, before the induction of anesthesia, followed by 0.15 μg/kg/min remifentanil infusion that was stopped after the skin incision.

2. group (B) – 27 patients received 1μg/kg remifentanil bolus, given over 30 s, just before the induction of anesthesia

3. control group (C) – 19 patients did not receive remifentanil until the delivery

After 3 minutes of preoxygenation through a facemask and remifentanil administration in A and B group, anesthesia was induced with thiopentone, starting with 3 mg/kg over 20 s, followed by additional boluses (if needed) of 25 mg until adequate dept of anesthesia has been reached (BIS values under 60, but not below 40); succinylcholine 1.5 mg/kg was administered and after 60 s endotracheal intubation was performed by the anesthetist blinded to group assignment, who also estimated and graded intubating conditions as excellent, good or poor (Table 1). The intubating score was evaluated according to the consensus conference on Good Clinical Research Practice in Pharmacodynamic Studies of Neuromuscular Blocking Agents [22]. Anesthesia was maintained with 1–1.5% end-tidal sevoflurane and 50% nitrous oxide in oxygen. Further muscle relaxation has been provided with rocuronium 0.6 mg/kg. The lungs were mechanically ventilated to maintain end-tidal PCO_2 of 28–32 mmHg, with fresh gas flow of 6 l/min.

Beginning from the induction of anesthesia until delivery SAP, DAP, MAP (systolic, diastolic, main arterial pressure respectively) and HR (heart rate), were measured and recorded at 2 minutes interval. We specially

Table 1 Scoring conditions for endotracheal intubation

Variables	Intubating conditions		
	Clinically acceptable		Clinically unacceptable
	excellent	good	poor
Laryngoscop			
Jaw relaxation	relaxed	not fully	poor
Resistance to laryngoscope	none	slight	active
Vocal cords			
Position	abducted	intermediate	closed
Movements	none	moving	closing
Reaction to tube insertion and cuff inflation			
Limb movements	none	slight	vigorous
Cough	none	slight	sustained (>10s)

Conditions: excellent – if all the answers are 'excellent'
good – if the answers are 'excellent' or 'good'
poor – if one or more answers are 'bad'

recorded values measured after induction to anesthesia (T1) and 30 s after endotracheal intubation (T2).

After delivery, pediatrician blinded to group assignment assessed the neonate and recorded the time to sustained respiration, Apgar score at 1st and 5th minute and resuscitative measures (if required), that might have included the use of tactile stimulation, beg-mask ventilation, endotracheal intubation or naloxone administration.

Statistical analyses

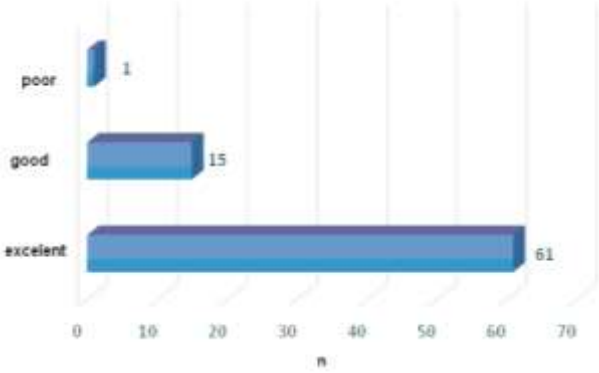
Statistical analysis was performed using SSPS statistic package, version 13. Normal distribution was evaluated with Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was used for parameters comparison between three groups, with subsequent post hoc analysis. In cases of irregular data distribution Kruskal-Wallis test was utilized, with subsequent post hoc analysis with Mann-Whitney U test. The Chi-square test was used to verify the relation between categorical variables. The statistic hypothesis was tested on the significance level for risk of α=0.05; the difference between samples was considered significant if p was < 0.05.

Results

Seventy seven ASA status I-II parturients were included in this study. Patient's characteristics and surgical details are summarized in Table 2; no differences between groups have been observed.

Hemodynamic variables measured after the induction to anesthesia did not show statistical difference between groups (Table 3). After the intubation all hemodynamic variables in group C have raised significantly compared to groups A and B. The increase in variables was also greater in group B compared to A, but the difference did not reach statistical significance (Table 3)

The intubation conditions were excellent in 61 parturient (79.2%), good in 15 (19.5%) and poor in one parturient (1.3%) (Graph 1).



Graph 1 Intubation condition in our patients

The intubation scores showed significant difference between groups (Table 4.).

Table 2 Parturients characteristics and surgical details

	Group			F	p
	A	B	C		
Age (years)	31.74 ± 4.46	31.22 ± 5.22	30.89 ± 1.04	0.202	0.818
Gestation weeks	38.94 ± 0.72	39.04 ± 1.09	39.47 ± 0.90	2.162	0.122
Weight (kg)	77.19 ± 13.27	82.37 ± 9.52	79.26 ± 11.84	2.216	0.918
I-D interval (minutes)	11.22 ± 1.67	10.04 ± 1.81	10.37 ± 1.71	3.639	0.031
U-D interval (seconds)	57.39 ± 18.93	58.00 ± 14.92	60.42 ± 22.25	0.165	0.848

F-ANOVA

Table 3 Hemodynamic variables after the induction to anesthesia (T1) and 30 s after the intubation (T2)

	T_1			F	p	Post Hoc
	A	B	C			
SAP1	110.03 ± 14.16	107.14 ± 12.59	116.89 ± 9.93	3.364	0.040	c
SAP2	119.61 ± 13.95	121.89 ± 13.82	149.00 ± 14.50	29.302	<0.001	b, c
DAP1	67.93 ± 10.99	71.28 ± 10.51	75.31 ± 14.60	2.313	0.106	
DAP2	75.71 ± 12.93	81.56 ± 10.65	98.21 ± 15.01	18.750	<0.001	b, c
MAP1	85.80 ± 13.21	84.22 ± 13.01	91.05 ± 13.17	1.590	0.211	
MAP2	91.06 ± 12.60	96.70 ± 12.49	116.68 ± 14.76	23.292	<0.001	b, c
HR1	97.06 ± 9.88	94.70 ± 9.96	103.15 ± 11.64	3.819	0.026	c
HR2	100.68 ± 8.92	102.41 ± 11.02	109.68 ± 9.61	5.165	0.008	b, c

F-ANOVA,
a (A vs B), b (A vs C), c (B vs C)
Abbreviations: SAP1 – systolic arterial pressure after the induction to anesthesia (mm Hg), SAP2 – systolic arterial pressure after the endotracheal intubation, DAP1 – diastolic arterial pressure after the induction to anesthesia (mm Hg), DAP2 – diastolic arterial pressure after the endotracheal intubation (mm Hg), MAP1 – main arterial pressure after the induction to anesthesia (mm Hg), MAP2 – main arterial pressure after the endotracheal intubation (mm Hg), HR1 – heart rate after the induction to anesthesia (beat per minute), HR2 – heart rate after the induction to anesthesia (beat per minute)

Table 4 Intubation conditions

Score (in % of patients)	Group			χ^2	p
	A	B	C		
Poor	0.0	0.0	5.3		
Good	6.5	18.5	42.1	13.276	0.010
excellent	93.5	81.5	52.6		

χ^2 – chi square test

Table 5 Newborns characteristics

		Group			χ_{KW}^2 /*F	p
		A	B	C		
Ap^1		8.81±0.55	8.81±0.48	8.63±0.49	2.969	0.227
Ap^5		9.03±0.31	8.93±0.26	8.89±0.32	2.972	0.226
Breathing*	immediately	77.4	81.4	73.7		
(% of newborns)	tactile stimulation	12.9	7.4	15.8		
	bag mask ventilation	9.7	11.1	10.5	4.365*	0.359

F-ANOVA

χ_{KW}^2– Kruskal-Wallis test

Abbreviation: Ap^1 – Apgar score in 1st minute, Ap^5 – Apgar score in 5th minute

The greater percent of patients in group A had excellent sore ($\chi^2 = 6.471$; $p = 0.011$), while in group C 42.1% of patients had good score ($\chi^2 = 5.617$; $p = 0.017$). Patients with good score in group A (two patients) had intermediate vocal cords position and moving. All patients with good score in group B (five patients) had intermediate vocal cord position and moving; two of them (7.4%) additionally had slight cough. All patients with good score in group C had intermediate vocal cord position; four of them (21.2%) additionally had not fully relaxed jaw, one (5.3%) slight cough. Patient with poor intubation score had poor jaw relaxation, vocal cords in closed position and slight limb movements.

Newborn characteristics are presented in Table 5, with no differences between groups in any of the estimated variables. All neonates were vital (Apgar score ≥ 8). The reanimation of neonates who did not start to breathe immediately consisted only of brief (1–2 minutes) tactile stimulation or beg- mask ventilation.

Discussion

The number of studies reporting the use of remifentanil during I-D period of caesarean section is increasing. The dosing regiments were different and so were maternal effects and neonatal outcomes [13–15, 17, 18, 21, 23]. The suppression of exaggerated neuroendocrine response to endotracheal intubation and surgical stress was sometimes achieved at the expense of maternal hypotension or neonatal respiratory depression and lower first minute Apgar scores, so optimal remifentanil dosing regimen was yet to be determined.

In presented article we compared the effects of two remifentanil dosing regimens with remifentanil-free control, (meaning traditionally performed anesthesia, with omission of opioids during I-D interval), hypothesizing that remifentanil beneficial effects could justify its use. Group B received remifentanil bolus just before the induction to anesthesia. In group A remifentanil bolus was followed by infusion, meant to extent its analgesic effect to whole I-D period. The infusion was interrupted after skin incision; taking into account the average length of I-D period of caesarean section performed at our Clinic (10–11 min) and remifentanil context sensitive half-time of 3 min, we believed this should leave enough time for remifentanil redistribution and metabolism in fetal circulation, thus diminishing the probability of neonatal respiratory depression.

According to our results both remifentanil dosing regimens successfully blunted maternal hemodynamic response to endotracheal intubation. SAP, DAP, MAP and HR, measured 30 s after the intubation, were significantly higher in group C than in groups A and B. The elevation of blood pressure after endotracheal intubation was even less in group A than in group B, but at this point of the operation the difference did not reach statistical significance (this will be reached as soon as at skin incision, but further analysis of maternal hemodynamic was beyond the scope of this article).

Intubating conditions (Table 5) in group A were also significantly better than in other groups. The excellent conditions were noted in 93.5% patients, compared to 81.5% in group B and 52.6% in group C; the difference between groups B and C was significant as well.

This finding did not come as a surprise, because it is known that remifentanil, used with propofol or thiopentone to facilitate endotracheal intubation, is an acceptable alternative to neuromuscular blocking drugs, since it may potentiate depression of the laryngeal reflexes [24]. Remifentanil boluses of 2–4 μg/kg (depending on a study) with propofol 2 mg/kg or thiopentone 5 mg/kg, provided satisfactory or excellent intubating conditions [25–28]. Remifentanil-hypnotics synergism could be particularly useful in cases in which muscle relaxants are contraindicated, e.g. myopathies or choline-esterase enzyme deficiency [28]. Alexander et

al. [29] reported successful use of 0.5 μg/kg remifentanil followed by 0.25 μg/kg/min, together with thiopentone for endotracheal intubation in a parturient with suxamethonium apnea.

Even when used with muscle relaxants, as in our study, remifentanil could be of great help in ameliorating intubating conditions, especially in cases where difficult intubation is anticipated. Due to physiologic changes of pregnancy, like airway edema, enlarged breasts, weight gain, change in Mallampati score, the risk of difficult/failed intubation is increased [1, 3]. The incidence of difficult and failed intubation in obstetric patients is 1–6% and 0.13–0.6% respectively (0.13–0.3% in general surgical population); additionally, the tolerance to apnea is reduced as a consequence of reduced pulmonal functional residual capacity and increased metabolic rate and oxygen consumption. This could lead to respiratory complications, like coughing, bucking, laryngospasm and bronchospasm, hypercarbia and hypoxia [1, 7, 10], making airway management problems one of the leading causes of anesthesia-related maternal mortality [2]. According to our results, the addition of remifentanil will help provide smooth endotracheal intubation, ameliorate intubation conditions and attenuate excessive hemodynamic response. This effect could be particularly beneficial in parturients with serious comorbidities, e.g. preeclampsia, but also in healthy obstetric population.

Opposite to the data from the literature [15, 17, 18, 21, 23], remifentanil regimens applied in our study did not affect neonatal outcome. First minute Apgar scores were ≥8 in all cases, without difference between groups. Majority of neonates (77.4% in group A, 81.4% in group B, 73.7% in group C) started breathing within few seconds after delivery. Resuscitative measures applied to the neonates with respiratory depression consisted of tactile stimulation and brief beg-mask ventilation with no significant difference between groups. There was no need for endotracheal intubation or for naloxone administration, and no muscular rigidity was observed.

Conclusion

Our dosing regimen of remifentanil 1μg/kg bolus given immediately before the induction followed by 0.15 μg/kg/min interrupted after skin incision provided the best compromise between achievement of excellent intubating conditions, attenuation of maternal blood pressure and heart rate response to endotracheal intubation, and avoidance of neonatal respiratory depression.

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Original Article

ACNE VULGARIS – ADEQUATE AND TIMELY THERAPY AS AN EARLY PREVENTION OF PSYCHOSOCIAL DISTURBANCES

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Abstract. *Acne is a polyetiological chronic disease of pilosebaceous units that affects 80% to 90% of teenagers and adolescents. It is manifested as mild, moderate, or severe form. Since adolescence is time of psychological, emotional and social personality development, the appearance of acne, most frequently on face, demands long-term treatment, significantly affects psychologic and emotional state, creating the feeling of being marked and leading to depression, anxiety, social isolation and negative effect on the quality of life. Timely education, with general information on the causes, duration of the disease and adequate treatment can significantly affect patients' relation to the disease and reduction of psycho-social problems. The analysis included 220, 39 (55%) males and 60 (45%) females, 14 to 30 years of age (or more), most frequently 16 to 20 years old with moderate form of the disease. Therapy was applied according to valid protocols. Each patient was given full attention with the explanation of the nature and course of the disease. The largest number of patients had the expected results, which was mutually appreciated. It was concluded that individual approach and cooperation during the treatment of each patient were necessary.*

Key words: *Acne vulgaris, therapy, psychosocial disturbances, quality of life.*

Introduction

Acne is a polyetiological chronic disease of pilosebaceous units. The etiopathogenesis involves the following: 1. elevated sebum production stimulated by the effects of androgynous hormone, 2. elevated follicular proliferation and keratinocyte differentiation, 3. colonization of *Propionibacterium acnes*, 4. inflammation induction. Acnes are manifested as: mild form (A. comedonica), moderate (A. papulosis and A. nodules-small nodule), and as a severe form (nodulo-cystica and A. conglobata). The presence of acne (most frequently on face, chest and back) demands long-term treatment. It has been estimated that 80–90% of teenagers and adolescents suffer from acne, although they can be present during adulthood as well [1, 2]. Adolescence is the period of psychic, emotional and social personality development [3, 4]. Acnes strongly affect [5, 6] psychological and emotional wellbeing, creating the self-image and the feeling of being marked, which leads to frustration, anger, depression, anxiety, social isolation, life-long problems with confidence and self-respect, with higher unemployment rate, limited choice of work place, inability of promotion, inappropriate comments. As a result, negative effect on the quality of life of the patient is registered.

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The Aim

The aim is the analysis of patients treated for acne (gender, age, disease type, applied therapy, treatment success).

Materials and Methods

The analysis included 220 outpatients. Therapy was applied according to the valid protocol: topical retinoids (keratinocyte proliferation, inflammation), oral retinoids (sebum production, keratinocyte proliferation, *Propionibacterium acnes* colonization, inflammation), azelaic acid (keratinocyte proliferation, *P. acnes* colonization and inflammation), topical and oral antibiotics (P acnes colonization and inflammation), benzoyl peroxide (sebum production, keratinocyte proliferation, *P. acnes* colonization), hormones (sebum production) and alpha HA and beta HA (keratinocyte proliferation). Each patient was given a detailed explanation of the nature of their disease, its mechanism of appearance, duration, therapeutic possibilities, results and their personal contribution to the successful treatment.

Results

Results are present in tables.

Table 1 presents the structure of treated patients according to gender. Out of 220 treated patients, 87 were male (39.55%) and 133 (60.45%) were female.

Table 1 Gender structure

Gender	Male	Female	Total
Number	87	133	220
Percentage	39.55	60.45	100.00

Table 2 Age structure

Age	Up to 15	16–20	21–25	26–30	Over 30	Total
Number	30	109	55	17	9	220
Percentage	13.64	49.54	25.00	7.73	4.09	100.00

Table 3 Type of the disease

Form	Mild	Moderate	Serious			Total
Type	Type I A. comedonica	Type II A. papulopustulosa	Type III A. nodularis	Type IV A. nodulocystica	Type IV A. conglobata	
Number	33	91	61	24	11	220
Percent.	15.00	41.36	27.73	10.91	5.00	100.00
Total/%	15.00	69.09		15.91		100.00

Table 4 Gender structure of the isotretinoin treated patients

Gender	Male	Female	Total
Number	13	3	16
Percentage	81.25	18.75	100.00

Table 5 Age structure of the isotretinoin treated patients

Age	Up to 15	16–20	21–25	Total
Number	3	9	4	16
Percentage	18.75	56.25	25.00	100.00

Table 6 Type of acnes in isotretinoin treated patients

Form	Moderate	Serious		Total
	Type II A. papulopustulosa	Type IV A. nodulocystica	Type IV A. conglobata	
Number	1	7	8	16
Percentage	6.25	43.75	50.00	100.00
Total/%	6.25	93.75		100.00

Table 2 presents the age structure of the treated patients. The majority of the treated patients was in the age group of 16–20 years (109 – 49.54%), then 21–25 years (55 – 25.00%) and to 15 years (30 – 13.64%), with reduced numbers for 26–30 years (17 – 7.73%) and over 30 (9 – 4.09%).

Table 3 presents the disease structure according to types depending on intensity. Most frequently it was Moderate, type II – Acne papulopustulosa (91 – 41.36%) and type III – Acne nodularis (61 – 27.73%), total 152 – 69.09%. Mild form (type I – Acne comedonica) was present in 33 – 15.00% of the treated patients. Serious forms: Acne nodulocystica (type IV) had 24 – 10.91% of the treated, and Acne conglobata (Type IV) 11 – 5.00%, total of 35 – 15.91% of the treated patients.

Table 4 shows the structure according to gender of those treated with isotretinoin. Out of 16 treated 13 patients (81.25%) were male and 3 (18.75%) were female.

Table 5 shows the age structure of the patients treated with isotretinoin. The majority, 9 patients(56.25%) was in the category of 16 – 20 years, then 4 – 24% from 21 – 25 years and 3 – 18,75% up to 15 years.

Table 6 presents the disease structure according to types depending on intensity. Moderate form –Type II, Acne papulopustulosa was found in 1 patient (6.25%). Serious forms – Type IV, Acne nodulocystica were found in 7 patients (43.75%) and Acne conglobata was present in 8 patients (50.00%), total 15 (93,75%) patients.

Great majority of patients had the expected results (figures) which was mutually appreciated. Figures: 1A, 2A, 3A (at the beginning of treatment); 1B (After one month), 2B (After 2 months), 3B (4 months after the treatment with isotretinoin).

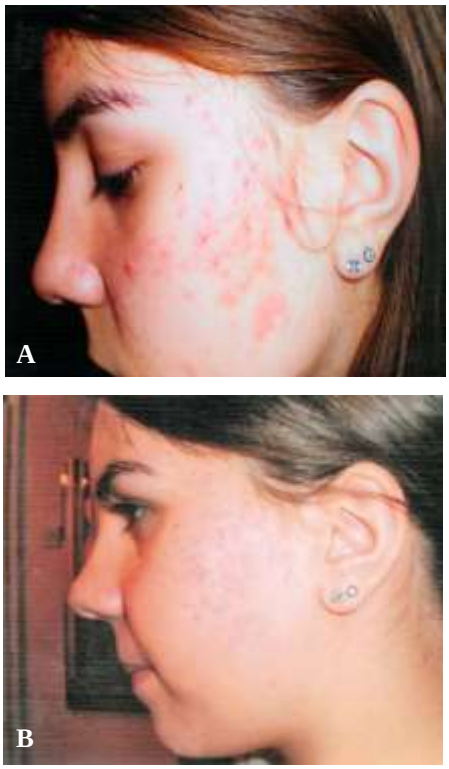

Fig. 1 A – At the beginning of the treatment, B – after one month

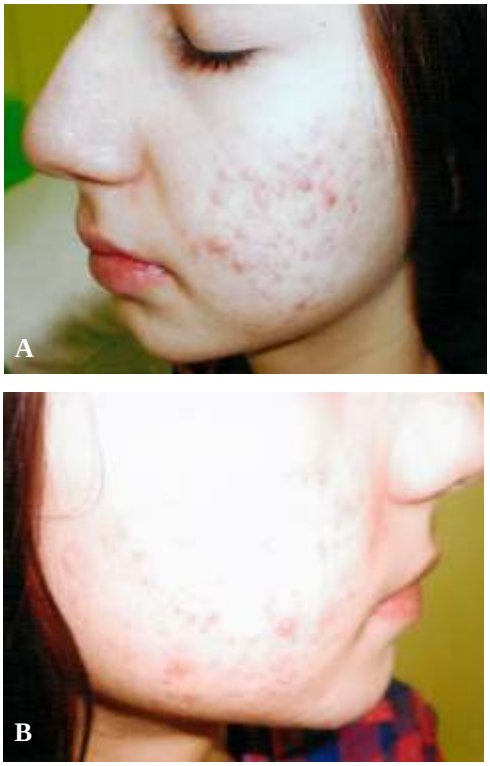

Fig. 2 A – At the beginning of the treatment, B – After 2 months

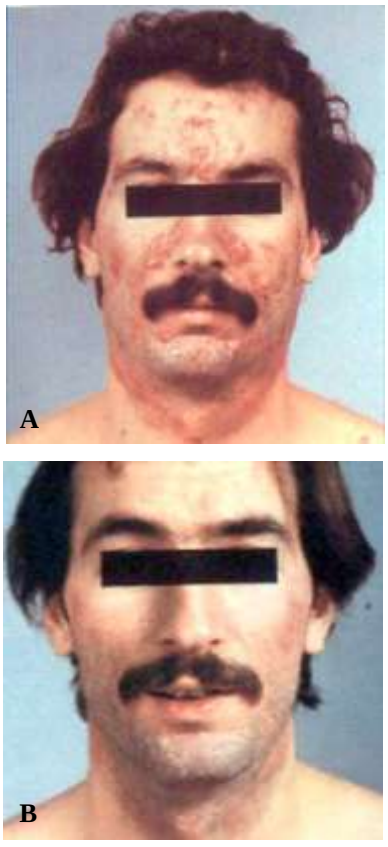

Fig. 3 A – Before the treatment, B – 4 months after the treatment with isotretinoin

Discussion

The treatment was conducted with 220 patients aged between 15 to 35. The largest number of patients was in the category of 16 to 20 years of age (49.54%). There were more female patients (f:m = 60, 45%:39, 55%). The greatest number had a moderate form (69. 9%). All the patients had full attention during the treatment and there was also an attempt to affect their behavior and acceptance of the reality with the aim of overcoming the present discomfort.

"There is no other disease which provokes so much psychological trauma, and lack of possibility to improve the relationship between parents and children, so much insecurity and inferiority feeling and so much psychological suffering as acne vulgaris" [7]. Social and economic effects of acne are usually related to a high prevalence of this pathology, which can be marked as a public health problem [8]. More than 2000 studies on the relationship between acne and psychological state of the patients were performed [9]. Emotional problems as well as behavioral ones are determined as double in those affected with acne [10]. Significantly elevated stress level in relationships with other people and in everyday life is evident [11]. People affected with acne have lower level of self-confidence and higher level of depression and anxiety [9]. Higher level of dissatisfaction is registered in persons with facial acne [12]. Clinically significant anxiety was registered in 44% of the affected, while clinically significant depression was registered in 18% [13]. Age, sex and seriousness of acne are closely related to depression [14]. The prevalence of acne grows with age, in girls when they get period. In moderate acne, the higher level of psychic symptoms is more frequent in later periods of puberty [15]. The prevalence of anxiety in the affected is 68.3%. Anxiety and depression rates were not related to the age, sex, marital status and the acne SCOR [16]. Suicidal ideas are not rare in dermatology patients and they can occur in patients with mild skin lesions, as well [17].

The highest prevalence of suicidal ideas is found in psoriasis patients (7.2%), acne (5.6%), atopic dermatitis (2.1%), and in none with alopecia areata [18].

The prevalence of suicidal ideas in the patients with acne is 8% [19] and 7% [17]. Some patients became suicidal even after successful dearmabrasion [20]. The suspicion that isotretinoin used for acne treatment, could provoke depression and suicidal ideas, is not supported by scientific evidence. Acnes are the primary cause of depression and efficient treatment can improve the depression symptoms and reduce the frequency of suicidal ideas [21]. The lack of serious acne treatment with isotretinoin is accompanied with a higher risk for suicide [22]. Stress is a leading factor in the appearance of a large number of chronic non-infectious diseases [23]. It seems that stress causes acne and that acnes cause stress. There is a vicious stress-acne circle [24]. The correlation between the perceived stress and the seriousness of clinical picture of papulopustular acne is

proven. The combination of treatment for acne and controlling stress affects both causes and helps the diseased. Anxiety prevention in adolescence means prevention of many mental disorders later in life [25]. Not only that it is evident that acnes are a visual condition which can provoke various psychosocial effects, but also that mental health problems provoke acne or make them worse [26]. However, the impact of acne on psychological implications of their treatment has not been fully understood, they are often considered to be a cosmetic temporary problem [12]. Considering acne as a cosmetic problem and neglecting psychosocial aspect can be dangerous [27, 28]. It is important to concentrate on the subjective perception of acne treatment without regard to the objective seriousness [11]. The reduction of psychological effects of acne is considered to be one of the leading priciples for their clinical treatment [29].

Timely education with general information on the causes and duration of the disease and early intervention accompanied with adequate treatment, probably psychotherapy and anti-anxiety drugs, can significantly affect patients' attitude towards the disease as well as avoidance and reduction of the psychosocial problems. The quality of life of the patients with acne is on the same level as that in patients with other chronic conditions (asthma, diabetes, epilepsy, arthritis) [30]. Acnes are accompanied with the disturbed quality of life of the affected [31[. Acnes negatively affect the quality of life [32]. In most students the quality of life is moderately harmed [33]. Only 17% of boys and 18% of girls perceive their problems with acne as huge. 15% of students felt depressed and miserable due to their acne [34]. Moderate disturbance of the quality of life was found regardless of sex, while it was worse in the case of a prolonged disease [35]. The effect of acnes on the quality of life of adolescents is more pronounced in serious clinical forms of acnes, more obvious in women [36]. The disease provokes higher psychosocial disturbance in women [37]. The quality of life depends on the degree of the disease as well as on its duration, although in people affected with mild forms of the disease and shorter duration the effect on the quality of life is also registered [38]. It is believed that there is a linear relationship between the clinical seriousness of acne and the quality of life [39]. However, the condition depends on the capability of a person to cope and sometimes persons with mild acnes can have serious subjective symptoms, which affects their quality of life [40]. Acnes can greatly affect patients' lives, regardless of how serious they are [27].

Efficient treatment of acne in combination with appropriate mental health support offers great opportunity for the improvement of the quality of life of people with acne [30].

Conclusion

Individual approach is necessary in the treatment of each patient, with the awareness of etiopathogenesis of the disease and wide array of drugs, with necessary psychotherapy accompanied with the information about the nature and the duration of the disease and the possibility of gaining good results, following necessary cooperation and discipline.

Note: This paper was presented at The First Regional Congress on The Health of the Young, Belgrade 2016.

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Highlights

SOME GUIDELINES FOR WRITING A LETTER TO THE EDITOR

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Abstract. *Letter to the editor is a tool offered to readers most often to react to articles published in a journal. From the standpoint of journals, this genre is very important as it prolongs the process of peer review and maintains the integrity of evidence. These letters have a specific structure often determined by the journals in terms of the number of words, authors, references, figures, and tables. With regard to the style, letters to the editor should be clear, precise and to the point, stating the purpose directly and avoiding unnecessary information. Compared to research articles, letters to the editor rarely use passive constructions and hedging, the most commonly used tense is the present simple and they are often laden with nouns and verbs belonging to the critical style and reflecting strong subjectivity. Although a tool for questioning previously validated research, letters to the editor need to be written in a respectful manner, maintaining the professional level of communication and always having in mind that the purpose is sharing and promotion of knowledge.*

Key words: *letter to the editor, writing skill, structure, style, grammar*.

Introduction

Discussion and exchange of ideas are fundamental to scientific research and progress. Letters to the editor can form an important aspect of the development of such ideas. They enable free expression of opinion, reveal the intellectual vigor of the community concerned, and help shape knowledge [1].

Scientific discourse occurs in many forms - among colleagues, at scientific meetings, during peer review, and after publication. Such discourse is essential to interpreting studies and guiding future research. Letter to the editor is a written way of talking to a journal, newspaper or other regularly printed publication. It is found in the first section of the journal or in the editorial page. For journals, these letters are very important [2]. They serve an important role in post-publication review by maintaining the integrity of evidence. The act of critical appraisal of the literature, an important step of evidence-based practice, may generate letters to the editor. Letters may serve to (1) identify errors or deficiencies and make a correction to the literature, (2) point out alternative theories or additional information not contained in the original article, (3) offer new, additional, or counterevidence to that of the original article, and/or (4) hold authors and journals accountable for their publications [2, 3].

The most frequent reason for writing a letter to the editor is to comment on a published article. Its purpose is to support or criticize the justification, analysis or outcome of the study. The letter should point out to new, not previously considered issues, and represent additional information which refute or support the assertions of other authors. The author of the letter should avoid assuming a personal and biased attitude but base all his suggestions and comments on scientific data and evidence. The criticism should be professional. The letter should contain objective, constructive interpretation or discussions on the area of interest. It should have an objective and transmit a message with brief, clear language. Materials published elsewhere should not be used [4].

In general, letter to the editor should be concise and to the point. The author ties the subject of the letter to a recent article and uses this article for communicating a message. As one of the widely read features in journals, these letters allow an author to reach a wide audience.

Letter to the Editor in Medical Journals

Among the genres identified in medical journals, along with research papers, review articles, editorials, book reviews, case studies, and the news section, letters to the editor are a tool offered to the community to react to other scientists' research and mainly to express personal opinions and disagreement. Letters to the editor offer a freer mode of expression than the classical scientific rhetoric, which is described as objective, purely referential, impersonal, and detached [5].

From mere clarifications aiming to provide further knowledge on a given research topic, letters to the editor gradually became a tool for questioning previously validated research. They have grown as a complementary, and sometimes alternative strategy used to establish a position, and defend it in the scientific community.

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Letters to the editor are a way to express one's opinion or to set something straight but they are not really letters, communication actually builds up between a scientist and his or her community and not between two individuals.

Letters to the editor usually represent a form of contradiction of the research paper. Controversy generally concerns the experimental method selected, the duration of the experiment, the number of experimental subjects, and results too flimsy or too frail to be exploited.

The letter writers require the entire community to witness and even take part in their public debate. Writing letters to the editor may appear to be an irrepressible need for some scientists because it allows them to react swiftly, personally, and sometimes contentiously to issues about which they feel strongly concerned [4].

The Structure and Style of Letters to the Editor

First of all, as with any other piece of writing, a journal may set certain limitations concerning the length of the letter, the number of authors, the number of figures and/or tables, the number of references.

An interesting point is the very low occurrence of passive structures in letters to the editor. Scientific discourse is generally characterized by a heavy use of the passive, especially in the 'Materials and Methods' and the 'Results' sections of papers. The reason for this is to focus on the object of the experiment, rather than on the subject, to give an objective value to the published research. In letters to the editor, on the contrary, the emphasis is put on the choices made by the criticized authors and thereby on the authors of the letters themselves. Thus, selecting active forms to build an argument strongly reinforces the contentious mode [6].

The most common grammatical tense used is the simple present tense, and this again contrasts sharply with the research article, in which majority of the verbs are in the simple past. In letters to the editor, the simple past is used only to report the experiments carried out by the criticized authors or by the authors themselves. The simple present is chosen to express the reality of the article in question and it refers to the established scientific fact.

Scientific discourse is generally used to weigh evidence and draw conclusions from data. Thus, uncertainty and doubt are necessarily present at least in the 'Discussion' section of experimental papers. This is expressed through hedges, which account for various degrees of probability. Scientific discourse deals with the problem of what is true or false. In letters to the editor, in contrast, epistemic modality (hedging) has a low occurrence. The most frequently used modals are *should, could, may,* and *would* and to a lesser extent *can, must, will,* and *might.*

In contrast to epistemic modals, root modals may convey a deontic meaning to indicate a form of moral advice, expressing strong pressure from the utterer on the criticized authors. Root modals serve to express orders, wishes, suggestions, causality, or capacity [4].

In contrast to the depersonalized style observed in the experimental paper, giving vent to direct criticism in letters to the editor is accepted by the community. Some of the commonly used terms include *poorly, mistakenly, biased, emotive, confusing, too simplistic, old and outmoded, artificial, vague, speculative*.

Letters also reveal a massive use of certain nouns and verbs that are absent from the research paper because they belong to the critical style and reflect strong subjectivity. Nouns such as *critique, rebuttal, borderline, reductionism* and *blurring* and verbs such as *refute, rebut, fail to, contend, disagree, reject, challenge* and *invalidate* are common in letters.

In scientific discourse, adjectives mostly express a quantitative value, whereas in letters to the editor, an extensive use of qualitative adjectives can be observed. Most of them carry a negative prefix whose aim to weaken the arguments set out in the paper. Examples of these prefixes are *in-* (inappropriate, inaccurate, inconsistent, incomplete, intemperate, incorrect, implausible, etc.), *un-* (unreliable, unexpected, unproven, unsupported, unclear, unaware, unfounded, unfortunate, etc.), *out-* (outmoded, etc.), *under-* (underpowered, understated, etc.) and *mis-* (misleading, misused, misdirected, etc.).

Specific markers are necessary to build an argument. In letters to the editor, these markers may be classified into four groups that all express disagreement but with different levels of intensity.

Concession - The weakest markers used to contradict somebody's opinion express concession. The most recurrent forms present in letters to the editor are *although, however, but, yet, nevertheless, nonetheless, even if, even though.* These markers are used to diminish or belittle the impact of published observations and conclusions.

Antithesis - In order to express the opposition in a stronger way, the following markers can be used: *but, while, whereas, conversely, by contrast, in contrast, otherwise, instead, unlike, opposite.*

Rewording - Some markers are used to reformulate a previous statement and incite the criticized authors to change their minds and possibly their methods or conclusions. Examples of these markers are *rather, better, more accurately, in other words.*

Doubt - The most subtle way of explicitly questioning a method is to raise doubts concerning the validity of the study. The most common words and expressions used in letters to the editor to mark this are *maybe, perhaps, probably, highly unlikely, wonder whether, far from verified*.

The use of implicit disagreement can be considered to be a less direct way to modulate contradiction. Examples of these forms are: *we find it surprising that, therefore we strongly suggest, therefore we think, I have several comments, I showed clearly, In my opinion, we believe, we are aware, we advocate doing this*.

Letters to the editor are a useful, and even necessary, but not self-sufficient communication tool within the scientific community. They reflect tensions in this community. Their most interesting role is to provide researchers with an outlet for oppositions, controversies, and disagreements [5].

Table 1 shows an example of a letter to the editor published in a scientific journal.

Table 1 Example letter to the editor.

FALLS AND MOBILITY LIMITATIONS IN OLDER PEOPLE: MEASURES OF HIGHER CEREBRAL INTEGRATION ARE ALSO IMPORTANT

To the Editor:

Ferrucci et al draw welcome attention to the importance of undiagnosed and subtle manifestations of neurological disease in older people (1). However, the authors fail to include two common neurological findings that are possibly as important, if not more so, than the individual signs that they describe. The detection of cognitive impairment by formal cognitive screening is a neurological finding in its own right. The very act of excluding 104 patients with cognitive impairment short of dementia (8% of the study sample) may have diluted the predictive power of their study. Cognitive impairment is a potent risk factor for gait imbalance and falls (2) and is often undetected in routine clinical practice (3). Although the reason for exclusion relates to the use of subject recall as an index of falls over the previous 12 months, it would be helpful for further studies to include older people with cognitive impairment that falls short of overt dementia.

An equally important neurological finding that is not included in the study is that of higher-level gait disorders or gait apraxia (4). These conditions have been characterized very well by Tallis and coworkers as a disorder of gait that is out of proportion to what would be expected on bedside neurological examination and is best explained by disorders of integration of cerebral activity (5). There is a reasonably high level of inter-rater reliability in detection of this finding between experienced doctors and physiotherapists (6). It is likely that the key cause in gait apraxia is silent and overt cerebrovascular disease. While Ferrucci et al suggest that radiological changes in white matter may be associated with the findings described in their study, it is even more likely that the changes are associated with gait apraxia in older people (7). In addition, many patients with subtle, undetected signs of upper motor neuron lesions (positive Babinski/Hoffman reflexes or increased tendon reflexes) may also have demonstrated gait apraxia. Conversely, could some of the 68 subjects with a history of falling and "no" neurological findings have had an element of gait apraxia?

The emphasis on careful neurological history and examination for older people with impairment of stability and mobility is welcome. However, it is important that it should routinely include measures of higher cerebral integration—in particular, cognitive function and assessment of gait apraxia.

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*Taken from The American Journal of Medicine, December 15, 2004 Volume 117, Issue 12, p. 971. [7]

After opening the letter, the next step is to grab the readers' attention by stating the reason for writing. The key point is given at the beginning and the importance of the issue is explained. This is immediately followed by stating the evidence for praise or criticism (Table 2). Good practice is to give suggestions about what could be done differently with better results.

Table 2 The opening paragraph illustrating the steps for starting a letter to the editor.

To the Editor:
Leung et all present a 10-year study of patients taking antihypertensives followed for hyponatremia. Their study is an important contribution to the literature on the comparative effectiveness of commonly used hypertensives. We are concerned, however, with 2 aspects of their design, both of which may induce selection bias with the potential to explain their observed results.

*The American Journal of Medicine, December 2012 Volume 125, Issue 12, p. e7. [8]

Some of the useful expressions for writing a letter to the editor are presented in Table 3.

Beside maintaining the clarity and precision in writing a letter to the editor, another very important thing that the author should keep in mind is the need for presenting the ideas and the point of view in a respectful manner, not using the letter simply to "vent". The comments should be objective and they should critically assess the published article, offering scholarly opinion and information relevant to the readers [9].

Table 3 Some expressions commonly used in letters to the editor.

To begin the letter: ▪ I have read with great interest … ▪ I am writing to express my support for / (dis)approval of … ▪ I am writing with regard to … ▪ I am writing about …	To state an opinion: ▪ In my opinion… ▪ I do not believe that … ▪ I strongly (dis)agree with … ▪ I am opposed to … ▪ I am in favor of …
To express consequences / results: ▪ Therefore, … ▪ As a result, … ▪ Consequently, … ▪ Obviously, … ▪ Clearly, …	To list points: - Firstly, … ▪ First of all, … ▪ Secondly, … ▪ Furthermore, … ▪ Finally, …

Conclusion

One of the purposes of letters to the editor is allowing the readers of a journal to comment on recently published articles. These letters may ask important questions of the author of published papers, request clarification about the content, request additional data, provide an alternative viewpoint or criticize [9].

The letter to the editor is important as it allows the peer review process to continue after an article is published. In that way, the authors are held accountable for the content of articles.

Journals typically have instructions for writing a letter to the editor in terms of limits on the number of words, references, tables, figures in a letter.

The writer should focus on the reason for writing, avoiding unnecessary information, assuring the statements are accurate, objective and supported by appropriate arguments and references. Furthermore, even though the purpose of the letter may be criticism, it is imperative to maintain professionalism in communicating ideas and opinions.

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