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Original papers

Reviews

Potential protective effect of N-acetyl cysteine in acoustic trauma: An experimental study using scanning electron microscopy

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Abstract

Background. Oxidative stress has been associated with pathological processes involved in acoustic trauma.

Objectives. In this prospective experimental study, we investigated the potential preventive effect of N-acetyl cysteine (NAC) in rats exposed to acoustic trauma (AT). Light microscopic and scanning electron microscopic (SEM) evaluations were performed.

Material and methods. Healthy Wistar albino rats (n = 18) were divided into 3 groups: group 1 (control group, n = 6), group 2 (acoustic trauma group, n = 6), and group 3 (AT+NAC group, n = 6). The rats in group 2 were exposed to AT. The rats in group 3 received NAC at a dose of 100 mg/kg/day by gavage for 7 days, and then 10 min after the 7th-day dose, they were exposed to AT.

Results. From light and scanning electron microscopy evaluations in the control group, the cochlear structure and epithelium were normal. In group 2 (AT group), extensive hair cell loss was observed in the cochlea by light microscopy evaluation. In the SEM evaluation, various epithelial damage and loss of stereocilia were also observed. In group 3 (AT+NAC group), decreased damage with preserved cochlear structures was seen by light microscopy. In the SEM evaluation, although stereocilia loss was also seen, nearly normal cell structures and vertical and symmetrical alignment of stereocilia structures were observed compared to the AT group.

Conclusions. NAC reduced cochlear damage due to acoustic trauma. Because NAC has antioxidant capacity, AT mat have caused an increase in free radicals and death of outer hair cells. NAC is an antioxidant agent and it prevented cochlear damage due to AT in rats.

Key words: N-acetyl cysteine (NAC), acoustic trauma (AT), light microscopy, scanning electron microscopy (SEM)

Oxidative stress has been associated with pathological processes involved in auditory trauma including mitochondrial injury, activation of cell death pathways, activation of mediators of inflammation, glutamate excitotoxicity, and increased levels of lipid peroxidase.[1–3] These findings suggest that antioxidants have the potential to block molecular cascades that are triggered by auditory trauma, which induces oxidative stress and results in permanent threshold shifts (PTS) and hearing loss.[4]

In blast-exposed ears, cochlear pathology includes scar formation replacing dead hair cells, fused and damaged stereocilia, and in some extreme cases, separation of the organ of Corti from the basilar membrane. Most of the hair cell loss was in the outer hair cell (OHC) region and generally in the middle or basal turn. Although hair cell loss was found in the apical turn, the damage was less pronounced. N-acetyl cysteine (NAC), which was used after blast exposure, significantly reduced the permanent structural and functional damage resulting from exposure to blast overpressure. Drug treatment suppressed both functional and physical damage to the OHCs.[4]

In the present study, we investigated the potential preventative effect of NAC in rats exposed to acoustic trauma (AT). In this experimental study, light microscopy and scanning electron microscopy (SEM) evaluations were performed.

Material and methods

The study occurred at Eskişehir Osmangazi University, Faculty of Medicine. The animals were maintained at the Experimental Animal Breeding and Experimental Studies Centre of Eskişehir Osmangazi University. Adaptation and care of the animals and experimental studies were performed at the same center in compliance with the principles of the Declaration of Helsinki.[5] Ethics committee approval was obtained from Eskişehir Osmangazi University.

Animal subjects

The study was performed in 18 healthy Wistar albino rats, weighing 190–220 g. Each group of rats was housed separately in a climate-controlled room at 20°C. The animals were randomly divided into 3 groups: group 1 (control; n = 6): rats in this group received no drug and were not subjected to AT. Group 2 (n = 6): rats in this group received no drugs and were exposed to AT. Group 3 (n = 6): rats in this group received NAC dissolved in distilled water at a dose of 100 mg/kg/day via gavage for 7 days. Then, 10 min after the 7th-day dose, they were exposed to AT (NAC+AT).

Anesthesia procedure

The rats were anesthetized during experiments via intramuscular injection of 40 mg/kg ketamine hydrochloride (Ketalar, Parke-Davis, USA), and 5 mg/kg xylazine hydrochloride (Rompun, Bayer, Germany). Eye-blink reflexes and respiratory rhythms were followed during the experiments, and deep anesthesia was achieved by repeated doses.

Acoustic trauma (AT)

To create AT in rats with 1–12 kHz band white noise, a MATLAB program that produced a single unit of variance sound was used. Then, the sound was filtered with

Fig. 1. Light microscopy views of cochlea in the control group, acoustic trauma group, and acoustic trauma+NAC groups. a. Control group: normal-appearing cochlea structure; b. Acoustic trauma group: extensive hair cell loss (double arrow), c. Acoustic trauma+NAC group: decreased damage with preserved cochlear structures was seen (sv – scala vestibuli, sm – scala media, st – scala tympani, vestibular membrane [▶], the organ of Corti [→]) (scale bar: 500 µm (a), scale bar: 200 µm (b), scale bar: 500 µm (c), hematoxylin and eosin (H&E) staining)

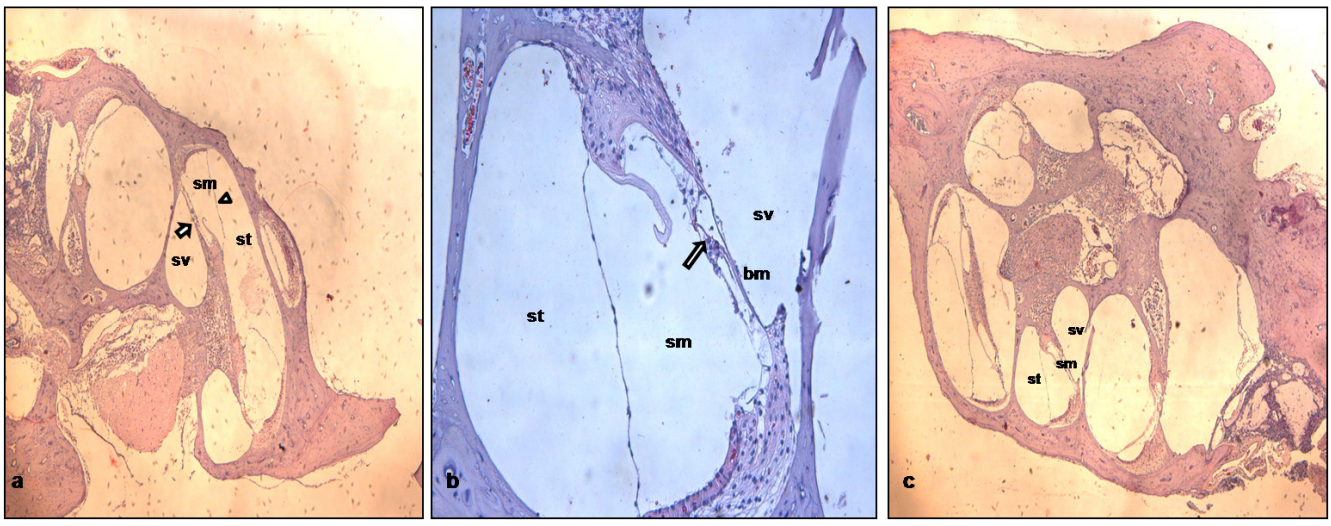
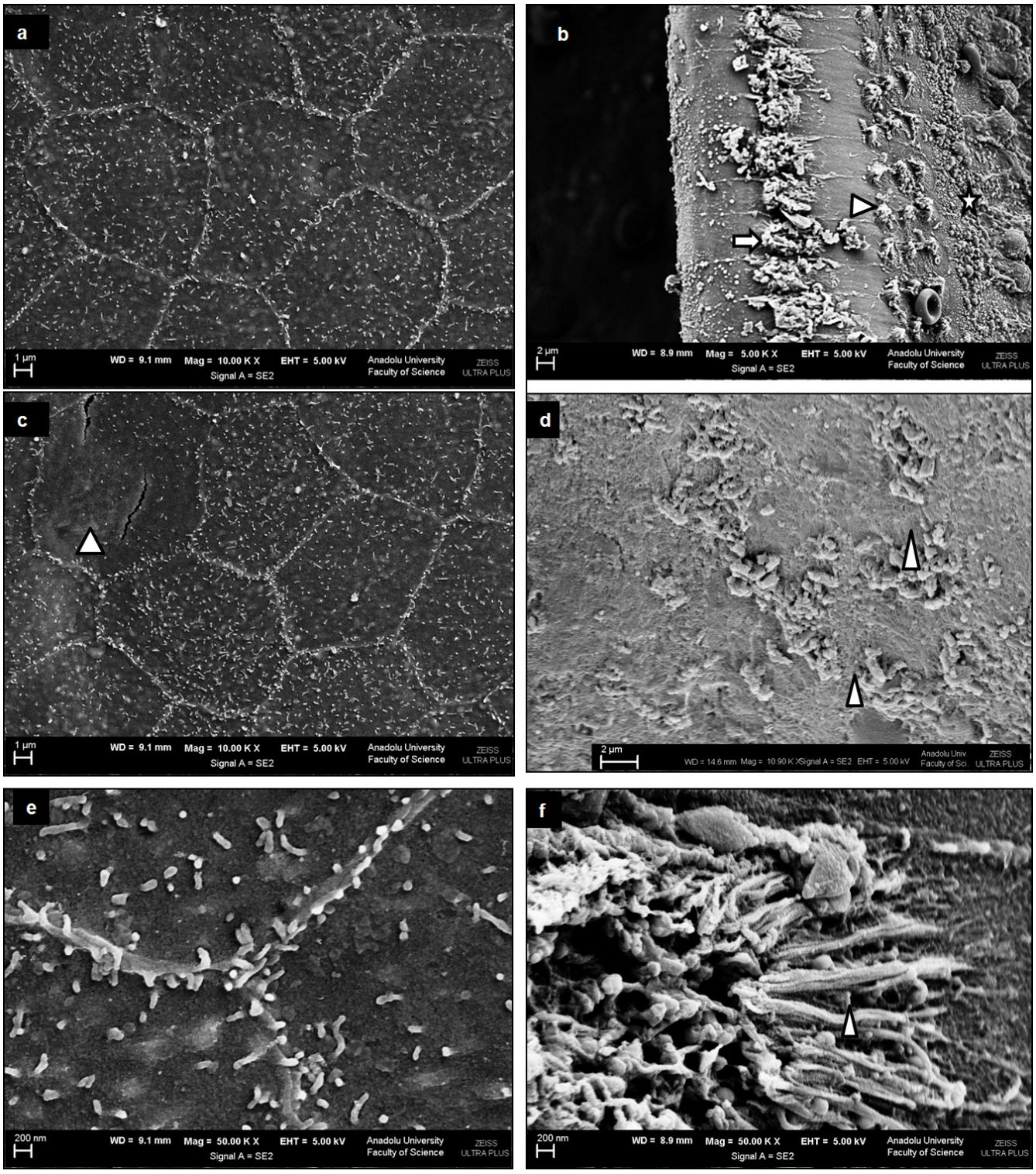


Fig. 2. Scanning electron microscopic (SEM) views of cochlea in the control group, acoustic trauma group, and acoustic trauma+NAC groups. a, b. Control group: in the control group, the SEM view of the epithelium showed a normal structure (a), with outer hair cell (▶), inner hair cells (→) and Hensen's cells (*) (b); c, d. Acoustic trauma group: in cross-sectional images, various epithelial damage and loss of stereocilia were observed (▶) (c, d); e, f. Acoustic trauma+NAC group: although stereocilia loss was seen, nearly normal cell structure and vertical and symmetrical alignment of stereocilia structures were also observed, vs the acoustic trauma group (▶) (e, f) (SEM)



a 1–12 kHz FIR type digital filter, and an additional filter at 200 Hz. Then, the filtered noise was recorded in a computer-based Wav file. Set with a decibel meter, the AT was 110 dB of noise for 6 h (continuous noise).

Electron microscopy evaluation

After the aforementioned procedures were performed, the rats were sacrificed using 80 mg/kg pentothal. The time of sacrifice was 30 min after AT. Immediately after death, the temporal bones were removed, and the otic bullas were excised.[6] Under a dissecting microscope, the bony capsule of the cochlea was carefully removed, and then the lateral wall was cut away to reveal the organ of Corti. We analyzed hair cell damage in 2 different areas of the middle and basal turns.

Scanning electron microscopy analysis

The cochlea and organ of Corti were dissected out and fixed for SEM preparation; all samples were fixed and post-fixed in the same way. Then, the cochlea was dehydrated through a graded ethanol series, prior to desiccation, using a critical-point drying method described by Lovell et al.[7] Fully desiccated samples were mounted on a specimen stub using a carbon tab. After critical-point drying using CO_2 and sputter-coating with gold according to standard procedures, specimens were investigated with a Zeiss Ultra 50 SEM operating at 5 kV accelerating voltage.[7]

Results

Light microscopy and SEM evaluations showed that there was no cochlear damage in group 1 (control group). In group 2 (AT-exposed group), cochlear damage was evident. Inner hair cell stereocilia were missing in some regions, and OHC stereocilia were severely damaged by the noise trauma, with a large number prostrate or missing. The damage was more evident in the OHCs in the basal turns of the cochlea. In group 3 (NAC+AT group), although there was cochlear damage, it was less than in group 2 (AT-exposed group without NAC). In group 3, there were fewer missing cells in the inner and OHC group. There was slight damage to the OHC stereocilia.

Light microscopic evaluation results of groups 1–3 (Fig. 1)

Control group: Normal-appearing cochlear structures were observed (Fig. 1a). Acoustic trauma group: Extensive hair cell loss was detected in the cochlea (Fig. 1b). Acoustic trauma+NAC group: Decreased damage with preserved cochlear structures was seen (Fig. 1c).

Scanning electron microscopy evaluation results of Groups 1–3 (Fig. 2)

Control group: In the control group, SEM views of epithelium showed normal structures (Fig. 2a), outer hair cells, inner hair cells, and Hensen's cells (Fig. 2b). Acoustic trauma group: In cross-sectional images of this group, various epithelial damage and loss of stereocilia were observed (Fig. 2c, d). Acoustic trauma+NAC group: Although stereocilia loss was also present in this group, nearly normal cell structures and vertical and symmetrical alignment of stereocilia structures were also observed versus the acoustic trauma group (Fig. 2e, f).

Discussion

Acoustic overstimulation of the cochlea generates free radicals and reactive oxygen species (ROS), which may induce OHC death.[8–10] Elevated levels of ROS activate the upregulation of cochlear antioxidant enzyme activity and modulate the key antioxidant compound, GSH.[11–13] Various agents with antioxidant properties have been shown to attenuate threshold shifts and/or hair cell loss after the exposure to damaging noise.[14]

Oxidative stress plays a crucial role in the pathogenesis of NIHL and NIT. During exposure to excessive noise, the OHCs suffer metabolic depletion, leading to the accumulation of ROS and reactive nitrogen species (RNS), which may ultimately lead to necrosis and/or apoptosis.[15] While necrosis is a passive form of cell death, usually occurring after gross physical or chemical insult, associated with cell swelling and eventually causing cell rupture and loss of function, apoptosis is an active process of cell death which also occurs under normal metabolic circumstances.[16] Oxidative stress from the production of excessive amounts of free radicals and nitric oxide and peroxynitrite is involved in noise-related injury.[8,16,17] Oxidative damage is also associated with medication-induced hearing impairment.[18]

In the present study, we investigated the potential preventative effect of NAC in rats exposed to AT. Transmission and scanning electron microscopy evaluations were performed. Our light microscopy and SEM evaluations showed no cochlear damage in group 1 (control group). In group 2 (AT-exposed group), cochlear damage was detected. The inner hair cell stereocilia were missing in some regions, and the OHC stereocilia were severely damaged by noise trauma with a large number prostrate or missing. The damage was more evident in the OHCs in the basal turns of the cochlea. In group 3 (NAC+AT group), although there was cochlear damage, it was less than in group 2 (AT-exposed group without NAC). In group 3, there were fewer missing cells in the inner and OHC group. There was slight damage in the OHC stereocilia.

In light microscopic and SEM evaluations of the control group, the cochlear structure and epithelium were normal. In group 2 (AT group), extensive hair cell loss was observed in the cochlea by light microscopy. In the SEM evaluation, various epithelial damage and loss of stereocilia were also observed. In group 3 (AT+NAC group), light microscopy showed decreased damage with preserved cochlear structures. In the SEM evaluation, although stereocilia loss occurred in this group, nearly normal cell structure and vertical and symmetrical alignment of stereocilia structures were also observed compared to the AT group.

Single agents have demonstrated some preventative benefits against noise-induced damage and medication-related ototoxicity. Vitamin E reduces cochlear damage from these factors.[19,20] NAC can also protect the inner ear apparatus.[21,22] Coenzyme Q10 and vitamin C both have shown damage-preventing properties.[23,24] High-dose NAC has been combined with acetyl-L-carnitine before and after noise exposure to attenuate hearing loss.[25,26]

The use of NAC has been validated in preventing noise-induced permanent threshold shift (NIPTS).[14] Ewert et al. reported that a combination of antioxidants, 2,4-disulfonyl α-phenyl tertiary butyl nitrone (HPN-07) and NAC could enhance temporary threshold shift (TTS) recovery and prevent permanent threshold shift (PTS) by reducing damage to the mechanical and neural components of the auditory system when administered shortly after blast exposure.[27]

In the present study, NAC reduced the cochlear damage due to AT. Because NAC has antioxidant capacity, it is possible that AT caused increased free radicals and death of OHCs. NAC is an antioxidant agent and prevents cochlear damage due to AT in rats. If it is used before AT, its preventative effects are more obvious. Thus, we recommend the use of NAC in places with high noise exposure.

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Chosen single nucleotide polymorphisms (SNPs) of enamel formation genes and dental caries in a population of Polish children

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Abstract

Background. It is increasingly emphasized that the influence of a host's factors in the etiology of dental caries are of most interest, particularly those concerned with genetic aspect.

Objectives. The aim of the study was to analyze the genotype and allele frequencies of single nucleotide polymorphisms (SNPs) in *AMELX*, *AMBN*, *TUFT1*, *TFIP11*, *MMP20* and *KLK4* genes and to prove their association with dental caries occurrence in a population of Polish children.

Material and methods. The study was performed in 96 children (48 individuals with caries – "cases" and 48 free of this disease – "controls"), aged 20–42 months, chosen out of 262 individuals who had dental examination performed and attended 4 day nurseries located in Poznań (Poland). From both groups oral swab was collected for molecular evaluation. Eleven selected SNPs markers were genotyped by Sanger sequencing. Genotype and allele frequencies were calculated and a standard χ^2 analysis was used to test for deviation from Hardy-Weinberg equilibrium. The association of genetic variations with caries susceptibility or resistance was assessed by the Fisher's exact test and $p \leq 0.05$ was considered statistically significant.

Results. Five markers were significantly associated with caries incidence in children in the study: rs17878486 in *AMELX* ($p < 0.0001$), rs34538475 in *AMBN* ($p < 0.0001$), rs2337360 in *TUFT1* ($p < 0.0001$), and rs2235091 ($p = 0.0085$) and rs198969 ($p = 0.0069$) in *KLK4*. Genotype and allele frequencies indicated both risk and protective variants for these markers.

Conclusions. Single nucleotide polymorphisms in *AMELX*, *AMBN*, *TUFT1*, *KLK4* genes may be considered as a risk factor for dental caries occurrence in Polish children.

Key words: children, single nucleotide polymorphism, dental caries, enamel formation genes

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Dental caries is a complex and multifactorial chronic disease that develops by the interaction of variables over time, and currently remains the most common disorder of childhood.[1,2] The pathology develops in abnormal conditions of environmental stability on the tooth surface, i.e. when there is imbalance between the process of demineralization and remineralization of the enamel.[2] At present, the disease remains a serious health and social problem.

It is increasingly emphasized that the influence of a host's factors in the etiology of dental caries are of most interest, particularly those concerned with genetic aspect. Literature data describes possible association between the genes responsible for production of different enamel proteins and dental caries occurrence.[1,4–13]

Mature tooth enamel consists almost completely of inorganic material (above 90%). However, during the development it is composed of organic matrix, which is mostly replaced by mineral compounds.

Numerous chemical and physiological processes, such as protein secretion, assembly, folding and degradation, mineral growth as well as gene expression are responsible for the production of the tooth enamel.[14] The extracellular proteins situated between dentin and ameloblasts control the initiation, organization and orientation of crystals in the enamel.[14]

Ameloblasts, which are cells producing the enamel, are of epithelial origin and their activity is strictly dependent on the differentiation of odontoblasts, i.e. dentin-making cells of mesenchymal origin.[14] Ameloblasts during the secretory stage produce and secrete proteins into the matrix of the enamel, which are exchanged by phosphate and calcium at the maturation stage.[15] The most significant secretory proteins may be divided into amelogenin and nonamelogenin proteins.[15] Amelogenin constitutes more than 90% of the extracellular matrix protein content and is suggested to control the oriented growth and organization of enamel crystals.[14,16] This protein is expressed from genes on the Y and X chromosomes, however, approx. 90% of all RNA transcripts are from the chromosome X.[17] Nonamelogenins include ameloblastin, enamelin and tuftelin. Ameloblastin is functioning as cell adhesion protein and is responsible for maintaining rod integrity and controlling cell differentiation.[14] Enamelin is involved, in cooperation with amelogenin, in controlling elongated growth and mineral nucleation[14], whereas tuftelin is proposed to be a potential nucleator of enamel crystallites.[18] It is interesting that currently 2 other enamel matrix proteins were discovered, odontogenic ameloblast-associated (ODAM/APIN) and amelotin.[15]

During the maturation stage the matrix is quickly degraded since at that time apatite crystals grow primarily in thickness and width, and finally it is removed from the extracellular space and the mineralization is completed.[15]

Ameloblasts secrete also proteinases, such as matrix metalloproteinase-20 and kallikrein-related peptidase-4, which function during different stages of amelogenesis and cause amelogenin and other enamel proteins degradation.[14]

The aim of the study was to analyze the genotype and allele frequencies of single nucleotide polymorphisms (SNPs) in *AMELX* (amelogenin, X isoform), *AMBN* (ameloblastin), *TUFT1* (tuftelin), *TFIP11* (tuftelin-interacting protein 11), *MMP20* (matrix metalloproteinase-20) and *KLK4* (kallikrein-related peptidase-4) genes and to prove their association with dental caries occurrence in a population of Polish children.

Material and methods

Determination of caries phenotype

Study subjects were recruited from 4 day nurseries which constitute 1 institution, located in the city of Poznan, in the Wielkopolska Province, Poland. Caries was diagnosed by visual examination, using a probe and a dental mirror to confirm such a change. It was carried out from April to June 2014 by 1 trained and calibrated dentist, a specialist in pediatric dentistry after calibration by an experienced specialist. Dental evaluation concerned the occurrence of teeth with carious cavities (dt) as well as teeth with initial (incipient) caries lesions (non-cavitated, white spot) (d_i). The intra-examiner agreement was evaluated by another dental examination in 10 children after 2 weeks, with a κ of 1.00. In total, 262 children who attended the day nurseries and whose parents gave written informed consent were examined. Oral swabs for DNA extraction were collected during the examination. In cases when the quality of the biological material for molecular analysis was poor, the sample was taken once again on subsequent visits, up to 2 or 3 times. In 48 subjects dental caries was diagnosed ($dt + d_i \geq 1$), and they were classified as the study group ("cases"). In those children 1 child had, apart from carious lesions, 1 filled tooth. From the remaining 214 subjects, who were free of the disease ($dt + d_i = 0$), and had no filled teeth, the control group of 48 counterparts was selected ("controls"). The age of all individuals was 20–42 months and the study and control subjects were matched by gender, age and the number of erupted teeth. The specimens from children of other than Caucasian ethnic group were excluded from this study.

Sample collection and genotyping

Oral swabs were provided to each subject in sterile packs. The inside of the mouth was rubbed at least 10 times from each side of both cheeks and then the swab was placed inside the 1.5 mL Eppendorf tube and placed at +4°C in a portable fridge. Genomic DNA was extracted from buccal cells using the column system from EXTRACTME DNA Swab & Semen Kit from Blirt S.A., according to the manufacturer's protocol. DNA extraction was carried out the same day when the oral examina-

tion was done and the DNA samples were frozen in -20°C until further analyses.

We performed genotyping of 11 single nucleotide polymorphisms (SNPs) in 6 enamel formation genes, as followed: rs17878486 in *AMELX*, rs34538475 and rs4694075 in *AMBN*, rs3790506, rs4970957 and rs2337360 in *TUFT1*, rs134136 and rs5997096 in *TFIP11*, rs1784418 in *MMP20*, rs2235091 and rs198969 in *KLK4*. All genetic markers were genotyped by the real-time polymerase chain reaction (PCR) using the TaqMan method (Life Technologies), in which 2 probes – each for 1 allele in a 2-allele system – discriminate between 2 polymorphic variants. SNPs selected for the study are listed in Table 1.

The quantitative real-time PCRs were performed on the 96-well optical reaction plates using the 7900HT real-time PCR system (Applied Biosystems), following the manufacturer's protocol. For each sample reactions were carried out in a 25 µL total reaction volume, using 12.5 µL of TaqMan genotyping master mix, 1.25 µL of ×20 probe and 11.25 µL of genomic DNA. DNA samples were adjusted to 10 ng per 11.25 µL by diluting the sample with appropriate amount of nuclease-free water (Ambion). The TaqMan probes were shipped in ×40 concentration and were diluted to ×20 working stocks with ×1 TE buffer, prior to the analyses. The cycling conditions were as follows: an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min. Allelic discrimination data was captured with ABI SDS software (Life Technologies).

Table 1. Single nucleotide polymorphisms analyzed for candidate genes in the study

No.	Gene	Genetic marker	Chromosome (Chr.) position	MAF (minor allele frequency)
1.	AMELX	rs17878486	Chr.X:11313948	0.11
2.	AMBN	rs34538475	Chr.4:71471176	0.19
3.		rs4694075	Chr.4:71466914	0.48
4.	TUFT1	rs3790506	Chr.1:151538366	0.25
5.		rs4970957	Chr.1:151517388	0.25
6.		rs2337360	Chr.1:151514603	0.25
7.	TFIP11	rs134136	Chr.22:26899474	0.34
8.		rs5997096	Chr.22:26895957	0.47
9.	MMP20	rs1784418	Chr.11:102484396	0.41
10.	KLK4	rs2235091	Chr.19:51410471	0.34
11.		rs198969	Chr.19:50910546	0.43

Statistical analysis

Genotype and allele frequencies were calculated and a standard χ^2 analysis was used to test for deviation from Hardy-Weinberg equilibrium. The association of genetic variations with caries susceptibility or resistance was assessed by the Fisher's exact test and $p \leq 0.05$ was considered statistically significant. Odds ratio (OR) values were calculated for the minor allele and for the heterozygote and minor homozygote. All calculations were performed so as to put the group expected to have higher number of observations in the first column, so the OR results could be interpretable directly.[19] In addition, we used HaploView 4.2 software to test pairwise association between studied single nucleotide variations, using Gabriel et al. algorithm and shown as R^2 value.[20] We defined SNP pairs to be in "strong LD" (LD-linkage disequilibrium) when the lower bound of R^2 (coefficient of determination) value was above 0.7 (70%).[20]

For a statistical analysis relating to the differences between study and control group, as to age the student's t-test was used, as to gender the Pearson's χ^2, whereas as to number of erupted teeth Mann-Whitney U test. The statistical analysis was done with the use of STATISTICA v. 8.0. The assumed level of statistical significance was $p < 0.05$.

Ethical Committee approval

The study was approved by the Ethics Committee of Poznan University of Medical Sciences.

Results

Homogeneity of the study and control group

In the study group there were 25 females (52.08%) and 23 males (47.92%) when in the controls respectively, 24 (50.00%) and 24 (50.00%), and the differences were not statistically significant ($p = 0.838$).

The statistically insignificant differences ($p = 0.532$) were observed in the children's age since in the study and control group it amounted from 20 to 42 months, but the mean ± SD (standard deviation) was respectively, 30.58 ± 5.91 and 29.85 ± 5.48. In the "cases", females were aged 20–40 months (30.88 ± 5.72), while males 20–42 months (30.26 ± 6.22), whereas in "controls" girls were 20–38 months old (28.33 ± 5.40) and boys were 20–42 months old (31.38 ± 5.23).

Cases had from 11 to 20 erupted teeth (mean ± SD = 18.56 ± 2.29), while controls had 12–20 erupted teeth (mean ± SD = 18.14 ± 2,16), and the differences were not statistically significant ($p = 0.265$).

Table 2. Summary of allele and genotype frequency related to caries experience in 11 studied SNPs

Genetic marker	Genotypes			Cases vs control		Alleles		Cases vs control	
				OR [CI 95%]	p-value			OR [CI 95%]	p-value
rs17878486 *AMELX* Caries n = 48 Control n = 48	CC	CT	TT			C	T		
	8 (16.7%)	10 (20.8%)	30 (62.5%)	CC: 0.11 [0.04–0.2]	< 0.0001***	26 (27.1%)	70 (72.9%)	T: 10.2 [5.2–20]	< 0.0001***
	31 (64.6%)	14 (29.2%)	3 (6.2%)	TT: 25 [6.8–92.4]	< 0.0001***	76 (79.2%)	20 (20.8%)		
rs34538475 *AMBN* Caries n = 48 Control n = 48	GG	GT	TT			G	T		
	27 (56.3%)	17 (35.4%)	4 (8.3%)	GG: 5.6 [2.2–14]	0.0003***	71 (74.0%)	25 (26.0%)	G: 7 [3.7–13]	< 0.0001***
				control vs cases				control vs cases	
	9 (18.8%)	10 (20.8%)	29 (60.4%)	TT: 17 [5.2–54.4]	< 0.0001***	28 (29.2%)	68 (70.8%)	T: 6.9 [3.7–13]	< 0.0001***
rs4694075 *AMBN* Caries n = 48 Control n = 48	CC	CT	TT			C	T		
	16 (33.3%)	20 (41.7%)	12 (25.0%)	CC: 1.5 [0.6–3.6]	0,3703	52 (54.2%)	44 (45.8%)	C: 1,5 [0.9–2.7]	0.1496
	12 (25.0%)	18 (37.5%)	18 (37.5%)	CT: 1.2 [0.5–2.7]	0.6765	42 (43.8%)	54 (56.2%)	T: 0.7 [0.4–1.2]	0.1496
rs3790506 *TUFT1* Caries n = 48 Control n = 48	AA	AG	GG			A	G		
	2 (4.2%)	18 (37.5%)	28 (58.3%)	AG: 0.8 [0.4–1.9]	0.6765	22 (22.9%)	74 (77.1%)	G: 1.5 [0.8–2.9]	0.1953
	5 (10.4%)	20 (41.7%)	23 (47.9%)	GG: 1.5 [0.7–3.4]	0.3074	30 (31.2%)	66 (68.8%)		
rs4970957 *TUFT1* Caries n = 48 Control n = 48	AA	AG	GG			A	G		
	18 (37.5%)	29 (60.4%)	1 (2.1%)	AG: 2.3 [1–5.3]	0.0428*	65 (67.7%)	31 (32.3%)	G: 1.5 [0.8–2.9]	0.2004
	27 (56.2%)	19 (39.6%)	2 (4.2%)	GG: 0.5 [0.04–5.6]	0.5651	73 (76.0%)	23 (24.0%)		
rs2337360 *TUFT1* Caries n = 48 Control n = 48	AA	AG	GG			A	G		
	36 (75.0%)	11 (22.9%)	1 (2.1%)	AG: 0.06 [0.02–0.16]	< 0.0001***	83 (86.5%)	13 (13.5%)	A: 4.6 [0.1–0.4]	< 0.0001***
	8 (16.7%)	40 (83.3%)	0 (0.0%)	AA: 15 [0.1–77.1]	< 0.0001***	56 (58.3%)	40 (41.7%)		
rs134136 *TFIP11* Caries n = 48 Control n = 48	CC	CT	TT			C	T		
	15 (31.2%)	25 (52.1%)	8 (16.7%)	CT: 1.2 [0.5–2.6]	0.6832	55 (57.3%)	41 (42.7%)	T: 1.2 [0.7–2.1]	0.5568
	18 (37.5%)	23 (47.9%)	7 (14.6%)	TT: 1.2 [0.5–2.6]	0.7788	59 (61.5%)	37 (38.5%)		
rs5997096 *TFIP11* Caries n = 48 Control n = 48	CC	CT	TT			C	T		
	36 (75.0%)	12 (25.0%)	0 (0.0%)	CT: 1.3 [0.5–3.3]	0.6276	84 (87.5%)	12 (12.5%)	T: 1.2 [0.5–3]	0.6508
	38 (79.2%)	10 (20.8%)	0 (0.0%)			86 (89.6%)	10 (10.4%)		
rs1784418 *MMP20* Caries n = 48 Control n = 48	CC	CT	TT			C	T		
	20 (41.7%)	23 (47.9%)	5 (10.4%)	CT: 0.8 [0.3–1.7]	0.5405	63 (65.6%)	33 (34.4%)	T: 0.9 [0.5–1.6]	0.6519
	17 (35.4%)	26 (54.2%)	5 (10.4%)	TT: 1 [0.3–3.7]	1	60 (62.5%)	36 (37.5%)		
rs2235091 *KLK4* Caries n = 48 Control n = 48	GG	GA	AA			G	A		
	10 (20.8%)	18 (37.5%)	20 (41.7%)	GA: 0.9 [0.4–2.1]	0.8339	38 (39.6%)	58 (60.4%)	G: 2.3 [1.2–4.4]	0.0085**
	1 (2.1%)	19 (39.6%)	28 (58.3%)	GG: 12.4 [1.5–101]	0.0189*	21 (21.9%)	75 (78.1%)		
rs198969 *KLK4* Caries n = 48 Control n = 48	CC	CG	GG			C	G		
	14 (29.2%)	23 (47.9%)	11 (22.9%)	CG: 0.9 [0.4–2]	0.8382	51 (53.1%)	45 (46.9%)	G: 2.4 [1.3–4.3]	0.0049**
	23 (47.9%)	24 (50.0%)	1 (2.1%)	GG: 14 [1.7–113.2]	0.0135*	70 (72.9%)	26 (27.1%)		

*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; OR – odds ratio; CI – confidence interval; A – adenine; G – guanine; C – cytosine; T – thymine.

SNPs and caries prevalence

All genotypes were in Hardy-Weinberg equilibrium. Table 2 summarizes the results of genotype and allele frequency comparisons calculated for all 11 studied SNPs. We found that the differences in genotype and allele prevalence were statistically significant in 6 SNPs, indicating possible correlation of these marker with caries in children. For rs17878486 in *AMELX* gene there was higher incidence of the minor TT homozygote in caries experienced children in comparison with caries free individuals (OR = 25, $p < 0.0001$). It was confirmed by the allele frequency analysis, as the prevalence of the alternative T allele was significantly more common in group with caries in comparison with the control group (OR = 10.2, $p < 0.0001$). Those results indicate the T allele and TT genotype as a putative strong risk variants for caries experience in the study group.

The distribution for genotypes and alleles for *AMBN* gene also revealed significant differences for 1 of the 2 studied SNPs. For rs34538475 there was higher prevalence of the minor TT homozygote and the minor T allele seen in controls in comparison to children with caries ($p < 0.0001$ for both calculations), which strongly indicates the T allele as a protective against caries variant in our study group. In the other studied SNP for *AMBN* gene, rs4694075, genotype and allele frequencies did not differ significantly between caries and caries-free children.

In rs2337360 for *TUFT1* gene there was also statistically significant frequency of the AG heterozygote and the minor G allele ($p < 0.0001$ for both calculations) between the groups. We did not observe differences in genotype and allele frequencies in the other 2 SNPs for *TUFT1* gene between caries and control children. Although, in rs4970957 there was a significant overrepresentation of the AG genotype in children with caries ($p = 0.0428$), distribution of both homozygotes and alleles did not differ between caries and caries-free participants.

No significant association with caries phenotype was found in SNPs for *TFIP11* and *MMP20* genes. On the other hand, there were significant associations in both rs2235091 and rs198969 for *KLK4* gene with caries occurrence. In rs198969, we observed significantly higher incidence of the major GG homozygote ($p = 0.0135$) and the major G allele ($p = 0.0049$) in children with caries than in controls. There was also a significantly different distribution of alleles ($p = 0.0085$) in rs2235091, however, the frequency of genotypes did not differ statistically between the groups.

Allelic association analysis

We conducted association test for each candidate gene (it was possible only when 2 or more SNPs for single gene were analyzed, *i.e.* for *AMBN*, *TUFT1*, *TFIP11* and *KLK4* genes) and for all markers, simultaneously. The analysis did not reveal presence of any haplotype block or significant association for studied markers. Interestingly, linkage disequilibrium, shown as R^2 values was different between caries-free and caries experienced children (data not shown). There was stronger association between rs4694075 and rs34538475 for *AMBN* gene in caries children ($R^2 = 19$) than in controls ($R^2 = 1$). The latter was significantly associated with caries, however no specific haplotype was revealed. We also observed weak association ($R^2 = 18$) between rs5997096 and rs134136 for *TFIP11* gene.

Discussion

Although there are several studies that strongly suggest a role for enamel formation genes in caries susceptibility, there are still some discrepancies in the results, mainly in relation to age, sex and ethnicity of the individuals studied.[1,4–12] The present study is limited to Polish Caucasian children and, to the best of our knowledge, our study is the first one concerning the genetic aspect of dental caries to be performed on such a homogenous population in Poland. It is well established that multiple environmental as well as genetic factors contribute to caries. In this report, we investigated the role of single nucleotide polymorphisms in several genes related to enamel formation and development to evaluate their possible association with susceptibility to caries. All markers were chosen on the basis of previous reports concerning the association between genetic variants and caries phenotype in children. Studied SNPs were located in intronic regions of candidate genes, however, they could influence mRNA expression process and alter functional activities of encoded genes.

In fact, 4 of 11 markers were overrepresented in genotype and allele transmissions to caries-experienced or caries-free individuals, indicating the markers to be associated with caries. We found significant association between the minor T allele and TT homozygote in polymorphism rs17878486 for *AMELX* and caries experience. There was 25-fold higher prevalence of the TT genotype and over 10-fold higher prevalence of the T allele in caries children than in caries-free children ($p < 0.0001$ for both calculations). Patir et al. observed an overrepresentation of the CC genotype in caries individuals (Turkish population), which is in contradiction with our results, where the major CC genotype turned out to be protective against caries occurrence.[4] On the other hand, Abbasoğlu et al. found no association between the same *AMELX* polymorphism and caries susceptibility in Turkish children.[5] There were also no associations between amelogenin genetic variants and dental caries experience in other studies performed on Polish, French, Caucasian, and Japanese populations.[6–8,12] However, studied SNPs for *AMELX* gene and the age of children were different

from those chosen for our study, also the studied populations were heterogeneous regarding demographic origins and cultural diversity.

Another major enamel protein, ameloblastin, is in synergy with amelogenin during the process of enamel development, but, in contrary to the latter, does not exhibit diversity in gene structure or expression level in reference to gender.[21] Abbasoğlu et al., Deeley et al. and Slayton et al. did not find association between *AMBN* genetic variants and caries experience in Turkish, Guatemalan-Mayan and mixed populations, respectively, although Slayton et al. noticed that out of the children who participated in the study, Caucasian children had the greatest severity of the disease.[5,7,11] On the other hand, Patir et al. observed an overrepresentation of the minor T allele in rs34538475 in caries experienced individuals in comparison to controls, regardless of the stage of the disease.[4] In our study, however, we observed an opposite association, as both the minor T allele ($p < 0.0001$) and the minor TT homozygote ($p < 0.0001$) were overrepresented in controls in comparison to cases with caries, which supported the T allele as a protective variant. There were no differences in allele and genotype frequency between caries experienced and caries-free children in rs4694075 for *AMBN* gene in our study. On the contrary, Schimizu et al. noticed an association between the major C allele and caries cases with higher dmft scores, however, the calculation was carried out for the caries group only and not for cases and controls.[10]

In 1 of 3 variants studied for *TUFT1* gene, i.e. rs2337360, we found a strong association between the overrepresented wild A allele and AA genotype ($p < 0.0001$ for both calculations) and caries incidence, which supports this SNP as a risk variant. Interestingly, Deeley et al. noticed significant association between this marker and individuals with different dmft scores, but not when caries-free children were included in the comparisons.[11] Several studies showed an association between other 2 markers (rs3790506 and rs4970957) for *TUFT1* gene and caries experience, while in our study there were no differences in genotype and allele distribution or the results were inconclusive.[4,5,10] It is worth mentioning that in most of the studies concerning genetic variation of tuftelin and caries, an association could only be detected when the interaction with *Streptococcus mutans* was included in the model. Therefore, it is difficult to estimate whether the contradictory results are due to the differences in ethnicity and age of the participants or to the lack of information about *S. mutans* level, which was not possible to analyze in our research because of some technical obstacles. Nevertheless, it is known that genetic changes in enamel genes could lead to some degree of enamel prisms disorganization and influence the enamel microhardness, that is why the strong association between tuftelin rs2337360 variant and caries incidence should be taken under consideration when concerning individual's susceptibility to caries.

While amelogenin, ameloblastin and tuftelin proteins are the main components of the tooth enamel and regulate its forming process directly, the other candidate genes included in this study play a role in the degradation of enamel proteins, i.e. matrix metalloproteinase *MMP20* and kallikrein 4 *KLK4*. *MMP20* is expressed during early stages of enamel development and, on the contrary, *KLK4* is expressed later during hardening of the enamel, so that genetic variants of those genes could alter protein function and disturb enamel mineralization throughout all the stages. Both genes are also implicated in hereditary defect of the enamel, i.e. amelogenesis imperfecta.[22] There was no association between *MMP20* variant and caries in our study, however, both SNPs for *KLK4* gene were significantly associated with caries experience and indicated risk variants. In rs198969 there was 14-fold higher prevalence of the minor GG homozygote ($p = 0.0135$) and over 2-fold higher prevalence of the minor G allele ($p = 0.0049$) in caries experienced in comparison to caries-free children, which supports this allele as a risk variant. The other *KLK4* marker, rs2235091, displayed significant differences in allele distribution only. Although not significant, there was a trend for higher prevalence of the minor AA homozygote in caries-free children. Our results remain in agreement with previous study of Abbasoğlu et al., who did not observe association between *MMP20* variant and caries, however, they found a protective variant for *KLK4* gene.[5] On the other hand, Wang et al. observed only borderline significant value for the role of *MMP20* gene during the enamel development, similarly to the study of Tannure et al., where there were no differences in allele and genotype distribution between caries cases and controls in general studied population.[9,22] Although, there were some differences in allele and genotype distribution in Caucasians only, those subjects were reported to have poor oral hygiene and health habits when compared to other participants.

Therefore, it is evident that environmental, as much as genetic factors play a role in the etiology of dental caries in children. Demographic origin also seems to be a factor of great importance, as numerous studies performed on populations of various ethnicity differ significantly in allele and genotype distribution. It is worth mentioning that genetic variants, even as subtle as single nucleotide polymorphisms in intronic region, in candidate enamel formation genes in this study, have been reported to influence other dental conditions, such as amelogenesis imperfecta or molar-incisor hypomineralization.[23] It strongly supports the hypothesis that even minor alterations in gene structure could influence protein function and impact the enamel growth and mineralization and, therefore, alter individual's susceptibility to caries alongside of the environmental factors. Another possibility is that the SNPs could be in linkage to genetic variants outside the region of interest, but testing the actual influence of non-coding SNPs on protein function and

analysis of linkage disequilibrium throughout the genome is still not a simple task.[10,24] Thus, screening known genetic variants that proved to be risk variants has the potential to identify young children who may be at risk of caries and make their parents pay more attention to improve children's eating habits and oral hygiene. Moreover, it could provide genetic information about the general population, making the results more replicated and contribute to a more precise caries phenotype and better understanding of the disease.[25]

In conclusions one may say that single nucleotide polymorphisms in *AMELX, AMBN, TUFT1, KLK4* genes may be genetic markers that contribute to dental caries occurrence in Polish children.

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Vitamin E (α tocopherol) attenuates toxicity and oxidative stress induced by aflatoxin in rats

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Abstract

Background. Aflatoxins are toxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* and are classified as group I carcinogens by the International Agency for Research on Cancer (IARC).

Objectives. The purpose of this study was to investigate the possible preventive role of vitamin E (Vit E) on aflatoxin (AF) induced toxicity by using biochemical and histopathological approaches.

Material and methods. Wistar-Albino rats were divided into 4 groups as follows: control group, Vit E group (Vit E was administered), AFB_1 group (a single dose of AFB_1 was administered), AF + Vit E group (AF and Vit E were administered). The effects of Vit E on AFB_1 induced tissue toxicity were evaluated by using malondialdehyde (MDA), reduced glutathione (GSH) levels, antioxidant enzyme activities, and histopathological examination in tissues.

Results. AF caused the oxidative stress by the increased MDA level and the reduced GSH level, glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase (G6PD) activities in tissues. Plasma aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities, creatinine, and urea concentrations significantly increased; whereas, chloride, phosphorus, and magnesium concentrations were insignificantly affected. Plasma glucose, protein and sodium concentrations significantly decreased. Administration of AF caused hepatotoxicity, cardiotoxicity, and nephrotoxicity. As far as histopathological changes are concerned, a statistically significant difference was found in AFB_1 group compared to the control group. Vit E considerably reduced plasma AST, ALT, ALP, LDH activities, and urea concentration and ameliorated the deleterious effects of AF on oxidative stress markers and pathological changes.

Conclusions. This data indicated that the natural antioxidant Vit E might have a protective effect against AF-induced toxicity and oxidative stress.

Key words: oxidative stress, antioxidant, aflatoxin, vitamin E

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Aflatoxins (AF) are polysubstituted bifuranocoumarins that are secondary fungal metabolites produced by parasiticus/ flavus group of the genus *Aspergillus*. AF-contaminated feeds may cause many health problems among livestock. AF is a carcinogen when administered in repeated doses in many animal species. AFB_1 is nephrotoxic, hepatotoxic, mutagenic, genotoxic, and immunotoxic.[1–5]

AFB_1 mediated cell injury may be due to the release of free radicals and these radicals initiate lipid peroxidation (LPO) and a damaging process in biological systems since all cell membranes contain the polyunsaturated fatty acids which are substrates for such a reaction.[6] Kodama et al. showed the formation of LPO by AFB_1. Oxidative stress is thought to play an important role in AFB_1 by increasing LPO and decreasing antioxidants in treated animals.[7-9]

Vitamin E (α-tocopherol) is a lipophilic alcohol and its food source is the root of wheat and vegetable oils. The most important part of this substance is the α-part because it constitutes 90% of the tocopherol composition of animal tissues. Many physiological functions including stabilization of membrane have been taken into consideration for this substance. This substance can absorb free radicals of oxygen and thus prevent the negative effects of LPO in the brain tissue.[10–12]

In the present study, the protective effect of Vit E was investigated by estimating malondialdehyde (MDA), reduced glutathione (GSH) levels and antioxidant enzymes such as glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase (G6PD) activities in liver, kidney and heart of AFB_1 treated rats.

Material and methods

Chemicals

AFB_1 (5 mg, Code 11293) was purchased from Cayman Chemical Company (Michigan, USA), Vit E (α-tocopherol from vegetable oil, Code T3634), GSH, glutathione reductase, thiobarbituric acid, hydrogen peroxide, nicotinamide adenine dinucleotide phosphate (NADPH) and other reagents were supplied from Sigma (St. Louis, MO, USA).

Preparation of AFB_1 and Vit E

AFB_1 was dissolved in dimethyl sulfoxide (DMSO), diluted with distilled water, and then administered to the experimental animals.[8] Vit E (α-tocopherol ampoule) was dissolved in corn oil and administered at the dose of 100 mg/kg.

Experimental groups and sample collection

Twenty-eight healthy male Wistar-Albino rats (300–350 g body weight) were used in this study. The protocol for the use of animals was approved by the National Institutes of Health and Committee on Animal Research. AFB_1 (1.0 mL) was a single intraperitoneal (i.p.) dose administered to the animals at the dose of 2.5 mg/kg b.w. Vit E (1.0 mL) was suspended in corn oil and administered to the animals by gavage at the dose of 100 mg/kg/day for 20 days. The doses of AF and Vit E used in this study were selected according to previous studies in which Vit E was administered together with AF.[13-16] The animals were randomly divided into 4 experimental groups including 7 rats in each. These groups were arranged as follows: control group, Vit E group (Vit E was administered), AFB_1 group (a single dose of AFB_1 was administered), AF + Vit E group (AF and Vit E were administered).

Biochemical analysis

Under ether anesthesia, blood samples were withdrawn by an injector from the heart of the animals and collected into tubes containing EDTA. At the end of the experiment, the rats in control and experimental groups were sacrificed by decapitation under ether anesthesia.

Plasma was used to measure MDA level as a marker of LPO and to determine aspartate transaminase (AST), alanine transaminase (ALT), alkaine phosphatase (ALP), lactate dehydrogenase (LDH) activities, glucose, protein, creatinine, urea, sodium, chloride, phosphorus, and magnesium concentrations. Tissue samples were quickly removed and perfused with ice-cold saline for biochemical and histopathological evaluation. The tissues were homogenized in distilled water by using a Potter-elvehjem Homogenizer. The homogenate was centrifuged (at 3.500 rpm for 15 min to MDA, GSH, GST, CAT, SOD analyze and at 14.000 rpm for 55 min to GSH-Px and G6PD at +4°C).

MDA and GSH levels, GST, CAT, GSH-Px, SOD and G6PD activities were analyzed in liver, kidney and heart tissues. While MDA level was measured according to the method developed by Placer et al., the GSH level was determined by the method developed by Ellman et al.[17,18] GST activity was measured by the method developed by Habig et al.[19] CAT activity was carried out by using Aebi's method.[20] GSH-Px activity was measured by the Beutler method.[21] SOD activity was measured by using xanthine and xanthine oxidases to generate superoxide radicals reacting with nitroblue tetrazolium.[22] G6PD activity was measured by the Beutler method.[21] The protein concentration determination was based on the method of Lowry et al.[23] The plasma glucose, protein, creatinine, urea, sodium, chloride, phosphorus and magnesium were measured by using an AutoAnalyzer (Olympus AU 600, Tokyo, Japan).

Histopathological examination

Necropsy of the rats was performed and liver, kidney, and heart tissue samples were fixed at 10% neutral buffered formalin. Paraffin embedded blocks were routinely

processed. 5-μm thick sections were stained with hematoxylin-eosin and examined under a microscope. Then random 10 microscopic fields were examined in ×40 magnification.

Statistical analysis

The results are expressed as mean ± standard error. Statistical significance between the different groups was determined by using a one-way analysis of variance (ANOVA) in the SPSS 21 software package. Post hoc test was performed for between-group comparisons by using the Tukey multiple comparison test. The level of significance was set at $p < 0.001$.

Results

MDA and GSH levels

Figures 1–3 show the tissue MDA levels, GSH levels and the activities of antioxidant enzymes such as GST, CAT, GSH-Px, SOD, and G6PD in the control and experimental groups. The data indicated that the AFB_1 group had a significantly higher MDA level than the control group in all the tissues. The Vit E group had a significantly lower MDA level than the AFB_1 group. Treatment with AFB_1 and Vit E provided apparent normalization in MDA level compared to the AF group. GSH level significantly decreased in the AFB_1 group compared to the control group. Upon supplementation of Vit E to the AFB_1 group, a significant increase was observed in the GSH level compared to that of the AFB_1 group. The GSH level was restored in the AFB_1 and the Vit E group.

GST, CAT, GSH-Px, SOD and G6PD enzyme activities

A significant reduction was found in GST, CAT, GSH-Px, SOD and G6PD activities in the AFB_1 group compared to that of the control group. Upon simultaneous supplementation of Vit E to the AFB_1 group, significant increases were observed in antioxidant enzyme activities compared to the AFB_1 group. With the addition of Vit E to AF, normal values were reached ($p < 0.001$, $p < 0.05$).

Biochemical parameters

Fig. 4 shows the biochemical parameters such as AST, ALT, ALP, LDH, glucose, protein, creatinine, urea, sodium, chloride, phosphorus and magnesium in the control and experimental groups. Plasma AST, ALT, ALP, LDH, creatinine, and urea concentrations were significantly higher after administration of AFB_1 compared to the control group. Administration of AFB_1 alone induced a significant decrease in plasma glucose, protein, sodium concentration, however; it did not induce a significant effect on plasma chloride, phosphorus, and magnesium concentrations. Plasma AST, ALT, ALP, LDH activities, glucose, protein and urea concentrations in AFB_1 + Vit E group were lower than those determined in AFB_1 group. Plasma creatinine concentration in AFB_1 + Vit E group was lower than AFB_1 group, but this value could not reach the control level. Administration of AFB_1 alone did not cause a significant change in plasma chloride, phosphorus, and magnesium concentrations ($p < 0.001$, $p < 0.05$).

Histopathological examination

The histological changes in the tissues were assessed as defined in Fig. 5–7. Control and Vit E groups had a normal histological appearance in liver, kidney, and heart tissues. As histopathological changes, a statistically significant difference was found in the AFB_1 group compared to the control group ($p < 0.05$).

While severe necrotic hepatocytes and hydropic degeneration were observed in liver tissues of the AFB_1 group, it was determined that these changes reduced in AFB_1 with Vit E group (Fig. 5). Administration of AFB_1 caused severe tubular necrosis, tubular degeneration, and hyaline droplets in kidney tissues. However, administration of Vit E ameliorated severe tubular necrosis and degeneration, and hyaline droplets (Fig. 6). Administration of AFB_1 caused a severe hemorrhage and degenerative changes in heart tissues. Rats treated with AFB_1 + Vit E had lower hemorrhage and degenerative changes (Fig. 7).

Discussion

One of causes for AFB_1 induced toxicity is the oxidative stress which caused improved reactive oxygen species (ROS) generation and oxidative DNA damage.[24,25] Previous studies, using different animal species, indicated that AF induced changes in oxidative stress markers.[6,26–29] This study was conducted to assess the effect of AF on oxidative stress signs in rat liver, heart and kidney tissues.

According to AFB_1 concentration, the organs were classed as follows: gonads, liver, kidney, spleen, bursa cloacalis, thymus, endocrine glands, muscles, lungs, and the brain.[30] Petr et al.[31] revealed that AFB_1 was determined in the blood, kidney, liver, and testis from minutes to maximum 8–10 h after a single i.p. injection at 0.1 mg/kg AFB_1.

The results showed that the administration of AFB_1 produced a marked oxidative impact as evidenced by a significant increase in MDA in the liver, kidneys and heart of AF-treated rats. These alterations might have been triggered either by the direct effects of AFB_1 or by the metabolites formed by AF and the free radicals, which were generated during the formation of these metabolites. This result is compatible with the results reported previously about rat tissues.[32,33] Initiation of LPO

Fig. 1. Effect of Vit E on oxidant-antioxidant status in liver tissue of AFB_1 treated rats. MDA (nmol/g tissue), GSH (µmol/mL), GST (U/mg protein), CAT (k/mg protein) GSH-Px (U/g protein), SOD (U/mg protein), G6PD (U/g protein)

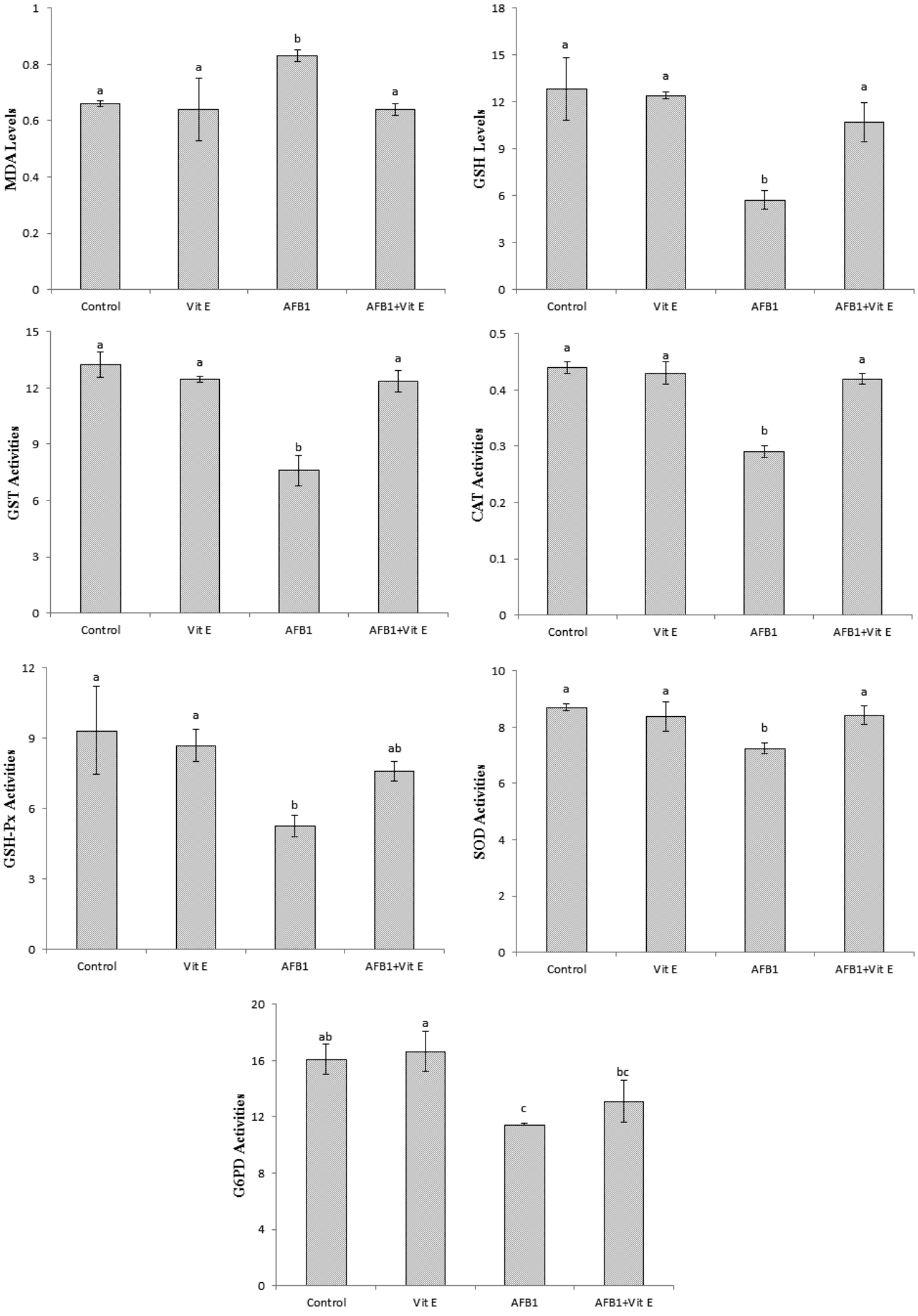


Fig. 2. Effect of Vit E on oxidant-antioxidant status in kidney tissue of AFB_1 treated rats. MDA (nmol/g tissue), GSH (µmol/mL), GST (U/mg protein), CAT (k/g protein) GSH-Px (U/g protein), SOD (U/mg protein), G6PD (U/g protein)

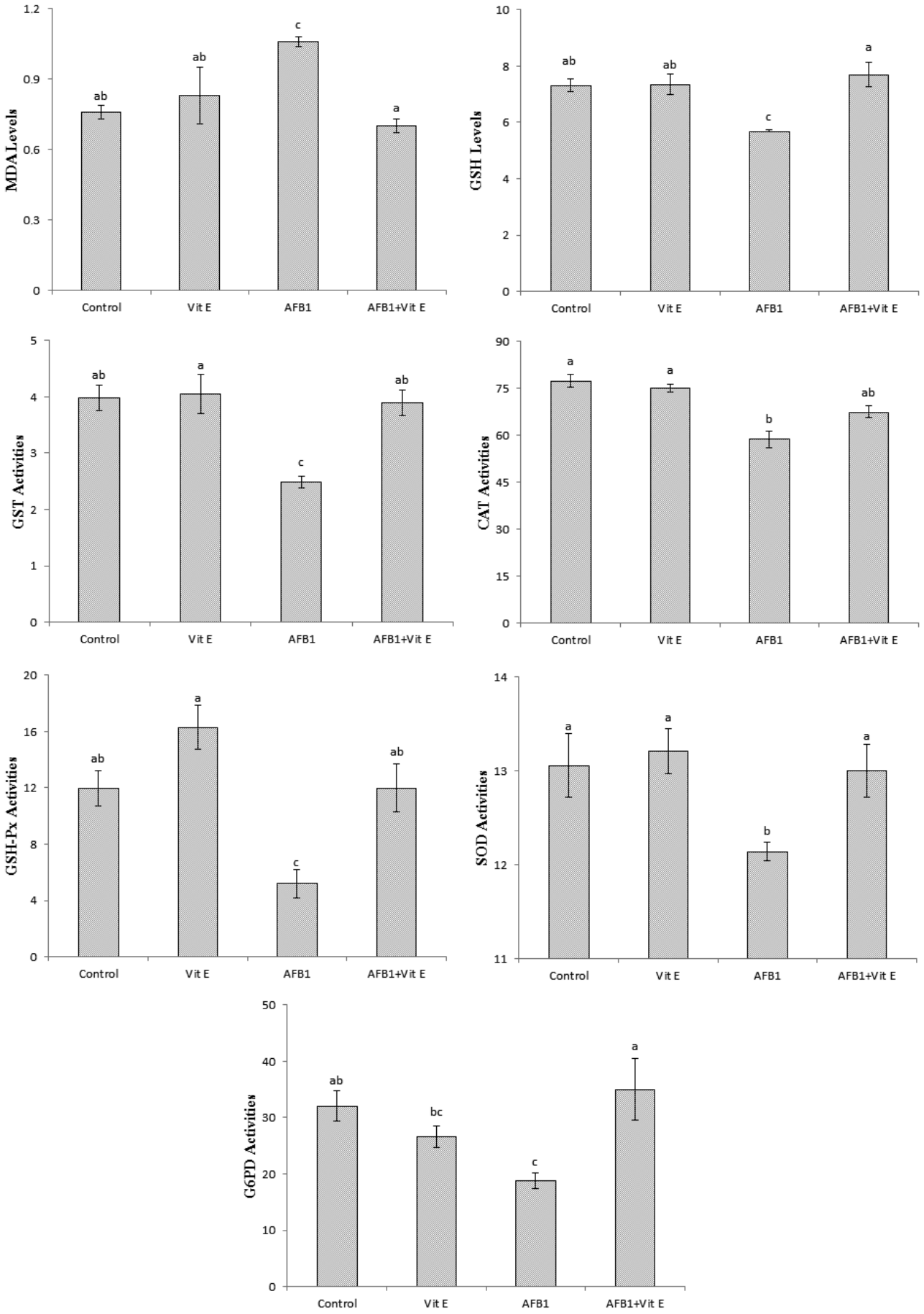


Fig. 3. Effect of Vit E on oxidant-antioxidant status in heart tissue of AFB_1 treated rats. MDA (nmol/g tissue), GSH (µmol/ml), GST (U/mg protein), CAT (k/g protein) GSH-Px (U/mg protein), SOD (U/mg protein), G6PD (U/g protein)

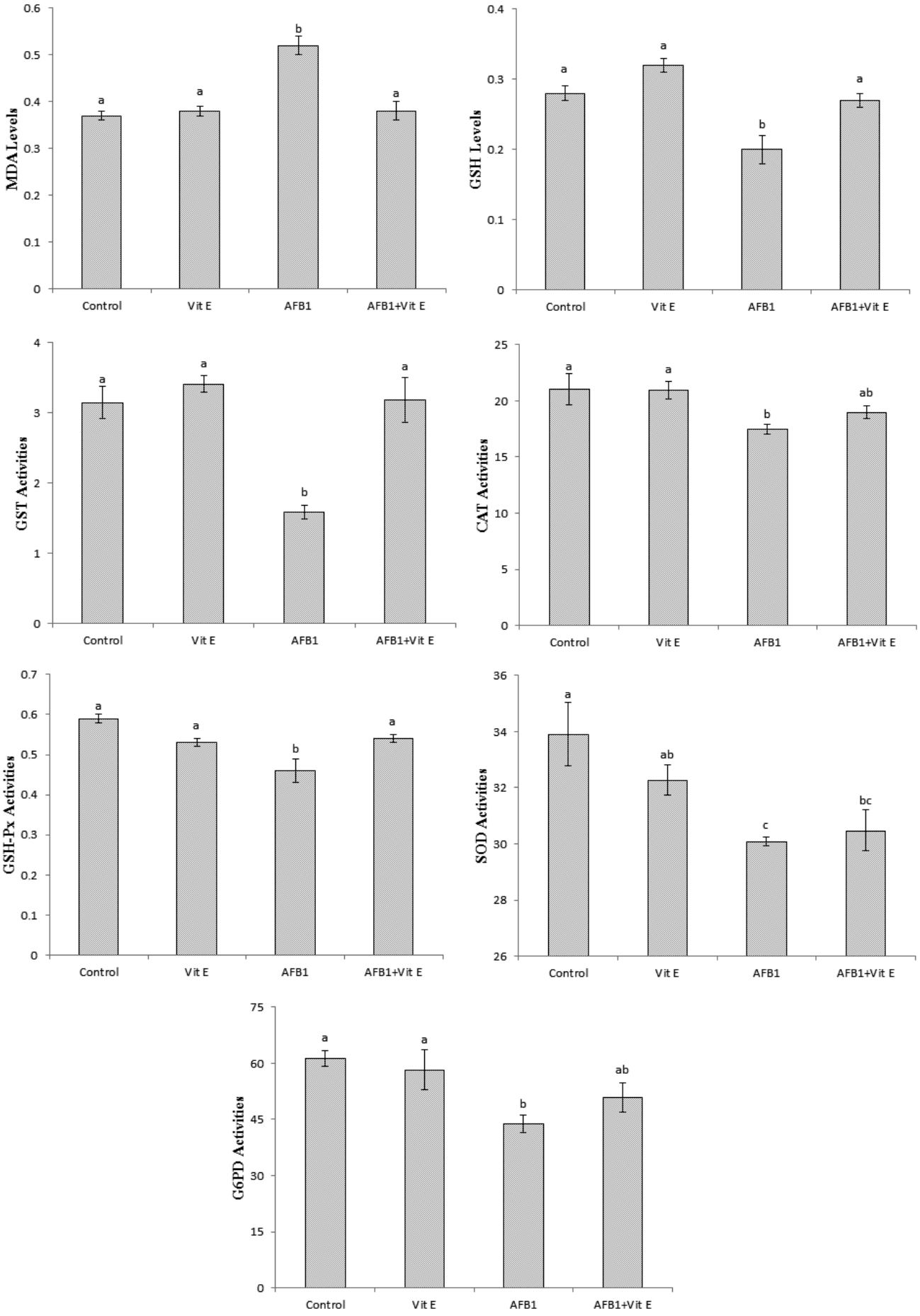
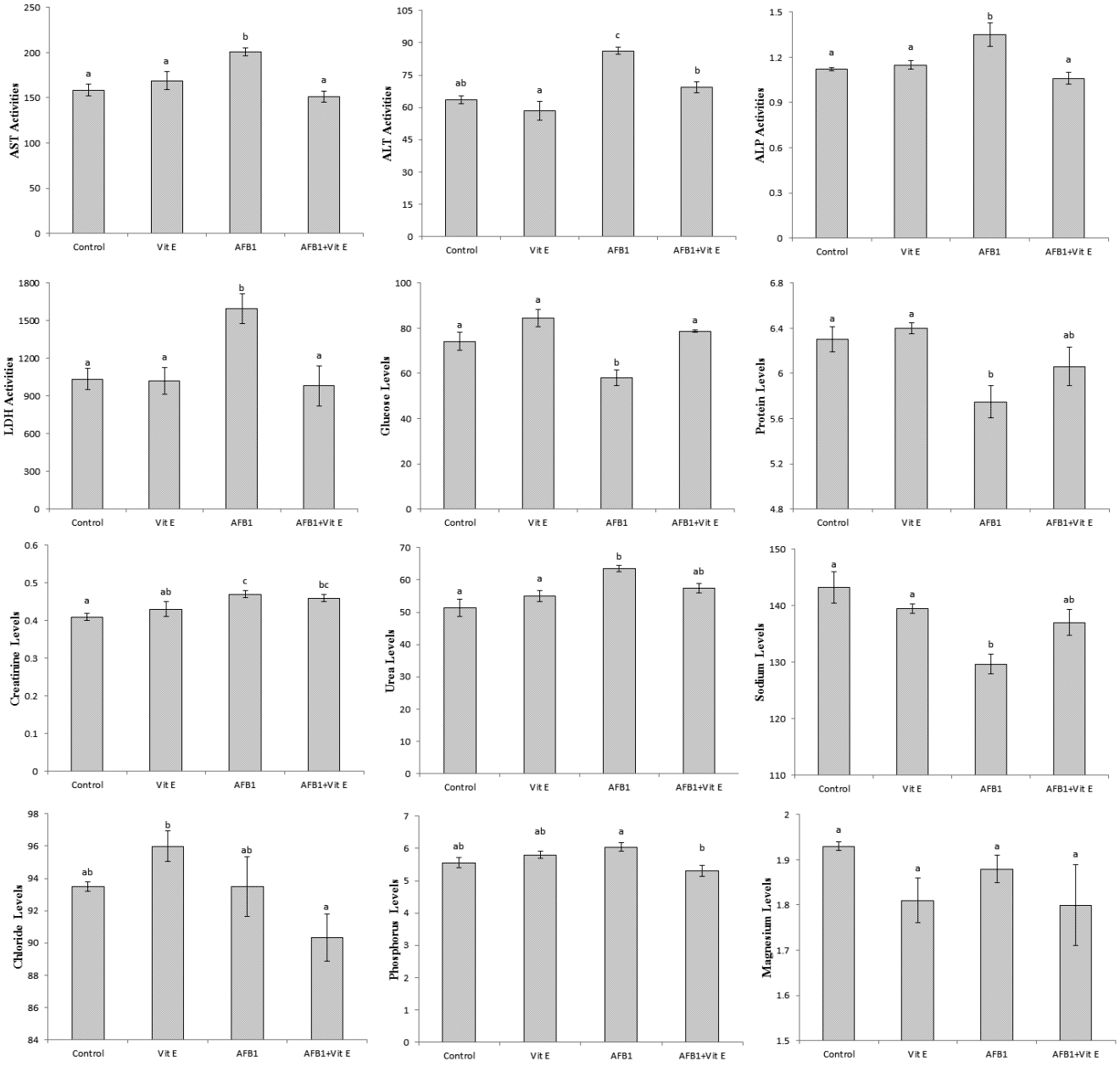


Fig. 4. Effect of Vit E on plasma AST (U/L), ALT (U/L), ALP (U/L), LDH (U/L), glucose (mg/dL), protein (g/dL), creatinine (mg/dL), urea (mg/dL), sodium (mmol/L), chloride (mmol/L), phosphorus (mg/dL), magnesium (mg/dL) in AFB_1 treated rats



by AFB_1 is noted as one of the principal appearances of ROS-induced oxidative damage. The mechanism of free radical damage also includes ROS-induced peroxidation of polyunsaturated fatty acids in the cell membrane lipid bilayer which causes a chain reaction of LPO, thus damaging the cellular membrane, causing further oxidation of membrane lipids and proteins, and leading to DNA damage.[34] It is revealed that AFB_1, with the help of microsomal cytochrome p-450 mediated oxidation, is biotransformed into AFB_1–8-9-epoxide, which is a reactive intermediate and highly toxic.[35]

The present study also showed that a significant increase in the oxidative stress was accompanied by a concomitant decrease in the enzyme activities involved in the disposal of superoxide anions and peroxides, namely CAT and SOD, as well as GSH levels and its related enzymes (GST, GSH-Px). A significant increase observed in tissue MDA levels in AFB_1 treated animals indicated that AF led to the generation of high level of free radicals, which could not be tolerated by the cellular antioxidant defense system. A significant decrease in these enzyme activities could be explained by their consumption during the conversion of free radicals into less harmful or harmless metabolites. The enzyme activities decreased by AFB_1 can be attributed to the lower ability of the tissue, which cannot scavenge free radicals and prevent the action of LPO. Similar results were also reported in previous studies.[8,36–38]

Fig. 5. Sections of the liver in AFB_1, Vit E treated rats. livers of control a), Vit E treated rats b) show normal histological appearance. However, liver of AFB_1 treated rats c) shows severe necrotic changes in hepatocytes (*) and hydropic degeneration (arrowhead) in the hepatocytes. The liver of AFB_1 plus Vit E treated rats d) shows moderate necrotic changes in the hepatocytes (*) (haematoxylin and eosin staining, magnification ×40)

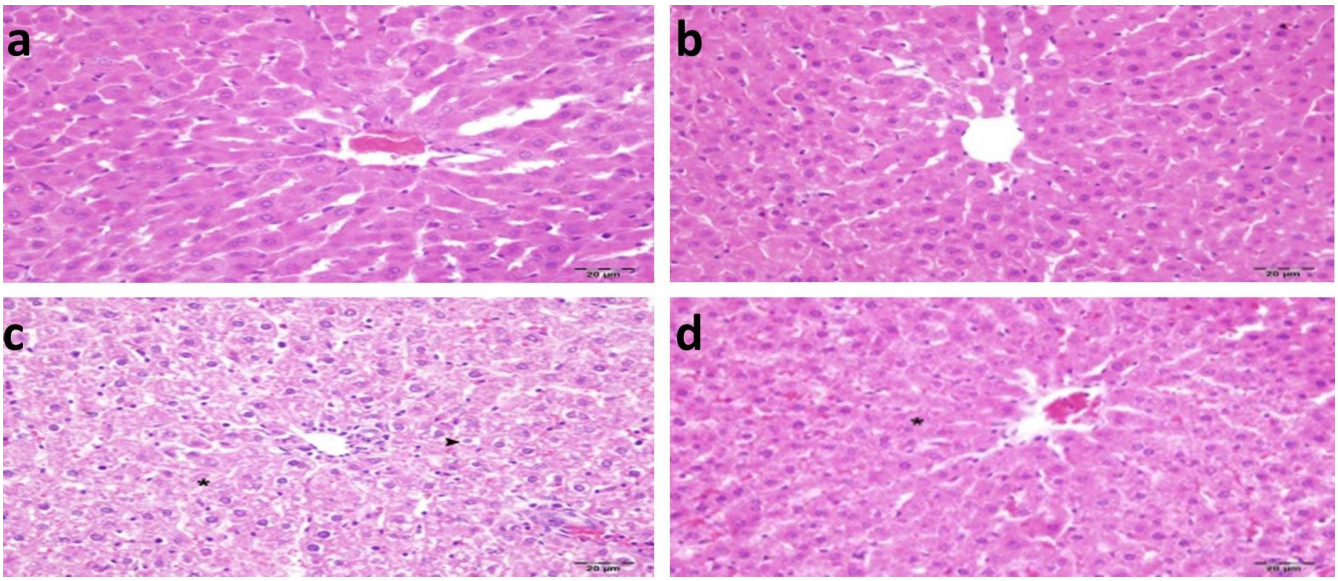
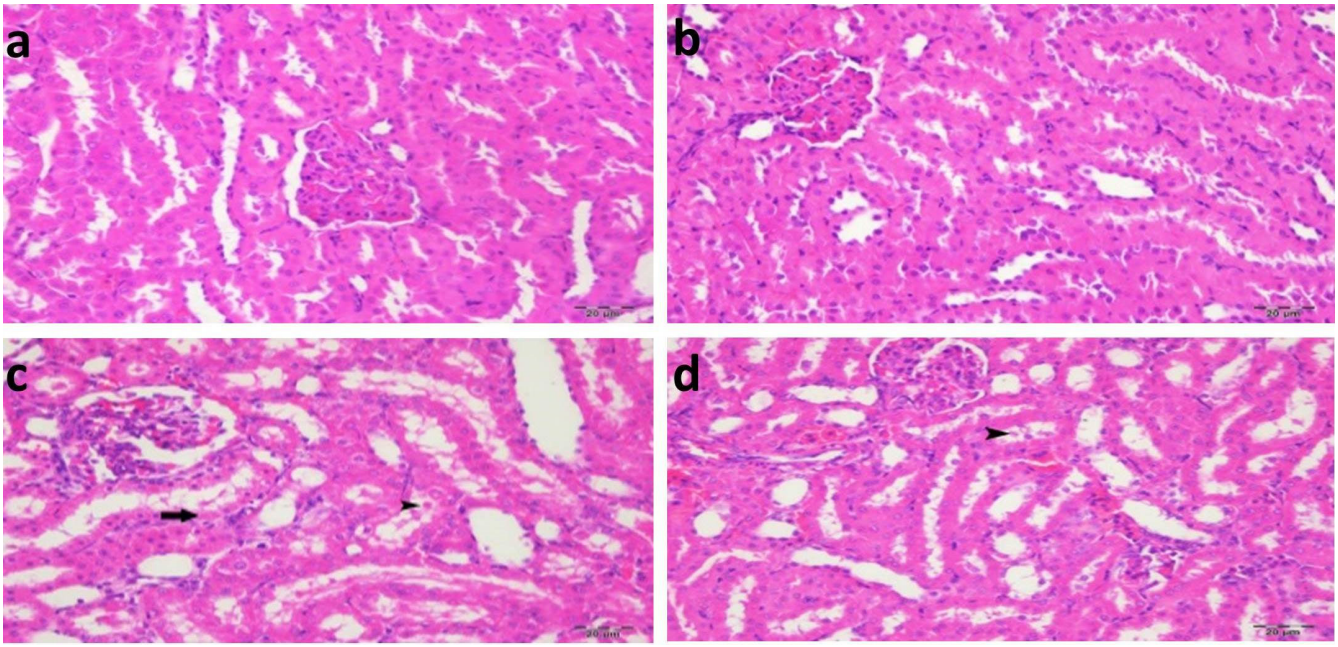


Fig. 6. Sections of the kidney in AFB_1 and Vit E treated rats. kidneys of control a), Vit E treated rats b) show normal histological appearance. However, kidney of AFB_1 treated rats c) reveals severe degenerative changes, necrotic tubular epithelial cells (*) and hyaline droplets in the tubules (arrowheads). The kidney of AFB_1 plus Vit E treated rats d) shows moderate degenerative changes, hyaline droplets in the tubules (arrowheads) (haematoxylin and eosin staining, magnification ×40)

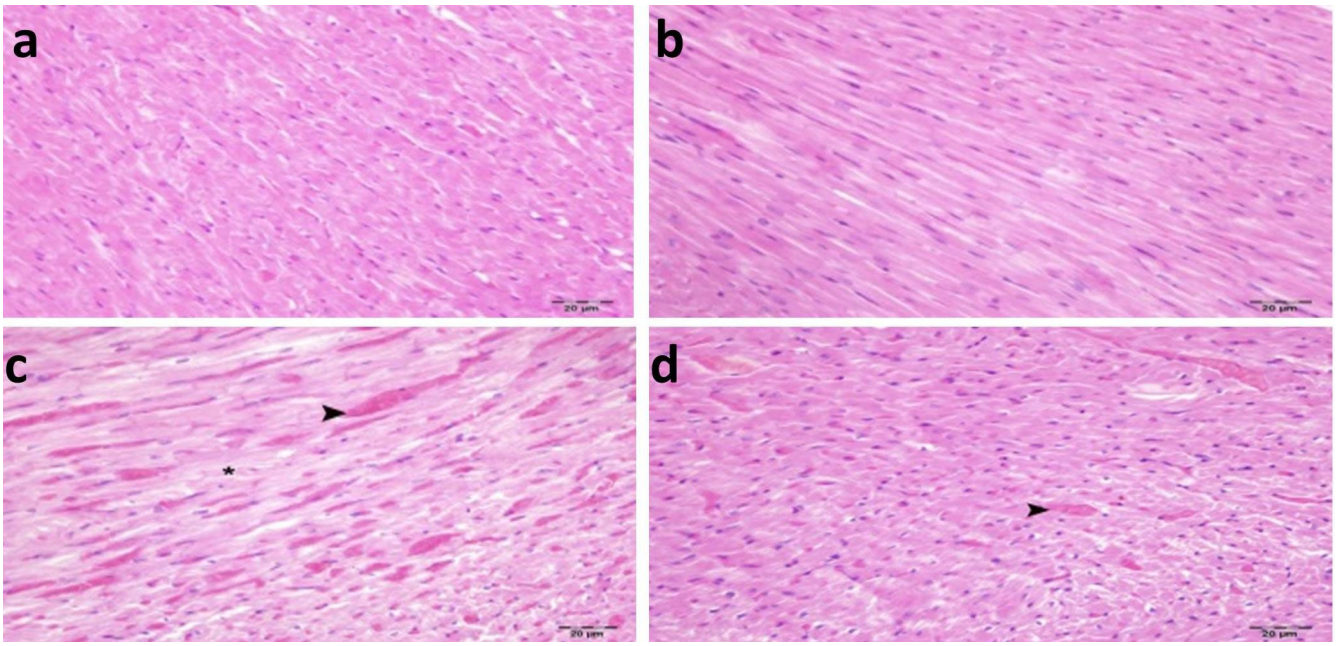


GSH and GST are effective in providing protection from the harmful effects on the tissues of AFB_1.[39] GST catalyzes the conjugation of AFB_1–8, 9-epoxide with GSH to form AFB_1-epoxide-GSH conjugate, thereby decreasing the intracellular GSH content.[40,41] The activity of GSH-Px, which is a constituent of GSH redox cycle decreased during AFB_1 administration. The reduction in the GSH-Px activity by AFB_1 may be due to a decrease in the availability of GSH and also alterations in their protein structure by ROS.[32,39,42,43]

The decreased G6PD activity as GSH metabolizing enzymes in AFB_1-treated animals occurs as a result of decreased supply of reduced NADPH for the conversion of oxidized glutathione to GSH. Under conditions of oxidative attack, the $NADP^+$/NADPH ratio changes in favor of $NADP^+$, indicating decreased G6PD activity.[44] The present study also showed a similar result in this enzyme activity and administration of Vit E significantly improved the G6PD activities.

Supplementation of Vit E to AFB_1 significantly reduced MDA level and eliminated the possibility of oxidative stress

Fig. 7. Sections of the heart in rats treated with AFB_1, Vit E. hearts of control a), Vit E treated rats b) show normal histological appearance. However, the heart of AFB_1 treated rats c) reveals degeneration, necrosis (*), severe hemorrhages in the myocardium (arrowhead). The heart of AFB_1 plus Vit E treated rats d) shows moderate hemorrhages in the myocardial tissue (arrowhead) (haematoxylin and eosin staining, magnification ×40)



due to the administration of AFB_1 to rats. The antioxidative function of Vit E is mainly due to its reaction to membrane phospholipid bilayers to break the chain reaction initiated by hydroxyl radicals.[11,45] Vit E may inhibit free radical generation by direct scavenging of the free radicals and subsequent transformation of the antioxidant species into less toxic product.[46,47] Vitamin E as a therapy for AF-treated rats can be effective to significantly increase GSH, CAT, GSH-Px and GST activities. Vit E increases the GSH levels, resulting in an increase in SOD activity, and thereby preventing the deleterious effect of superoxide radicals. The restoration of antioxidant enzyme activities and the GSH level of Vit E indicates that they play an important role in mitigating AFB_1 induced oxidative stress and subsequent damage to the tissue.[48] The results of the present study revealed that AF was potent hepatotoxic, nephrotoxic, and cardiotoxic and Vit E showed protection against AF-induced nephrotoxicity, hepatotoxicity, and cardiotoxicity.

The liver is considered the principal target organ for AF.[1] The result of the study clearly indicated that the administration of AF caused a significantly higher level of plasma AST, ALT and ALP in rats. Increased activities of these enzymes are well known to be diagnostic indicators of hepatic injury. ALP is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce a derangement in the transport of metabolite. The results of the present study indicated a significant improvement in these marker enzyme activities in plasma, which is compatible with the previous studies.[49–52] In the present study, LDH levels increased significantly in AFB_1-treated rats which indicated the presence of myocardial infraction, cardiac injury, and toxicity to cardiac tissue and was compatible with the previous studies suggesting that exposure to AFB_1 causes heart defects.[53–55] Increased levels of these enzymes might also be due to the leakage of enzymes from the kidney as a result of necrosis induced by AFB_1. Administration of Vit E significantly reduced these enzyme levels. The morphological changes included severe necrotic hepatocytes and hydropic degeneration. The histopathological and biochemical actions may be due to its antioxidant effects. Histopathologically, the livers of AFB_1 treated rats were observed to be extensive hepatocellular necrosis, fatty infiltration, and bile duct proliferation.[56] Vayalil[57] showed that the date fruit extract (*Phoenix dactylifera* L. *Arecaceae*) induced liver protection against aflatoxicosis occurred via decreased liver enzyme activity as well as decreased free radical propagation, and also by its lowering the pathological lesions resulting from AFB_1.

Histopathologically, the liver and kidney treated with AFB_1 and Vit E showed less morphological changes compared to the changes seen in AFB_1 alone, which was an indication of partial protection. The results support the presence of hepatoprotective and nephroprotective roles of Vit E and this might be due to the membrane stabilizing and antioxidant activity of Vit E.

A significant decrease was found in plasma glucose and protein levels in AF-treated rats. This result is supported by the results of Abdulmajeed.[53] who indicated that aflatoxicosis affects the cellular energy supply of rat hearts by causing its inhibitory effects on some markers of the energy metabolism due to a decrease in glucose and glycogen contents of the heart tissue and a reduction in the activities of some glycolytic enzymes such as phosphogluco isomers and glyceraldehyde-3-phosphate dehydrogenase.

Decreased biosynthesis and secretion of proteins might be due to formation of AF adducts with DNA, RNA and protein. AFs were previously shown to lower the total protein concentration in serum of rabbits and broilers.[58,59]

Several studies have reported that the intoxication of AFB_1 causes nephrotoxicity.[60] Administration of AF caused a significantly higher concentration of plasma creatinine and urea. This result is compatible with results of Rati et al.[61] The heightened appearance of plasma creatinine of AFB_1-treated rats indicates the increased transformation of phosphocreaine to creatinine in the muscle, which might be due to utilization of phosphocreatine in muscular contraction. Thus, the significant increase in creatinine concentration in plasma could be due to the increased release of muscles and/or decrease excretion from the kidney. The increased levels of plasma urea and creatinine with decreased levels of plasma protein may indicate protein catabolism and kidney dysfunction.[62] Verma and Raval[63] reported the occurrence of nephrotoxicity and the elevation of creatinine in serum and urine of rabbits receiving 15 mg/kg AF-contaminated feed for 60 days. Histopathological studies revealed tubular necrotic degeneration in the kidney of AFB_1-treated rat. A histological examination of kidneys demonstrated previously mentioned microscopic changes that were compatible with the kidney dysfunction showed by biochemical tests. It could be concluded that nephrotoxicity induced by AFB_1 affects the tubulointerstitial structure histologically. Vit E ameliorates changes in experimental glomerulopathy, which is associated with increased renal oxidant injury. Preservation of the tubular architecture was evident in AFB_1 + Vit E group in the present study.

In conclusion, results of the present suggest that AF has a harmful and stressful effect on hepatic, renal and cardiac tissue. Treatment of Vit E may alleviate AF toxicity by the reduction of oxidative damage of AF in liver, renal, and cardiac tissues and alterations of cardiac energy metabolism. For this reason, Vit E can be regarded as a good therapeutic agent against aflatoxicosis.

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Effect of nebivolol on fracture healing: An experimental rat model

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Abstract

Background. Bone metabolism is a complex system, and fracture healing is one of its most important functions. Many circumstances can influence this process. Chronic drug use in elderly populations can affect bone healing, and inadequate tissue perfusion, increased free radicals and adverse drug effects can negatively influence fracture healing. Nebivolol, an anti-hypertensive drug that selectively blocks β1 receptors, effectively reduces blood pressure by inducing peripheral vasodilation. Nebivolol also exerts anti-oxidant effects by stimulating nitric oxide (NO) synthesis. Many studies show that NO protects the vascular endothelium and improves fracture healing.

Objectives. In this study, the histological and radiological effects of intraperitoneally administered nebivolol on fracture healing were evaluated.

Material and methods. Twenty-one Sprague Dawley rats were divided into 3 (nebivolol 1, 2 and control) groups. Sterile nebivolol solution (1 mL = 0.017 mg nebivolol) was given to the rats in group 1 every day for 4 weeks, while the rats in nebivolol group 2 were given 2 mL per day, beginning after the production of an open, displaced unilateral femur fracture. Radiographic and histological studies were used to evaluate fracture healing.

Results. Histological and immunohistochemical analysis showed osseous healing with woven bone at the fracture site and only minimal amounts of cartilage in nebivolol 1 and 2 groups. Radiological grading was not different between the control and the nebivolol groups.

Conclusions. This study suggests that nebivolol, a selective β blocker, has positive effects on fracture healing through anti-oxidative effects via the NO pathway and direct vasodilator effects.

Key words: experimental model, nitric oxide, fracture healing, nebivolol

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Fracture healing is a complex process that requires a local hematoma rich in mesenchymal cells and cytokines. In the fracture healing phase, there is a strong correlation between angiogenesis and osteogenesis. Transportation of biomaterials through the blood to the fracture site is necessary for adequate and prompt healing. The process of converting the organized hematoma to bone callus includes endochondral and intramembranous ossification stages. In addition, chondrocyte differentiation and mineralization require adequate nutrition from the vascular bed.[1–3]

Nebivolol is a highly selective β 1-adrenergic receptor antagonist that is used for antihypertensive therapy.[4] In many individuals, a dose of 5 mg effectively reduces blood pressure over a 24 h period and has been shown to cause endothelium-dependent vasodilation.[5,6] Nebivolol demonstrates systemic anti-oxidant effects by stimulating nitric oxide (NO) release.[7] NO is produced by endothelial NO synthase (eNOS) from L-arginine.[6,8] The l-nebivolol enantiomer stimulates NO release, and the d-nebivolol enantiomer is responsible for a β1-selective blockade.

The aim of this study was to evaluate the effect of intraperitoneally administered nebivolol on fracture healing. Histological and radiological evaluation of healing was performed in nebivolol and control groups using a rat open femoral fracture model.

Material and methods

Twenty-one adult Sprague Dawley rats (average weight 250 g) were used in this study. All in vivo study protocols were approved by the Institutional Laboratory Animal Care and Use Ethical Committee. The animals were housed in the Laboratory Animal Care-Augmentation Facility of Dumlupınar University in a temperature-controlled room (room temperature 20–22°C) on a 12 h light-dark cycle and were provided with rat pellets with water ad libitum. There were 7 animals in each standard cage.

Nebivolol preparation

Five-milligram nebivolol tablets (Vasoxen, Menarini Group, I. E. Ulagay, Germany) were pulverized and dissolved in distilled water to obtain a 0.017 mg/mL solution. This was equivalent to a 5 mg adult human (70 kg) dose.

Surgical procedure

The 21 rats were randomly divided into 3 groups: nebivolol 1, nebivolol 2, and the control. Following the induction of anesthesia with intraperitoneal injection of ketamine (50 mg/kg) and xylazine hydrochloride (10 mg/kg), the right hind limb of each animal was shaved and prepared with chlorhexidine gluconate for aseptic surgery. Using an aseptic technique, a longitudinal incision was made on the lateral aspect of the right hind limb. An open femoral fracture was created on the midshaft of the femur using a 1.0 mm sterile drill (Aysam Samsun, Turkey). Two perpendicular drill holes were made in the middle of the shaft, and the bone was manually broken. An intramedullary fixation was performed using a 1.50 mm (0.057 inch)-diameter stainless Kirschner wire (Aysam Samsun, Turkey). The wound was closed using 5–0 vicryl sutures (Pegelac, Doğsan Trabzon, Turkey), and the skin was closed with polypropylene 4–0 sutures (Propylene, Doğsan Trabzon, Turkey). The rats were left without cast immobilization for 4 weeks (Fig. 1).

Nebivolol treatment

Sterile nebivolol solution (1 mL) was given to the rats in group 1 every day for 4 weeks, while the rats in nebivolol group 2 were given 2 mL per day. At the end of the 4th week, all rats were sacrificed, and the broken femurs were removed. Radiological examinations were first performed before fixing the femurs for 2 days in 10% buffered formaldehyde solution for histological examination.

Histological and immunohistochemical examination

The specimens were decalcified before embedding in paraffin. They were then sectioned (4 μm), placed on slides, and stained with hematoxylin & eosin (H&E) and Masson's trichrome. CD34 immunostaining was used to detect angiogenesis. Each specimen was graded based on 10 sections. Grades were determined based on the visual field. Slides were examined using a light microscope (Olympus BX51, Tokyo, Japan) for fracture healing and new bone formation by pathologists who were blinded to the groups. The histological assessment scale (Table 1) described by Allen et al. was used for this study.[9]

Table 1. Histological fracture healing scale

Histological grade	Score
Pseudoarthrosis	0
Incomplete cartilage union	1
Complete cartilage union	2
Incomplete bone union	3
Complete bone union	4

Fig. 1. Surgical procedure; open femoral fracture model and pinning

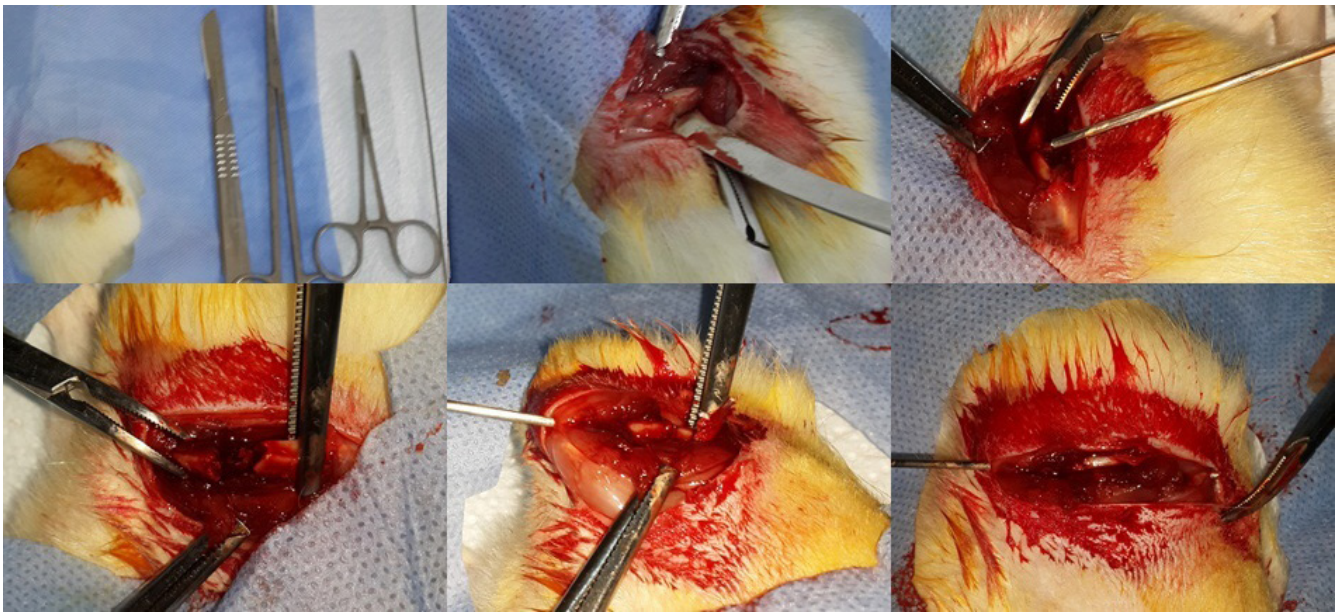
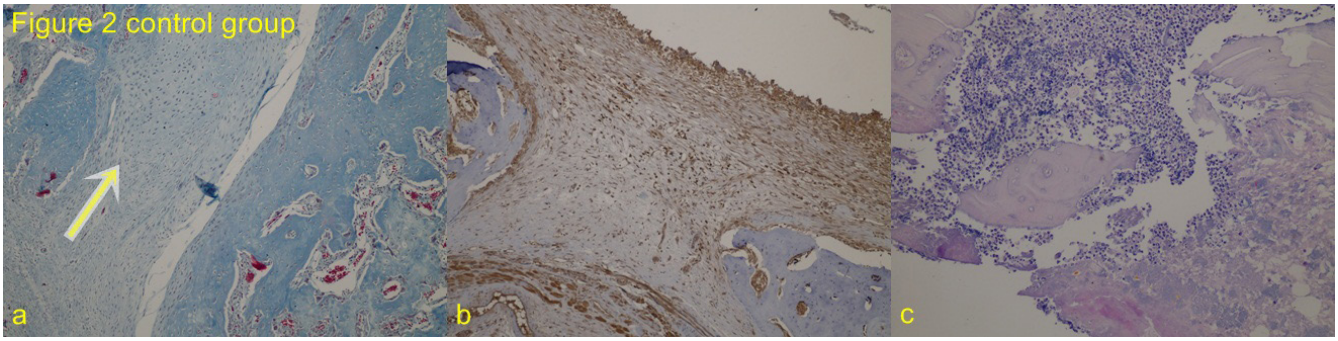


Fig. 2. Control group histological sections: a) Masson's trichrome staining showed large amounts of cartilage (yellow arrow) in the callus; b) No new vascular bed was shown with CD34 immunostaining; c) Minimally trabecular bone visualized with hematoxylin & eosin (H&E) staining (×40)



Radiological examination

Standard anteroposterior roentgenograms were taken. An orthopedic surgeon evaluated the radiographic data according to the scale. Fracture healing was radiographically evaluated and graded into Class 0 (non-union), Class I (mild union), and Class II (union) groups.[10]

Statistical analysis

Radiologic and histologic scores were determined at the end of the 4th week. Statistical analyses were performed using GraphPad Prism v. 6.05 (GraphPad Software, Inc., La Jolla, USA). All data was expressed as mean ± standard deviation (SD). Because of the small experimental groups, we used nonparametric statistical tests. The differences among the multiple groups were analyzed with the Kruskal-Wallis test. The differences between 2 groups were analyzed with Dunn's post-hoc test. A p-value < 0.05 was considered as statistically significant.

Results

All rats completed the 4-week study. K-wire migration was detected in the abdominal region of one rat in nebivolol group 1; the rat was not excluded from the study because it had no negative impact on animal welfare. The histological findings are shown in Fig. 2–4.

Histological findings

The average histological scores for the control (Fig. 2), and nebivolol groups 1 and 2 (Table 2) were 1.71, 2.28, and 2.85, respectively. Histopathological scoring results revealed that statistically significant differences were observed between the study groups ($p = 0.007$), as well as between nebivolol groups 1 and 2 ($p < 0.005$). Vascularity (red arrows) was remarkable in nebivolol group 2 (Fig. 4). Trabecular bone formation was significant in nebivolol groups 1 and 2 (Fig. 3, 4). Cartilage formation occurred at a higher rate in the control group (yellow arrow), compared with the nebivolol groups (Fig. 2).

Fig. 3. a) Masson's trichrome stained micrographs at the fracture site; cartilage formation on the left side and trabecular bone on the right; b) CD34 immunostaining showed mild vascularity; c) Hematoxylin & eosin staining showed increased trabecular bone formation in the nebivolol group 1 (×40)

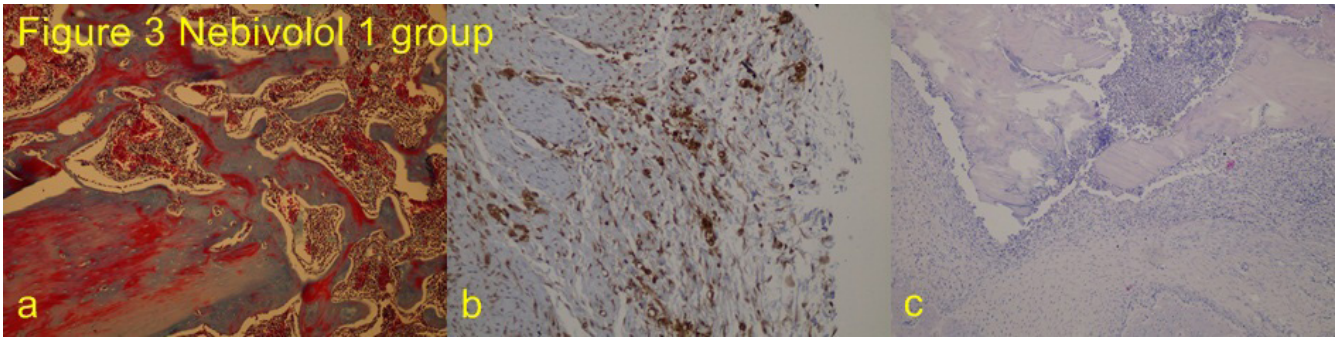


Fig. 4. a) Masson's trichrome and c) hematoxylin & eosin micrographs showed predominantly trabecular bone and less cartilage formation; b) CD34 immunostained micrographs showed increased vascularity in nebivolol group 2 (×40)

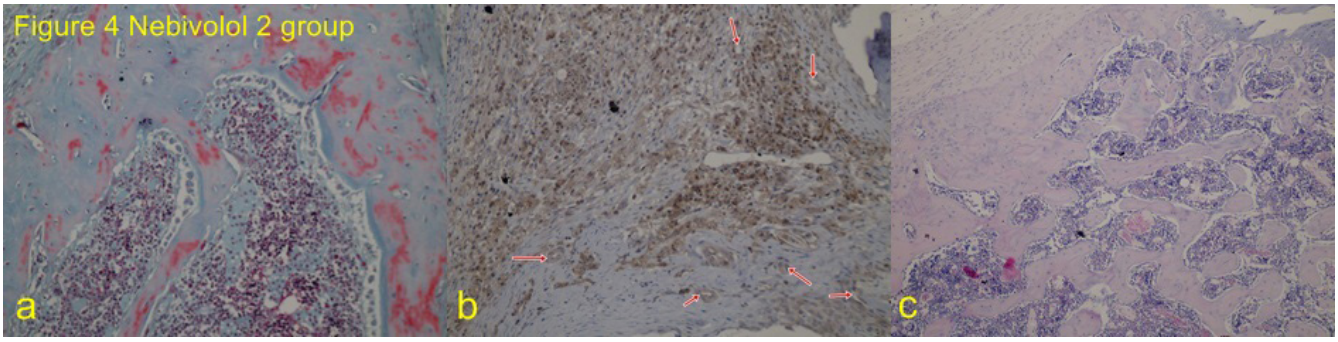


Fig. 5. Radiological findings of the 3 groups

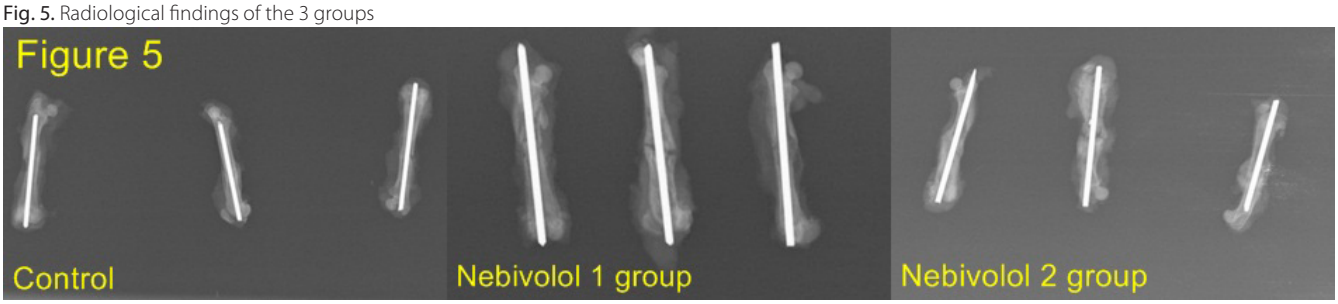


Table 2. Statistical evaluation of histologic and radiological scores

Parameters	Control	Nebivolol 1	Nebivolol 2	p-value
Histological score	1.71 ± 0.75a	2.28 ± 0.75	2.85 ± 0.78a	0.007
Radiological score	1.14 ± 0.69	1.28 ± 0.53	1.71 ± 0.27	0.088

Data are presented as mean ± standard deviation (SD); data were tested using the Kruskal-Wallis test and the Dunn's method was used for post hoc testing; p-value < 0.05 was considered statistically significant; $p^a < 0.05$, compared to the control group.

Radiological findings

The average radiological scores were 1.14, 1.28, and 1.71 in the control and nebivolol groups 1 and 2, respectively. There were no statistically significant differences in radiographic results between the 3 groups and 2 groups (Fig. 5).

Discussion

Hypertension and osteoporosis that results in poorly healing fractures are frequently seen in elderly populations. In addition, elderly populations typically have higher and more continuous drug use, due to the presence of chronic conditions. Chronic drug use can positively or negatively affect bone metabolism. Heparin and its derivatives have negative effects on the bone microstructure, while angiotensin-converting enzyme inhibitors can reduce fracture risk.[11,12] The selective β1-blocker nebivolol, which is frequently used for hypertension, has both vasodilation and anti-oxidation effects. Recent studies suggest that high concentrations of nebivolol result in healing through the increased release of NO.[13] Gülcan et al. and Schaffer et al. previously showed that nebivolol had positive effects on wound healing.[14,15] However, according to our knowledge, this study is the first to investigate the effects of nebivolol on fracture healing.

The histochemical results of this study clearly show the positive effects of nebivolol on fracture healing. Complete bone union and trabecular bone formation were significantly higher in nebivolol group 2 (0.007) (Fig. 4). There were significant differences between the 5 mg/kg (nebivolol 1) treatment and the 10 mg/kg (nebivolol 2) treatment groups ($p < 0.005$). This suggests that nebivolol has a dose-dependent effect on healing. β1-receptors cause vascular endothelial injury, and nebivolol protects endothelial cells exerting anti-oxidative effects and prevents atherosclerosis.[16] However, nebivolol can also prevent osteoporosis by selectively inhibiting sympathetic nervous system β1 adrenergic receptors.[17] Nebivolol inhibits NO synthase uncoupling and produces systemic antioxidant effects. Thus, the beneficial activity of nebivolol is attributed to both the inhibition of eNOS uncoupling and endogenous antioxidant properties that lead to free-radical scavenging.[6,18] Interestingly, nebivolol is the only selective β1-blocker that has anti-oxidative properties.[16] Turner et al. showed that treatment with an NOS inhibitor decreased new bone formation in a rat tibia model.[19] In addition, supplementation with NO induces new bone formation, and NO-mediated vasodilation increases blood flow during the early phases of fracture healing.[7,20]

There was no significant difference in radiographic results; however, nebivolol has a significantly positive effect on bone microstructure healing by inducing NO release. Therefore, the histological results obtained in this study could not be confirmed radiologically.

The results of this study suggest that nebivolol has positive effects on bone healing and thus can be used to treat hypertension in elderly populations. The limitations of the present study include the small sample size, which was a result of ethical considerations. In addition, bone healing was not determined through micro-computed tomography or positron emission tomography due to cost restrictions. These additional tests may demonstrate the vasodilatory effects of nebivolol for further support of our findings.

Conclusions

This study suggests that nebivolol, a selective β blocker, has positive effects on fracture healing through anti-oxidative effects via the NO pathway and direct vasodilator effects.

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The effects of crocin on the symptoms of depression in subjects with metabolic syndrome

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Abstract

Background. Studies have suggested that metabolic syndrome (MetS) is associated with increased depressive symptoms, and reducing depression in subjects with MetS is important. Crocin, an active component of saffron, has useful properties for subjects with MetS, including antidepressant properties.

Objectives. The aim of the study was to assess the effect of a preparation of crocin on the symptoms of depression in subjects with MetS, and the relationship between changes in those symptoms and the serum pro-oxidant/anti-oxidant balance (PAB).

Material and methods. This sub-study was carried out on 34 subjects with MetS from the authors' previous randomized double-blind controlled clinical trial (RCT), all of whom met the inclusion criteria for this study. The subjects were randomly assigned to treatment and placebo groups ($n = 17$ in each group) and received each 30 mg of crocin (2 tablets of 15 mg) or placebo for 8 weeks. Depressive symptoms were assessed using the Beck Depression Inventory (BDI). The BDI questionnaire was completed for each subject at the baseline and at the end of the 8th week of treatment. Blood samples were taken from the subjects before and after the intervention period. Statistical analyses were performed using the SPSS for Windows, v. 16 (SPSS Inc., Chicago, USA).

Results. Out of the 34 participants enrolled, 33 completed the trial. The degree of depression decreased significantly in the crocin group ($p = 0.005$), but not in the placebo group ($p > 0.05$), and the difference between the 2 groups was statistically significant ($p = 0.013$). No significant relationship was observed between changes in depression symptoms and changes in the serum PAB ($p > 0.05$).

Conclusions. This study demonstrates that at a dose of 30 mg per day for 8 weeks, crocin reduced the symptoms of depression in subjects with MetS compared to the control group, and this effect was independent of its effect on the serum PAB.

Key words: crocin, metabolic syndrome, depression, saffron

Metabolic syndrome (MetS) is a common disorder that increases the risk of cardiovascular disease and type 2 diabetes. MetS is defined by a set of cardiovascular risk factors including abdominal obesity, hypertension, hyperglycemia and dyslipidemia (high blood triglycerides, low high-density lipoprotein).[1] MetS has high prevalence worldwide, and also in Iran.[2] Some studies show that MetS is associated with an increased risk of depressive symptoms.[3,4] Epidemiological studies show that the chances of patients with MetS developing depression are high and that there is a positive association between components of the metabolic syndrome, including abdominal obesity, hypertriglyceridemia and low HDL cholesterol, and depression.[5,6] Previous studies have pointed to the importance of early detection and management of depression in patients with MetS.[7]

There is evidence suggesting that a significant multifaceted and possibly reciprocal relationship exists between depression and MetS.[7–9] First, increased inflammatory cytokines and leptin resistance occur in patients with MetS, which increases their chances of developing depression.[5,7] Evidence also suggests that some metabolic abnormalities in MetS, such as impaired glucose homeostasis and mitochondrial respiration can contribute to the pathophysiology of depression.[10] It has also been suggested that possible subclinical vascular damage in Mets can contribute to the development of depressive symptoms.[11] Also, certain components of MetS, including obesity, may be associated with decreased self-esteem and increase the chances of depression.[12]

At the same time, MetS may be characteristic for subjects with depression leading an unhealthy lifestyle, because their diet often contains large amounts of fats and carbohydrates but only small amounts of vegetables, fruits and whole grains. Also, they do not engage in enough physical activity, and these factors make them predisposed to metabolic syndrome.[5,7,13] Depression is also associated with an increase in chronic inflammation and insulin resistance, and some neuroendocrine effects that can increase the risk of MetS.[7]

Oxidative stress is an imbalance between the production of pro-oxidants and anti-oxidants, with an excess of pro-oxidants. Studies have confirmed the contribution of oxidative stress in the etiology of both depression and MetS.[14,15] Therefore, reducing oxidative stress could be one of the important goals in the treatment of both MetS and depression. The pro-oxidant/anti-oxidant balance (PAB) assay is a new and simple method to measure the balance of pro-oxidants and anti-oxidants concurrently. Evidence has shown that PAB results are comparable with approved standard assessments of oxidative stress and that they correlate with protein oxidation markers such as carbonyl, advanced glycation end products (AGEs) and advanced oxidative protein products (AOPPs).[16,17]

Previous studies have provided evidence that saffron can be effective in improving depressive symptoms.[18] Crocin is one of main active components of saffron, and animal studies have confirmed its antidepressant properties.[19] In addition, previous studies have shown that crocin has anti-oxidant properties as well.[19]

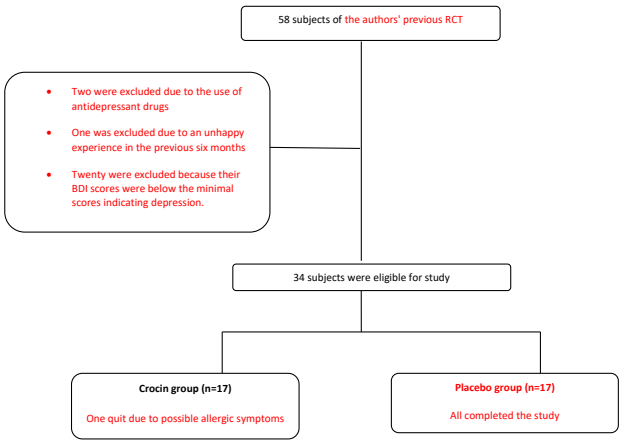
One of crocin antidepressant mechanisms may be related to its anti-oxidant property. The first objective of this study was to evaluate the effects of crocin on the symptoms of depression in subjects with metabolic syndrome. The second objective was to assess the association between changes in the symptoms of depression and the serum PAB, in order to evaluate the relationship between the anti-oxidative and antidepressant effects of crocin. As far as the authors are aware, there has been no previous research similar to this study, and the are no reports in the literature yet on the effects of crocin on depressive symptoms in humans.

Material and methods

This study is a substudy of the authors' previous randomized double-blind controlled clinical trial (RCT) on subjects with Mets, conducted at the Nutrition Clinic of Qaem Teaching Hospital, Mashhad, Iran, from April to June 2014.[20] The clinical trial was an approved and registered RCT, compliant with ethical standards, and written consent was obtained from all the participants. Volunteers with MetS according to the International Diabetes Federation (IDF) criteria were randomly assigned to a treatment group and a placebo group, and received either 30 mg of crocin or a placebo for 8 weeks. All subjects received similar dietary advice based on the American Heart Association (AHA) guidelines. This substudy involved 34 of the eligible RCT subjects (Fig. 1).

Exclusion criteria included pregnancy, lactation, age under 18 or over 70 years, use of antidepressant drugs, under 10 points on the Beck Depression Inventory (BDI) questionnaire, grief or unpleasant event during the previous 6 months, and a lack of compliancy in taking the pills regularly.

Fig. 1. Flow chart of the eligibility of study participants



The scores of less than 9 points on the BDI scale indicate a minimal degree of depressive symptoms. Since a drug shows its therapeutic potential in subjects that suffer from a complication rather than in non-affected subjects, this study (like the previous one) included subjects whose BDI score was 10 points or higher.[21]

Symptoms of depression

The BDI, which was used to assess depressive symptoms, includes 21 questions with 4 options for each question.[22] The responses to the questions are scored from 0 to 3 according to their intensity, and the total score is used to estimate the degree of depression. The cut-off values of BDI scores to define 4 degrees of depression are 0–9 (grade 1: minimal depression), 10–18 (grade 2: mild depression), 19–29 (grade 3: moderate depression) and 30–63 (grade 4: severe depression). The BDI questionnaire was completed for each subject at the baseline and at the end of week 8 of the study.

Crocin tablets

The extraction of crocin from saffron was done according to a previously published work by the current authors, and the pill manufacturing was undertaken by the School of Pharmacy, Mashhad University of Medical Science (MUMS), Iran.[23] Each crocin tablet contained 15 mg of crocin; the placebo pills were given a similar appearance, but contained only starch and food coloring. Quality control tests were conducted under the supervision of an industrial pharmacy specialist.[20]

Anthropometric and blood pressure assessment

Anthropometric measurements performed on all the participants included height, weight, waist circumference and hip circumference. Standing height (cm) was measured without shoes, with a wall-mounted stadiometer. Maximum hip circumference and minimum waist circumference were measured with a tape to the nearest 0.1 cm. A BC-418 bioelectrical impedance analysis device (Tanita, Tokyo, Japan) was used to measure body weight and body mass index (BMI). Blood pressure was measured on the right arm in the sitting position after at least 15 min of rest.

Blood sampling and laboratory tests

Blood samples were taken in the morning after 12 h of fasting, before and after the intervention. After centrifugation, the separated serum was stored at -80°C until the tests were run. Fasting blood lipid profile and blood glucose (FBG) were measured to diagnose metabolic syndrome; the fasting lipid profile included total cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). These blood factors were measured enzymatically according to standard protocols and using the relevant commercial kits (Pars Azmoon, Tehran, Iran). The serum PAB measurement was done according to the method described in previous studies.[16,17]

Statistical analysis

Statistical analyses were performed using the SPSS for Windows v. 16 software package (SPSS Inc., Chicago, USA). The data was analyzed using the Kolmogorov-Smirnov test to assess normality; Student's t-test or the Mann-Whitney test were used to compare baseline characteristics; the χ^2 test, Wilcoxon and Mann-Whitney tests were used to assess depression. A 2-tailed p-value < 0.05 was considered statistically significant.

Results

No significant differences were observed in the baseline characteristics of the drug group and the placebo group (Table 1). Similarly, no significant differences were observed in the baseline degree of depression between the 2 groups before the intervention (Table 1).

Out of the original 34 volunteers, 33 subjects completed the trial. One participant was excluded due to potential allergic symptoms.

The results of the analyses show that the degree of depression decreased in 68.8% of the crocin group and 29.4% of the placebo group (Fig. 2). The reduction of depression in the crocin group was statistically significant, while the reduction in the placebo group was not statistically significant. The difference in the changes in the degree of depression between the 2 groups was statistically significant at the 0.05 level (Table 2).

Fig. 2. Changes in the degree of depression in the 2 groups

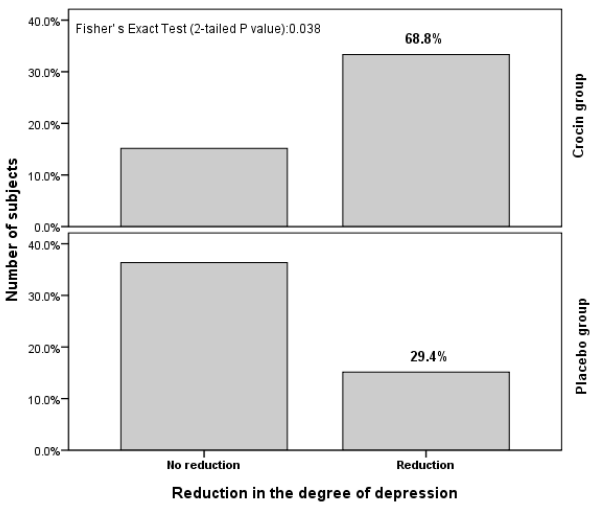


Table 1. Baseline characteristics of the study participants

Features	Case group	Control group	*p-value
Female/male (n)	12/4	10/7	p > 0.05
Age (years)	45 (34.5, 50)	48 (37, 55)	p > 0.05
Weight (kg)	83.4 (79.2, 102.2)	85.9 (79.3, 102.2)	p > 0.05
Height (cm)	159.5 (153.5, 166.2)	156 (153, 167)	p > 0.05
WC (cm)	110.7 (100.7, 120.5)	107 (102, 122.5)	p > 0.05
HC (cm)	113.0 (112.0, 121.7)	113.2(108.5, 124.5)	p > 0.05
BMI	34.25 (31.27, 36.2)	34.6 (31, 38.3)	p > 0.05
Smokers % (n)	6.2% (1)	0% (0)	p > 0.05
Diabetics** % (n)	18.7% (3)	23.5% (4)	p > 0.05
Hypertensive*** % (n)	18.7% (3)	18.7% (3)	p > 0.05
Cardiovascular disease**** % (n)	6.2% (1)	0% (0)	p > 0.05
Degree of depression*****	n (%)	n (%)	*p-value
Degree 2 (scores 10-18)	8 (50%)	10 (58%)	p > 0.05
Degree 3 (scores 19-29)	5 (31.2%)	6 (35.2%)	
Degree 4 (scores 30-63)	3 (18.7%)	1 (5.8%)	
BDI Score	18.5 (12.5, 26.5)	17 (15, 22)	

WC – waist circumference; HC – hip circumference; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; FBS – fasting blood glucose; DBP – diastolic blood pressure; SBP – systolic blood pressure; BMI – body mass index. Values are expressed as number, median (Q1, Q3) and percentage. *According to the non-normal distribution of data with the Kolmogorov-Smirnov test, non-parametric tests were used. **Diabetes was diagnosed from the medical history, treatment with diabetic drugs or high blood sugar (FBS ≥ 126). ***Hypertension was diagnosed from the medical history and treatment with blood pressure drugs. ****Heart disease was diagnosed from the medical history and treatment with heart disease medications. *****Based on the results of the Beck Depression Inventory (BDI).

BDI scores were significantly reduced in the crocin group (p = 0.009) but not in the placebo group (p > 0.05). However, the difference between the 2 groups in terms of the magnitude of the changes in BDI scores did not reach statistical significance (p = 0.09).

There was no significant association between changes in serum PAB values and the severity of depression. The magnitude of changes in PAB values was comparable between the subgroups with and without improvement in their BDI scores in both the crocin group and the placebo group (p > 0.05) (Table 3).

Discussion

The results of the present study showed that at a dose of 30 mg per day for 8 weeks, crocin was associated with a reduction in the degree of depressive symptoms in subjects with MetS. But no association was found between the reduction in the degree of depression and the reduction of serum PAB.

Some previous animal studies have shown that saffron had a much greater antidepressant effect than a placebo; a 2004 study by Hosseinzadeh et al. found that this effect occurs through inhibition of the uptake of dopamine, norepinephrine and serotonin by safranal and crocin, components of saffron.[19,24] Also, some human clinical trials have compared the antidepressant effect of saffron on mice with standard medications, including imipramine and fluoxetine, and found that saffron can be as effective as standard medications in treating depression.[25,26] In 2010 Wang et al. found that the antidepressant effect of extract of stigmas of saffron on mice is associated with the presence of crocin.[27]

As noted earlier, studies have confirmed that MetS is associated with depression and that oxidative stress is implicated in the etiology of both depression and metabolic syndrome.[14,15] The antidepressant mechanisms of saffron and its components are not still clear. In vitro and animal studies have shown the effectiveness of crocin in reducing depression, as well as its anti-oxidant properties.[19] Therefore, crocin may be effective in treating both depression and oxidative stress in patients with Mets.

As far as the authors are aware, no study has previously been published about the effect of crocin on depression in human subjects. In 2011 Shemshian et al. conducted a study involving saffron and the BDI questionnaire.[21] They assessed the effect of 100 mg of saffron per day for 12 weeks, comparing it with a placebo on 60 patients with depression and anxiety. They found that saffron significantly reduced the BDI scores of depression in the patients, and concluded that saffron has a therapeutic antidepressant effect on patients with a clinical diagnosis of depression. The present study, on the other hand, assessed the effect of crocin on the symptoms of depression in subjects with MetS who have a mild to moderate degree of depression according to the BDI scale. Shemshian et al. used 100 mg saffron per day and the present study used 30 mg of crocin per day. Crocin comprises 10% of the dry weight of saffron, so the crocin dose in the present study was 3 times higher than in their study. However, in the study by Shemshian et al. there were other active components of saffron in addition to crocin.

The present study found no association between the reduction in the degree of depression and the serum PAB. This means that the results of this study did not support the theory that anti-oxidant action of crocin is involved in reducing the symptoms of depression in subjects with

Table 2. Comparison of changes in the degree of depression in the 2 groups

Study group and statistical test	Degree of depression* median (Q1–Q3) week 0	Degree of depression* median (Q1–Q3) week 8	Reduction in the degree of depression n (%)	Wilcoxon test (2-tailed p-value)
Crocin group (n = 16)	2.5 (2–3)	2 (1–2.75)	11 (68.8%)	p = 0.005
Placebo group (n = 17)	2 (2–3)	2 (2–3)	5 (29.4%)	p > 0.05
Mann-Whitney test for changes in the degree of depression in the 2 groups	p = 0.013 (2-tailed p-value)			

*The degree of depression based on the results of the Beck Depression Inventory (BDI) as follows: degree 1 – minimal depression (scores 0–9); degree 2 – mild depression (scores 10–18); degree 3 – moderate depression (scores 19–29) and degree 4 – severe depression (scores 30–63).

Table 3. Serum PAB changes in relation to reductions in the degree of depression

The study groups	Reduction in the degree of depression (n %)	PAB changes median (Q1, Q3)
Crocin group (n = 16)	reduction (n = 11, 68.8%)	-17.7 (-51.9, 5.2)
	no reduction (n = 5, 31.2%)	-4.9 (-22.31, 8.98)
Placebo group (n = 17)	reduction (n = 5, 29.4%)	-15.9 (-23.9, 9.6)
	no reduction (n = 12, 70.6%)	-6.82 (-34.3, 35.7)
Mann-Whitney test for the relation between changes in depression and PAB†	ns*	
Spearman correlation for changes in depression and PAB in the 2 groups	ns*	

*ns – not significant (p > 0.05); †In this Mann-Whitney test, reduction in the degree of depression (as two variables: 1 – no reduction, 2 – reduction) was the grouping variable, and change in serum PAB was the test variable.

MetS. It is likely that the main antidepressant mechanism of crocin is preventing the uptake of the neurotransmitters such as dopamine, norepinephrine and serotonin, as previous evidence has suggested, but this needs to be further explored in future clinical studies.

The main limitation of this study is that it included subjects who did not have a clinical diagnosis of depression, because it was a substudy of research that was not primarily designed to assess the impact of crocin on the participants' psychological status.[20] Other limitations include the small sample size, the evaluation of the effects of only 1 dosage (30 mg per day), and the relatively short duration of the treatment and follow-up. All of these limitations need to be addressed in future studies.

Conclusions

The results of this study showed that supplementation with crocin at a daily dose of 30 mg for 8 weeks could reduce the symptoms of depression in subjects with MetS, an effect that appears to be independent of changes in plasma PAB status.

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Soluble ST2 protein and hospitalizations due to worsening chronic heart failure during a one-year follow-up in a population with reduced ejection fraction

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Hospitalizations due to worsening chronic heart failure (CHF) are common. However, the relationship between a single measurement of soluble ST2 protein (sST2) and the necessity of hospitalization in CHF is still unclear.

Objectives. The aim of this study was to determine the association between a single measurement of sST2 concentration and hospitalizations due to worsening CHF during a one-year follow-up.

Material and methods. The study involved 167 consecutive patients (mean age 63 years, 83% males) with CHF in stable NYHA classes I-III with left ventricular ejection fraction (LVEF) $\leq$ 45% (median 29.65%, range 13–45%). Fifty-six variables were analyzed (clinical factors, basic laboratory results on admission, standard 12-lead ECG, echocardiography and coronary arteriography results). Information about hospitalizations due to worsening CHF was obtained during telephone interviews conducted 12 months after discharge from the cardiac ward. In order to define factors associated with hospitalization, uni- and multivariate regression analyses were performed.

Results. A total of 53 patients from the study group (38%) were hospitalized due to worsening CHF. They included a higher percentage of males ($p = 0.042$), higher concentrations of sST2 ($p = 0.049$), and glucose ($p = 0.010$). The multivariate analysis (for model $\chi^2 = 17.235$; $p < 0.001$) revealed that glucose and sST2 were independently associated with hospitalization due to worsening CHF during the 1-year observation ($p < 0.001$).

Conclusions. In patients with stable mild to moderate CHF with reduced EF, a single measurement of sST2 protein and glucose were independent variables for hospitalization due to worsening CHF over a 1-year follow-up period. The defined prognostic model including sST2 and fasting glucose better identified patients without HF-related hospitalizations.

Key words: prognosis, biomarkers, hospitalization, heart failure, ventricular dysfunction

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Chronic heart failure (CHF) is becoming an increasing clinical and economical problem. According to the estimates of the European Society of Cardiology, heart failure (HF) absorbs approx. 2% of the funds allocated to health care, including a significant proportion for repeated hospitalizations.[1] In the USA, unplanned rehospitalizations cost Medicare 17.4 billion dollars, and HF is the most common cause.[2] Decompensation of CHF represents 80% of all hospitalizations due to HF.[3] One of the most important aspects is the frequency of hospitalization and readmission of patients with HF. The rate of rehospitalization within 30 days of discharge ranges from 15 to 25%.[2,4,5] In other published studies, the incidence of hospital readmission approaches 45% at 6 months.[6]

Although some biomarkers have established roles in the prognosis for CHF, improvement of risk stratification in HF continues to be developed using new markers, and among these, soluble ST2 protein (sST2) has come of age. sST2 is part of the cardioprotective signaling pathway involving interleukin-33. Biomechanical strain in cardiomyocytes and remodeling fibrosis affect sST2 concentration in the serum. The prognostic utility of sST2 protein has recently been documented in acute and chronic heart failure. There have been several papers suggesting the usefulness of sST2 in predicting adverse outcomes, but the relationship between a single measurement of sST2 protein and the necessity of hospitalization in CHF still remains unknown.[7–20] The aim of this study was to determine the association between a single measurement of sST2 concentration and hospitalizations due to worsening CHF in a 1-year follow-up period.

Material and methods

The study included 167 consecutive patients diagnosed at the Department of Noninvasive Cardiology at the Medical University of Lodz (Poland). The inclusion criteria were as follows: stable (defined as at least 4 weeks without CHF exacerbation); mild to moderate CHF categorized as NYHA (New York Heart Association) class I-III; left ventricular ejection fraction (LVEF) lower than or equal to 45%; optimal treatment of HF according to the guidelines of the European Society of Cardiology (ESC).[1] The patients' hemodynamic state was stable and the reason for admission to the hospital was to determinate HF etiology and to establish appropriate management. The exclusion criteria were NYHA class IV, acute heart failure, acute coronary syndrome, inflammatory states and thyroid dysfunction.

The study was approved by the Bioethics Committee at the Medical University of Lodz (no. RNN/79/10/KE) and all the patients signed informed consent form to participate in the study.

The patients' clinical history, basic laboratory results and standard 12-lead ECG were assessed upon their admission to the hospital. The results of echocardiography and coronary arteriography were obtained according to American Society of Echocardiography/European Association of Echocardiography (ASE/EAE) and ESC recommendations.

The analyses included 58 variables:

- clinical features such as NYHA class, age, body mass index (BMI), arterial blood pressure, smoking status; coincidence of arterial hypertension, diabetes mellitus (DM), stroke, myocardial infarction (MI), peripheral arterial disease or chronic lung diseases;
- selected laboratory results such as morphology, sodium, creatinine, estimated glomerular filtration rate (e-GFR), serum lipid concentrations, C-reactive protein (CRP), high-sensitivity troponin T (hsTnT) and N-terminal prohormone B-type natriuretic peptide (NT-proBNP);
- electrocardiographic variables: heart rhythm (sinus rhythm or atrial arrhythmias), heart rate, left bundle branch block (LBBB), QRS duration, QTc;
- selected echocardiographic results (M-mode, 2-dimensional and Doppler echocardiographic examinations): left atrial diameter (LA), left ventricular systolic dimensions (LVESD), left ventricular diastolic dimensions (LVEDD), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), and left ventricular ejection fraction (LVEF);
- coronary arteriography results (stenosis > 50% of left main coronary artery, > 75% of other coronary arteries; analyzed as 1-, 2-, or 3-vessel disease) to determine the etiology of HF (ischemic vs non-ischemic). Coronary arteriography was performed by a radial or femoral approach with visual quantification of sclerotic alteration.

Blood samples (5 mL) to determine sST2 concentration were collected upon admission to the hospital using standard collection techniques into vacuum tubes containing a clot activator. The samples were centrifuged for 5 min at 3000 rpm after the formation of a clot, and the supernatant (serum) was immediately separated and frozen at -76°C. Then, after thawing the serum, the soluble ST2 level was measured by quantitative assay using a sandwich ELISA kit (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) validated for use with human serum. This kit uses 2 monoclonal antibodies against 2 different epitopes of human sST2. The serum samples were incubated in microwells coated with the first antihuman sST2 monoclonal antibody. After the washing stage, the second incubation with the peroxidase conjugated antihuman sST2 monoclonal antibody was conducted. After further washing, the peroxidase substrate was added to each well and the optical density was measured at 450 nm using a microplate reader.

A telephone interview with each patient was conducted by the cardiologist 12 months after discharge from the

cardiac ward, and information about hospitalizations due to decompensation of CHF was collected; the patients were always asked to read the discharge diagnosis aloud. The patients were divided into groups and analyzed depending on the occurrence of hospitalization due to decompensation of CHF.

The statistical calculations were performed using the STATISTICA PL v. 9.0 software package (StatSoft, Inc. Tulsa, USA) and IBM SPSS Statistics for Windows v. 19 (IBM Corp., Armonk, USA).

For measurable variables, basic descriptive characteristics – the mean, median, maximum and minimum values, interquartile range (IQR, Q25 - Q75) and standard deviation (SD) – were provided. For qualitative variables, the number of observations (N) and the corresponding percentage (%) were indicated. Normality was tested using the Shapiro-Wilk test.

For comparison of 2 independent groups of quantitative variables, Student's t-test (in the case of normal distribution in both groups) or the nonparametric Mann-Whitney U-test (in the absence of normal distribution) was used. To verify the relationship between qualitative variables, a χ^2 test of independence or a χ^2 test of independence with Yates' correction (in lower numbers) was used.

For statistically significant quantitative variables, ROC curves were drawn and the optimal cut-off points were determined. The sensitivity, specificity, positive and negative predictive value (PPV and NPV), odds ratio (OR) and a 95% confidence interval (95% CI) were also assigned.

In order to determine the factors related to hospitalizations, univariate and multivariate forward stepwise regression analyses were performed. Variables that reached $p < 0.1$ in univariate analyses were used in the stepwise logistic regression models. Results were considered statistically significant at $p < 0.05$. Kaplan-Meier survival curves were created for rehospitalization.

Results

The mean age of the patients included in the study was 63 years +/- 11.58. The majority of patients were men (83%), mainly in NYHA class III (72%), with mean LVEF 29.65% +/- 7.83 (range: 13–45%). The patients studied were burdened with many comorbidities: coronary artery disease (54%), hypertension (53%), chronic kidney disease (22%), chronic lung disease (12%) and disorders of carbohydrate metabolism (37%). The mean sST2 concentration was 0.65 ng/mL +/- 0.7. The only factor independently associated with the level of sST2 was the QRS complex duration (OR = 1.013; 95%CI [1.001–1.025]; p = 0.03).

The patients who were rehospitalized during the follow-up period due to decompensation of CHF (n = 53; 38%) were mostly male and had higher levels of sST2 and glucose (Table 1). The average time to the first rehospitalization was 6.7 months +/- 3.45. The rehospitalization rates in the first month, 3 months and 6 months after discharge were 5.6%, 20.7% and 52.8% respectively. Among all the patients, 24 died due to HF (n = 20) or MI (n = 4) during the observation period and were excluded from the analysis in the present study; the data on those patients were analyzed in detail in a previous paper.[17]

In a multi-variate forward stepwise regression analysis both sST2 and glucose are independently associated with hospitalization due to decompensation of CHF during the 1-year observation period (Table 2). If sST2 protein concentration increased by one unit, the risk of hospitalization increased more than twofold. On the other hand, if the concentration of serum fasting glucose increased by 1 unit, the risk of hospitalization increased by 50.5%.

Using ROC analysis, a cut-off point value for sST2 ≥ 0.3389 ng/mL was determined (AUC [95%CI] = 0.599 [0.503–0.696]; p = 0.049) with a sensitivity of 58.49%, specificity 55.17%, and positive and negative predictive values of 44.3% and 68.6% respectively. From the ROC analysis a cut-off point value for glucose > 5.918 mmol/L was determined (AUC [95%CI] = 0.637 [0.535–0.739], p = 0.01) with a sensitivity of 62.5%, specificity 55%, and positive and negative predictive values of 45.45% and 70.97% respectively. A prognostic model containing both variables had a NPV of 88.7% and a PPV of 39.1%.

Survival curves for hospitalization during the 1-year observation period were determined using the Kaplan-Meier method (Fig. 1). In comparison to a high concentration of sST2 ≥ 0.3389 ng/mL, low concentrations of sST2 < 0.3389 ng/mL, below the threshold from the ROC curve, indicated a better prognosis, and the probability of non-occurrence of HF-related hospitalization increased from 0.58 to 0.7. This model showed a trend toward statistically significance (p = 0.065) in the study population.

Fig. 1. Kaplan-Meier curves for hospitalizations based on sST2 concentrations

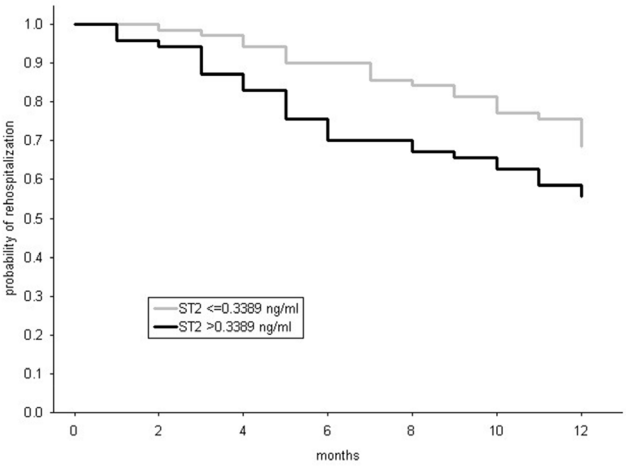

Table 1. Selected patient characteristics in relation to hospitalization due to decompensation of chronic heart failure

Variables	Without hospitalization n = 114		Hospitalization n = 53		p-value
	n or mean +/- SD (range)	% or median (IQR)	n or mean +/- SD (range)	% or median (IQR)	
Demographic and clinical					
Male.	70	80.46	50	94.34	0.042
Age [years]	63.33 +/- 12.2 (24–87)	64 (55–73)	62.3 +/- 10.42 (38–82)	63 (57–67)	0.610
Body mass index [kg/m^2]	26.53 +/- 3.98 (16.6–39.26)	26.5 (23.67–29.07)	26.92 +/- 3.65 (20.05–35.32)	26.4 (24.3–29.36)	0.568
NYHA class. I II III	 4 25 58	 4.60 28.74 66.67	 2 11 40	 3.77 20.75 75.47	0.540
Ischaemic HF	48	55.17	32	60.38	0.546
Former or current smoking	46	62.16	33	68.75	0.457
HA	42	50.00	32	62.75	0.149
DM	27	32.14	23	45.10	0.130
Peripheral sclerosis	9	10.59	9	17.65	0.240
MI	45	51.72	31	58.49	0.436
Chronic lung disease	10	11.90	4	7.48	0.646
Systolic blood pressure [mm Hg]	120.41 +/- 17.97 (90–190)*	120 (110–130)	120.93 +/- 17.69 (85–164)	120 (110–130)	0.761
Diastolic blood pressure [mm Hg]	72.18 +/- 9.37 (55–100)*	70 (70–80)	75.32 +/- 10.79 (60–100)*	75 (70–80)	0.102
ECG					
Atrial fibrillation	22	26.19	10	18.87	0.324
LBBB	23	27.38	14	27.45	0.993
Heart rate [bpm]	77.16 +/- 20.17 (53–170)*	70 (65–83)	79.15 +/- 15.32 (55–120)*	80 (65–87)	0.133
QRS duration [ms]	109.77 +/- 28.4 (60–180)*	102 (80–120)	116.04 +/- 29.51 (80–200)*	100 (100–136)	0.368
QTc [ms]	387.1 +/- 52.45 (260–520)	380 (340–420)	395.2 +/- 44.1* (280–520)	400 (360–420)	0.294
Echocardiography					
LVEF (%)	30.18 +/- 7.75 (13–45)	30 (24–36)	29.02 +/- 7.33 (14–45)	29 (24–34)	0.380
LVESD [cm]	5.35 +/- 0.9 (3.5–8)	5.33 (4.7–5.94)	5.59 +/- 0.88 (3.4–7.2)	5.8 (4.9–6.18)	0.165
LVEDD [cm]	6.64 +/- 0.84 (5–9.2)	6.5 (6–7.2)	6.89 +/- 0.87 (4.7–8.7)	7 (6.4–7.5)	0.123

The table shows selected variables relevant to the context of the article. *variables with non-parametric distribution; SD – standard deviation; NYHA – New York Heart Association; HF – heart failure; HA – hypertension; DM – diabetes mellitus; MI – myocardial infarction; ECG – electrocardiogram; LBBB – left bundle branch block; LVEF – left ventricular ejection fraction; LVESD – left ventricular end-systolic diameter; LVEDD – left ventricular end-diastolic diameter; LVESV – left ventricular end-systolic volume; LVEDV – left ventricular end-diastolic volume; LA – left atrium; CAD – coronary artery disease; hsTnT – high sensitivity troponin T; hsCRP – high-sensitivity C-reactive protein; e-GFR – estimated glomerular filtration rate.

Table 1. Selected patient characteristics in relation to hospitalization due to decompensation of chronic heart failure (cont.)

Variables	Without hospitalization n = 114		Hospitalization n = 53		p-value
	n or mean +/- SD (range)	% or median (IQR)	n or mean +/- SD (range)	% or median (IQR)	
Echocardiography					
LVESV [mL]	134.87 +/- 55.89* (46–343)	122 (92–170)	156.02 +/- 69.54 (34–364)	145 (114–187)	0.081
LVEDV [mL]	190.71 +/- 68.49* (87–477)	182 (140–230)	215.1 +/- 83.74 (55–449)	206 (169–245)	0.076
LA [cm]	5.88 +/- 0.98 (3.5–8.6)	5.7 (5.3–6.4)	6.09 +/- 0.78 (3.5–7.8)	6 (5.7–6.6)	0.206
Coronarography (extent of CAD)					
1 vessel.	11	18.97	11	28.95	
2 vessels.	16	27.59	10	26.32	0.611
3 vessels.	8	13.79	6	15.79	
Biochemical					
sST2 ng/mL	0.5 +/- 0.4 (0.19–2.25)*	0.31 (0.27–0.53)	0.81 +/- 0.96 (0.25–4.3)*	0.38 (0.29–0.85)	0.049
NT-proBNP (mg/dL)	2759 +/- 3041 (197–13863)*	1881 (761–3078)	3445 +/- 5399 (333–35000)*	1912 (1001–3985)	0.408
hsTnT [mg/L]	0.048 +/- 0.072 (0.004–0.36)*	0.023 (0.014–0.053)	0.039 +/- 0.033 (0.004–0.14)*	0.032 (0.016- 0.045)	0.396
hsCRP [mg/L]	7.15 +/- 14.46* (0.2–97)	3 (0.8–7)	6.44 +/- 9.66* (0.5–47.8)	3.4 (0.9–6.7)	0.683
Creatinine [mg/L]	89.46 +/- 22.13* (49–168.11)	88.48 (71.5–97.33)	89.61 +/- 26.78* (53.09–194.66)	85 (70.78–97.33)	0.576
e-GFR [mL/min/1.73 m^2]	84.22 +/- 29.29 (27–156.32)	81 (63.66–105.2)	94.55 +/- 36.45* (27.9–223.5)	91 (70.486–113)	0.151
Sodium [mmol/L]	136.95 +/- 3.09* (127–143)	137 (136–139)	137.04 +/- 3.28 (129–144)	138 (135–139)	0.743
Glucose [mmol/L]	5.89 +/- 0.93* (4.37–9.3)	5.82 (5.26–6.13)	6.89 +/- 2.49* (2.63–16.2)	6.05 (5.63–7.39)	0.010

The table shows selected variables relevant to the context of the article. *variables with non-parametric distribution; SD – standard deviation; NYHA – New York Heart Association; HF – heart failure; HA – hypertension; DM – diabetes mellitus; MI – myocardial infarction; ECG – electrocardiogram; LBBB – left bundle branch block; LVEF – left ventricular ejection fraction; LVESD – left ventricular end-systolic diameter; LVEDD – left ventricular end-diastolic diameter; LVESV – left ventricular end-systolic volume; LVEDV – left ventricular end-diastolic volume; LA – left atrium; CAD – coronary artery disease; hsTnT – high sensitivity troponin T; hsCRP – high-sensitivity C-reactive protein; e-GFR – estimated glomerular filtration rate.

Table 2. Model of multivariate stepwise logistic regression analysis for hospitalization due to worsening chronic heart failure

Variables	B – parameter estimation	Standard error (SE)	p-value	Exp(B) – odds ratio OR	95% confidence interval for OR	
sST2	0.801	0.335	0.017	2.228	1.156	4.296
Glucose	0.408	0.149	0.006	1.505	1.123	2.015

Discussion

The present study revealed that in a population with stable mild-to-moderate chronic heart failure and EF ≤ 45%, a single measurement of sST2 protein concentration is an independent variable for HF-related hospitalization during a 1-year observation period.

In most studies HF-related hospitalization occurs in conjunction with death as a composite endpoint, and most studies have been performed in populations that have experienced an HF event, to assess the prognostic role of biomarkers. Only Felker et al. described an association of ST2 and long-term outcomes (over a period of 2.5 years) including death, hospitalization and functional capacity ambulatory population with CHF and EF < 35%.[21]

In the Controlled Rosuvastatin Multinational Trial in Heart Failure (the CORONA study) on 1449 patients with CHF of ischemic origin with LVEF ≤ 40%, it was found that sST2 is an independent predictor of hospitalization due to worsening CHF (HR 1.30, 95% CI [1.04–1.62], p = 0.02); of death in the course of increasingly severe CHF (HR 1.57, 95% CI [1.05–2.34, p = 0.03); and of hospitalization due to cardiovascular events (HR 1.28, 95% CI [1.07–1.52], p = 0.006).[22] In the present study group, there was a wide spectrum of patients with CHF of both ischemic and non-ischemic etiology. The HF etiology was not associated with rehospitalization, and sST2 was not related to the etiology of HF. In the Valsartan Heart Failure Trial sST2 was significantly (p < 0.0001) associated with the risk of hospitalization for heart failure.[23]

In CHF, subsequent measurements of either BNP or NT-proBNP levels provide independent information regarding the risk for disease progression across a wide spectrum of adverse outcomes: ventricular remodelling, malignant ventricular arrhythmias, hospitalization for HF, the need for transplantation, as well as death.[24,25] In CHF, measurements of natriuretic peptide (NP) levels provide independent information regarding the risk for hospitalization for HF, and NP-guided therapy can reduce all-cause mortality and HF-related hospitalizations.[24,26] The present study did not find a correlation between hospitalization and NTproBNP. In contrast to other studies, in which patients entering the survey were hospitalized due to HF decompensation, in the present study they were in clinical stable CHF. Therefore further study of this issue is needed in mild-to-moderate CHF.

An interesting finding is the lack of association of traditional risk factors and the risk of HF-related hospitalization. Similar findings were also revealed in a previous publication by the present authors.[27] It should be emphasized that the study population had stable mild-to-moderate CHF and EF ≤ 45% that was optimally treated. The hospitalization during which the patients were enrolled in the study was not due to exacerbation of HF (it was to determinate HF etiology and to establish appropriate management), and the period of observation was short (1 year).

In the authors' opinion, these 4 aspects (the population, the optimal treatment, the nature of the primary hospitalization and the short-term observation) were the reason for the absence of any relationship between other risk factors and hospitalization due to worsening HF. Moreover, according the International ST2 Consensus Panel, sST2 is one of the most powerful predictors of HF complications.[28] According to that document, sST2 is considered to be especially useful in the prognosis of HF events such as worsening HF and the risk of hospitalization, arrhythmia or death from HF. This emphasizes even more the role of sST2 as a prognostic factor for hospitalization due to worsening HF in the patients in the current study.

The present study is based on a single measurement of sST2 and NT-proBNP in stable CHF patients, whereas most studies on the prognostic utility of NP were conducted using serial measurements of NP.

Another important finding of the present study is the independent correlation between the fasting glucose serum level and HF-related hospitalization. This is consistent with other studies showing an association between high concentrations of glucose and a worse prognosis in CHF.[29,30] In a study by Gotsman et al., impaired fasting glucose was a predictor of increased cardiac-related hospitalizations (HR 1.17, 95% CI 1.00–1.35, p < 0.05).[30]

Recently more and more attention is being paid to the problem of hospitalization due to CHF decompensation, which means that identifying factors that affect its occurrence is very important. Reports of found new variables associated with hospitalization have been published, such as heart rate[31], which was not a factor influencing hospitalization in the present study. This was probably due to the study population undergoing optimal treatment that resulted in satisfactory heart rates.

Among biomarkers, NP has an established position as a predictor of HF-related hospitalization. Beside NP, more and more has been written about novel biomarkers related to cardiac remodeling and fibrosis. Gaggin et al.[32] confirmed that sST2 appears to be a more useful biomarker than others, such as GDF-15 and galectin-3 in the population of patients with CHF.[33]

In addition, the present study found that QRS duration is independently associated with sST2 levels. It is well known that the duration of the QRS has prognostic value in HF and is a criterion in qualification for electrotherapy.[34] Thus, a relationship between sST2 and QRS is plausible, but until now nothing has been stated about that relationship in the literature.

One of the limitations of the present study is the use of an older generation of sST2 test, which is less sensitive than newer ones. Among several highly sensitive assays, the most popular is the Presage® ST2 Assay (Critical Diagnostics, San Diego, USA), which has FDA approval. The cut-off point for adverse outcomes in HF is ≥ 35 ng/mL, which resulted in

this ST2 assay being taken into account in the ACCF/AHA Guideline for the Management of Heart Failure 2013.[35] The present study used another cut-off point for sST2 concentration, which was due to the methodology of sST2 measurement (the MBL ST2 assay).

It is also worth mentioning the Aspect Plus Reader ST2 test (Critical Diagnostics, San Diego, USA), a rapid lateral flow immunoassay for the quantitative determination of ST2 in venous EDTA anticoagulated plasma, intended for clinical practice. The test can clarify ST2 levels at the bedside, guide clinical practice and improve risk stratification in CHF patients.

Another limitation of this study is the relatively small group of patients and the lack of control group; and some of the results are borderline significant, which is probably also due to the relatively small population. Also, follow-ups based on telephone contact without the possibility of firsthand examination of the discharge cards from HF hospitalizations is a limitation.

It is possible that the impact of the HF drugs used by the patients enrolled in the study could have influenced the number of hospitalizations due to worsening HF. However, neither the HF drugs nor the doses were included in the analysis, which is also a limitation of the study.

In the current literature the cut-off point for ST2 ≥ 35 ng/mL was described for ambulatory CHF-patients with the risk of hospitalization.[21] The present study, however, revealed that sST2 concentration better identifies patients without HF-hospitalizations. There is a need for further investigation of the role of sST2 in CHF, based on personalized cardiovascular care with the Aspect Plus Reader ST2 test.

In patients with stable mild to moderate chronic heart failure with reduced EF ≤ 45%, single measurements of sST2 protein and fasting glucose were independent variables for hospitalization due to worsening CHF during the 1-year follow-up period. In the study population the defined prognostic model consisting of sST2 and glucose better identified patients without HF-related hospitalizations.

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Oxidative damage of DNA in subjects occupationally exposed to lead

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Abstract

Background. Exposure to lead (Pb) in environmental and occupational settings continues to be a serious public health problem and may pose an elevated risk of genetic damage.

Objectives. The aim of this study was to assess the level of oxidative stress and DNA damage in subjects occupationally exposed to lead.

Material and methods. We studied a population of 78 male workers exposed to lead in a lead and zinc smelter and battery recycling plant and 38 men from a control group. Blood lead levels were detected by graphite furnace atomic absorption spectrophotometry and plasma lead levels by inductively coupled plasma-mass spectrometry. The following assays were performed to assess the DNA damage and oxidative stress: comet assay, determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG), lipid peroxidation and total antioxidant status (TAS).

Results. The mean concentration of lead in the blood of the exposed group was 392 $\pm$ 103 µg/L and was significantly higher than in the control group (30.3 $\pm$ 29.4 µg/L, $p < 0.0001$). Oxidative DNA damages measured by comet assay showed no significant differences between populations. The concentration of 8-OHdG was about twice as high as in the control group. We found a significant positive correlation between the level of biomarkers of lead exposure [lead in blood, lead in plasma, zinc protoporphyrin (ZPP)] and urine concentration of 8-OHdG. The level of oxidative damage of DNA was positively correlated with the level of lipid peroxidation (TBARS) and negatively with total anti-oxidative status (TAS).

Conclusions. Our study suggests that occupational exposure causes an increase in oxidative damage to DNA, even in subjects with relatively short length of service (average length of about 10 years). 8-OHdG concentration in the urine proved to be a sensitive and non-invasive marker of lead induced genotoxic damage.

Key words: oxidative stress, lead exposure, DNA damage, comet assay, 8-hydroxy-2´-deoxyguanosine

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Exposure to lead (Pb) in the environmental and occupational settings continues to be a serious public health problem. Pb causes a number of adverse effects on many systems of the body like hematopoietic, renal, hepatic, bone, nervous, cardiovascular and reproductive systems.[1] Exposure to Pb may pose an elevated risk of genetic damage. Subjects occupationally exposed to lead compounds have an increased risk of cancer, lung and gastric in particular.[2,3]

One of the recognized mechanisms of lead toxicity is the induction of oxidative stress. Excessive generation of ROS (Reactive Oxygen Species) is caused by the inhibition of δ-ALAD (δ-aminolevulinic acid dehydratase) by lead. As a result of this, there is an increased accumulation of δ-ALA (aminolevulinic acid), which undergoes enolization and auto-oxidation and generates hydrogen peroxide and superoxide radical, and also interacts with oxyhemoglobin. The final oxidation product of δ-ALA, 4,5-dioxovaleric acid, is an effective alkylating agent of the guanine in DNA. The generation of potentially genotoxic compound is a possible mechanism for the metal-dependent DNA carcinogenicity of lead.[1,4] The lead-induced generation of ROS results in the attack of polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. The main product of peroxidation process is malondialdehyde (MDA). MDA can react with DNA bases (G, A and C) and form adducts. M1G (pyrimido [1,2-a] purin-10(3H)-one), a major endogenous DNA adduct in humans is mutagenic and may lead to the formation of DNA-DNA crosslinks or DNA-protein crosslinks.[5] Significantly elevated levels of lipid peroxide in the plasma, even among workers exposed to low concentrations of lead, has been observed.[6]

Most oxidative DNA damage is caused by very reactive hydroxyl radical, which is known to react with all components of the DNA molecule. The effects of oxidative DNA damage are GC/TA transversions, single strand breaks, double strand breaks, generation of apurinic/apyrimidinic sites and DNA-DNA crosslinks. This kind of damages can result in the arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis and mutagenesis.[5,6]

Comet assay is a simple, rapid and sensitive method for measuring DNA damage, such as single-strand DNA breaks (SSB), double-strand DNA breaks (DSB), alkali labile sites (apurinic/apyrimidic sites), crosslinks and incomplete DNA repair sites.[7,8] A modified version of comet assay with an addition of formamidopyrimidine glycosilase (FPG) can be used for the detection of DNA damage induced by reactive oxygen species.[9,10]

Hydroxyl radical reacts with all the nucleobases of DNA, but most frequently causes damage to guanine. This leads to the formation of C8-hydroxyguanine (8-OHGua) or deoxyguanosine (8-hydroxy-2'-deoxyguanosine, 8-OHdG), its nucleoside form. The formation of 8-OHdG is caused by the weakening of the antioxidant system of the organism, as well as the imprecise repair of the damaged nucleic acid. 8-OHdG is promutagenic, and thus may be used as a potential biomarker of increased risk of carcinogenesis.[11]

Detection of those early, adverse changes in cells caused by exposure to lead allows us to undertake prevention measures reducing the health risks for employees. For this reason, the present study was carried out to assess the level of oxidative stress, in markers of oxidative DNA damage (urinary 8-OHdG levels, comet assay) in subjects occupationally exposed to lead.

Inhibition of ferrochelatase activity by lead prevents the incorporation of iron into protoporphyrin and the formation of heme. This leads to binding of zinc (Zn) and the production of zinc protoporphyrin (ZPP). An increase in ZPP production has been recognized to be an early biological effect of lead exposure and has been frequently used in health effects monitoring for lead exposure.[1,4,12] Concentration of lead in blood is a result of both current and long term exposure.[13]

Another aim of our study was to analyze the correlation between the Pb exposure markers (lead in blood (B-Pb), ZPP) and markers of oxidative stress, DNA damage and level of antioxidative defence (total antioxidative status, TAS).

Subjects and methods

Study population

We studied a population of 78 male workers (aged from 20 to 62 years, mean age 36.4 ± 8.6 years) employed in lead and zinc smelter and battery recycling plant, exposed to lead. They were exposed to lead for 10.5 ± 8.3 years (0.8–35 years). The control group consisted of 38 men (aged from 19 to 61 years, mean age 35.0 ± 10.4 years) with no history of occupational exposure to lead, who were working for 11.6 ± 9.0 years (0.5–38 years). Only environmental exposure to lead occurred in the control group. Based upon questionnaire data, neither the exposed nor control group suffered from any acute or chronic disease, nor were alcohol addicted.

We used urine and blood samples collected in PHIME project (FOOD-CT-2006–016253) in 2009–2010. Blood was collected by venipuncture into sterile tubes containing either lithium heparin or ethylenediamine-tetraacetic acid (EDTA) solution as an anticoagulant. Blood samples were stored at -20°C and urine at -80°C until required for analysis. The protocol for this project has been approved by Bioethics Committee at Institute of Occupational and Environmental Health. All subjects gave informed consent.

Lead intoxication parameters

B-Pb levels were detected by graphite furnace atomic absorption spectrophotometry using Perkin-Elmer 4100ZL instrument. The laboratory participates in internal and inter-laboratorial proficiency tests (CDC in Atlanta, USA). ZPP levels were measured in whole blood using a haematofluorometer. The concentrations of B-Pb were quantified as µg/l and ZPP as µg/g Hb.

The concentrations of lead in plasma (P-Pb) were determined in samples diluted 5 times with an alkaline solution, according to Barany et al., by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo X7, Thermo Elemental, Winsford, UK).[14] The detection limit, calculated as 3 times the standard deviation (SD) of the blank, was 0.06 µg/L. All analyzed samples were prepared in duplicate and the method imprecision (calculated as the coefficients of variations in measurements of duplicate preparations) was 2.5%. To ensure analytical accuracy, quality control samples were analyzed along with the collected samples. The results obtained (mean ± SD) were 0.99 ± 0.08 µg/L (n = 9) vs recommended 1.02 µg/L (Seronorm Elements Serum, lot 0903106; SERO AS, Billingstad, Norway) and 9.3 ± 0.46 µg/L (n = 9) vs recommended 8.2 ± 0.83 (Centre de Toxicologie du Quebec, International Comparison Program, Quebec, Canada; lot QMEQAS068–06).

Comet assay method

The DNA damage was analyzed in whole blood using alkaline comet assay according to the method by Singh et al. with some modification and FPG-modified comet assay, as previously described.[15,16] Briefly, 40 µL of whole blood in 1% low melting point agarose (Sigma) was placed on a microscope slide that had been precoated with 0.5% normal melting point agarose (Sigma). Coverslips were placed on the gels, and the slides with coverslips were put on ice. Then coverslips were removed and the slides were submersed in lysis solution (2.5 mol L^{-1} NaCl, 10 mmol L^{-1} EDTA, 1 mmol L^{-1} Tris, 1 % Tritron X-100, pH = 10) at 4°C in the dark. After 1 hour, the slides were washed 3 times with enzyme reaction buffer (4 mmol L^{-1} Hepes, 0.1 mol L^{-1} KCl, 0.5 mmol L^{-1} EDTA, 0.2 mg/mL BSA) at 4°C for 5 min each. Then slides were treated with 70 µL of FPG solution (New England Biolabs) or buffer alone as control. The enzyme was diluted right before use. The slides were placed in a humid chamber at 37°C for 30 min and then washed with cold PBS solution. Afterwards, the slides were placed in a horizontal electrophoresis tank filled with electrophoresis buffer (30 mmol L^{-1} NaOH, 1 mmol L^{-1} EDTA) for 40 min at 4°C to DNA unwinding and denaturation. Electrophoresis was carried out for 30 min at 1.2 V/cm. In order to reduce light-induced DNA damage, all steps were performed under red light. After electrophoresis, the slides were washed 3 times with a neutralization buffer (0.4 mol L^{-1} Tris-HCl, pH = 7.5), dried and stained with DAPI (4,6-diamidino-2-phenylindole) solution (1 µg/mL). The slides were stored in a closed humid chamber at 4°C for 20 h. Slides were prepared in duplicate per person and analyzed by image analysis system Comet v. 5.5 (Kinetic Imaging Ltd., Liverpool, UK). 75 cells were calculated per 1 person (2 slides). To quantify DNA damage, the following comet parameters evaluated the percentage of DNA in the tail (relative fluorescence intensity of tail; TI), tail length (distance from head center to the end of the tail; in m) and tail moment (TM), which was calculated as tail length × percentage of DNA in tail (in arbitrary units). The control slides (no enzyme treatment) provided an estimate of the background of DNA strand breaks. The enzyme-treated slides revealed strand breaks and oxidized bases (total DNA damage). Differences in the tail length (ΔTL), tail intensity (ΔTI), and tail moment (ΔTM), between samples obtained with standard alkaline comet assay and FPG-modified comet assay were considered as oxidative DNA damage.

DNA damage in urine

Detection of 8-OHdG was performed using ready-to-use ELISA-based assay (Cat. RSCN213100R, BioVendor) according to the manufacturer's instructions. Samples of urine and 8-OHdG standards were added to a 96-well plate which has been precoated with 8-OHdG. Then, the 8-OHdG monoclonal antibody was added and the plate was incubated at 37°C for 1 h. Next, the plate was washed and incubated with secondary antibody labeled with horseradish peroxidase at 37°C for 1 h. The plate was washed thrice and substrate solution was added and incubated for 15 min at room temperature before a stop solution (1M phosphoric acid) was added. Spectrophotometric readings were obtained at 450 nm by using BIO-TEK PowerWave XS microplate reader (BIO-TEK Instruments). The concentration of 8-OHdG in urine was determined using a standard curve. The level of 8-OHdG was expressed as ng/g creatinine.

Determination of lipid peroxidation

Urine levels of lipid peroxides were determined as TBARS using Cayman TBARS Assay Kit (Cat. 10009055 Cayman Chemical, USA). The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90–100°C) and acidic conditions were measured colorimetrically at 530–540 nm using the BIO-TEK PowerWave XS microplate reader (BIO-TEK Instruments). The concentration of MDA was determined using an MDA standard curve. Results were expressed as µmol MDA per liter and µmol MDA per gram of creatinine. Typically, normal human urine has a lipid peroxide level of 0.8–2.0 µmol/g creatinine.

Total antioxidant status (TAS)

TAS was measured in urine using the Antioxidant Assay Kit (Cat. 709001, Cayman Chemical) following the manufacturer's instruction. This assay relies on the ability of the antioxidants in the sample to inhibit the oxidation of 2,2-azino-di- [3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS+ radical by metmyoglobin. The amount of ABTS+ was monitored by reading the absorbance at 750 nm. The antioxidants in the sample caused the suppression of the absorbance to a degree which was proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue and was quantified as milimolar Trolox equivalents. The plate reader used was BIO-TEK PowerWave XS (BIO-TEK Instruments).

Statistical analysis

The STATISTICA v. 9.PL was used for data analysis. The Shapiro-Wilks test was used to verify normality. The significance of difference between the exposed and control group was determined using the Mann-Whitney U test as the data distribution was non-parametric. Spearman's non-parametric correlation was used to find out the correlation between study parameters. The probability values of $p < 0.05$ were considered significant.

Results

Table 1 presents the characteristics of the exposed and control groups. No statistical differences in age and body weight were found between the groups. The percentage of smokers was evenly distributed within both groups.

The mean concentration of lead in blood in exposed group was 392 ± 103 µg/L and was significantly higher than in control group (30.3 ± 29.4 µg/L, $p < 0.0001$).

The differences between study groups were also significant ($p < 0.001$) according to lead level in plasma and ZPP, which were higher among occupationally exposed subjects (Table 2). The results of biomarkers of oxidative stress demonstrated that subjects exposed to lead had a lower level of antioxidant status and higher levels of TBARS and 8-OHdG, but only the latter was significantly different compared to control group ($p = 0.01$).

Oxidative DNA damages measured by FPG-modified comet assay showed no significant differences between populations.

Correlations between the studied parameters are shown in Table 3. Lead intoxication parameters significantly correlated with each other. We found a positive correlation between biomarkers of lead exposure (B-Pb, P-Pb, ZPP) and DNA damage in urine. The level of lipid peroxidation (TBARS) was found to be significantly correlated with oxidative DNA damage measured by comet assay. The negative correlations were observed between tail intensity, tail length, tail moment and TAS.

Table 1. Characteristic of exposed and control groups

Variable	Exposed group (n = 78)	Control group (n = 38)	p-value
Age (years)	36.5 ± 8.6	35.0 ± 10.4	ns
Weight (kg)	86.7 ± 13.2	85.2 ± 15.5	ns
Years of exposure to lead	10.5 ± 8.3	–	–
Smoking habit: Yes No	 26 (33%) 52 (67%)	 15 (39%) 23 (61%)	 ns ns

Mean (SD) or n (%); ns – non significant.

Table 2. Comparison of studied groups

Variable	Exposed group (n = 78)	Control group (n = 38)	p-value
B-Pb (µg/L)	392 ± 103	30.3 ± 29.4	< 0.001
P-Pb (µg/L)	1.53 ± 0.66	0.080 ± 0.11	< 0.001
ZPP (µg/g Hb)	5.5 ± 3.9	2.05 ± 0.92	< 0.001
8-OHdG (ng/g creatinine)	41.5 ± 36.8	24.6 ± 29.1	0.01
TAS (mmol/ g creatinine)	2.13 ± 1.75	2.57 ± 2.18	ns
TBARS (µmol/g creatinine)	6.74 ± 6.20	5.46 ± 5.65	ns
TI (% DNA)	14.1 ± 8.8	16.2 ±12.8	ns
TM	6.5 ± 8.4	10.2 ± 15.7	ns
TL (µm)	28.4 ± 13.5	31.9 ±24.4	ns
TI – FPG (% DNA)	66.1 ± 13.8	65.3 ± 13.2	ns
TM – FPG	42.2 ± 12.9	46.7 ± 18.3	ns
TL – FPG (µm)	59.5 ± 10.5	65.3 ± 22.0	ns
ΔTI (% DNA)	52.0 ± 15.9	49.1 ± 18.8	ns
ΔTM	35.7 ± 14.3	36.5 ± 16.1	ns
ΔTL (µm)	31.1 ± 15.1	33.4 ± 15.6	ns

Mean (SD) or n (%); ns – non significant; 8-OHdG – 8-hydroxy-2'-deoxyguanosine; Δ – change; B-Pb – blood lead level; FPG – formamidopyrimidine glycosilase assay modification; P-Pb – plasma lead level; TAS – total antioxidative status; TBARS – thiobarbituric acid reactive substances – lipid peroxidation; TI – tail intensity; TL – tail length; TM – tail movement; ZPP – zinc protoporhyrin.

Discussion

A safe level of lead exposure is very hard to define because health risks associated with lead are found at even low doses.[13] The permitted biological concentration of

Table 3. Correlation between study parameters in studied population

Variable	B-Pb	P-Pb	ZPP	8-OHdG	TAS	TBARS
B-Pb	1.00					
P-Pb	0.90***	1.00				
ZPP	0.64***	0.71***	1.00			
8-OHdG	0.19*	0.20*	0.27**	1.00		
TAS	ns	ns	ns	ns	1.00	
TBARS	ns	ns	ns	0.43***	ns	1.00
TI	ns	ns	ns	ns	0.18*	ns
TM	ns	ns	ns	ns	0.20*	ns
TL	ns	ns	ns	ns	ns	ns
TI – FPG	ns	ns	0.19*	ns	ns	0.29**
TM – FPG	ns	ns	ns	ns	ns	0.19*
TL – FPG	ns	ns	ns	ns	ns	ns
ΔTI	ns	ns	ns	ns	-0.21*	0.26**
ΔTM	ns	ns	ns	ns	-0.23*	0.22*
ΔTL	ns	ns	ns	ns	-0.24**	ns

Spearman's R values *p < 0.05; **p < 0.01; ***p< 0.001, ns – non significant; 8-OHdG – 8-hydroxy-2'-deoxyguanosine; Δ – change; B-Pb – blood lead level; FPG – formamidopyrimidine glycosilase assay modification; P-Pb – plasma lead level; TAS – total antioxidative status; TBARS – thiobarbituric acid reactive substances – lipid peroxidation; TI – tail intensity; TL – tail length; TM – tail movement; ZPP – zinc protoporhyrin.

lead in blood in Poland, according to the regulation of Ministry of Health, is 500 μg/L of blood and 300 μg/L of blood for women under 45 years of age. In other countries, like Germany, those levels are lower, at 400 μg/L for men and 100 μg/L for women. Researchers suggests a negative effect of Pb on kidneys, nervous, hematopoietic and cardiovascular systems even at lower levels; therefore, organizations such as ACGIH (American Conference Governmental and Industrial Hygienists) and SCOEL (Scientific Committee on Occupational Exposure Limit Values) recommend reducing the permitted concentration of Pb in blood to 300 μg/L.[1]

Vaglenov et al. were measuring the levels of DNA damage in peripheral blood lymphocytes of subjects exposed to lead, based on increases in frequency of binuclear cells with micronuclei (BNMN).[17] They observed that even at low lead concentrations B-Pb 1.2–1.9 μmol L^{-1} (B-Pb 248–393 μg/L), where no clinical changes are seen, an increased risk of genetic material damage in the cells occurs. The average concentration of lead in the blood of subjects analyzed by us was within the upper limit suggested by Vaglenov et al. and was equal to 392 ± 103 μg/L.[17] It was, however, 13 times higher than that of the control group. The concentration of ZPP and 8-OHdG was about twice as high as in the control group. We found also a positive correlation between lead exposure markers (B-Pb, P-Pb and ZPP) and the level of oxidative damage of DNA in urine (concentration of 8-OHdG). Our results are in line with earlier findings that occupational exposure even to low levels of lead as well as to levels not exceeding the permissible levels increases the risk of genetic damage.[17,18]

We have observed also an inverse relationship between oxidative damage, measured as change in tail intensity, tail length, tail moment between FPG modification and standard comet assay, and antioxidative potential (TAS) in serum, and positive correlation between level of lipid peroxidation (TBARS) and levels of DNA damage (measured based on both comet method as well as based on 8-OHdG levels in urine). Those results confirm the relationship between a weakened antioxidative defence and oxidative stress induced by various factors, including Pb, and the effects of genotoxic, observed also by other authors.[5,19]

Genotoxic effects may be modulated by many factors affecting studied subjects, such as cumulated exposure to lead, genetic predispositions, health, nutrition, smoking, drinking alcohol and other present in the environment, causing damage to the genetic material.[20]

Polymorphic genes taking part in pollution metabolism or in DNA repair processes may control the degree of damage caused by exposure to genotoxic factors and increase the risk of cancer and other diseases.[21] Coelho et al.

observed significantly increased levels of DNA damage biomarkers in people environmentally and occupationally exposed to metals/metaloids.[21] Those effects were correlated with internal doses of exposure and were more common in sensitive genotypes. Gene polymorphisms may influence the rate and efficiency of recovery and thus shape the individual sensitivity to Pb in the environment.

The choice of the method of measurement used to assess the level of DNA damage is equally important. The comet method gives the possibility of detecting DNA damage at the single cell level, identifying DNA breaks and chemical and enzymatic modifications that can be converted into breakage of DNA or chromatids. The comet assay can analyze any tissue which can provide cell suspension.[7,10]

The comet assay has been used in numerous studies to evaluate genotoxicity associated with lead exposure.[16,22–26] Most of them reported increased levels of DNA damage measured by comet assay parameters in exposed workers with regard to controls. In our study, the comet assay did not show a significantly higher incidence of oxidative DNA damage in exposed workers compared to the control group. Although the tail length was widely used in different biomonitoring studies, it has been criticized due to the sensitivity to the background and threshold setting of the image analysis program.[9,10] The divergences can also be explained by differences in the methodology, the characteristics of the populations studied and different biological material used for analysis. We have performed our assay in whole blood, while in the above-mentioned reports the damage was studied in isolated leukocytes.

Steinmetz-Beck et al. suggest a positive linear correlation between the concentration of lead in the blood and the values of comet parameters.[27] We did not observe this relation; however, we found a positive correlation between B-Pb and 8-OHdG concentration in urine. In our study, the exposed group mainly consisted of young people with a rather short length of service and the B-Pb was lower than in the group measured by Steinmetz-Beck and coauthors. The average time of exposure to lead was equal to 10.5 ± 8.3 years and the age 36.5 ± 8.6 years vs 19 ± 7.1 (length) and 46.7 ± 8.6 (age) respectively. Also, Olewińska et al. observed associations between B-Pb and DNA damage measured by comet assay but in their study the mean B-Pb was higher (457 μg/L), the population was older (mean age 39.2 years) and the duration of exposure to lead was longer (14.2 years).[16] The other possible explanation is that in most of the studies the isolated leukocytes were used for comet assay, while we used fresh whole blood. The advantage of using fresh whole blood is that the amount of blood which is used is 40 μL, while for the isolation of leukocytes a bigger volume is required. It is possible that the comet assay reflects the damage which can be repaired relatively quickly, especially in young, healthy people with efficient recovery mechanisms, while urinary 8-OHdG reflect the change more intractable to repair. Studies by Kim et al. also have indicated a positive association between Pb exposure and oxidative DNA damage (measured as urine concentration of 8-OHdG) in relatively young and healthy cohort of workers exposed to high levels of metal-containing particulate matter.[18]

B-Pb reflects to a greater extent the current rather than long-term exposure and as such does not show the cumulated levels of exposure to Pb.[13] However, Pb concentration in blood increases with years of exposure.[19] Occupational exposure to Pb is associated with decreased repair capacity of DNA.[28] The increase in comet parameters observed at higher Pb concentrations due to prolonged exposure can be associated with less efficient and more error-prone mechanisms of repair of DNA.

Other studies also have not found significant differences in the level of DNA damage measured by the comet method between the group exposed and the control one. In the study by García-Lestón et al., the average concentration of lead in the blood of subjects heavily exposed to lead (workers from chemical plants and producing rechargeable batteries) was 312 ± 21 μg/L, and in the control group (administrative staff) was about 9 times lower (35.4 ± 4.3 μg/L); however, there are no differences in the values of the comet parameters between these groups.[29] There was a significant increase in the frequency of mutations in the T-cell receptor (TCR -MF) in subjects exposed to Pb compared to control group. According to these authors, the changes detected in the TCR mutation assay are a result of an earlier long-term exposure taking place from several months to 2–3 years before the measurement, and the comet assay reflects the more recently formed damage, which is quickly repaired. Thus, the differences between the control group and the Pb exposed, assayed by comet method, are not always significant.

The urinary excretion of 8-OHdG has been used as a biomarker to assess the extent of repair of ROS-induced DNA damage in both the clinical and occupational setting. 8-OHdG forms a alkali pair with cytosine and adenine and increases the rate of spontaneous mutations incorporated into DNA. 8-OHdG is considered to be a useful marker of oxidative damage of DNA, increased risk of carcinogenesis and the development of degenerative diseases.[11,18]

In our study, 8-OHdG analysis in the urine was a more sensitive method of measuring genetic damage associated with occupational exposure to Pb compared to the comet method. As previously mentioned, 8-OHdG level in urine was significantly higher in Pb exposed subjects when compared to control subjects, and additionally this level positively correlated with the concentration of lead in plasma and the level of lipid peroxidation.

In summary, our study suggests that occupational exposure to Pb causes an increase in oxidative damage to DNA, which, in turn, increases the risk of carcinogenesis. These changes were observed in subjects with relatively short length of service (average length of about 10 years) and with significantly lower than the permissible limit

of lead concentration in the blood. 8-OHdG concentration in the urine proved to be a sensitive and non-invasive marker of lead induced genotoxic damage.

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Computational fluid dynamics in the assessment of patients' postoperative status after glottis-widening surgery

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Computational fluid dynamics (CFD), a rapidly developing instrument with a number of practical applications, allows calculation and visualization of the changing parameters of airflow in the upper respiratory tract.

Objectives. The aim of this study was to demonstrate the advantages of CFD as an instrument for non-invasive tests of the larynx in patients who had undergone surgical treatment due to bilateral vocal fold paralysis.

Material and methods. Surface measurements of the glottic space were made during maximum adduction of the vocal folds. Additionally, the following spirometric parameters were determined: forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), and peak expiratory flow (PEF) rate. Based on the measurements, commercial mesh generation software was used to develop a geometrical model of the glottic space. The computations were carried out using a general purpose CFD code. The analysis included patients who were surgically treated for BVFP in the authors' department between 1999 and 2012. The study group consisted of 22 women (91.67%) and 2 men (8.33%).

Results. It was observed that the pressure drop calculated for free breathing depends on the area of the glottis and is independent of its shape. Importantly, for areas below approx. 40 mm^2, a sudden rise occurred in the resistance to flow; for the smallest glottic areas studied, the pressure drop was almost 6 times higher than for an area of 40 mm^2. Consequently, in cases of areas below 40 mm^2 even minor enlargement of the glottic opening can lead to a marked improvement in breathing comfort.

Conclusions. Computational fluid dynamics is a useful method for calculating and visualizing the changing parameters of airflow in the upper respiratory tract.

Key words: CFD, bilateral vocal fold paralysis, air flow

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The larynx is an organ that fulfills vital functions. It is part of the upper respiratory tract, a speech organ and a protective barrier for the lower respiratory tract against aspiration and choking on food.[1] Impaired morphology of the larynx or the mobility of the structures result in the impairment of at least one of the functions of the organ. Bilateral vocal fold paralysis (BVFP) is an example of a condition in which both airway hydrodynamics and phonation are affected. It is a symptom of bilateral damage to the recurrent laryngeal nerve (RLN), which is responsible for the mobility of the abductor and adductor muscles of the glottic space.[2,3] The most common cause of RLN damage is surgical intervention in either the neck or the chest (thyroid gland, cardiac and thoracic surgical procedures).[2,4] Shortness of breath, especially during the inspiratory phase, is a symptom accompanying BVFP, which can be concurrent with stridor and hoarseness.[5] Some patients adapt quickly to diminished glottic space, while others require immediate tracheotomies followed by glottis-widening surgery.[6] In BFVP, 2 categories of reconstructive surgical procedures are performed, i.e. surgery which models the glottic space and procedures that change the glottic space permanently by removing some laryngeal structures.[7] Improvement of ventilation is almost always associated with deterioration of the quality of the patient's voice.[8]

Currently, 2 main types of surgical procedures widening the glottic space are performed at the Department of Otorhinolaryngology and Laryngological Oncology, Medical University of Silesia in Zabrze (Poland): arytenoidectomy with posterior cordectomy using a CO_2 laser, and laterofixation with the Lichtenberger needle carrier. The aim of an arytenoidectomy with posterior cordectomy is to widen the glottis by removing the posterior part of the vocal fold and the arytenoid on the same side.

In everyday practice, there is no test that could determine the morphology of the larynx and the dynamic properties of the airflow, such as air velocity, pressure and resistance in the upper respiratory tract. Moreover, some tests are invasive, causing the patient's discomfort. Computer simulation with computational fluid dynamics (CFD) is an alternative to invasive tests. This method allows non-invasive calculation and visualization of the dynamically changing parameters of the laryngeal airflow.

Computational fluid dynamics is a rapidly developing instrument with a number of practical applications. CFD simulations have been used for years in aerodynamics, engineering, hydraulics, meteorology, construction and a number of other fields.[9] In medicine it has been used primarily in pulmonology and cardiology – areas in which the dynamic properties of gas and liquid play an important role in the proper functioning of the body.

The aim of the present study was to demonstrate the advantages of CFD as an instrument for non-invasive tests of the larynx in patients treated surgically due to BVFP. Because of the complexity of the method, its implementation requires cooperation between medical doctors and engineers, and this was the case in this study as well.

Material and methods

The study included patients who were surgically treated for BVFP in the authors' department between 1999 and 2012. The study group consisted of 22 women (91.67%) and 2 men (8.33%). The average age of the patients was 66.13 years (range: 49–82). In 20 cases, BVFP occurred as a complication following a thyroidectomy (83.33%). In 3 cases, the reason for BVFP was prolonged intubation (12.5%), and in 1 case the cause remained unknown (4.16%). These patients underwent arytenoidectomy with posterior cordectomy. The control group was comprised of 3 women and 2 men (mean age 33.2 years).

The study protocol was approved by the local Bioethics Committee (decision no. KB/157/12, October 2nd, 2012).

Laryngeal examinations were performed during routine visits to the laryngological outpatient clinic. Video images of the glottis during phonation were recorded using indirect laryngoscopy – a Karl Storz Telecam 20212030 video laryngoscope (Karl Storz GmbH & Co., Tuttlingen, Germany) and a monitor. The data was analyzed using IRIS 2.2.0 software (MediCom, Wrocław, Polska) While recording the video of the glottis during phonation, a freeze-frame of the maximum vocal fold adduction was captured. Surface measurements of the glottic space were made during the maximum adduction of the vocal folds. In order to calibrate the measurements, the "length of the glottis" was taken as a constant parameter, as proposed by Eckel and Sittel, who reported measurements of 20 larynges obtained from fresh corpses.[10] Among the parameters measured, the length of the glottis, defined as the distance from the base of the vocal folds to the mucosa of the posterior commissure, is regarded as particularly reproducible. This distance was 17.55 ± 0.92 mm and 20.09 ± 3.07 mm in women and men, respectively.

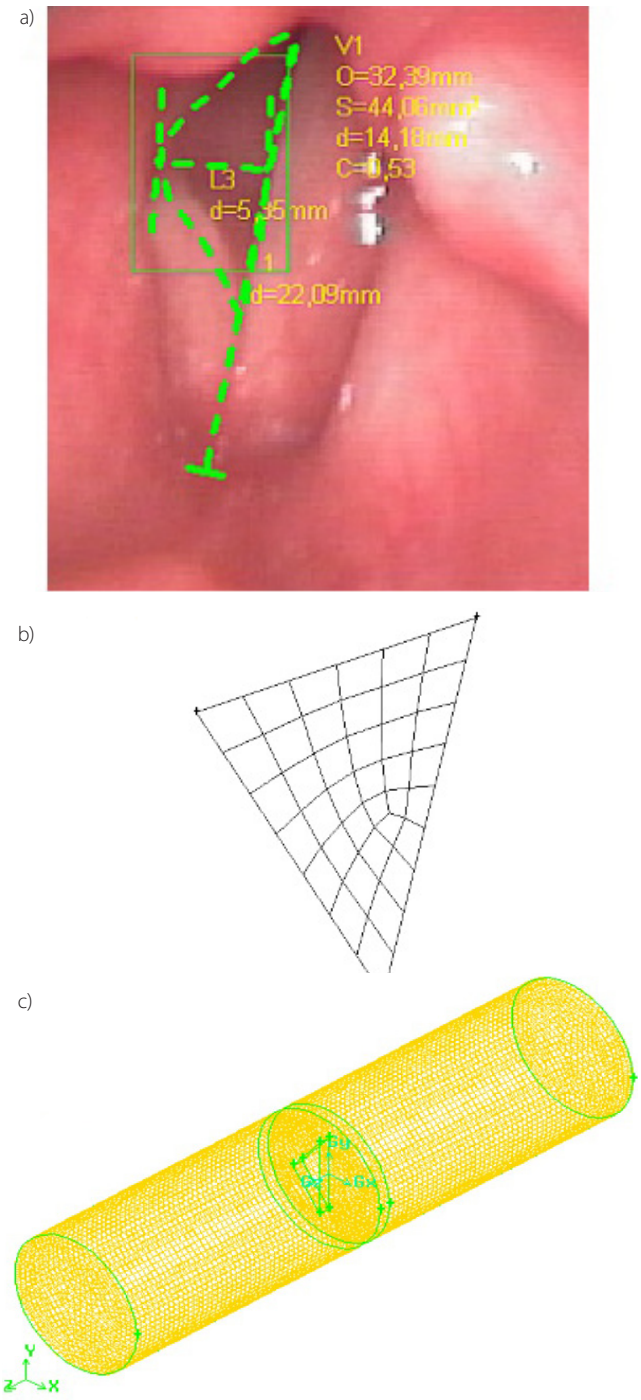
Subsequently, the contours of the glottic space were outlined using the IRIS program and the following geometric parameters were calculated: O – the circumference of the outlined figure (mm); S – the surface area of the outlined figure (mm^2); and D – the length of the segment connecting 2 most distant points on the perimeter (mm). The width of the glottic space was measured at the largest point of the lumen.

Additionally, the following spirometric parameters were determined: forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1) and peak expiratory flow (PEF) rate. Since it was not possible to compare the airflow before and after surgery, appropriate imaging studies and spirometry were performed for 5 healthy controls to compare with the results from the patients.

Based on the measurements, Gambit 2.4.6 (Ansys Inc., Canonsburg, USA) commercial mesh generation software was used to develop a geometrical model of the glottic space. Since the larynx has an irregular structure, some simplifications had to be made in formulating the model (Fig. 1). The shape of the glottic opening was analyzed in both postoperative patients and the controls during the largest abduction of the vocal folds. The glottis was represented by means of straight lines and curves in the Cartesian coordinate system. The shapes were approximated with isosceles and right triangles, and with semi-ellipses. The surface areas obtained were 18–64 mm^2 for the postoperative patients and 48–143 mm^2 for the controls. The regions above and below the glottic opening were modeled as a cylinder with a diameter of 25 mm and a length of 50 mm. It was assumed that these regions were the same for all patients. The length of the section analyzed was 102 mm. Based on this geometrical model, a numerical grid comprising about 54,000 cells was generated. The actual number of grid elements depended on the glottic area. Although the assumed 3-dimensional geometry is simplified compared with images yielded by computed tomography, it is quite sufficient for the purpose of analyzing the effect of the glottic shape on airflow hydrodynamics.[11] The boundary conditions associated with the grid were as follows: no slip of the fluid at the wall, flat velocity profile at the inlet, and no velocity gradient across the outlet. All the results were obtained during the expiratory phase, so the inlet quantities are those on the tracheal side of the glottis.

The computations were carried out using the general purpose CFD code Fluent (Ansys Inc., Canonsburg, USA). A steady flow of air was assumed with the atmospheric pressure prevailing in the larynx (101.325 Pa). The initial conditions were defined by the airflow rate, and the minimum value was set at 5L/min (tidal volume, TV). The maximum value of this flow was calculated separately for each patient, based on the PEF rate obtained using spirometry. These velocities correspond to turbulent flow. Laminar flow occurs at relatively low flow rates, and the velocity profiles become parabolic. For turbulent flow, the fluid elements rotate in various directions rather than moving parallel to one another, which leads to flattening of the velocity profiles. The mode of the flow (turbulent or laminar) is determined by the Reynolds number (Re): below Re = 2.400, the flow is laminar, while above this value the flow becomes turbulent. In the present study, the transition occurred at an inlet velocity of about 1.5 m/s. Therefore, for w < 1.5 m/s, the laminar model was used in the calculations, whereas for the velocities exceeding 1.5 m/s, the computations were done by employing the turbulence model k-ε standard, available in the Fluent software package. The convergence criterion was based on the normalized sum of numerical residues and was set at 10^{-3}.

Fig. 1. Geometrical model: a) measurement of the area of the glottic opening; b) numerical grid; c) simplified geometrical representation of the larynx and the glottic opening



Results

For velocities associated with normal, unobstructed breathing (w = 0.169 m/s), the lowest pressure drops were obtained for the controls (0.5–4.1 Pa). The values documented postoperatively were higher, ranging from 2.4 Pa to 29.7 Pa. Figure 2 illustrates the results. It can be observed that the pressure drop calculated for free breath-

Fig. 2. Pressure drop (ΔP) vs the area of the glottic opening (S) at an inlet velocity of w = 0.169 m/s. Solid symbols – postoperative patients; outline symbols – controls

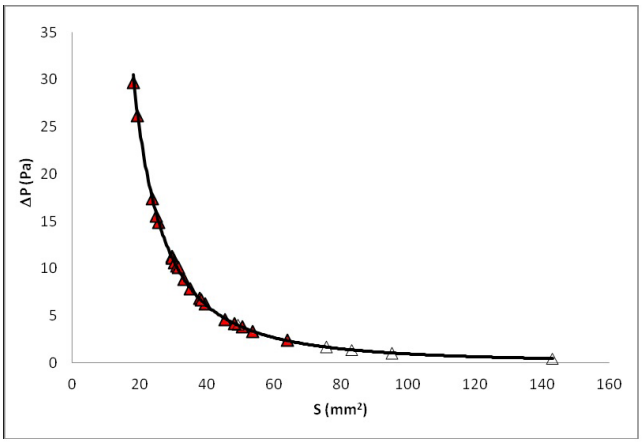
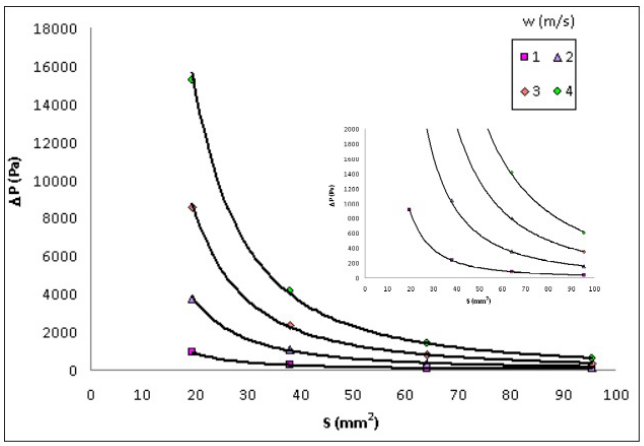


Fig. 3. Pressure drop vs the area of the glottic opening for the various inlet velocities



ing depends on the area of the glottis and is independent of its shape. Importantly, for areas below approx. 40 mm^2, a sudden rise occurred in the resistance to flow – for the smallest glottic areas studied, the pressure drop was almost 6 times higher than for an area of 40 mm^2. Consequently, in cases of areas lower than 40 mm^2 even minor enlargements of the glottic opening over this range may lead to a marked improvement in breathing comfort. Conversely, above 40 mm^2 the pressure drops are lower than 7 Pa, and increases in the glottic area have a relatively small effect on breathing. Furthermore, as shown in Fig. 3, the limiting area of the glottis grows as the inlet velocity increases, finally reaching values matching those of the controls.

The pressure drop over the glottis was correlated with the area of the opening, and the following relationship was calculated: $\Delta P = 1.4315 (Q^2/S^2)$, with $R^2 = 0.99$, where ΔP represents the pressure drop across the glottis (Pa), Q is the airflow rate (m^3/s), and S is the area of the opening (m^2). This correlation is quite general in the sense that it is possible to quantify a decrease in the pressure drop following an enlargement of the glottic area without the necessity of using CFD simulations. Noticeably, the present results are consistent with both experimental and CFD data published previously.[12,13]

The effect of the glottic area on the maximum air velocity in the larynx with inlet velocity w = 0.169 m/s was also analyzed. For a given flow, mass flow rates through the various openings are identical. Therefore, if the opening area is smaller, the linear velocity of the flow increases so that the mass of air passing through the larynx in a unit of time remains constant. This velocity is the highest at the smallest cross-sectional area of the larynx. Maximum velocities (w_{max}) are shown in Fig. 4 as a function of the area (S). The maximum velocity depends not only on the area of the glottic opening but also, to a certain extent, on its location within the larynx. Following partial arytenoidectomy with posterior cordectomy, the glottic area was unilaterally enlarged. Consequently, the opening became asymmetrical, which led to some minor differences in velocities for the same areas of the opening. Again, for areas below about 40 mm^2, there is a visible increase in the maximum velocity, albeit less pronounced than in the case of ΔP. For areas between 20 and 40 mm^2, w_{max} increased from 2.5 to 5 m/s.

The measured PEF rates were 1.22–6.59 L/s for postoperative patients and 4.1–12.7 L/s for the controls. The calculated pressure drops across the glottis for 3 different ranges of PEF (expressed as corresponding inlet velocities) are shown in Fig. 5 as a function of the glottic area. The function illustrates that ΔP depends on both PEF and S. It should be noted that patients with the same opening area usually had different PEF values during spirometry.

Figure 6 shows a comparison between the local pressure and velocity fields in the larynx for a selected postoperative patient (S = 31.6 mm^2) and a control subject (S = 48.3 mm^2) for the same value of PEF (4.1 L/s). Due to the reduction in the area available for the flow, the pressure in the larynx increases before the glottis and decreases on the other side of the opening. The pressure drops are 24 kPa and 10.3 kPa, respectively. The highest pressure drops across the glottis occur in the corners of the glottic opening. As for the velocity field, it can be noted that narrowing generates a considerable temporary increase in the air velocity. For a postoperative patient, this momentary velocity was as high as 163 m/s, whereas for a healthy subject it was 112 m/s.

Discussion

Damage to the RLN during surgical intervention in the thyroid gland was the most common reason for BVFP in the patients in the present study (20 out of 24 cases). Three cases of BVFP due to prolonged intubation were noted; the cause of 1 case remains unknown. The majority of

Fig. 4. Maximum air velocity in the larynx vs the area of the glottic opening for an inlet velocity of w = 0.169 m/s. Solid symbols – postoperative patients; outline symbols – controls

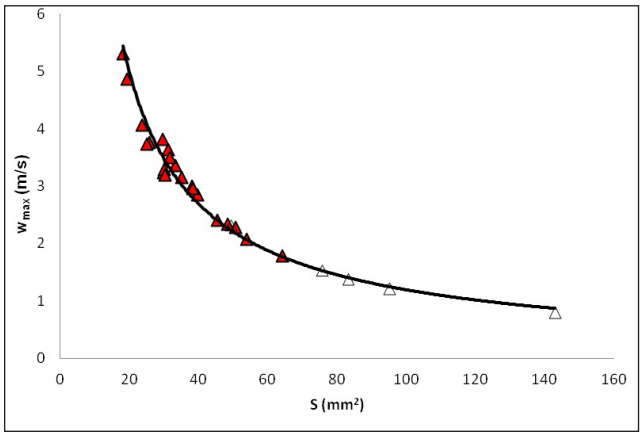
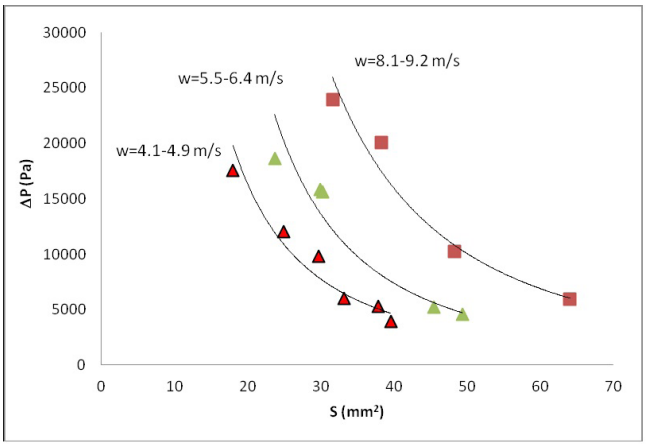


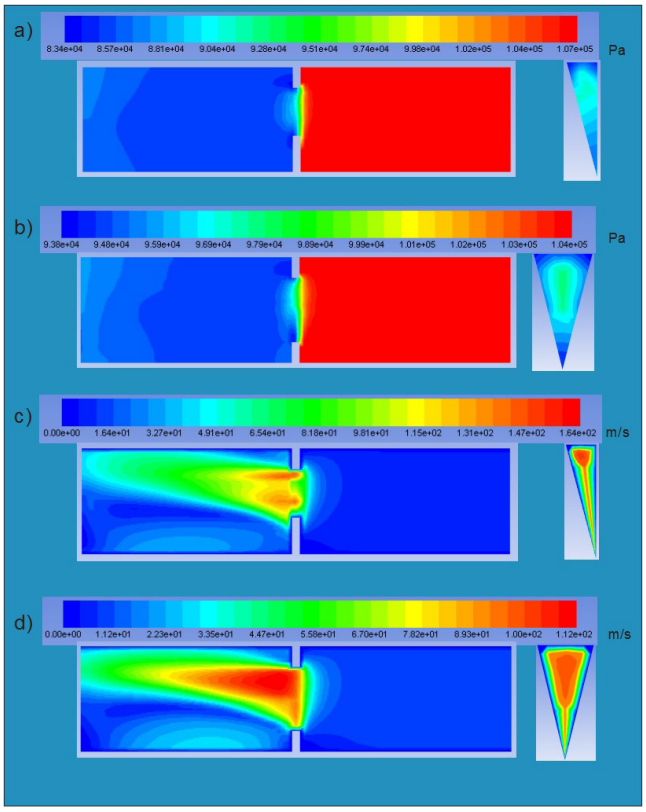
Fig. 5. ΔP vs S for the various PEF values (expressed as inlet velocities)



patients were women (22 out of 24 cases), which is consistent with other authors.[14–18] Feehery et al. and Rosenthal et al. showed the highest percentage of paralysis due to the neoplastic process in the neck or the mediastinum.[4,19] In contrast, Sharan et al. reported a predominance of men in their study, and Plouin-Gaudon et al. reported the same proportion of men and women. In both of those studies, damage to the RLN during thyroidectomy was the most frequent etiology.[20,21] Dursun et al. reported an overwhelming inflammatory etiology of the paralysis, and a preponderance of women.[22] The frequency of reported significant majorities of women is associated with the more frequent incidence of thyroid disease among women, and hence the necessity to perform thyroid surgery.[23]

During the flow of air across the glottis a pressure drop (ΔP) occurs, which is caused by perturbations of the flow. The pressure losses are caused by the resistance to the flow produced by the changing cross-section of the larynx. The pressure drop is an important parameter, covered extensively in a number of papers that discuss airflow in the upper respiratory tract.[11–13,24–26] The calculations by means of the Fluid software yielded the values

Fig. 6. Local pressure (a, b) and velocity (c, d) fields in the larynx for the same PEF value in a selected postoperative patient (a, c) and a healthy subject (b, d)



of the pressure drop over the glottic opening, as well as the local pressure and velocity fields in the larynx.

Conclusions

Computational fluid dynamics is a useful method that allows the changing parameters of the airflow in the upper respiratory tract to be calculated and visualized. This method comprises the core of the Fluent software package, which can be efficiently used to analyze various problems associated with normal or impaired breathing. This study presented simulations of airflow through the larynx in patients who had undergone laser arytenoidectomy with posterior cordectomy and in control subjects. The calculations were done for velocities ranging from those corresponding to tidal volume (TV) up to values coinciding with the PEF obtained from spirometry. It was determined that resistance to airflow, expressed as the pressure drop ΔP, depends on the area of the glottic opening and is virtually independent of its shape. During free breathing, a steep rise in ΔP occurs for areas below about 40 mm^2. It can be concluded that below this area even a minor enlargement of the glottis may significantly improve quality of breathing. Above 40 mm^2 widening the glottic space begins to lose its therapeutic value. With increases in inlet velocities to the glottis, this boundary

shifts toward larger values. Based on CFD analyses, it was possible to correlate ΔP with the area of the glottic opening. This correlation is a useful instrument in evaluating the change in airway sufficiency resulting from the altered geometry of the glottis.

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The evaluation of the changes in enzymatic antioxidant reserves and lipid peroxidation in chosen parts of the brain in an animal model of Parkinson disease

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Abstract

Background. Parkinson's disease is a progressive neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. The causes of Parkinson's disease are not fully understood; however, increasing evidence implicates oxidative stress.

Objectives. The study was aimed at assessing the nature of the changes in the oxidation-antioxidant balance in the cerebral cortex, striatum, hippocampus, thalamus, and cerebellum in a rat model of Parkinson's disease (PD).

Material and methods. Sixteen male Wistar rats were divided into 2 groups: I- control, II- Parkinson's disease. The 8-weeks-old animals were decapitated, their brains removed and the following structures dissected and then frozen for further biochemical assays: cerebral cortex, striatum, hippocampus, thalamus and cerebellum. The activities of: the catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), superoxide dismutase (SOD) and the isoenzymes: Cu/ZnSOD and MnSOD; together with the malondialdehyde (MDA) and the total oxidative status (TOS) concentrations were measured in each structure.

Results. A significantly increased activities of SOD, Cu/ZnSOD, GST and reduced GR activity and an increase of MDA concentration were observed in the striatum of PD rats, comparing to the control group, combined with a significantly reduced activities of GR,SOD, Cu/ZnSOD and an increased GPX activity and MDA concentration in the hippocampus, a significantly lower GR, SOD, MnSOD, Cu/ZnSOD, and GST activities in the cerebral cortex. A significantly lower GR activity, higher CAT activity and MDA concentration in the thalamus and a significantly increased GR activity in the cerebellum were observed in PD rats compared to the corresponding control group.

Conclusions. Oxidative stress in PD involves many brain structures and various antioxidant enzymes and oxidative status parameters become dysfunctional, depending on the area of the brain, which might reflect the complexity of the clinical symptoms of PD.

Key words: oxidative stress, Parkinson disease, brain, antioxidant

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Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the substantia nigra of the midbrain, which results in a significant reduction in dopamine levels in the striatum. PD was the subject of many studies over the years; however, the factors causing selective neuronal cell death have not been clearly defined. Numerous studies suggest an increased exposure to free radicals, in combination with deficient antioxidant enzyme, might play an important role in neurodegeneration.[1,2] PD is a multifactorial disease, and different mechanisms may be involved in the production of reactive oxygen species (ROS), and the interactions between them contribute to the severity of the degenerative processes of nerve cells.[3] It is clear that the substantia nigra of the midbrain and the adjacent striatum are the most affected structures in the course of the analyzed disease. It should be noted, however, that the neurodegeneration in PD is not only limited to the dopaminergic pathways, but also include changes in other trails and structures of the brain, which can be damaged to varying degrees, reflecting the emergence of a variety of clinical symptoms.[1,4]

6-hydroxydopamine (6-OHDA) used in the experiment is a neurotoxin, which, applied to the lateral ventricles of the brain, causes a persistent, lifelong destruction of the dopaminergic nigrostriatal pathway and profound deficits of dopamine in the striatum. The resulting damage is proposed as a near-ideal model of advanced stage Parkinson's disease, which mimics human PD.[5]

The "free radical" hypothesis in PD has become the object of a number of experimental and clinical studies, but the role of oxidative stress and its devastating effects on different brain structures in PD have not been fully explained. Moreover, the results of studies on oxidative stress in PD are not homogeneous, and do not fully specify which antioxidant enzymes deplete and which indicators of oxidative stress are essential for the PD.[2,4] In connection with data mentioned above, which is not fully precise in the available literature, this study was aimed to assess the role played by oxygen free radicals in the pathogenesis and course of PD, and to thoroughly evaluate the oxidation-antioxidant balance in the different structures of the brain (in the striatum, frontal cortex, hippocampus, thalamus and cerebellum), in the experimental model of PD.

Material and methods

Animals

This experiment was performed on 16 male Wistar rats, weighing 180–200 g. The animals were housed under standard conditions of humidity (55–60%), temperature (21–22°C) and lighting (day-night: 12/12 h) throughout the experimental period, with unlimited access to food and filtered water. Experimental procedures with the use of animals were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the Animal Experiments Local Ethics Committee of the Medical University of Silesia in Katowice (permit no. 33/2013). All efforts were made to minimize suffering.

Research project

Wistar newborns were divided into 2 groups and subjected to the following procedure:

- Group I: control rats: the 3-days-old neonatal rats were given desmethylimipramine in a dose of 20 mg /kg, intraperitoneally (IP) in a volume of 1.0 mL/kg and, after 60 min, 10 mL of 0.1% solution of ascorbic acid intraventricularly (ICV).
- Group II: rats with PD: the 3-days-old neonatal rats were given 20 mg/kg of desmethylimipramine IP in a volume of 1.0 mL/kg and, after 60 min, 6-hydroxydopamine in a dose of 15 mg/5 mL in 0.1% ascorbic acid solution into the right and left lateral ventricle of the brain.

The newborns were housed with their mothers until the age of 4 weeks, separated by gender and placed in separate cages for breeding thereon. After reaching the 8-week, the rats were decapitated. Then, after the opening the skull, brains were removed and the following structures of the brain were dissected: frontal cortex, striatum, hippocampus, thalamus, and cerebellum. The tissues were weighed and frozen on solidified CO_2 (so-called "dry ice") and stored for further biochemical assays at -80°C.

Tissues preparation

Tissues were homogenized on ice, an UP50H ultrasonic processor (Hielscher) was used. The homogenates were centrifuged at 3000 rpm for 10 min, and supernatant was used for assay of the oxidant-antioxidant parameters.

Biochemical analysis

Glutathione reductase (GR) activity was measured by the modified method by Richterich.[6] This method is based on a determination of changes in the concentration of reduced NADPH, which reacts with oxidized glutathione. The activity of GR was expressed as μmol of NADPH per minute per gram of protein (IU/g protein). The method is absolutely specific for glutathione reductase. Glutathione peroxidase (GPx) activity was measured by the kinetic method of Paglia and Valentine.[7] In this method, GPx catalyzes the reaction between reduced glutathione (GSH) and t-butyl hyperoxide. The resulting oxidized glutathione (GSSG) is then converted back to the reduced form (GSH) by a NADPH-dependent glutathione reduc-

tase (GR). The activity of GPx was expressed as micromoles of NADPH oxidized per minute normalized to gram of protein (IU/g Hb). Glutathione transferase (GST) activity was determined by kinetic method according to Habig.[8] GST activity was expressed as μmol of thioether formed within 1 min per gram of protein (IU/g Hb). The method of Oyanagui was used to measure the activity of superoxide dismutase (SOD) and its izoenzymes: the cytoplasmic Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and the mitochondrial Mn-superoxide dismutase (MnSOD) in brain's homogenates.[9] In this method, xanthine oxidase produces superoxide anions which react with hydroxylamine forming nitric ions. These ions react with naphthalene diamine and sulfanilic acid generating a colored product. The concentration of this product is proportional to the amount of produced superoxide anions and negatively proportional to the activity of SOD. The enzymatic activity of SOD was expressed in nitric units. The activity of SOD is equal to 1 nitric unit (NU) when it inhibits nitric ion production by 50%. Activities of SOD were normalized to milligram of protein in homogenates (NU/mg protein). Catalase (CAT) was measured by the Aebi kinetic method.[10] The rate of decomposition of hydrogen peroxide was measured spectrophotometrically. The activity of catalase was expressed as units per milligram of protein (IU/mg protein). The malondialdehyde (MDA) level was determined by the Ohkawa method using a Perkin Elmer LS45 spectrofluorometer.[11] The concentration of MDA was expressed as mol/g of protein.

Statistical analysis

All statistical analyses were based on STATISTICA v. 10 program (StatSoft, Polska). The normality of the results distribution was verified using the Kolmogorov-Smirnov test, whereas Levene's test was used to verify homogeneity of variances. The data was analyzed using non-parametric Mann-Whitney U test and they were presented as median with the first and fourth quartiles. Value of $p < 0.05$ was considered to be statistically significant.

Results

In the cortex of the studied rats we have observed statistically significant decrease in GR (29%), SOD (29%), MnSOD (25%) and CuZnSOD (31%) activities in the 6-OHDA group compared to that of the control rats (Table 1).

Results observed in the striatum showed us statistically significant increase in GST (25%), SOD (25%), CuZnSOD (15%) activities and increase in MDA concentration (18%) activity in the 6-OHDA group compared to that of the control rats. GR activity decreased in the 6-OHDA group (11%) compared to that of the control rats (Table 2).

Table 1. Antioxidant enzymes activity and malondialdehyde concentration in cerebral cortex of studied groups of rats (U Mann–Whitney test)

Cortex	Control	6–OHDA	p–value
GR [IU/g protein]	14.18 [12.63–15.08]	10.67 [8.83–12.46]	0.009
GPX [IU/g protein]	7.02 [6.70–8.50]	7.14 [5.65–8.50]	ns
GST [IU/g protein]	4.31 [4.21–4.78]	3.22 [3.12–4.01]	0.001
CAT [kIU/g protein]	29.59 [28.30–34.00]	25.63 [22.43–35.60]	ns
SOD [NU/mg protein]	31.76 [22.61–36.68]	18.33 [15.6–28.5]	0.03
MnSOD [NU/mg protein]	12.65 [9.50–14.50]	7.99 [6.87–11.45]	0.04
CuZnSOD [NU/mg protein]	19.11 [13.11–22.11]	10.34 [8.72–17.04	0.03
MDA [umol/g protein]	1.22 [1.13–1.23]	1.06 [0.93–1.34]	ns

Table 2. Antioxidant enzymes activity and malondialdehyde concentration in striatum of studied groups of rats (U Mann-Whitney test)

Striatum	Control	6–OHDA	p–value
GR [IU/g protein]	12.46 [10.94–12.99]	10.89 [10.54–11.59]	0.03
GPX [IU/g protein]	11.59 [11.40–13.11]	12.48 [12.17–13.15]	ns
GST [IU/g protein]	3.39 [3.03–3.49]	4.16 [3.72–4.49]	0.0008
CAT [kIU/g protein]	45.92 [44.13–48.42]	46.25 [36.41–50.22]	ns
SOD [NU/mg protein]	16.90 [15.96–18.33]	20.77 [18.63–23.40]	0.009
MnSOD [NU/mg protein]	7.77 [7.08–8.28]	7.95 [7.39–9.59]	ns
CuZnSOD [NU/mg protein]	9.96 [8.71–10.46]	11.45 [10.77–13.37]	0.02
MDA [umol/g protein]	0.83 [0.8–0.87]	0.96 [0.88–1.09]	0.03

In the hippocampus we have observed increase in GPX activity (15%) and MDA concentration (10%) and decrease in GR (18%), SOD (14%), CuZnSOD (16%) activity in the 6-OHDA group compared to that of the control rats (Table 3).

Results observed in the thalamus showed us statistically significant decrease in the GR activity (16%) and increase in CAT activity (30%) and MDA concentration (14%) in the 6-OHDA group compared to that of the control rats (Table 4).

In the cerebellum we have observed statistically significant increase in GR activity (26%) in the 6-OHDA group compared to that of the control rats (Table 5).

Table 3. Antioxidant enzymes activity and malondialdehyde concentration in hipocampus of studied groups of rats (U Mann-Whitney test)

Hipocampus	Control	6–OHDA	p–value
GR [IU/g protein]	11.45 [11.34–12.15]	10.20 [8.70–10.80]	0.003
GPX [IU/g protein]	8.47 [8.02–9.06]	9.86 [9.18–10.83]	0.02
GST [IU/g protein]	4.57 [4.41–4.96]	4.61 [4.24–5.04]	ns
CAT [kIU/g protein]	42.36 [39.86–43.14]	41.73 [40.10–43.70]	ns
SOD [NU/mg protein]	18.57 [17.56–18.86]	17.77 [16.51–18.50]	0.05
MnSOD [NU/mg protein]	8.23 [7.78–8.89]	7.58 [5.87–8.18]	ns
CuZnSOD [NU/mg protein]	10.36 [9.89–11.05]	9.44 [6.52–10.07]	0.03
MDA [umol/g protein]	1.02 [0.96–1.05]	1.07 [0.90–1.20]	ns

Table 4. Antioxidant enzymes activity and malondialdehyde concentration in thalamus of studied groups of rats (U Mann-Whitney test)

Thalamus	Control	6–OHDA	p–value
GR [IU/g protein]	7.43 [6.79–8.04]	6.58 [6.03–6.60]	0.03
GPX [IU/g protein]	6.67 [5.93–7.31]	6.71 [6.46–6.82]	ns
GST [IU/g protein]	2.56 [2.39–3.17]	1.73 [2.45–3.19]	ns
CAT [kIU/g protein]	25.22 [22.96–35.01]	36.09 [30.60–41.10]	0.04
SOD [NU/mg protein]	12.89 [11.02–14.05]	13.18 [10.95–14.30]	ns
MnSOD [NU/mg protein]	5.95 [5.24–6.68]	5.90 [5.20–6.50]	ns
CuZnSOD [NU/mg protein]	6.86 [5.78–7.48]	7.12 [5.78–7.90]	ns
MDA [umol/g protein]	0.57 [0.51–0.67]	0.59 [0.54–0.68]	ns

Table 5. Antioxidant enzymes activity and malondialdehyde concentration in thalamus of studied groups of rats (U Mann-Whitney test)

Cerebellum	Control	6–OHDA	p–value
GR [IU/g protein]	7.74 [6.86–8.24]	10.23 [8.03–11.65]	0.03
GPX [IU/g protein]	10.00 [9.23–10.52]	9.95 [7.90–11.30]	ns
GST [IU/g protein]	2.22 [2.13–2.35]	2.09 [1.59–2.36]	ns
CAT [kIU/g protein]	51.33 [48.65–61.50]	50.48 [47.81–65.45]	0.04
SOD [NU/mg protein]	11.65 [10.72–14.08]	13.58 [9.84–14.79]	ns
MnSOD [NU/mg protein]	5.51 [5.17–6.56]	6.04 [4.52–6.98]	ns
CuZnSOD [NU/mg protein]	6.14 [5.66–7.51]	7.17 [5.32–7.75]	ns
MDA [umol/g protein]	0.75 [0.69–0.86]	0.61 [0.46–0.76]	ns

Discussion

The balance of the oxidation-antioxidant system in neurodegenerative diseases has been the subject of intense experimental and clinical studies over the past few years. Plentiful evidence suggests that the cell aging processes and progressive neurodegeneration are closely associated with oxidative stress. Although the definitive cause of nigral dopamine neuron loss remains unknown, oxidative stress is the greatest risk factor for PD. Oxidative stress has been defined as a state of impaired oxidation-antioxidant balance, in the direction of oxidation, which is caused by excessive production of ROS and antioxidant mechanisms inability to detoxify them.[12] Even though there are numerous defense mechanisms against free radicals in the body, it appears that the brain is exposed to an increasingly damaging effects of ROS compared to peripheral organs.[1] Although the adult human brain represents only 2% of the body weight, it consumes almost 20% of the total amount of oxygen absorbed by the body. Such excessive oxidative metabolism favors the generation of oxygen free radicals. Excessive ROS generation is harmful to the cell membrane and may damage the nerve cells. Additionally, the high levels of polyunsaturated fatty acids in cell membranes of neurons and relatively low levels of antioxidants in brain tissues, as compared to other organs, make them more susceptible to the damage caused by ROS.[3,12]

Nerve cells are protected from the damaging effect of ROS under physiological conditions, but in the course of PD the depletion of the reserves of the antioxidant mechanisms is observed. Our results show that in the striatum of the rats with PD, the severity of oxidative stress is observed, as evidenced by the significantly higher level of MDA compared to healthy individuals. MDA is a marker of lipid peroxidation, which is significantly accumulated in striatum of the subjects with PD, as compared to the other areas of the brain and comparing to the control group.[13] In addition, we observed a significant increase in the activity of SOD, Cu/ZnSOD, GST, and a serious reduction in the activity of the GR in the striatum of the subjects with PD, compared to the control group. On the other hand, another study has shown that the antioxidant activity is reduced in the striatum of the rats with PD and it appears to be a key determinant of the susceptibility to damage of the dopaminergic neurons.[14] These differences between the results can be explained on the basis of the duration of the test, because the longer the observation

time, the greater is the extent of the marked symptoms of the increased oxidative stress. Our results indicate a stimulation of the antioxidant system in response to the increased lipid peroxidation and an increase in the free radicals levels, which is aimed to protect the nerve cells from oxidative damage. It is believed that this increase may be temporary. The defense mechanisms are depleted as a result of continuously sustained oxidative stress, which is observed in PD.

An important role for the protection of neurons against oxidative stress is played by SOD and its 2 fractions: Cu/ZnSOD, and MnSOD. Those enzymes catalyze the removal of the toxic superoxide radical by converting it into hydrogen peroxide and molecular oxygen.[15] Research indicates that overexpression of SOD and its isoenzymes may protect efficiently against neuronal loss.[16] Our findings also support this hypothesis. Moreover, the study by Choi et al. has shown that oxidative modifications of Cu/ZnSOD might contribute to the pathogenesis of PD.[17]

In turn, glutathione reductase catalyzes the reduction of the oxidized form of glutathione (GSSG) in the presence of NADPH, the donor of electron necessary to restore the reduced form of glutathione (GSH). GSH depletion rate correlates positively with the severity of the disease and the ratio of GSH/GSSG is an important indirect indicator of the loss of dopaminergic neurons. The GSH deficiency causes an increase of the H_2O_2 concentration, which is continuously produced by the mitochondria in the brain, and its detoxification in the brain is carried out by GSH-dependent mechanisms; therefore, GR activity impairment causes GSH deficiency, and this contributes to the inability of H_2O_2 neutralization by GPx, which requires the presence of GSH.[18] A previous study by Pearce et al. also showed GSH depletion in the substantia nigra of the midbrain of patients with PD. The depletion of GSH might indicate clinical susceptibility to the development of PD. The authors of the research indicate that GSH can be used as a biomarker for the diagnosis of these neurodegenerative diseases. Therefore, the activity of GSH-dependent enzymes may indirectly indicate the progression of the disease.[19] The study by Zhou et al. suggests a possible correlation between severe dopamine oxidation and reduced protective potential of GSH. Additionally, the reduced GSH level significantly impairs the protective capacity of the brain from the toxic impacts of the oxidized dopamine molecules and ROS.[20] The other enzyme along with the increased activity, which has been observed in the striatal of the subjects with PD, also plays an important role in protecting the tissues from free radicals. GST catalyzes the conjugation of glutathione with nucleophiles and electrophiles, resulting in the formation of glutathione conjugates.[21]

In addition to the degeneration of dopaminergic neurons in the striatum, extensive neurodegeneration and atrophy is observed in other types of nerve cells in the brain, including areas such as hippocampus, thalamus, cortex and cerebellum. This damage may cause the appearance of symptoms other than characteristic motor symptoms, which include, inter alia, cognitive impairments, affective changes, depression, psychosis, memory deficits and other non-motor symptoms.[22] The chronic exposure to ROS can cause atrophy and abnormal functioning in many key areas of the brain structures, including the hippocampus and frontal cortex.[23] It has been proved that atrophy of the hippocampus may be a biomarker of the early stages of cognitive disorders and memory disorders in patients with PD.[24] Furthermore, cell damage in various regions of the brain reduces cognitive ability, causes deterioration of memory function, an increase of anxiety and depression levels in older rats with reduced dopamine and serotonin levels, in comparison with the group of young subjects.[25] It seems that ROS may be involved in the processes damaging the hippocampus and cerebral cortex, because their long lasting high levels lead to the failure of antioxidant systems. Our studies also confirm this theory. They have also shown a significant reduction in the activity of GR, SOD, Cu/ZnSOD and higher GPx activity and an increase of MDA production in the hippocampus of rats with PD, compared to the control group, and a significantly lower activity of GR, SOD, MnSOD, CuSOD, and GST in the cerebral cortex of rats with PD group, as compared to healthy rats. A study conducted by Che et al. has shown that chronic stress resulting in ROS overproduction might damage the cerebral cortex and the hippocampus, which manifests in the reduced activity of antioxidant enzymes in the brains of rats exposed to chronic stress compared to the control group. Moreover, this study showed that reducing the antioxidant ability and induction of the lipid peroxidation in the cerebral cortex and hippocampus correlates positively with the severity of memory impairment, a decrease in learning abilities and the severity of depression.[26] Looking at this study, it appears likely that the overproduction of free radicals and the progressive nature of PD supports the possibly negative impact of ROS in many areas of the brain, which reflects the appearance of clinical symptoms independent from the deficiency of dopamine and dopaminergic neurons. It is suggested that the overproduction of ROS is associated with a decrease in the antioxidant defense system during aging or as a result of an ongoing disease, which leads to the failure of cell homeostasis. Reduced expression or antioxidant deficiency may lead to excessive ROS production, and there is no possibility of removing them.[27,28] Thalamus plays an important role in the proper communication between each brain structure. It is also responsible for the initial evaluation of sensory stimuli and then communicating this information from the first-order kernel to the cortex, while the higher-order kernel transmits the information from one area of the cortex to another. Thalamus plays a key role in the integration of sensory and motor information and attention processes, using cortico-thalamic loops. Thalamus also

has connections to the hypothalamus and hippocampus.[29] The cerebellum receives information from multiple brain systems; it is also responsible for maintaining balance of the body, coordination and dexterity and maintaining proper muscle tone, among other things.[30] Our study shows that a significantly lower activity of GR, higher CAT activity and an increase in the concentration of MDA is observed in the thalamus of rats with PD, and a significantly higher activity of GR is detected in the cerebellum of rats with PD, compared to the corresponding control group. Our study indicates that in the case of a reduction in GR activity in the striatum, cerebral cortex, hippocampus and thalamus, which might be one of the possible markers of PD, a compensatory increase in the activity of other antioxidant enzymes is observed in brain structures including the striatum, hippocampus, thalamus, and cerebellum, which is aimed at protecting brain cells from damage caused by ROS.

Our research shows that oxidative stress in PD involves many brain structures and various antioxidant enzymes and oxidative status parameters become dysfunctional, depending on the area of the brain. The differences in the scope of changes in the antioxidant enzymes activity in various brain structures indicate that the mechanisms of oxidative damage are regional and specific. It seems that every cell or tissue has its own ROS defense system and in various areas under oxidative stress, different antioxidant enzymes, specific to the area of may deplete. It appears that the damage of various structures of the brain reflects the complex nature of the clinical symptoms of PD. Thus, the study on PD should not be limited to the analysis of changes in the substantia nigra and the striatum, but should also take into account other brain structures, the failure of which manifests itself in the form of clinical symptoms other than those of locomotion disorders. Future clinical and experimental studies should be directed to investigate molecular mechanisms of the neuronal damage by ROS. Understanding them may help in developing new approaches to neuroprotection. Further research, aimed at extending the knowledge about the specific mechanisms responsible for the modulation of ROS production in specific areas of the brain, is also required. In the available literature, only a few studies focus on the role of oxidative stress in damaging the brain structures other than the striatum in PD; therefore, there is a need for further studies, which will aim at expanding the concept presented by us.

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Gene expression profile of collagen types, osteopontin in the tympanic membrane of patients with tympanosclerosis

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Abstract

Background. Tympanosclerosis is a pathological process involving the middle ear. The hallmark of this disease is the formation of calcium deposits. In the submucosal layer, as well as in the right layer of the tympanic membrane, the calcium deposits result in a significant increase in the activity of fibroblasts and deposition of collagen fibers.

Objectives. The aim of our study was to examine the expression level of genes encoding collagen type I, II, III and IV (*COL1A1*, *COL2A1*, *COL3A1*, *COL4A1*) and osteopontin (*SPP1*) in the tympanic membrane of patients with tympanosclerosis.

Material and methods. The total RNA was isolated from middle ear tissues with tympanosclerosis, received from 25 patients and from 19 normal tympanic membranes. The gene expression level was determined by real-time RT-PCR. The gene expression levels were correlated with clinical Tos classification of tympanosclerosis.

Results. We observed that in the tympanic membrane of patients with tympanosclerosis, the expression of type I collagen is decreased, while the expression of type II and IV collagen and osteopontin is increased. Moreover, mRNA levels of the investigated genes strongly correlated with the clinical stages of tympanosclerosis.

Conclusions. The strong correlations between the expression of type I, II, IV collagen and osteopontin and the clinical stage of tympanosclerosis indicate the involvement of these proteins in excessive fibrosis and pathological remodeling of the tympanic membrane. In the future, a treatment aiming to modulate these gene expressions and/or regulation of the degradation of their protein products could be used as a new medical approach for patients with tympanosclerosis.

Key words: osteopontin, tympanosclerosis, collagen types

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Tympanosclerosis is a chronic disease of the tympanic membrane and middle ear manifested by the accumulation of collagen in the elastic and fibrous layer of the lamina propria of the tympanic membrane, submucosal membrane of the tympanic cavity, auditory ossicles and mastoid cavity. The disease process most often refers to the eardrum.[1] Tympanosclerosis limited to the tympanic membrane is called myringosclerosis and occurs in 24–82% of patients with tympanosclerotic lesions.[1,2] The initiation of the immune response leads to the formation of deposits of hyaline with subsequent calcification and sometimes ossification of tympanosclerotic foci.[3,4] These changes often lead to restricted mobility of the eardrum and ossicles, resulting in hearing loss. The tympanosclerotic plates formed from deposits of calcium, collagen fibers and hyaline masses result in conductive and mixed hearing impairment, by reducing the mobility of hearing elements, or in rare cases through the perforation of the eardrum, which exacerbates the hearing loss. Tympanosclerosis is characterized by dynamic ischemia and stiffening, and even the disappearance of the middle ear tissue.[1,5]

The etiology of tympanosclerosis is still not fully understood. A widely-accepted consensus recognizes the tympanosclerotic changes as a complication of acute inflammation or injury within the middle ear.[2,6–10] A special role is attributed to otitis media with effusion, particularly if patient treatment involved the insertion of a catheter vent.[11,12] Other factors that are taken into account in the etiology of tympanosclerosis include immunological processes, genetic predispositions, hypertension and hypercalcemia.[13–16]

Under the influence of cytokines and other regulatory factors, the fibrosis is initiated in the lamina propria of the tympanic membrane, involving degradation and vacuolization of fibrocytes. This results in the disintegration of cells and accumulation of deposits in the spaces between the collagen fibers. Endoplasmic reticulum released from the destroyed cells is equipped with calcium-binding receptors. This leads to an accumulation of calcium deposits and eventual calcification of tympanosclerotic foci.[17] The activated immune cells as well as activated fibroblasts promote tissue remodeling. Our previous in vitro study on fibroblasts isolated from tympanosclerotic lesions demonstrated an up-regulated sensitivity of these cells to mast cell stimulation, which could significantly contribute to the ongoing fibrosis and pathological remodeling of the tympanic membrane.[18]

Our present study aimed to evaluate the expression level of genes encoding type I, II, III and IV collagen, and osteopontin in the tympanic membrane of patients with tympanosclerosis. Moreover, we determined the correlations between the gene expression levels and clinical stages of tympanosclerosis.

Material and methods

Material

Tympanic membranes were obtained from 25 patients with tympanosclerosis who underwent tympanoplasty in the local Department of Otolaryngology of the Medical University. Patients with accompanied inflammatory disease, with immune deficiency or those taking antibiotics or glucocorticosteroids within the last two weeks were excluded. The clinical stage of the tympanosclerotic lesion was assigned based on the intraoperative evaluation of tympanosclerotic changes according to a modified Tos classification as described previously.[19] Morphologically normal tympanic membranes were dissected from 19 people who had died suddenly. The institutional review board at the Medical University previously approved all procedures (NKEBN/432/2009), and written consent was obtained from all patients. Immediately after resection, tissues were placed in RNAlater stabilization solution (Thermo Fisher Scientific, Walther, Massachusetts, USA) and stored at -20°C until the isolation of RNA.

Isolation of total RNA

Isolation of total RNA was carried out in accordance with the Chomczyński procedure, with our own modifications.[20] A TRI reagent and suspended material were vortexed briefly and then left standing for 10 min at 4°C. Next, chloroform (250 µL) was added, and the samples were vigorously shaken, incubated at 4°C for 15 min and centrifuged (10,000 × g for 15 min at 4°C). The upper aqueous phase was removed into a new Eppendorf tube, an equal volume of isopropanol was added and RNA was precipitated by overnight incubation at -20°C, followed by centrifugation (10,000 × g for 15 min at 4°C). RNA pellets were washed first with 96% and then with 70% (v/v) ethanol, air-dried and resolved in diethyl-pyrocarbonate-treated thermo-sterilized water, and stored at -20°C until further analysis.

mRNA level determination

The gene expression level was determined by real-time PCR performed in a Light Cycler 480 II (Roche Diagnostic GmbH, Manhein, Germany) using Path-IDTM Multiplex One-Step RT-PCR Kit and appropriate Universal ProbeLibrary Set, Human (Roche Applied Science). Transcript levels were normalized to that of the β-actin gene (*ACTB*). The primer sequences, TaqMan probes and cycling conditions used are listed in Table 1.

Statistical analysis

Statistical analysis was performed using STATISTICA v. 12.0 (StatSoft, Inc., Tulsa, USA). The level of gene expres-

Table 1. Primers and TaqMan probes and cycling conditions used for RT-PCR

Gene transcript	Primers	TaqMan probe
COL1A1	(F) gggattccctggacctaaag (R) ggaacacctcgctctcca	Universal ProbeLibrary Probe # 67 (Roche)
COL2A1	(F) tggtgctaatggcgagaag (R) cccagtctctccacgttcac	Universal ProbeLibrary Probe # 4 (Roche)
COL3A1	(F) agctggaaagagtggtgacag (R) ccttgaggaccaggagcac	Universal ProbeLibrary Probe # 18 (Roche)
COL4A1	(F) tggtgacaaaggacaagcag (R) ggttcacccttttggacctg	Universal ProbeLibrary Probe # 81 (Roche)
SPP1	(F) cccacagacccttccaagta (R) acactatcacctcggccatc	Universal ProbeLibrary Probe # 18 (Roche)
ACTB	Universal ProbeLibrary Reference Gene Assay Roche, Human ACTB Gene Assay	Universal ProbeLibrary Reference Gene Assay Roche, Human ACTB Gene Assay

Reverse transcription: 48°C (10 min), 95°C (10 min).
Amplification: 95°C (10 s), 60°C (45 s).

sion in a normal tympanic membrane (control) and the tympanosclerotic samples were analyzed with a nonparametric Mann-Whitney U test. The correlations between the level of gene expression and clinical classification of tympanosclerosis were analyzed using Spearman's R ratio.

Results

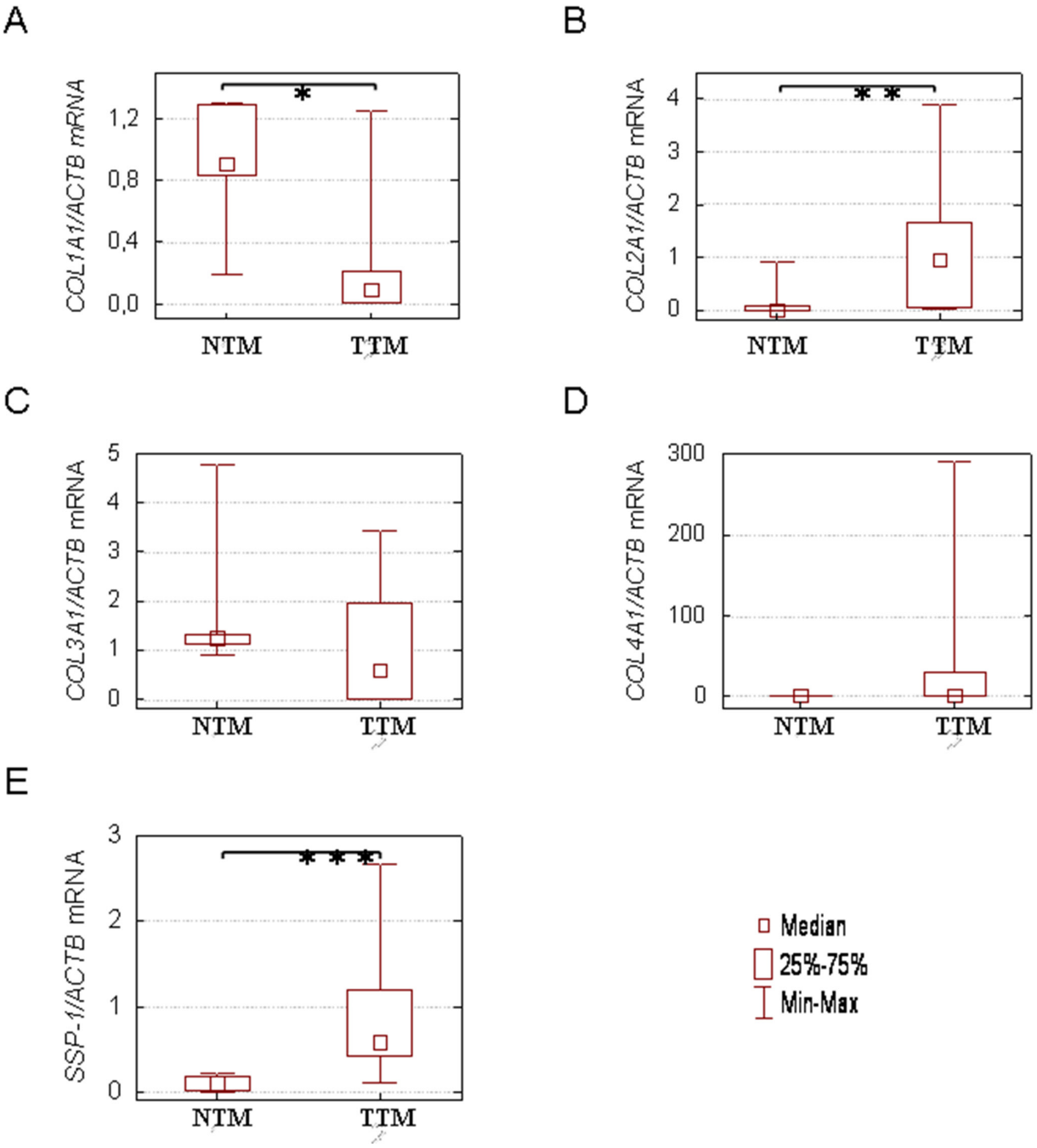
To evaluate the contribution of collagens and osteopontin to the pathogenesis of sclerotic changes in the tympanic membrane, we determined the gene expression profile of *COL1A1, COL2A1, COL3A1, COL4A1* and *SPP1* in the tympanic membrane of patients with tympanosclerosis. The performed real-time PCR analysis showed the presence of *COL1A1, COL2A1, COL3A1, COL4A1* and *SPP1* in normal and sclerotic tympanic membranes, but the level of *COL2A1* mRNA was barely detectable in the normal tympanic membrane. The level of gene profile expression of the collagen types and osteopontin in the selected structures of the middle ear with tympanosclerosis differed significantly compared to the healthy tympanic membranes (control group) (Fig. 1). We observed that the level of *COL1A1* transcript was significantly decreased in tympanic membrane subjects with tympanosclerosis (Fig. 1A), whereas the expression level of *COL2A1* was higher in sclerotic lesions of the tympanic membrane (Fig. 1B). The transcript levels of *COL3A1* and *COL4A1* were not altered in sclerotic tympanic membranes compared to controls (Fig. 1C, D). The expression level of the osteopontin gene (*SPP1*) was 7-fold higher in tympanosclerotic membranes compared to that determined in normal tympanic membranes (Fig. 1E).

Moreover, we compared the mRNA levels of the investigated genes within the patient groups arranged on the basis of Tos clinical classification of tympanosclerosis with our own modification. Our modification of the Tos classification concerns the study group division into 2 (instead of 3), i.e. group I – myringosclerosis with/or without perforation; group II – tympanosclerosis with/or without perforation. We observed a much lower mRNA level of the *COL1A1* gene in patients with stage II of tympanosclerosis. Expression of the *COL1A1* gene was inversely correlated with the degree of tympanosclerotic changes (Spearman's rank correlation (R) = -0.82, $p < 0.05$, Fig. 2A). Moreover, increased expression of *COL2A1,* and *SPP1* genes strictly correlated with the severity of the disease ($R = 0.8$ and $R = 0.82$, $p < 0.05$, respectively, Fig. 2C, D). Interestingly, the mRNA level of the *COL4A1* gene, which was not different in the study group compared to the control group, was correlated with the degree of tympanosclerotic changes ($R = 0.48$, $p < 0.05$, Fig. 2B).

Discussion

Tympanosclerosis (TS) is a chronic disease that is encountered at any age, but usually occurs in the years 30–50. However, as many as 87% of patients are over 40 years old.[21,22] The pathogenesis of TS is not clear. It usually develops following middle ear infection during the resolution phase of chronic otitis media. The clinical observations indicate that there are some differences in the disease procession between old and young patients. Tympanosclerosis in children is associated with secretory otitis media and the lesions are mostly limited to the tympanic membrane, whereas the tympanosclerotic changes in the elderly are also observed in other middle ear sites: the ossicular chain or the mastoid cavity. These changes are often accompanied by perforation of the tympanic membrane, varying degrees of destruction of the ossicles and accompanied diseases, such as hypercalcemia, hyperlipidemia or hypertension and atherosclerosis.[1,9] The risk of TS especially increases in children who had ventilation tubes inserted.[12,23] The clinical observations indicate that the frequency of myringosclerosis is much higher in tympanic membranes with tympanostomy tube insertion than in tympanic membranes with no tympanostomy tube insertion (23–70% and 0–13%, respectively).[5,11,23–30] It was also observed that the myringosclerosis rate increased with a larger size of tube, several tube insertions and time of tympanostomy tube stay.[25,29,31,32] Based on computer modeling, it has been shown that ventilation tube insertion induces shear stress in the structure of the tympanic membrane. The areas of maximal shear stresses have been found in the same positions as tympanosclerosis. It has been proposed that such stresses could damage the fibrils that connect the fibrous layer of the lamina propria and lead to TS.[33] Furthermore, hyperoxic conditions, for-

Fig. 1. Altered expression of genes (*COL1A1*, *COL2A1*, *COL3A1*, *COL4A1*) encoding collagens: type I (A), type II (B), type III (C), type IV (D), and gene (*SSP-1*) encoding osteopontin (E) in sclerotic lesion of tympanic membranes (TTM) and normal tympanic membranes (NTM). The data is means from at least 3 independent measurements performed on isolated total RNA from tympanic membranes, *p < 0.0007; **p < 0.002; ***p < 0.00004.

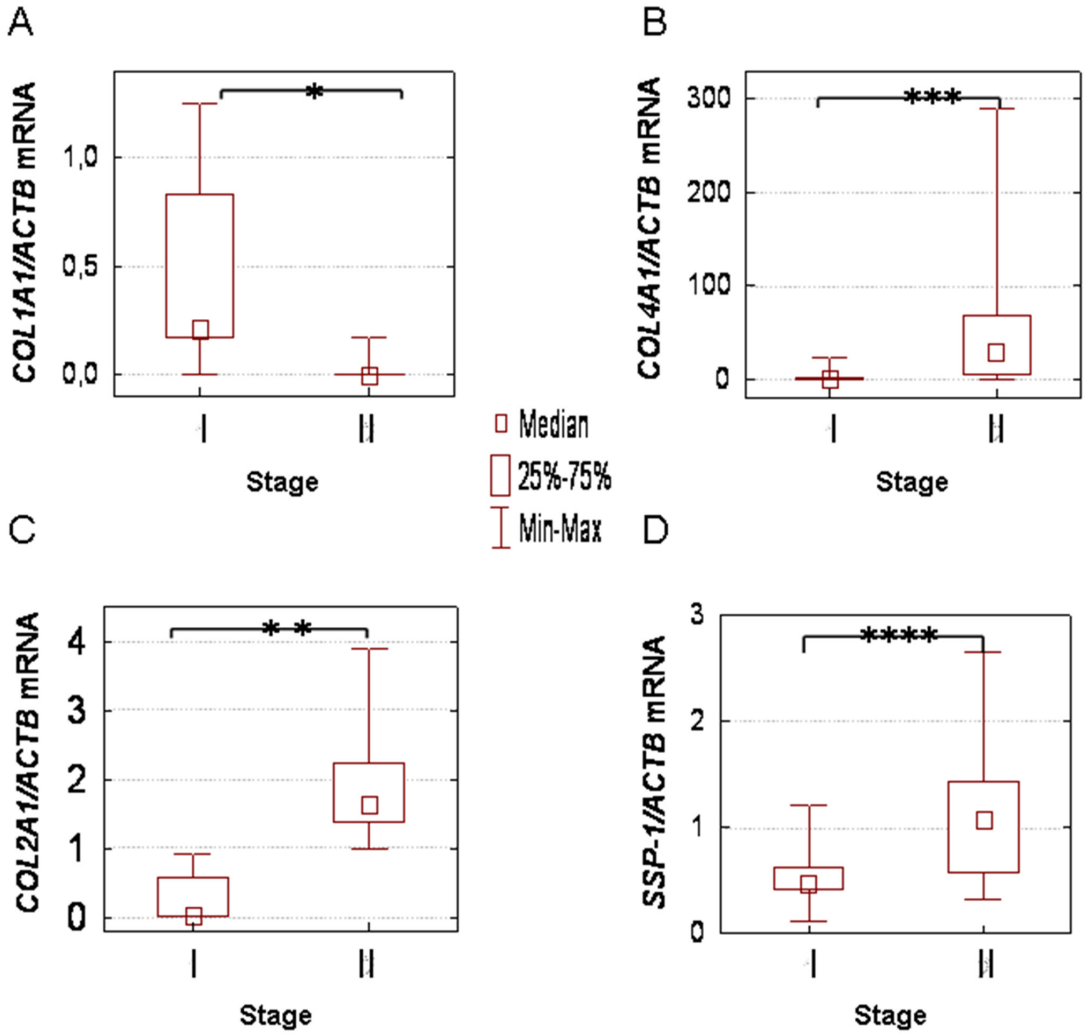


eign body reaction, inflammation, fibrous hyperplasia, hemorrhage and the release of free hemoglobin between the layers of the tympanic membrane are considered as factors involved in the development of myringosclerosis following tympanostomy tube insertion.[25,29,34]

Typically, tympanosclerotic changes proceed via the destruction of connective tissue followed by fibrosis resulting in elevated deposition of collagens and subsequent calcification of tissues in the middle ear. A recent histological study demonstrated that a healthy human tympanic membrane consists of collagen type I, II, III and IV.[35] All these collagen types have different mechanical properties. The type I collagen fibers are resistant to force, and type II fibers are resistant to deformation. The type III collagen fibers are flexible and elastic, whereas type IV collagen provides support and transport.[36]

Earlier studies showed that an autoimmune reaction (both cellular and humoral) to type II collagen, an essential component of ear tissue, leads to, among other things,

Fig. 2. Relation of the tympanosclerotic stage and the expression level of genes (*COL1A1*, *COL2A1*, *COL4A1*) encoding collagens: type I (A), type II (C), type IV (B), and gene (*SSP-1*) encoding osteopontin (D). The data is means from at least 3 independent determinations performed on isolated total RNA from tympanic membranes, *p < 0.0007; **p < 0.0002; ***p < 0.02; ****p < 0.05.



sensorineural hearing loss, vestibular dysfunction, endolymphatic hydrops, Eustachian tube inflammation and otitis media with effusion (not infectious). The tympanosclerotic membrane also has C3 and Ig deposits, which may suggest that tympanosclerosis is also induced by type II collagen immunization, especially in patients undergoing surgical incision of the tympanic membrane.[37,38] The mechanisms of type II collagen autoimmune-mediated middle ear disease are not clear. In our study we observed that a normal tympanic membrane exhibits a very low level of *COL2A1* mRNA, which tremendously increased (4.9-fold) in tympanosclerotic foci.

Our study has not shown any significant changes in *COL4A1* expression in the whole group of tympanosclerotic lesions, but we did observe an increase in type IV collagen expression in a subgroup of patients with stage II of tympanosclerosis compared to patients with stage I. This might indicate that the expression of *COL4A1* increases with the propagation of the disease.

Osteopontin (OPN), also known as bone sialoprotein I, is a universal regulator of inflammation, biomineralization and tissue remodeling. Osteopontin is expressed by a variety of cell types including fibroblasts, osteoblasts, osteocytes, odontoblasts, hypertrophic chondrocytes, dendritic cells and macrophages. The elevation of OPN level accompanies the exposure of cells to pro-inflammatory cytokines (e.g. TNFα, IL-1β, TGFβ). Several studies indicate that OPN is also up-regulated at sites of pathologic, ectopic calcification.[29] A study by Makiishi-Shimobayashi et al. suggested that macrophage-derived increased expression of *SPP1* in inflammatory tissues of the middle ear is involved in the development of tympanosclerosis.[40] Our present study showed that the expression of *SPP1* is significantly higher in tympanosclerotic foci and positively correlated with the degree of tympanosclerosis changes. Therefore, the manipulation of local OPN levels may be useful in the treatment of tympanosclerosis.

Conclusions

Tympanosclerosis is a result of post-inflammatory fibrosis characterized by elevated deposition of collagens, and calcification. The present study, with a detailed analysis of the expression of collagen types and osteopontin during the tympanosclerotic process, shows that in the tympanosclerotic membrane the expression of type I collagen is decreased, and the expression of type II and IV collagen and osteopontin is increased.

The altered secretory phenotype of cells from the middle ear induces histological remodeling of the tympanic membrane and correlates with the progression of tympanosclerosis.

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The sequence of lanugo pattern development on the trunk wall in human fetuses

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Abstract

Background. Due to a growing interest in developmental disorders, and in the long-term skin appendage diseases, both in the cosmetic industry and among specialists in dermatology (broadly defined), there is an increasing number of papers on hair development. The publications by the present team of authors are part of this trend.

Objectives. The aim of the study was to describe the topography and typology of skin pilosity patterns in human fetuses.

Material and methods. A total of 278 fetuses (141 male and 137 female) were qualified for the study. The gestational age ranged from 69 to 226 days after conception. All fetuses were taken from a local collection.

Results. The study revealed that the first single hairs occur on the posterior wall of the trunk in the 17th week of fetal life, and on the anterior wall between the 18th and 19th week. It was found that in human fetuses lanugo appears statistically significantly later on the skin of the anterior of the trunk than on its posterior. The difference in absolute time is almost 2 weeks of fetal life. No other differences were found in the development cycle of lanugo on the anterior and posterior walls of the trunk. A full pattern was first observed on the posterior wall of the trunk in a fetus in the 19th week, and on the anterior wall in the 21st week. It was found that the process of lanugo development was completed on the posterior wall in the 23rd week, and on the surface of the abdomen in the 26th week.

Conclusions. The lanugo developmental cycle, consisting in the appearance of the first single hairs, then partial hair and subsequently the formation of final patterns, is the same on both walls of the trunk.

Key words: lanugo, human fetuses, hair pattern, hair development

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Lanugo is a form of human pilosity in prenatal ontogenesis. It is the first developmental form of hair, usually unpigmented, which is softer and gentler than the later types referred to as vellus and pili.[1]

Due to the considerable interest in developmental disorders and skin appendage diseases in the cosmetic industry and among physicians specializing in dermatology, there has been a growing number of scientific works concerning hair development.[4-6] The present authors' previous publications on the topography and typology of human fetal hair patterns are part of that trend.[2,3] Reviewers, editors and scientists who commented on those works encouraged further study to determine the exact time when fetal pilosity appears on the trunk.[2]

Available literature data on this subject are scarce and inaccurate. Ludwig only states that lanugo patterns are "clear and explicit" in fetuses at the 5th–6th month of gestation.[7] According to Muller et al., fetal hair begin to develop at the early embryological stages, presumably between the 9th and 12th week of pregnancy.[8] Irmak et al. noticed that when the adrenal cortex starts to produce dehydroepiandrosterone sulfate (DHEA-S) at the early stage of pregnancy (8th–10th week), the initiation of hair and sebaceous gland differentiation follows.[9]

The sebaceous glands secrete an oily substance called sebum. It mixes with exfoliated epidermal cells to form vernix caseosa, which protects the embryonic skin from the direct influence of the amniotic fluid. Androgen production in the adrenal glands is reduced to a minimal level shortly after labor, which results in lanugo hair loss and sebaceous gland shrinkage.

The aim of this study was to provide a precise and objective assessment of when lanugo hair patterns first appear on the trunk of the human fetus.

Material and methods

The study was carried out on a collection of fetuses from Department of Normal Anatomy, Wroclaw Medical University (Poland). A total of 278 fetuses (141 males, 137 females) were qualified for the study. The gestational age ranged from 69 to 226 days after conception. All the fetuses investigated had complete case histories and no malformations.

Basic anthropological measurements were made (using Mitutoyo digital calliper 500–709–11, IP67, Kabushiki Kaisha Mitsutoyo, Kanagawa, Japan) to determine the precise morphological age of all the fetuses (Table 1).

In a previous study 3 main types of human lanugo patterns on the anterior trunk wall were defined: the cross pattern (Fig. 1), thoracic whorl pattern (Fig. 2) and abdominal whorl pattern (Fig. 3); 2 main types of lanugo patterns on the posterior trunk wall were also identified: the vertebral column path pattern (Fig. 4) and whorl pattern.[3]

STATISTICA PL software (StatSoft Polska, Kraków, Poland) was used for that statistical analysis. The analysis of the level of dimorphic differences using the χ^2 independence test did not reveal any statistical significance. Therefore, all the material could be analyzed together, without any gender differentiation.

The photographs were taken with a Sony Alpha 37 camera (Sony Corp., Tokyo, Japan) with a Kenko automatic extension tube set (Kenko Co. Ltd., Tokyo, Japan) and a Minolta AF 28–80 zoom lens (Konica Minolta Inc., Tokyo, Japan).

Results

The results of the study indicate that the appearance of lanugo patterns proceeds similarly in all the types that have been distinguished. No differences were found in the time of appearance of different lanugo patterns of varying degrees of complexity. Hair growth occurs in a certain rhythm, distinctive for both the anterior and posterior walls of the trunk.

The study results allowed fetal hair growth to be assessed from the time of its appearance on the skin surface to the final lanugo pattern formation. Four phases of lanugo development were distinguished:

- No visible lanugo (Fig. 5);
- Trace hair: isolated hairs about 1 mm long, fastened obliquely; no hair streams; their arrangement is disordered and their length is slightly diverse (Fig. 6);
- Partial pattern: hair is arranged in non-continuous streams forming fragments of patterns; hair length undergoes further differentiation and some single hairs reach over 15 mm;
- Complete pattern: complete patterns are visible regardless of the type (Fig. 7, 8).

Anterior trunk wall

No lanugo is found on the anterior trunk wall until the end of the 4th month of fetal life (Table 2). The first vestigial hair appears in a fetus on the 128th day of pregnancy. This type of hair was found in 5 fetuses in the 5th month of pregnancy (7.9%). In this age group, 5 cases of the incomplete cross pattern and 4 cases of the complete cross pattern (including 1 case with the whorl pattern) were observed. The cross patterns were found in fetuses between the 135th and 140th day of fetal life. A lack of hair was found in 7 fetuses and trace hair in 4 fetuses in the 6th month of pregnancy; their age ranged from 141 to 150 days after conception. No hairless fetuses were found in the study population beyond the 150th gestational day. Partial and complete patterns occurred in 51 fetuses at the age over 150 days after fertilization. This constitutes 82.5% of the study population in the 6th month of fetal life. They were predominantly the cross pattern, with

Table 1. Basic characteristics of the study group

No. of fetuses	Mean menstruation age [days] (SD)	v-pl [mm] (SD)	v-tub [mm] (SD)	Body mass [g] (SD)	Mean morphological age [days]	Age [days]
25	75.5 (5.83)	78.0 (18.33)	61.0 (14.97)	28.6 (10.24)	77.0	56–84
53	101.9 (8.40)	128.9 (29.61)	95.3 (20.05)	61.6 (31.76)	103.4	85–112
63	127.4 (7.19)	199.1 (25.10)	140.9 (17.23)	198.8 (65.18)	129.1	113–140
63	157.7 (8.19)	265.4 (26.61)	186.3 (17.49)	538.5 (132.66)	156.0	141–168
38	176.4 (7.68)	312.1 (22.45)	214.3 (17.10)	882.8 (164.92)	178.2	169–196
36	234.5 (9.46)	253.3 (35.43)	245.0 (24.83)	1017.7(279.39)	235.5	197–256

SD – standard deviation; v-pl – vertex:plantare length; v-tub – vertex:tuberale length.

Table 2. Time of the appearance of lanugo patterns on the anterior wall of the trunk in human fetuses

Age [days]	No. of fetuses	No lanugo		Trace hair		Partial pattern		Complete pattern	
		n	%	n	%	n	%	n	%
69–84	25	25	100						
85–112	53	53	100						
113–140	63	49	77.9	5	7.9	5	7.9	4	6.3
141–168	63	7	11.1	4	6.4	21	33.3	31	49.2
169–196	38					10	26.3	28	73.7
197–226	36							36	100
SUM	278	134	48.3	9	3.2	36	12.9	99	35.6

Fig. 1. Diagram of the cross pattern on the anterior wall of the thorax

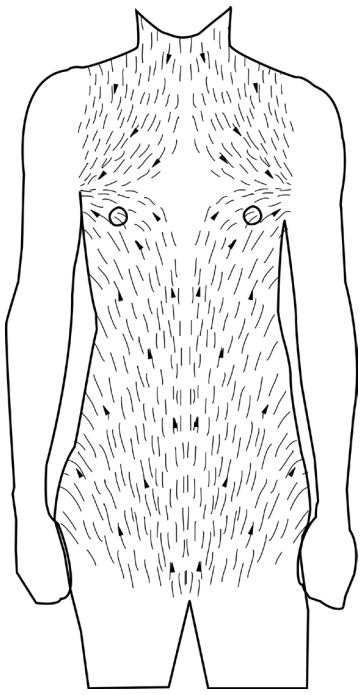


Fig. 2. Diagram of the thoracic whorl pattern on the anterior wall of the thorax

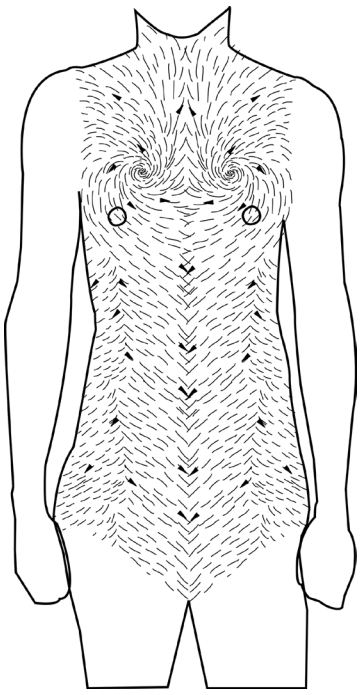


Table 3. Time of the appearance of lanugo patterns on the posterior wall of the trunk in human fetuses

Age [days]	No. of fetuses	No lanugo		Trace hair		Partial pattern		Complete pattern	
		n	%	n	%	n	%	n	%
69–84	25	25	100						
85–112	53	53	100						
113–140	63	43	68.3	6	9.5	7	11.1	7	11.1
141–168	63	7	11.1	4	6.4	21	33.3	31	49.2
169–196	38							38	100
197–226	36							36	100
SUM	278	128	46.0	10	3.6	28	10.2	112	40.2

Fig. 3. Diagram of the abdominal whorl pattern on the anterior wall of the thorax

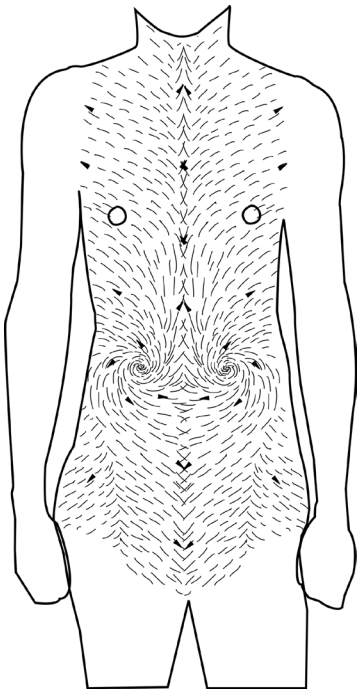
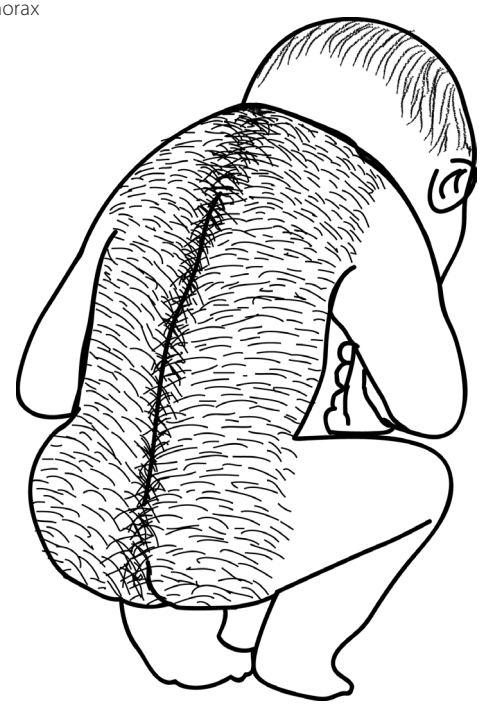


Fig. 4. Diagram of the vertebral column path pattern on the anterior wall of the thorax



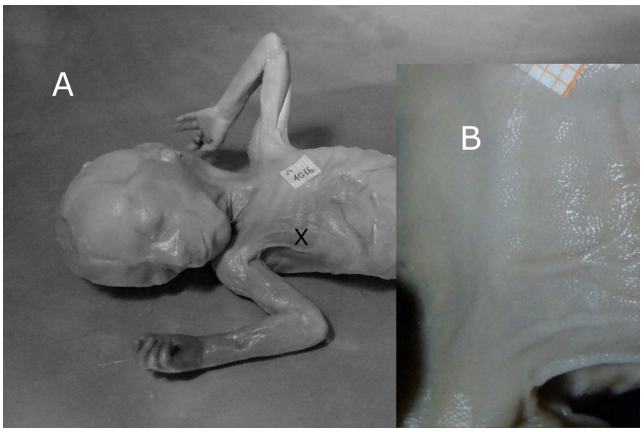
isolated cases of the whorl pattern and the cross pattern with whorls. In all the fetuses examined, lanugo formed patterns on the anterior wall of the trunk from the 7th month of fetal life. In 25% of the cases these patterns are incomplete. Complete patterns were found in the fetuses from the 26th week of fetal life.

Posterior trunk wall

As with the anterior wall, there was no presence of lanugo on the posterior wall in the first two age brackets (i.e., up to 84 days and 85–112 days). The first single lanugo hairs appear on the 116th day of pregnancy. It is significantly earlier than on the anterior wall (χ^2 test; $p < 0.05$).

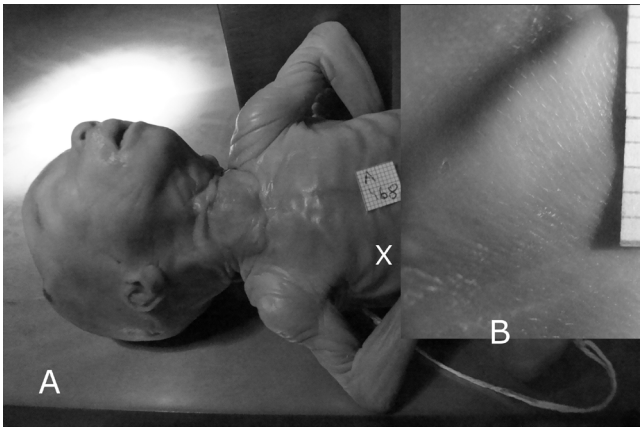
A partial vertebral column path was first observed in a fetus in the 129th day of pregnancy. Altogether, lanugo patterns were present in 22.2% of the 5-month fetuses. A predominance of the vertebral column path was observed; single-whorl patterns are numerous and multi-whorl patterns are rare. From the 156th day of pregnancy, i.e. from the end of the 6th month, complete hair patterns were found on the posterior trunk wall. This was significantly earlier than on the anterior trunk wall. A detailed analysis of the time of hair appearance is presented in Table 3.

Fig. 5. A photo of a fetus without visible lanugo (fetus no. A-1016, morphological age: 5 months after conception)



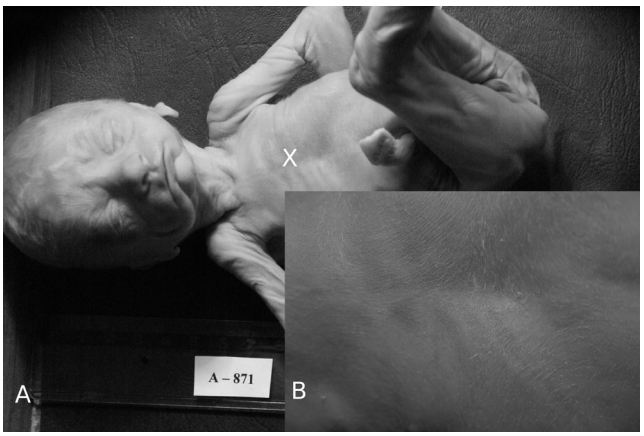
A – main picture; X – the location of the enlarged area; B – the enlarged area (magnification: x4.1).

Fig. 6. A photo of a fetus with trace hair (fetus no. A-468, morphological age: 6 months after conception)



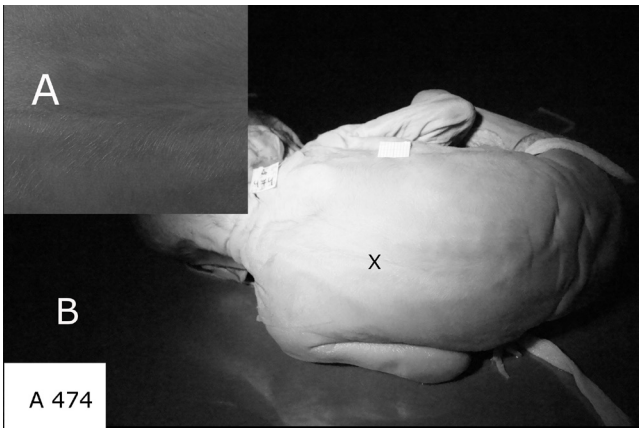
A – main picture; X – the location of the enlarged area; B – the enlarged area (magnification: x4.5) showing the hair pattern.

Fig. 7. A photo of a fetus with a "thoracic delta" pattern (fetus no. A-871, morphological age: 6 months after conception)



A – main picture; X – the location of the enlarged area; B – the enlarged area (magnification: x4.1) showing the hair pattern.

Fig. 8. A photo of a fetus with a "vertebral column path" pattern (fetus no. A-474, morphological age: 6 months after conception)



A – main picture; X – the location of the enlarged area; B – the enlarged area (magnification: x3.2) showing the hair pattern.

Discussion

The appearance of lanugo on human skin is determined by the developmental processes during embryogenesis. The first stage of this process is the creation of rudimentary hair follicles, starting with the organization of epidermal keratinocytes into hair placodes. This is followed by the accumulation of specialized fibroblasts with inductive properties in the underlying mesenchyme.[12] The hair buds that are formed by quickly proliferating epidermal cells bulging towards the mesenchyme begin to appear around the 8th or 9th week of fetal development. Their development and maturation is controlled by various signalling substances such as lymphoid enhancer factor 1 (LEF-1), bone morphogenic protein 4 (BMP4) and transforming growth factor beta (TGF-beta). These substances are responsible for hair growth dynamics and for the correct order of development of the individual elements. The main gene controlling the in-growth of the growing hair papilla is the hedgehog gene, which codes the polypeptide, singaling ligand of the hedgehog signaling pathway. It probably plays a key role in the fusion of the ectodermal part of a hair with the mesenchyme.[13]

Single hair follicles are very precisely located within the developing skin. Their position is a result of interactions between Wnt proteins and beta-catenin.[14] These relations are the reason why the hair follicles that begin to appear on the human fetus skin at around the 10th week after fertilization are equidistant from each other. Further maturation of the hair follicle is the effect of the interaction among various chemical factors. As a result of the whole multistage process, approx. 5 million hair follicles are created. Paus and Cotsarelis suggested that new hair follicles are not subsequently created and that observed hair changes are the result of the influence of hormonal factors on hair follicles located in various parts of the human body.[15]

According to the literature, the time lanugo appears on human fetuses is specified at the 18th–20th gestational week.[14,16] In 1921 Ludwig also indicated that lanugo patterns become "clear and explicit" in the 5th month of fetal life.[7]

The present study shows that the first single hairs appear on the posterior wall of the trunk in the 17th gestational week, and on the anterior wall between the 18th and 19th gestational weeks. Plikus and Chuong suggested that the first developmental hair cycle in a mammal's life starts in the cephalic part of the mammal, then "wanders" distally, first to the back, then the abdomen and finally reaching the limbs. They referred to this process as "the developmental wave".[17] The same researchers, whose findings are based on the studies of genetically modified mice, indicated that this mechanism is responsible for the creation of hair whorls, and that it is a result of the activity of various genes and epigenetic factors discovered during an analysis of the *Drosophlila melanogaster* genome.

The results of the present study confirm the suggestions of Plikus and Chuong, revealing a statistically significant difference in the timing of lanugo appearance in humans. Hair streams first become visible on the dorsum, and then appear on the abdominal wall. The difference in actual time is almost 2 gestational weeks. No other differences in the lanugo developmental cycle were observed.

The developmental cycle, consisting first in the appearance of single hairs, then incomplete patterns and finally complete lanugo patterns, is equivalent on the anterior and posterior walls of the trunk. The first complete pattern was observed in the 19th gestational week on the posterior trunk wall, and in the 21st week on the anterior wall. Complete lanugo patterns were observed in 100% of the examined fetuses on the dorsal side in the 23rd gestational week, and on the ventral side in the 26th gestational week.

Verification of the "developmental wave" theory in humans will be possible shortly, because the present authors are currently carrying out a study evaluating the morphology of fetal pilosity within the head and neck.

Conclusions

The developmental cycle, consisting first in the appearance of single hairs, then incomplete patterns and finally complete lanugo patterns, is equivalent on the anterior and posterior walls of the trunk.

The first single hairs appear on the posterior wall of the trunk in the 17th week of fetal life, and on the anterior wall between the 18th and 19th weeks.

It was found that lanugo development was complete on the back in the 23rd week and on the abdomen in the 26th week.

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Body composition in 13-year-old adolescents with abdominal obesity, depending on the BMI value

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Excessive adipocyte growth during the pubertal transition predisposes to the development and persistence of obesity in adulthood. Visceral accumulation of body fat is particularly disadvantageous when it is correlated with insulin resistance, secondary hyperinsulinaemia, dysglicaemia, and atherogenic dyslipidemia.

Objectives. The aim of this study was to conduct a nutritional status assessment and body composition analysis in 13-year-old adolescents of both genders with visceral fat accumulation (WC $\geq 90^{th}$ percentile) and different BMI values.

Material and methods. The evaluation of state of nutrition of 1,738 Polish boys (n = 882) and girls (n = 856) aged 13 was done based on anthropometric measurements and calculated BMI (body mass index), WC (waist circumference) and WHtR indices (waist-to-height ratio). Taking into consideration the value of WC $\geq$ 90 pc, 353 people were designated (20.3 % of the total) with visceral obesity (but with various BMI), whose body composition was examined by the method of bioelectric impedance analysis (BIA). A total of 249 adolescents of both sexes (70.5% of the selected, 102 boys and 147 girls) and their parents agreed to the study.

Results. In adolescents with visceral obesity a significant change of body content was ascertained depending on the value of the BMI. Even in the people with a proper value of the BMI, a significantly higher than standard increase of the percentage of total body fat (TBF) and decrease of both the percentage of body lean (BL) and the content of total body water (TBW) in the body was observed. The values of the BMI, WC and WHtR in adolescents were significantly correlated with each other as well as with TBF, BL and TBW, and the strength of correlation was dependent on sex.

Conclusions. The state of nutrition in adolescents with visceral obesity, even with a proper BMI, might contribute to the development of a metabolic syndrome

Key words: body composition, bioimpedance, adolescents

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The presented results are part of a research project constituting a unique 4-year program co-funded by the Department of Health and Social Policy of the City of Szczecin, which evaluates the nutritional status, the nutrition methods in Szczecin's secondary schools, nutrition education and the effects of this education by means of a questionnaire.

The age of 13 years was chosen for this study and for health-oriented nutrition education due to 4 major aspects. Firstly, young people of this age are at puberty, which is associated with a number at changes, including changes in hormone levels that affect the functioning of the whole organism. Secondly, they change school during this period, which is conventionally associated with "being no longer a child, and becoming a youth". Thirdly, the change of school means also a new environment, making new acquaintances, the need for adaptation and adjustment to a new school environment, which significantly affects the emotional state of young people. And fourthly, this difficult stage of puberty, associated with physiological, social and psychological changes, may considerably affect youth's eating behaviors, possibly resulting in the use of different types of diets (including slimming diets), excessive binge eating, or complete rejection of food. And especially in this period, food is one of the major environmental factors of the development that is compliant with the genetic potential of adolescents. Irregularities in their eating habits constitute a threat to their health that manifests during the growth period in somatic development disorders, including abnormalities in the content and distribution of fat tissue, which predispose to the development of undernourishment, overweight or obesity. Excessive adipocyte growth during the pubertal transition predisposes to the development and persistence of obesity in adulthood. Deposition of body fat in adolescents results mainly from a positive energy balance and low physical activity, which overlap genetic predisposition to obesity. The abnormal nutritional status of adolescents not only poses an increased risk of cardiovascular complications, but also reduces the quality of life as a result of disapproval of their own appearance, low self-esteem, a sense of isolation, difficult relationships with their peers and even depressive states.[1] Visceral accumulation of body fat is particularly disadvantageous when it is correlated with insulin resistance, secondary hyperinsulinaemia, dysglicaemia, and atherogenic dyslipidemia.[2] Also, normal-weight patients with visceral fat accumulation, referred to as MONW (metabolically obese normal-weight), have an increased prevalence of the above-mentioned disorders.[3] Assessment of nutritional status in children and adolescents includes anthropometric measurements and indicators calculated on their basis, such as BMI, WC and WHtR, as they were shown to correlate strongly with risk factors for cardiovascular diseases.[4] Therefore, during pubertal growth spurts, screening tests are important for identifying people with abnormal nutritional status, in order to expose them to health-oriented nutrition education (which modifies abnormal eating habits) and to individual diet correction, as well as to encourage them to take part in more physical activity in order to reduce their weight.

This study aimed at nutritional status assessment and body composition analysis in 13-year-old adolescents of both genders with visceral fat accumulation (WC ≥ 90th percentile) and different BMI values.

Material and methods

After obtaining the approval of Local Ethical Commission (BN-001/93/07), the research was conducted in the years 2007–2010 among n = 1738 13-year-old Polish adolescents (856 girls and 882 boys) of 87 first classes, in 31 randomly selected junior high schools in Szczecin.

Anthropometric measurements

The children were anthropometrically examined, that is, their body mass was measured with medical scales (legalized and standardized to 0.1 kg) without shoes and in light clothes, and their body height was measured in Frankfurt position with height meter attached to mobil stadiometr Seca 215 within an accuracy of 0.1 cm. The waist circumference (WC) measurement was taken within an accuracy of 0.1 cm, midway between the 10th rib and the iliac crest; an anthropometric Gulick tape measure was employed with the subject remaining in a standing position.[5]

From the obtained measurements, BMI was calculated according to the formula: body mass (kg)/height (m^2). WHtR was calculated according to the formula: waist circumference (cm)/height (cm). The obtained values of the BMI were referred (according to sex and age) to Polish centile charts, and the BMI value was assumed as: ≤ 5th percentile as underweight; > 5th to < 85th percentile as normal weight, ≥ 85th to < 95th percentile as overweight, ≥ 95th percentile as obesity.[6] The values of WC and WHtR indices were also referred to Polish centile charts, and for both indices the assumed values were ≥ 90th percentile as a criterion of the location of the visceral fat tissue, a risk of the development of heart and cardiovascular diseases and impaired glucose tolerance.[7] Table 1 shows the average values of anthropometric features in 13-year-old adolescents from the groups under evaluation.

Despite the fact that the average values of BMI, WC, and WHtR did not indicate anything improper, their thorough analysis based on centile charts showed a significant percentage of people with improper nourishment. Based on the values of the WC ≥ 90th percen-

Table 1. Values of anthropometric attributes and the BMI, WC, WHtR indicators in 13-year-old adolescents (M ± SD; n = 1738)

Attributes and indicators	Girls (n = 856)	Boys (n = 882)
Body weight (kg)	52.2 ± 10.5	53.3 ± 12.0
Body height (cm)	160.2 ± 6.2	161.8 ± 11.4
BMI (kg· m^{-2})	20.2 ± 3.3	20.1 ± 3.5
WC (cm)	70.3 ± 8.2	72.2 ± 9.3
WHtR (cm/cm)	0.442 ± 0.05	0.455 ± 0.05

tile, a group of 353 participants (20.3% of all children under evaluation, that is, 209 girls and 144 boys, which is 12.0% and 8.3% of total number of children under evaluation) was picked from the group of 1,738 children with visceral location of fat tissue, which had different values of BMI index (Table 2). Upon parental consent, all the children were asked to have their body composition measured by bioelectrical impedance analysis (BIA).

Assessment of bioelectrical impedance analysis (BIA)

From among the selected 353 adolescents with abdominal obesity, only 249 of them and their parents agreed to carry out a study of body composition. A total of 249 children entered this study (70.5% of previously selected), including 147 girls (59.0% of previously selected) and 102 boys (41.0% of previously selected), and they were divided according to BMI into 3 groups (Table 3).

The measurements were made by means of Bodystat® 1500MDD in tetra-polar system configuration, hand-to-foot with Body Manager software adjusted for the analysis of children's body composition, i.e. using regression equations for body composition (TBF – total body fat, LBM – lean body mass, TBW – total body water) of children at the age of 11–19 years.[8] The measurements were taken in the school nurse's room, following all the recommendations for correct measuring procedure. As the children were required to refrain from eating prior to the measurement, once measured, each child received a nutritious breakfast.

Statistical analysis

The significance of differences (at the level $p \leq 0.05$ and $p \leq 0.01$) in the values of anthropometric characteristics, indicators and parameters of body composition was estimated using 2-way factorial ANOVA with the aid of STATISTICA® 9.0 software. Pearson's correlation was used to assess the relationships among BMI, WC and WHtR and their association with TBF, LBM and TBW.

Results

An analysis of the data obtained from anthropometric measurements of abdominally obese adolescents (Table 4) has revealed that the mean body weight, as well as the values of BMI, WC and WHtR exceeded the 85th or 90th percentile in both girls and boys. Differences in the values of these parameters within genders and groups were statistically significant ($p \leq 0.01$).

An analysis of TBF, LBM, and TBW (Table 5) in viscerally obese girls and boys has revealed significant differences among groups depending on the BMI value. Of interest, however, was that girls with normal BMI had higher TBF percentage (26.9%), which exceeded by 1.9% the recommended upper limit (25%), while the maximum TBF percentage (33.1%) exceeded the limit by as much as 8.1%.

This was accompanied by LBM, the mean value of which remained within the recommended standards (72–82%), but some individual values (66.9%) were below the lower threshold (72%). The average TBW in girls (55.2%) was below the recommended lower limit (57%), and in none of the girls TBW exceeded the upper limit (62%).

Table 2. The value of the BMI, WC and WHtR indicators in 13-year old adolescents, n = 1738

Percentile level	BMI				WC				WHtR			
	Girls (n = 856)		Boys (n = 882)		Girls (n = 856)		Boys (n = 882)		Girls (n = 856)		Boys (n = 882)	
	n	%	n	%	n	%	n	%	n	%	n	%
≤ 5th pc (underweight)	40	4.7	54	6.1	27	3.2	31	3.5	57	6.7	39	4.4
> 5th to < 85th pc (normal)	562	65.6	669	75.8	620	72.4	707	80.2	609	71.1	695	78.8
≥ 85th to < 95th pc (overweight)	144	16.8	74	8.4	84	9.8	56	6.3	95	11.1	58	6.6
≥ 95th pc (obesity)	110	12.9	85	6.6	125	16.6	88	10.0	95	11.1	90	10.2

Table 3. Percentage of 13-year old adolescents with WC ≥90th percentile index value, depending of BMI index value, n = 249

Range of BMI	Total (n = 249)		Girls (n = 147)		Boys (n = 102)	
	n	%	n	%	n	%
≤ 5th pc (underweight)	71	28.5	55	37.4	16	15.7
> 5th to < 85th pc (normal)	82	32.9	42	28.6	40	39.2
≥ 85th to < 95th pc (overweight)	96	38.6	50	34.0	46	45.1
≥ 95th pc (obesity)	110	12.9	85	6.6	125	16.6

Also, boys with normal BMI had a higher average TBF (25.6%), which exceeded the recommended upper limit (18%) by 7.6%, while the maximum TBF (35%) exceeded this limit by as much as 17%. This was accompanied by a lower LBM, the mean value of which (74.4%) was below the recommended lower limit (82%), although some individual values were close (87.2%) to the upper limit (88%). Similarly, the mean TBW in boys (57.0%) was below the recommended lower limit (61%), although in individual cases, water content exceeded the upper limit (66%) by even as much as 13.1%.

An analysis of Pearson's linear correlation coefficient (Table 6) has revealed that the BMI was significantly positively correlated with TBF in girls and boys ($r = 0.62$, $p < 0.001$; $r = 0.59$, $p < 0.001$), and negatively correlated with LBM (analogically $r = -0.62$ and $r = -0.59$, $p < 0.001$) and TBW. Considering BMI values, this index was significantly correlated with the body composition of only obese girls, and of both overweight and obese boys. A stronger correlation of BMI with TBF, LBM and TBW occurred in girls.

WC values were also significantly ($p < 0.001$) positively correlated with TBF in girls ($r = 0.46$, $p < 0.001$) and boys ($r = 0.59$, $p < 0.001$), and significantly negatively correlated with LBM (analogically $r = -0.46$ and $r = -0.59$, $p < 0.001$) and TBW ($r = -0.46$ and $r = -0.57$, $p < 0.001$). A significant increase in WC was combined with body composition only in obese boys and girls. A stronger correlation of WC with TBF, LBM and TBW occurred in boys.

WHtR was significantly positively correlated with TBF in girls ($r = 0.53$, $p < 0.001$) and boys ($r = 0.70$, $p < 0.001$), and significantly negatively correlated with LBM (analogically $r = -0.53$ and $r = -0.67$, $p < 0.001$) and TBW (analogically $r = -0.53$ and $r = -0.60$, $p < 0.001$). A significant increase in WHtR was combined with body composition only in obese girls, while stronger correlations of WHtR with TBF, LBM and TBW occurred in boys.

Significant ($p < 0.001$) positive correlations were found between BMI and WC, and between WC and WHtR, and were stronger in boys. Moreover, significantly ($p < 0.01$) positive correlations occurred in both overweight and obese girls and boys.

Discussion

The simplest and most frequently used methods of nutritional status assessment are anthropometric measurements of body weight and height, which are the basis for calculating BMI – an index showing a strong correlation ($r = 0.80$) with body fat tissue content.[9] The results obtained in this study of anthropometric measurements of Polish 13-year-old boys and girls have confirmed the epidemiologic reports, which show increasing incidence of overweight and obesity among adolescents in Europe, including Poland.[10,11] In our studies on a population accounting to 1,738 individuals, the percentage of overweight adolescents equaled to 12.5%, and obese adolescents – 11.2%. A higher percentage of Polish adolescents (13–15 years of age) with overweight (13.8%) and obesity (2.8%) was reported by Jodkowska et al.[11] In our study, overweight and obesity occurred more frequently in girls (16.8% and 12.9%) than in boys (8.4% and 9.6%). This phenomenon is disadvantageous, since it has been demonstrated that children who are overweight and obese during the period of puberty (10–15 years of age) have the tendency to obesity in adulthood reaching 75% and 83%, respectively. For this reason, adolescence is referred to as a critical period for the development of obesity. The puberty period brings an increased, compared to other life stages, risk of disorders in the structure or function of organs, as well as in the development of the tissues and systems of the body.

The studies by Lee et al. have shown insulin resistance in approx. 46–52% obese adolescents and in 11–16% overweight ones, while Jago et al. reported lipid abnormalities in 12–17% of children with excess body weight.[12,13] Moreover, fat tissue accumulation in teenagers often contributes to distorted body perception, which results in mood changes and depression. Excessive body weight diverges from the images created by the mass media, of a slim woman and a muscular man, to whom positive qualities are attributed, such as success, health, self-confidence, and sexual attraction.[14]

Studies have shown that the BMI alone is not a useful index of fat tissue location in children and adolescents. The BMI may also deliver false information in the case of strongly muscular people. Therefore, it is important to determine both fat tissue location and its percentage in body composition. The visceral location of fat tissue revealed in the developmental age is a significant factor in the development of metabolic syndrome. Hence, the importance of the early detection of patients with visceral fat accumulation, as it enables taking preventive measures to reduce the risk of developing metabolic syndrome. Between 10 and 16 years of age, the basis for recognizing metabolic syndrome is the waist circumference that exceeds the 90th

Table 4. Anthropometric parameters, the BMI, WC and WHtR indices in the examined girls (n = 147) and boys (n = 102), Mean ± SD, n = 249

Parameters and indices		Reference level > 5th to < 85th pc	Sex M ± SD	Groups			Effect		
				BMI > 5th to < 85th pc	BMI ≥ 85th to < 95th pc	BMI ≥ 95th pc	Sex (S)	Group (G)	S x G
Body weight (kg)	Girls	38.0–59.9	63.7 ± 9.8	57.3Bb ± 5.2	61.6^{Ba} ± 6.2	72.6^{A} ± 9.8	**	**	**
	Boys	36.8–64.9	70.7 ± 12.3	55.3 ± 8.6^{B}	66.2 ± 6.3^{A}	80.1 ± 9.5^{B}			
Body height (cm)	Girls	1.50–1.66	163.2 ± 7.2	169.8 ±7.0	162.2 ± 6.9	162.3 ± 7.4	–	–	**
	Boys	1.49–1.69	165.2 ± 8.1	160.9 ± 9.4	165.5 ± 8.0	166.5± 7.3			
BMI (kg·m^{-2})	Girls	15.9–21.8	23.9 ± 3.2	21.1 ± 1.0^{C}	23.4 ± 0.8^{B}	27.5 ± 2.4^{A}	**	**	–
	Boys	15.9–22.9	25.8 ± 3.6	21.2 ± 1.6^{C}	24.1 ± 1.1^{B}	28.9 ± 2.8^{A}			
WC (cm)	Girls	58.2–75.5	82.6 ± 7.2	78.6 ± 3.5^{B}	80.5 ± 4.7^{B}	88.8 ± 7.7^{A}	**	**	–
	Boys	59.8–80.8	89.5 ± 8.3	82.1 ± 2.8^{B}	85.2^{AB} ± 3.8	95.9 ± 7.9^{A}			
WHtR (cm/cm)	Girls	0.370–0.473	0.506 ± 0.04	0.477^{Bb} ± 0.02	0.496^{Ba} ± 0.02	0.547^{A} ± 0.04	**	**	–
	Boys	0.380–0.495	0.543 ± 0.05	0.512 ± 0.04^{B}	0.516 ± 0.03^{AB}	0.577 ± 0.06^{A}			

a, b – means denoted with the different letters are statistically significant difference p ≤ 0.05; A, B, C –means denoted with the different letters are statistically significant difference p ≤ 0.01; statistically significant difference *p ≤ 0.05; statistically significant difference **p ≤ 0.01.

Table 5. Body mass composition in 13-year old girls (n = 147) and boys (n = 102) with WC ± 90th percentile, n = 249

Parameters and indices		Girls (n = 147)			Boys (n = 102)		
		BMI > 5th to < 85th pc	BMI ≥ 85th to < 95th pc	BMI ≥ 95th pc	BMI > 5th to < 85th pc	BMI ≥ 85th to < 95th pc	BMI ≥ 95th pc
Total body fat (kg)	range	10.9–19.4	12.3–25.2	15.4–39.1	4.2–18.9	7.7–23.1	14.9–42.9
	M ± SD	15.5 ± 2.4B	20.7 ±13.0A	23.9 ± 5.4A	14.3 ± 4.0B	17.6 ± 3.3B	20.7 ± 13.0A
Total body fat (%)	range	19.5–33.1	22.6–38.7	22.5–42.4	12.8–35.0	10.5–38.0	19.4–44.9
	M ± SD	26.9 ± 3.3C	30.3 ± 4.1B	33.0 ± 4.2A	25.6 ± 6.3B	26.8 ± 5.3B	31.7 ± 5.3A
	reference level	18–25			12–18		
Lean body mass (kg)	range	33.7–51.1	34.3–65.0	35.0–65.0	28.6–50.2	36.2–65.8	42.7–77.0
	M ± SD	41.8 ± 4.0B	43.1 ± 5.3B	48.1 ± 5.7A	41.1 ± 6.8C	48.6 ± 6.7B	54.4 ± 6.8A
Lean body mass (%)	range	66.9–80.5	61.3–77.4	57.6–77.5	65.0 –87.2	62.0–89.5	55.1–80.6
	M ± SD	73.1 ± 3.3A	69.6 ± 4.2B	67.0 ± 4.2C	74.4 ± 6.3A	73.2 ± 5.3A	68.3 ± 5.3B
	reference level	72–82			82–88		
Total body water (l)	range	25.4–38.6	25.9–49.1	26.4–49.1	21.4–37.5	27.0–49.2	31.9–57.5
	M ± SD	31.6 ± 3.0B	32.4 ± 4.0B	36.3 ± 4.3A	30.7 ± 5.1C	36.3 ± 5.0B	40.7 ± 5.0A
Total body water (%)	range	50.5–60.8	46.3–58.5	43.5–58.5	48.6–79.1	46.8–66.9	41.2–60.4
	x ± SD	55.2 ± 2.5A	52.6 ± 3.1B	50.5 ± 3.2C	57.0 ± 7.6A	54.6 ± 4.0A	51.0 ± 4.0B
	reference level	57–62			61–66		

a, b – means denoted with the different letters are statistically significant difference p ≤ 0.05; A, B, C –means denoted with the different letters are statistically significant difference p ≤ 0.01.

percentile. Visceral obesity may occur not only in patients with an abnormal BMI, but also in those with a normal one, referred to as MONWs. In this study, visceral obesity occurred, regardless of the BMI value, in 20.3% of participants, more frequently in girls (23.2%) than in boys (16.3%), while among normal-weight participants – 28.5% were MONWs, also more frequently girls (37.4%) than boys (15.7%). Higher values of waist circumference were found in boys. A study by Przybylski et al. has shown a lower percentage (4.4%) of MONWs among Polish adolescents aged 16–18 years; however, it was higher in girls.[15] The greater waist circumference in boys, compared to girls, is a consequence of the fact that healthy boys during puberty tend to increase the lean body mass percentage, and reduce total body fat percentage, which is dependent on androgen concentration. Differently in girls, estrogens increase the accumulation of adipose tissue around the breasts, hips and buttocks, and consequently increase its percentage in the total body mass. The risk of becoming overweight during puberty is higher for girls than for boys, which was confirmed in our studies.[16]

WHtR is an indicator that reflects a relationship, changing with age, between the rate of waist circumference increase and body height. It is a simple indicator for estimating the risk factors of cardiovascular disease in clinical screening, and it seems to be even more reliable than WC and BMI. In our study, this ratio was related to centile charts, and has revealed higher risk of cardiovascular disease (WHtR ≥ 90th precentile) in 19.4% of adolescents, more frequently in girls (22.2%) than boys (16.8%), differently from the study by Kromeyer-Hauschild et al., with the results analogically 11.1% and 12.1%.[17]

The nutritional status assessment is not always tantamount to body fat assessment, especially in children during the pubertal transition. For this reason, selected volunteers with abdominal obesity had their body composition measured by BIA method, applied in both children and adolescents, since this method was proven by de Faria et al. as being more reliable for body fat assessment at the age of 10–19 years, compared to the BMI.[18]

Higher fat tissue accumulation in the bodies of people with a high BMI is a well-known fact. However, the body composition of viscerally obese individuals, with regard to the BMI and gender, was interesting, since even children with the same BMI value may considerably differ in body fat content.[19] Obese or overweight participants with WC ≥ 90th percentile were found to have higher body fat percentage and lower fat-free mass percentage and total water content. But what was especially interesting was TBF percentage, which was significantly higher than the standard one, in viscerally obese girls (26.9%) and boys (25.6%) with a normal BMI. These values were higher compared to 13-year-olds with a normal BMI and normal WC (27.1% in girls and 22.3% and boys).[20]

This study has demonstrated strong and significant correlations ($p < 0.001$) among the values of BMI, WC and WHtR in 13-year-old girls and boys, similarly to what Savva et al. demonstrated in children aged 10–14 years: between BMI and WC ($r = 0.91$), BMI and WHtR ($r = 0.92$), and WC and WHtR ($r = 0.95$).[4] Also, studies on 12–13-year-old Australian adolescents[21] revealed a significant correlation ($p < 0.001$) between BMI and WC ($r = 0.09$) – stronger in boys than in girls, which was in accordance with our results.

A study on adolescents by Noevius et al. showed a significant positive correlation ($r = 0.68–0.73$, $p < 0.0001$) between WC and TBF.[22] Similarly, Foo et al. found significant positive correlations between BMI and TBF ($r = 0.85–0.87$, $p < 0.001$), and WC ($r = 0.80–0.78$, $p < 0.001$) and WHtR ($r = 0.85–0.77$, $p < 0.001$) in 12–19-year-old Chinese and Malaysian boys and girls.[23] Our study has also revealed significant correlations among BMI, WC, WHtR and TBF, as well as LBM and TBW in adolescent girls and boys. A weaker positive correlation between WHtR and TBF ($r = 0.30–0.41$, $p < 0.0001$) in adolescents was reported by Noevius et al.[22] Our study has shown no correlations of BMI, WC, and WHtR with the body composition of adolescents with a normal BMI. According to Mehdad et al., who examined Moroccan adolescent girls and boys (11–17 years of age), correlations of the BMI and WC with TBF were dependent on body weight, and were stronger in girls than in boys, similarly as in our study.[24]

Knowing the role of fat tissue in the endocrine system, it is possible to predict how severe health consequences may result from its excess in adolescents with both normal and high BMI, and visceral fat accumulation. As demonstrated in a Danish study by Baker et al., the BMI of adult patients positively correlated with the BMI in 7–13-year-old boys and 10–13-year-old girls, so overweight and obesity prevention between 7 and 13 years of age seems to be well founded.[25]

Therefore, after diagnosing abnormal nutritional status in adolescents, all the examined children (1,738 individuals) were subjected to health-oriented education in a live workshop with food products, during which they learned about the principles of proper nutrition tailored to their developmental age and gender in the context of further physical and intellectual development, and the prevention of civilization diseases. Young people were acquainted with recommended sources of nutrients, interpreting information on packaging, trendy food ingredients, as well as health-oriented culinary processes and nutrient losses during food preparation. Parents of adolescents who underwent BIA measurement received a proposal of individual diet correction for their children; however, only 13% of them came forward. Such little interest of parents in their children's health and development was confirmed in numerous conversations with school directors, teachers, and guidance counselors.

In conclusion, the values of WC ≥ 90th percentile may indicate changes in body composition in adolescents of both genders. Body composition in adolescents of both

genders was significantly correlated with BMI, WC and WHtR indices, and the strength of these relationships was gender-dependent. The body composition in adolescents with abdominal obesity, even with a proper BMI, might make them susceptible to metabolic disorders; therefore, a health-oriented nutrition education is necessary in order to modify dietary habits.

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Application of the functional capacity scale in the early assessment of functional efficiency in patients after aneurysm embolization: Preliminary reports

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Abstract

Background. While data on the long-term (e.g., 1 year and subsequent years) outcomes of intracranial aneurysms treatment is relatively well-documented mainly in the clinical aspect (comparability of treatment, mortality, and complications), little is known about the early results, in terms of the functional outcome.

Objectives. The aim of the study was to analyze the use of Functional Capacity Scale (FCS) in the evaluation of patients in the early period after endovascular treatment of intracranial aneurysms.

Material and methods. The study was conducted in the Neurosurgery Clinic, University Hospital Collegium Medicum in Bydgoszcz, on a group of 118 consecutively admitted patients with the diagnosis of intracranial aneurysm, qualified for treatment using the endovascular method (embolization). The assessment was performed twice. In the clinical assessment the Glasgow Coma Scale (GCS) was used to evaluate the level of consciousness and the Hunt and Hess Scale (H&H) to assess the patient's condition. To assess the final outcome and early functional capacity Glasgow Outcome Scale (GOS), Barthel Index (BI), Modified Rankin Scale (mRS) and the new Functional Capacity Scale were used.

Results. The assessment performed with the FCS was comparable to the assessment conducted with standardized tools such as BI, mRS or GOS. The clinical condition assessed with the GCS ($p < 0.001$) and H&H ($p < 0.001$) differentiates the functional condition assessed using the FCS. Statistically significant correlations were found between FCS and BI ($r = -0.78$), GOS ($r = -0.69$) and mRS ($r = 0.68$).

Conclusions. The study indicates that the FCS correlates with other scales used in the assessment of patients with intracranial aneurysm, which means that the proposed tool can be applied successfully in practice. However, further randomized multicenter studies are necessary in order to clarify the final conclusion.

Key words: functional assessment, outcome, aneurysm, subarachnoid hemorrhage

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The death rate caused by SAH from ruptured intracranial aneurysm decreased in the recent decades by ca. 17% and the overall survival rate amounts to ca. 65%.[1,2] This is due to, among others factors, aneurysms being secured earlier, better understanding of the pathophysiology of angiospasm, as well as more aggressive and earlier praphylaxis and treatment of systemic complications.[2] Undoubtedly, considerable progress in the intensive treatment, diagnostics and accessibility to intracranial treatment contributed to the marked improvement in the results of treatment in patients after SAH caused by ruptured intracranial aneurysm, mainly from the long-term aspect.[3–5]

While the data on long-term results (i.e. after a year and longer) of intracranial aneurysm treatment are fairly well documented[6–9], mainly from the clinical aspect (comparison of treatment methods, mortality, complications occurrence), there is a lack of data on early results from the functional aspect. The immediate period following the procedure (time until the patient's discharge) is particularly dangerous for the patient due to the possibility of various complications appearing that may affect his or her functional capacity. A well-performed early functional assessment may provide prognosis regarding long-term results.

Research carried out for many years showed that one of the most important factors conditioning the result of treatment, as well as the functional result of patients with aneurysm (with/without SAH), is the patient's clinical condition (including consciousness level) prior to treatment. The analysis of the clinical condition and consciousness of patients with an aneurysm is usually conducted with scales such as the Glasgow Coma Scale (GCS), Hunt and Hess Scale (H&H) and the World Federation of Neurological Surgeons Scale (WFNS).[10–12] Their functional condition, on the other hand, is usually assessed with scales such as the Barthel Index (BI), Modified Rankin Scale (mRS), and Glasgow Outcome Scale (GOS).[13–15]

The main aim of the study was to analyze the performance of the Functional Capacity Scale (FCS) in the assessment of patients in the early period following the endovascular treatment of intercranial aneurysm. Possible correlations between the scales used for the assessment of the functional condition of a patient were investigated. Moreover, an attempt was made to determine whether clinical condition assessed with the Glasgow Coma Scale and the Hunt and Hess Scale provide similar results to the Functional Capacity Scale, Barthel Index, Modified Rankin Scale and the Glasgow Outcome Scale.

Material and methods

Setting and sample

The study was conducted at the Neurosurgery Clinic of the University Hospital Collegium Medicum (CM) in By-

Table 1. Demographic data regarding the study participants N (%)

Gender	
Female	73 (61.9)
Male	45 (38.1)
Age	
≤ 49	51 (43.2)
50-64	55 (46.6)
≥ 65	12 (10.2)
Location of aneurysms*	
ACoA	42 (35.6)
ICA	33 (28.0)
MCA	22 (18.6)
VBA	21 (17.8)
Clinical display	
Unruptured aneurysm	42 (35.6)
Ruptured aneurysm – SAH	76 (64.4)
Time for embolization	
≤ 24	85 (72.0)
25-71	15 (12.7)
≥ 72	18 (15.3)
GCS	
I (15-13)	100 (84.8)
II (12-9)	11 (9.3)
III (8-3)	7 (5.9)
H&H	
0	42 (35.6)
I-II	58 (49.2)
III	11 (9.3)
IV-V	7 (5.9)

*ACoA – anterior communicating artery (and location on the anterior cerebral artery); ICA – internal carotid artery; MCA – medial carotid artery, VBA – vertebral-basilar artery.

dgoszcz, Nicolaus Copernicus University (NCU) in Toruń. The study involved patients admitted with a diagnosed intracranial aneurysm and qualified for endovascular treatment (embolization). The patients chosen for the study had to meet the following criteria: 1) be diagnosed for intracranial aneurysm and 2) undergo a successful embolization procedure (for the first time) 3) with no fatal complications. The criteria that eliminated a patient from the study included: 1) being diagnosed with more than 1 intracranial aneurysm (consecutive aneurysm) or other vasal anomaly (i.a. arteriovenous angioma), 2) having undergone consecutive and/or unsuccessful embolization or surgery procedure (clipping, wrapping, trapping), 3) fatal complications.

Among the 118 patients there were 73 women and 45 men. The age of the patients ranged from 18 (the youngest) to 82 (the oldest). The average age was 51.4 ± 11.4, with patients of age 50–64 being the most numerous group. All the patients were hospitalized for intracranial aneurysm and all of them underwent an embolization procedure. Seventy-six patients were diagnosed with ruptured aneurysm (with subarachnoid haemorrhage – SAH), and 42 were suffering from non-ruptured aneurysm (i.e. cold). The aneurysm was usually located in the complex of the anterior communicating artery (42 persons) and the internal carotid artery (33 persons). The average time preceding the embolization procedure was 43 h. The largest number of patients (85) underwent embolization up to 24 h after having been admitted to the ward (Table 1).

Instruments

The study involved a two-time assessment. The first assessment (measure 1 – prior to the procedure) was conducted on the first day of patient's hospitalization. The clinical assessment involved the use of the Glasgow Coma Scale (GCS) to measure the patient's level of consciousness, and the Hunt and Hess Scale (H&H) to assess the patient's condition.[10,11] The second assessment (measure 2 – after the procedure) was carried out on the day of the patient's discharge from the ward. The end results and the patient's functional capacity were assessed with the Glasgow Outcome Scale (GOS), Barthel Index (BI), Modified Rankin Scale (mRS), and the proposed Functional Capacity Scale (FCS) (Table 2).[13–17]

Table 2. Condition on the day of discharge (after the procedure) N (%)

FCS	
I	98 (83.1)
II	11 (9.3)
III	4 (3.4)
IV	5 (4.2)
BI	
V	52 (44.1)
IV	41 (34.7)
III	13 (11.0)
II	6 (5.1)
I	6 (5.1)
mRS	
0	52 (44.1)
1	37 (31.4)
2	9 (7.6)
3	8 (6.8)
4	8 (6.8)
5	4 (3.4)
GOS	
5	65 (55.1)
4	34 (28.8)
3	16 (13.6)
2	3 (2.6)
1	0 (0)

Ethical considerations

The study obtained the consent of the Bioethics Commission of the Nicolaus Copernicus University in Toruń at Ludwik Rydygier Collegium Medicum in Bydgoszcz (KB no. 297/2008, no. 291/2013, and no. 564/2014).

Data analysis

The statistical analysis was conducted with the Microsoft Excel programme (licence CM NCU LOGON S.A., Bydgoszcz, PL) and STATISTICA v. 10.0. (licence CM NCU, StatSoft, Kraków, PL). The analysis of conformity of quantitative variables to normal distribution was carried out with the Shapiro-Wilk test. Following the accepted parameters in biomedical sciences, unless stated differently, the results were acknowledged as statistically significant at a value of 0.05. The analysis used the Kruskal-Wallis non-parametric Anova test and a post-hoc test to determine any statistically significant differences between the

assessment of the patient's consciousness level (GCS) and his or her clinical condition (H&H), and the patient's functional capacity after treatment. Moreover, the study involved the use of Spearman's rank correlation coefficient (r_S) to indicate the relationship between its value and the individual tools used to assess the patient's functional capacity (FCS, BI, mRS and GOS).

Results

On the day of the patient's discharge from the ward (Table 2), in accordance with the FCS classification adopted, the largest number of patients was found in group I (98 persons – 83%). This means that the early results regarding the functional capacity in the analysed group are successful. The fact is further confirmed by the results obtained with the standardized functional scales. The BI identified 93 persons as "able" (BI group V and IV). Similarly, the mRS estimated the number of people in good condition to 89 (mRS group 0 and 1). The Glasgow Outcome Scale, on the other hand, qualified 99 persons to group 5 and 4, indicating satisfactory result of treatment.

Furthermore, we analysed the influence of the consciousness level according to the GCS scale and clinical condition in the H&H scale (prior to the procedure) on the patient's functional capacity after receiving endovascual treatment of aneurysm as assessed with the functional scales (FCS, BI, mRS, GOS). The value of test statistics in the analysis of the influence of consciousness level (GCS) on the patient's functional capacity (Table 3) is statistically significant at $p < 0.001$. Statistically significant differences among all the analysed groups in accordance with the FCS, BI, mRS and GOS scales were found, which means that patients with a higher level of consciousness obtained better functional result. Similarly, the patient's clinical condition prior to the procedure assessed with the H&H (Table 4), significantly ($p < 0.001$) differentiated functional capacity after the procedure; thus, the patients in better clinical condition obtained better functional result.

Individual tools for the functional assessment were compared (Table 5). Each case provided a statistically significant value of the Spearman's rank correlation coefficient in the range from 0.68 to -0.88. The highest coefficient in the correlation analysis with the FCS was obtained for the BI (r_S = -0.78), and the lowest for the mRS (r_S = 0.68). A comparison of the remaining scales' correlations showed the highest coefficient (r_S = -0.88) for the GOS and mRS. Thus, there is a relationship between the FCS results and the results obtained with each individual scale (BI, mRS, GOS), which means that they influence one another in a statistically significant ways; hence, they are mutually dependant.

Table 3. Dependency of functional capacity (FCS, BI, mRS, GOS) from the level of consciousness (GCS)

Scale	GCS		
	I (15–13)	II (12–9)	III (8–3)
FCS – test ANOVA rank Kruskal-Wallis H (df = 2, n = 118) = 45.17; p < 0.001			
x ± SD	46.1 ± 2.9	32.6 ± 8.1	21.1 ± 6.4
Me	47	33	19
Range	35–48	16–40	15–31
BI – test ANOVA rank Kruskal-Wallis H (df = 2, n = 118) = 49.92; p < 0.001			
x ± SD	92.8 ± 10.6	47.7 ± 11.7	22 ± 3.9
Me	100	50	20
Range	60–100	20–70	20–30
mRS – test ANOVA rank Kruskal-Wallis H (df = 2, n = 118) = 47.37; p < 0.001			
x ± SD	0.65 ± 0.84	3.18 ± 0.98	4.43 ± 0.53
Me	0	3	4
Range	0–4	2–5	4–5
GOS – test ANOVA rank Kruskal-Wallis H (df = 2, n = 118) = 44.41; p < 0.001			
x ± SD	4.60 ± 0.59	3.36 ± 0.50	2.57 ± 0.53
Me	5	3	3
Range	3–5	3–4	2–3

x – average; SD – standard deviation; Me – median; range - range of points for the scale.

Discussion

The study involved an early assessment of functional capacity in patients with intracranial aneurysm treated by means of endovascular embolization. The authors believe this is the first such study regarding early-term results from the point of view of functional assessment, which is particularly significant for making prognoses on long-term results (e.g. 3-6-12 months after the procedure). Apart from the applied standardized functional scales (BI, mRS, GOS), the results were supplemented with the proposed FCS scale.[17]

The research tools used in the study consisted of the most popular and reliable scales applied in the assessment of patients with aneurysm and SAH. It was confirmed by Al-Khindi et al.[18], who presented a literature review on Cognitive and Functional Outcome After Aneurysmal

Table 4. Dependency of functional capacity (FCS, BI, mRS, GOS) from clinical condition (H&H)

Scale	H&H			
	0	I-II	III	IV-V
FCS – test ANOVA rank Kruskal–Wallis H (df = 3, n = 118) = 60.76; p < 0.001				
x ± SD	47.1 ± 2.2	45.3 ± 3.1	32.6 ± 8.1	21.1 ± 6.4
Me	48	46	33	19
Range	35–48	35–48	16–40	15–31
BI – test ANOVA rank Kruskal–Wallis H (df = 3, n = 118) = 64.39; p < 0.001				
x ± SD	96.4 ± 9.4	90.2 ± 10.6	47.7 ± 11.7	22.1 ± 3.9
Me	100	95	50	20
Range	60–100	70–100	20–70	20–30
mRS – test ANOVA rank Kruskal–Wallis H (df = 3, n = 118) = 60.66; p < 0.001				
x ± SD	0.26 ± 0.54	0.93 ± 0.92	3.18 ± 0.98	4.43 ± 0.53
Me	0	1	3	4
Range	0–2	0–4	2–5	4–5
GOS – test ANOVA rank Kruskal–Wallis H (df = 3, n = 118) = 50.02; p < 0.001				
x ± SD	4.79 ± 0.47	4.47 ± 0.63	3.36 ± 0.50	2.57 ± 0.53
Me	5	5	3	3
Range	3–5	3–5	3–4	2–3

x – average; SD – standard deviation; Me – median; range – range of points for the scale.

Subarachnoid Hemorrhage. In the opinion of the authors, patients after SAH tend to experience memory deficit, as well as a decline in executive and language functions. Cognitive disorders affect everyday functioning, employability and the quality of life.

In most cases the functional assessment is carried out alongside multicentre trials, such as the International Subarachnoid Aneurysm Trial (ISAT)[6–8] and the Barrow Ruptured Aneurysm Trial (BRAT)[9], which feature very long monitoring periods but are predominantly oriented towards comparing the results of intravascular and surgery treatment with respect to mortality and compilations occurrence.

One of the main factors that affects the results of treatment in patients suffering from intracranial aneurysm is their clinical conditions prior to the treatment[19–21]. The majority of the conducted analyses indicate that the clinical condition and the level of consciousness of patients on the day of admission constitute an independent prognostic factor for long-term results of treatment.

The presented material shows a significant relationship between the clinical condition assessed with the use of the H&H and the patient's consciousness level assessed with the GCS on the day of admission, and the functional result of discharge. Both the H&H and GCS scales showed that the more severe condition upon patients' admission translated into worse functional capacity upon their discharge, which was confirmed with three different measuring instruments (mRS, BI and FCS).

The research by Haug et al.[22] showed that 1 in 5 patients admitted to hospital after SAH in coma recover without any marked neurological deficits and cognitive functions disorders. Van Heuven et al.[23], on the other hand, indicated that patients who are admitted to hospital and give no verbal or motor response have a 5% chance to recover and function independently.

Our study showed statistically significant correlations between the applied functional scales, which is confirmed in the research conducted by other authors who

Table 5. Results of Spearman's rank correlation analysis

Scale	FCS	BI	mRS	GOS
FCS	–	r_S = -0.78, p = 0.0003*	r_S = 0.68, p = 0.0001*	r_S = -0.69, p = 0.0001*
BI	r_S = -0.78, p = 0.0003*	–	r_S = -0.83, p = 0.0001*	r_S = 0.83, p = 0.0001*
mRS	r_S = 0.68, p = 0.0001*	r_S = -0.83, p = 0.0001*	–	r_S = -0.88, p = 0.0004*
GOS	r_S = -0.69, p = 0.0001*	r_S = 0.83, p = 0.0001*	r_S = -0.88, p = 0.0004*	–

r_S – correlation coefficient; * – statistically significant.

use various measuring instruments in clinical (Hunt and Hess Scale, World Federation of Neurological Surgeons Scale, Glasgow Coma Scale, Fisher Scale)[7,24,25] and functional assessment (Barthel Index, Rankin Scale, Glasgow Outcome Scale, SF-36)[26,27] carried out both in the early and late period after the treatment of intracranial aneurysm.

Conclusions

The study shows that the FCS correlates with other scales used in the assessment of the condition of patients with intracranial aneurysm, which means that the proposed tool can be used in practice.

The clinical condition and consciousness level of a patient before the procedure constitutes a factor that differentiates his or her functional condition after the procedure.

The FCS should be further subjected to multicentre randomized research in order to provide more detailed conclusions.

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Rheumatoid factor and anti-cyclic citrullinated peptide (anti-CCP) antibodies with hepatitis B and hepatitis C infection: Review

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Abstract

Background. Viruses are common and are involved in the etiology of idiopathic rheumatological diseases. Hepatitis B virus (HBV), a member of the family *Hepadnaviridae* and hepatitis C virus (HCV), play an important role in the undetermined etiology of arthritis. The clinical manifestations of hepatitis B and C show similarities with various diseases, such as rheumatic diseases. Anti-cyclic citrullinated peptide (anti-CCP) is a specific serological marker for rheumatoid arthritis.

Objectives. The aim of this study was to analyze anti-CCP and rheumatoid factor (RF) levels in patients with a hepatitis B and C infection.

Material and methods. Forty-four patients with hepatitis B, 43 patients with hepatitis C, 25 patients with rheumatoid arthritis, and 46 healthy control serums and their RF and anti-CCP levels were compared. RF was measured by the nephelometer, which detects IgM-RF. Anti-CCP was measured using enzyme-linked immunosorbent assay (ELISA) that is included in the second-generation anti-CCP antibody assays (anti-CCP2).

Results. The anti-CCP positivity levels were 20.5%, 32.5%, 72.4% and 10.9% for HBV, HCV and RA groups and healthy control group, respectively. When the groups were compared based on their RF positivity and anti-CCP positivity while the values for HBV and HCV group and healthy control group were the same, in RA group there is a significant difference to the rest of the groups ($p < 0.01$).

Conclusions. Anti-CCP may be positive for HBV and HCV as well, but it is a sensitive and specific immunological marker for RA diagnosis, especially in high-titres.

Key words: rheumatoid arthritis (RA), hepatitis, anti-CCP

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Anti-citrullinated protein antibodies (ACPA) and RF are autoantibodies present in the majority of rheumatoid arthritis patients. Among these, anti-cyclic citrullinated peptide (anti-CCP) antibodies are widely known to be an important diagnostic and prognostic tool due to their high specificity. It has been determined that anti-CCP has a sensitivity level between 69.6% and 77.5% and a specificity between 87.8% and 96.4% in RA diagnosis.[4] anti-CCP and RF are important indicators in RA diagnosis, but they can also be positive for various infections and connective tissue diseases.[5–9] Anti-CCP and rheumatoid factor (RF) may be traced in some viral infections just as in hepatitis B (HBV) and hepatitis C (HCV) infections respectively.[14]

The diagnosis of chronic HBV and HCV infections is based on the HBsAg and anti-HCV positivity persisting for more than 6 months.[2] Certain characteristics of chronic HBV and HCV infections are similar to those of rheumatic and renal diseases. Any of the symptoms of chronic HBV and HCV infections such as arthralgia, peripheral arthritis or laboratory findings like elevated acute phase reactants including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and positivity of RF are important markers for the diagnosis of inflammatory rheumatic diseases.[3] The aim of this study was to evaluate the rate of anti-CCP and RF positivity in hepatitis B and C infection.

Material and methods

The study subjects consisted of 44 patients with hepatitis B (male/female: 18/26), 43 patients with hepatitis C (male/female: 10/33), 25 patients with rheumatoid arthritis (male/female: 9/16) and 46 healthy individuals as the control group (male/female: 18/28). All patients with RA fulfilled the 1987 ACR criteria for RA.[10] The diagnosis of chronic HBV infection is based on the persistence of the hepatitis B surface antigen (HBsAg) for more than 6 months and patients were determined to be carriers of inactive HBV (level of HBV DNA below 2000 IU/mL and serum levels of ALT that remained normal). Chronic hepatitis C infection is defined by the presence of anti-HCV and HCV-RNA positivity for at least 6 months. Serum levels of ALT remained normal in all patients with HCV group. No patient received antiviral therapy in both HBV and HCV groups. No specific musculoskeletal symptoms and findings in all patients carrying chronic phase hepatitis B and chronic hepatitis C have been defined yet. Serum samples were obtained from venous blood, frozen and stored at -80° for future analysis. Anti-CCP and RF were studied on these samples. RF was measured by the Nephelometer, which detects IgM-RF and the normal range was between 0 and 20 IU/mL. Anti-CCP was measured using enzyme-linked immunosorbent (ELISA), which is included in the second generation anti-CCP antibody assays (anti-CCP2) and manufacturer's cut-off for positivity was ≤ 25 U/mL. Anti-CCP levels were classified in titre as low, moderate and high for values 25–50 U/mL, 50–75 U/mL and 75 U/mL respectively. All participants were informed on the study, and the procedures complied with the Declaration of Helsinki and institutional guidelines. The Ethics Committee of Gaziantep University approved the study, and informed consent was obtained from all patients.

As for the statistical analysis, the Statistical Package for Social Sciences (SPSS) was used to analyze the data. A one-way ANOVA test was used for multiple comparisons. In addition, the χ^2 and Fisher's exact tests were used for categorical variables, and the Tukey test was used to compare mean values. P values < 0.05 were considered as statistically significant.

Table 1. Demographic features of all study groups

Patients	Chronic hepatitis B n = 44	Chronic hepatitis C n = 43	Rheumatoid arthritis n = 25	Healthy control n = 46	p-value
Age (years)	34.2 ± 7.4	34.3 ± 6.0	38.4 ± 7.8	33.9 ± 7.2	0.079
Sex (male/female)	18/26	10/33	9/16	18/28	0.068

Table 2. Laboratory findings of study population

Study groups	Levels of RF	RF positivity	Levels of anti-CCP	Anti-CCP positivity
Hepatitis B	14.5 ± 11.0	5 (11.4%)	22.1 ± 23.7	9 (20.5%)
Hepatitis C	16.0 ± 11.0	7 (16.3%)	28.7 ± 24.3	14 (32.5%)
Rheumatoid arthritis	60.4 ± 82.5	15 (60%)	141.1 ± 137.4	18 (72.4%)
Healthy control	7.8 ± 4.1	1 (2.2%)	18.7 ± 4.4	5 (10.9%)
p-value	< 0.001	< 0.001	< 0.001	< 0.001

RF – rheumatoid factor; anti-CCP – anticyclic citrullinated peptid.

Results

The average ages in the study groups for chronic hepatitis B, chronic hepatitis C, RA groups and healthy control group were 34.2 ± 7.4, 34.3 ± 6.0, 38.4 ± 7.8 and 33.9 ± 7.2 respectively. There were no meaningful differences of age or gender in the groups (p = 0.079, p = 0.068 respectively). Demographic characteristics of the patients and healthy controls are presented in Table 1.

Table 3. Comparisons of anti-CCP positivity levels of groups

Anti-CCP positivity	Hepatitis B (%)	Hepatitis C (%)	Rheumatoid arthritis (%)	Healthy (%)	p-value
Low level positivity	11.4	20.9	8.0	10.9	0.373
Moderate level positivity	6.8	9.3	8.4	0	0.248
High level positivity	2.3	2.3	56	0	0.00

anti-CCP – anticyclic citrullinated peptid; anti-CCP 25–50 U/mL low; 50–75 U/mL moderate; above 75 U/mL high titre positivity.

Table 4. Published reports of the prevelance of anti-CCP antibodies in HBV-infected patients

Studies	HBV		RA	
	n	anti-CCP positivity (%, no)	n	anti-CCP positivity (%, no)
Sang-il Lee 2007 *16	176	0.6%, (1)	**	**
Lim MK 2009 *17	240	4.5%, (11)	10	9% (90)
Zhou RF 2012 *22	280	5.7%, (15)	15	13% (86.7)
Our study	44	20.5%, (9)	25	18% (72)

*reference no; **not studied.

HBV and HCV groups and healthy control group have not presented a statistically significant difference between their RF and anti-CCP positivity. However, RA group had higher RF and anti-CCP levels and displayed a striking difference to the rest of the groups ($p < 0.001$ for each). Group laboratory results are presented in Table 2. A comparison of anti-CCP positivity levels of groups (Table 3) presented that hepatitis B, hepatitis C and healthy controls had lower levels of positivity (11.4%, 20.9%, 10.9% respectively). However, RA group showed mostly high levels of anti-CCP positivity (56%).

Discussion

In this study, we have analyzed anti-CCP positivity in asymptomatic chronic hepatitis B and C patients. We determined the anti-CCP positivity to be 20.5% and 32.5% in HBV and HCV patients respectively. However, most of these were low-level positivity.

Autoantibodies are widely used in rheumatoid disease diagnostics. Antinuclear antibody (ANA), RF and anti-CCP are among the most commonly used autoantibodies in clinic practice. RF has been used in RA diagnostics since 1970s and is amongst 1987 ACR criteria and 2010 classification criteria.[36]

According to the literature, RF positivity changes between 17.5–42.7% in HBV patients and between 9.7–54% in HCV patients. A correlation between RF, arthralgia and arthritis has been determined.[16,17,19,20,22,24,25] Our study also resulted in 11.4% RF levels in HBV patients and 16.3% for HCV patients, results not falling far from previous studies.

RF can also be induced in chronic infections (such as osteomyelitis, tuberculosis and subacute bacterial endocarditis) and acute infections besides chronic hepatitis. We had determined in a previous study that Brucellosis patients with arthritis had an RF level of 20%. In addition to those, RF could also be positive in rheumatoid diseases such as Sjögren's Syndrome, systemic lupus erythematosus, cryoglobulinemia and in healthy individuals.[37,38]

Anti-CCP was first used in the 2000s as a RA diagnosis tool and took its place amongst classification criteria in 2010. Previous studies show anti-CCP levels of HBV patients as between 0–13.9% (Table 4), and between 0–33% in HCV patients. Similarly, there has been a correlation detected between arthritis and anti-CCP positivity in these patients. Riccio et al. and Bassyouni et al. have determined 33% and 20%, respectively, of anti-CCP positivity in HCV patients with musculoskeletal symptoms.[19,29] In asymptomatic HCV patients, Liu Feng-Cheng et al. found 5.2% and Orge et al. 4.9% of anti-CCP positivity.[14,19,20,24,25] We determined a 20.5% anti-CCP positivity in HBV and a 32.5% in HCV in our study. Our study showed higher Anti-CCP positivity than previous studies, but significant part of them in low titers (11.4% in HBV, 20.9% in HCV in low titer).

Anti-CCP antibodies can be positive in other infections like tuberculosis (37%) and Lyme disease (2%) besides chronic hepatitis. As much as it is a sensitive and specific marker in RA diagnosis, it still can be found positive in systemic lupus erythematosus (8–17%), Sjögren's Syndrome (3–7.5%), scleroderma (5–10.6%), ulcerative colitis (3%), fibromyalgia (3%) and polymyositis or dermatomyositis (14%).[5,7–9,14,34]

There are some limitations to our study. First of all, none of the patients had symptoms. An evaluation of symptomatic hepatitis patients could have provided more detailed information. Secondly, patients were not observed over a long period of time, so there was no data about long-term consequences in our findings.

In conclusion, we determined that RF and anti-CCP positivity is between 11.4% and 20.5% in HBV patients and between 16.3% and 32.5% in HCV patients. Therefore, chronic hepatitis should be considered a possibility particularly in patients with positive anti-CCP antibodies and low RF titer.

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Filaggrin loss-of-function mutations as a predictor for atopic eczema, allergic sensitization and eczema-associated asthma in Polish children population

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Abstract

Background. Loss-of-function mutations in the filaggrin (FLG) gene were identified as a major risk factor for atopic eczema.

Objectives. The aim of the study was to investigate the importance of 4 common FLG null mutations in the susceptibility to atopic eczema and other allergic phenotypes in Polish children population.

Material and methods. The FLG mutations were determined in 158 children younger than 2 years of age. All subjects were selected using a detailed questionnaire and blood samples for total and specific IgE measurements were obtained. Cases of atopic eczema were diagnosed according to the criteria of Hanifin and Rajka and skin examination. All FLG mutations were genotyped by real-time PCR assays with a subsequent melting curve analysis using a SimpleProbe® probes.

Results. The combined genotype of all 4 mutations (carriage of ≥ 1 FLG mutation) was significantly associated with atopic eczema ($p = 0.016$). The odds ratio (OR) for individuals carrying 1 of these 4 null mutations was 5.52 (95% CI; 1.11 ÷ 37.12). The significant association between either the combined FLG genotype or 2282del14 deletion and eczema was seen only in the allergic group. The association with asthma was restricted to asthma occurring in the context of eczema (OR, 6.27; 95% CI, 0.89 ÷ 53.56; $p = 0.042$).

Conclusions. Our study confirms the previous findings that FLG mutations are strongly associated with atopic eczema and confer a significant risk of allergic sensitization and asthma in the context of eczema. These results underline the role of the epidermal barrier and filaggrin insufficiency in the pathogenesis of atopic eczema and eczema-associated asthma.

Key words: asthma, atopic dermatitis, atopic eczema, genotype, mutation

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Atopic eczema (or atopic dermatitis) is a chronic inflammatory skin disease with the onset typically occurring in early childhood and is the most common chronic inflammatory skin disease affecting children in the industrialized country. The worldwide cumulative prevalence of atopic eczema is approx. 10–20% in this age group with a steady increase over the past decades. Around 50% of the cases are diagnosed by the age of 1 year, with 1/3 of patients having disease persists throughout adulthood.[1] A multifactorial background for atopic eczema has been suggested with genetic as well as well environmental factors influencing disease development. Atopic eczema is associated with a number of abnormalities in skin barrier function owing to the mutations in cornified envelope genes, reduced ceramide levels, increased levels of endogenous proteolytic enzymes and enhanced transepidermal water loss.[2] Several recent studies have shown that the loss-of-function mutations of the epidermal barrier protein filaggrin are a major predisposing factor for atopic eczema.[3–5] The filaggrin gene is found on chromosome 1q21 which also contains genes of the epidermal differentiation complex that are expressed during the terminal differentiation of the epidermis.[6] Filaggrin deficiency has been shown experimentally to lead to the failure of the barrier function of the skin in humans.[7] An impressive series of replication studies that have followed the initial publication from Palmmer et al.[3] showed FLG to be the gene with the most widely replicated association with atopic eczema.[4,8,9] An association with asthma was also suggested and confirmed in subsequent reports on asthma occurring in association with eczema.[3,8–13] However, these studies exhibited considerable heterogeneity concerning the study design and magnitude of the genetic effect.

In the current study, we aimed to assess the importance of filaggrin loss-of-function mutations in the susceptibility to atopic eczema and disease severity in Polish children younger than 2 years of age. Moreover, we evaluated the association of the FLG mutations with other atopic phenotypes, such as eczema-associated asthma and allergic sensitization.

Material and methods

Study population

A total of 158 unrelated children (94 males) younger than 2 years at the time of recruitment were enrolled and followed at yearly intervals thereafter until age 4 years. The study subjects were recruited from the general population. All study participants were of Caucasian ethnicity. All subjects were selected using a detailed questionnaire that included questions on symptoms of eczema and asthma, sociodemographic information and family history of allergic diseases. The diagnosis of eczema was made according to the criteria of Hanifin and Rajka and visible symptoms of disease at the time of recruitment and follow-up.[14] Disease severity was assessed by using the SCORing Atopic Dermatitis index (SCORAD) and the patients were divided into mild (< 15 points), moderate (15–40 points) or sever (> 40 points) disease groups. The subjects with eczema were divided into atopic and non-atopic on the basis of the presence of allergic sensitization. Asthma was defined by the presence of 1 or more wheezing episodes during the previous 12 months at the age 3 or/and 4 years or a physician's diagnosis of asthma by 4 years of age. The control subjects met the following criteria: absence of symptoms of atopic eczema and asthma and negative family history of allergic diseases.

In all recruited subjects, serum measurements for total and specific IgE levels were performed, including IgE specific for inhalant mix *(Dermatophagoides pteronyssinus, Dermatophagoides farina,* cat, dog and horse epithelia, birch pollen, Timothy grass pollen, mugwort pollen, *Aspergillus fumigatus, Cladosporium herbarum*) and food mix (peanut, milk, egg white, egg yolk, potato, carrot, cod, apple, soya, wheat flour). The concentration of total serum IgE was measured by using a commercially available kit IMMULITE 2000 Total IgE (Diagnostic Products Corporation (DPC), USA). The levels of specific IgE were determined using a standard enzyme immunoassay (Polycheck, BIOCHECH, Germany). Allergic sensitization was defined as the presence of specific IgE level of ≥ 0.7 kU/L (class II) or greater to at least 1 tested allergen.

The study was approved by the ethics committee, and an informed written consent, including consent for genetic studies, was obtained from all of the subjects before testing.

Genotyping

The samples of the 158 subjects were genotyped for the FLG mutations R501X, 2282del4, R2447X and S3247X. Children with a mutation in any of these positions were classified as a loss-of-function mutation. Genomic DNA was obtained from EDTA whole blood samples using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Germany). All mutations were determined using LightSNiP assay (TibMolbiol, Berlin, Germany). PCR was performed in a final volume of 10 µL containing 1 µL of DNA at a concentration of 15–60 ng/µL, 0.5 µL of reagent mix containing specific primers and SimpleProbes® probes at optimized concentrations, 0.8 µL of MgCl2 and 1 µL of LightCycler® FastStart DNA MasterHybProbe (Roche Applied Science, Mannheim, Germany). Reactions were performed on a Light Cycler 1.5 platform (Roche Applied Science, Mannheim, Germany). For quality control of genotyping procedures, positive controls of each genotype, as well as negative controls, were included in each reaction.

Statistical analysis methods

The Hardy-Weinberg equilibrium was tested using the χ^2 goodness-of-fit test to compare the observed genotype frequencies with the expected frequencies among the controls. Differences in genotype frequencies or demographic characteristics between case and control groups were evaluated using the χ^2 test or the Fisher exact test as appropriate. The associations of genotypes or alleles with patient groups vs control subjects were determined by computing the odds ratio (OR), its 95% confidence interval (95%CI) and p-values using the logistic regression analysis for crude ORs. The predictive value of risk factors was assessed through analyzing the sensitivity, specificity, and the positive and negative predictive values. Risk factor's interaction was investigated by using logistic regression models for atopic eczema and asthma with interaction terms (SPSS). Statistical significance was set at a p value < 0.05. The statistical analyses were carried out using the program package STATISTICA v. 9.0 (StatSoft, Inc., Tulsa, USA) and the SPSS Statistics software package v. 11.1 (SPSS Inc., Chicago, USA).

Table 1. Characteristics of the study group. Polisensitization: The presence of specific IgE level of ≥ 0,7 kU/L (class II) or greater to more than 1 tested allergen

Variable	Eczema n = 87	Control n = 71
Age: month (mean ± SD)	13.2 ± 6.7	15.3 ± 5.6
Gender (male/female)	56/31	38/33
Allergic sensitization (%)	42 (48.3%)	8 (11.3%)
Asthma (%)	26 (29.9%)	0
SCORAD mild moderate sever	 54 (62%) 33 (38%) 0	 0 0 0
Polisensitization (%)	23 (26.4%)	0
Atopic hereditary (%)	49 (56%)	0
Serum total IgE IU/mL geometric mean 95%CI	26.68 (12.72 ÷ 40.63)	14.99 (11.15 ÷ 18.83)

Table2. Frequencies of FLG mutations and the combined FLG genotype in eczema cases and controls

Genotype	Control n(%)	Eczema		
		n(%)	p-value	OR (95% CI)
R501X normal null	 70 (98.6%) 1 (1.4%)	 86 (98.8%) 1 (1.2%)	p = 0.888	1.22 (0.03 ÷ 45.8)
2282del14 normal null	 70 (98.6%) 1 (1.4%)	 80 (92.0%) 7 (8.0%)	p = 0.058	6.12 (0.72 ÷ 135.7)
R2447X normal null	 71 (100%) 0	 86 (98.8%) 1 (1.2%)	p = 0.365	–
S3247X normal null	 71 (100%) 0	 84 (96.6%) 3 (3.4%)	p = 0.114	–
Combined FLG genotype normal null	 69 (97.2%) 2 (2.8 %)	 75 (86.2%) 12 (13.8%)	p = 0.016	5.52 (1.11 ÷ 37.12)

Table 3. Associations between FLG mutation 2282del14 and the combined FLG genotype and eczema severity

Genotype	Mild eczema n (%)	Moderate eczema n(%)	p-value	OR (95% CI)
2282del14 normal null	 49 (92.5%) 4 (7.5%)	 30 (90.9%) 3 (9.1%)	p = 0.779	1.22 (0.20 ÷ 7.31)
Combined FLG genotype normal null	 46 (85.2%) 8 (14.8%)	 29 (87.9%) 4 (12.1%)	p = 0.724	1.26 (0.30 ÷ 5.54)

Results

Baseline characteristics of patients with eczema and controls are given in Table 1. There were no significant differences between the cases and the controls in regards to age and gender. The allele and genotype distributions for the FLG mutationsR501X, 2282del14, R2447X and S3247X in the cases and controls are shown in Table 2. The genotype frequencies of all 4 investigated mutations were in agreement with the Hardy-Weinberg equilibrium in both groups.

An analysis of the combination of all 4 tested FLG mutations revealed a significant association with susceptibility to eczema (p = 0.016). In other words, an individual carrying one of these 4 mutations has an approx. 5 times greater chance of having eczema when compared with an individual who does not carry any of these variants. When analyzing distribution of individual variants an association between 2282del14 deletion and eczema was seen, although the statistical significance of this association was borderline. The common R501X variants were not significantly associated with eczema when analyzed individually. Neither R2447X nor S3247X were associated with susceptibility to eczema (Table 2).

Furthermore, there were no significant associations between eczema severity and either the 2282del14 deletion or the combined FLG variants. In our study there were no patients with severe

Table 4. Associations between the combined FLG genotype and serum total IgE

Genotype	Control			Eczema		
	n (%)	serum total IgE IU/mL, geometric mean, 95%CI	student's t-test	n (%)	serum total IgE IU/mL, geometric mean, 95%CI	student's t-test
Combined FLG genotype normal null	69 (97.2) 2 (2.8)	15.30 (11.37 ÷ 19.23) 7.39 (1.64 ÷ 13.14)	t = 0.666 p = 0.507	75 (86.2) 12 (13.8)	28.97 (24.47 ÷ 34.31) 40.28 (22.94 ÷ 70.74)	t = 16.59 p = 0.0001

Table 5. Associations between the combined FLG genotype and allergic sensitization and eczema plus asthma

Genotype	Total n (%)	Combined FLG genotype status			
		FLG normal (%)	FLG null (%)	p-value	OR (95% CI)
Allergic sensitization	50/158 (31.6 %)	40/144 (27.7%)	10/14 (71.4%)	p = 0.002	6.50 (1.73 ÷ 26.33)
Polisensitization (Sepc. IgE > 1)	23/158 (14.5%)	16/144 (11.1%)	7/14 (50.0%)	p= 0.001	8.00 (2.16 ÷ 29.98)
Allergic sensitization in subjects with eczema	42/87 (48.3%)	32/75 (42.6%)	10/12 (83.3%)	p = 0.009	6.71 (1.24 ÷ 47.94)
Allergic sensitization in subjects without eczema	8/71 (11.3%)	8/69 (11.6%)	0/2 (0%)	p = 1.000	–
Eczema plus asthma	26/158 (16.5%)	22/144 (15.3%)	4/14 (28.6%)	p = 0.042	6.27 (0.89 ÷ 53.56)
Eczema without asthma	61/158 (38.6%)	53/144 (36.8%)	8/14 (57.1%)	p = 0.044	5.20 (0.96 ÷ 37.17)
Eczema plus asthma vs eczema without asthma	42/87 (48.3%)	22/75 (29.3%)	4/12 (33.3%)	p = 0.746	1.20 (0.27 ÷ 5.08)

Table 6. Associations between FLG mutation 2282del14 and the combined FLG genotype and atopic and non-atopic eczema

Genotype	Control n(%)	Atopic eczema			Non-atopic eczema		
		n (%)	p-value	OR (95% CI)	n (%)	p-value	OR (95% CI)
2282del14 normal null	70 (98.6%) 1 (1.4%)	45 (90.0%) 5 (10.0%)	p = 0.030	7.78 (0.83 ÷ 181.9)	35 (94.6%) 2 (5.4%)	p = 0.230	4.00 (0.27 ÷ 115.7)
Combined FLG genotype normal null	69 (97.2%) 2 (2.8%)	40 (80.0%) 10 (20.0%)	p = 0.002	8.62 (1.64 ÷ 60.2)	35 (94.6%) 2 (5.4%)	p = 0.605	1.97 (0.18 ÷ 20.6)

Table 7. Prevalence and predictive values of risk factors (FLG, allergic sensitization) for eczema

Predictor	True-positive	False-positive	True-negative	False-negative	Sensitivity % (95% CI)	Specificity % (95% CI)	Positive predicitve value (PPV) % (95% CI)	Negative predictive value (NPV) % (95% CI)
FLG mutation	12	2	69	75	13.7 (7.3 ÷ 22.8)	97.1 (90.1 ÷ 99.5)	85.7 (57.1 ÷ 97.8)	47.9 (39.5 ÷ 56.3)
Allergic sensitization	42	8	63	45	48.2 (37.4 ÷ 59.2)	88.7 (78.9 ÷ 94.9)	84.0 (70.9 ÷ 92.8)	58.3 (48.4 ÷ 67.7)
FLG muatation and allergic sensitization	10	0	60	43	18.8 (9.45 ÷ 31.9)	100.0 (93.9 ÷ 100.0)	100.0 (68.9 ÷ 100.0)	58.2 (48.1 ÷ 67.9)

Table 8. Prevalence and predictive values of risk factors (FLG, allergic sensitization) for eczema plus asthma phenotype

Predictor	True-positive	False-positive	True-negative	False-negative	Sensitivity % (95% CI)	Specificity % (95% CI)	Positive predicitve value (PPV) % (95% CI)	Negative predictive value (NPV) % (95% CI)
FLG mutation	4	2	69	22	15.4 (4.4 ÷ 34.8)	97.2 (90.1 ÷ 99.5)	66.6 (22.7 ÷ 94.7)	75.8 (65.7 ÷ 84.2)
Allergic sensitization	15	9	62	11	57.7 (36.9 ÷ 99.6)	88.3 (77.3 ÷ 94.0)	62.5 (40.6 ÷ 81.2)	84.9 (74.6 ÷ 92.2)
FLG muatation and allergic sensitization	3	0	60	10	23.1 (5.3 ÷ 53.8)	100.0 (93.9 ÷ 100.0)	100.0 (30.4 ÷ 100.0)	85.7 (75.3 ÷ 92.9)

Table 9. Interaction between FLG mutation and allergic sensitization in eczema plus asthma phenotype

Risk factors	Eczma plus asthma n(%)	Control n(%)	p-value	OR (95% CI)	RR (95% CI)
FLG mutation (-) Allergic sensitization (-)	10 (38.5%)	60 (84.5%)	Ref.	1.00	1.00
FLG mutation (-) Allergic sensitization (+)	12 (46.2%)	9 (12.7%)	p < 0.001	8.00 (2.4 ÷ 27.9)	4.00 (1.6 ÷ 8.0)
FLG mutation (+) Allergic sensitization (-)	1 (3.8%)	2 (2.8%)	p = 0.392	3.00 (0.09 ÷ 49.6)	2.33 (0.11 ÷ 7.26)
FLG mutation (+) Allergic sensitization (+)	3 (11.5%)	0 (0%)	p < 0.001	–	7.00 (1.86 ÷ 7.00)

eczema and no significant differences between the patients with mild and moderate eczema could be observed in genotype frequencies for either 2282del14 mutation or the combined FLG mutations (Table 3). However, patients who carried at least one risk of FLG mutation alleles displayed a trend of disease onset at an earlier age compared with wild-type genotype (mean age 2.5 and 4.7 months, respectively; p = 0.045).

We also assessed the association between FLG mutations and eczema-associated phenotypes including elevated total IgE level and allergic sensitization. We found a significant difference in total serum IgE levels among genotypes in subjects with eczema (p = 0.0001), whereas such a difference was not present in healthy control (p = 0.507) (Table 4). We observed a highly significant association of the combined FLG null mutations with allergic sensitization and allergic sensitization with eczema. The association with allergic sensitization was stronger for multiply sensitized children. In contrast, this association was not evident in the absence of eczema (Table 5).

When the atopic and non-atopic eczema groups were analyzed separately, the atopic eczema patients had at least one risk of FLG mutations alleles significantly more frequently than the control group. We noted that the frequency of at least one risk of FLG mutations alleles was significantly increased only in atopic eczema patients when compared to controls (p = 0.002; OR 8.62 (95% CI 1.64, 60.2). No significant differences between the patients with non-atopic eczema and the healthy controls could be observed. The non-atopic and atopic eczema groups differ in term of the frequency of the combinations of these FLG mutations, although the statistical significance of this association was borderline (p = 0.05; OR 4.37 (95% CI 0.81, 31.15). Concerning the 2282del14 mutation, a similar trend was observed; children who were carriers of the 2282del14 deletion had an almost 8-fold higher risk of having atopic eczema when compared to controls. No significant association was seen in the case of the non-atopic eczema group. The non-atopic and atopic eczema groups did not differ in term of frequency of this mutation (p = 0.607; OR 1.94 (95% CI 0.30, 15.50) (Table 6).

We next evaluated the association between the combined FLG mutations and the presence of eczema and asthma combination. This analysis revealed that the combined FLG mutations predispose patients to eczema plus asthma, increasing the risk of this complex phenotype more than 6-fold (p = 0.043; OR 6.27 (95% CI 0.89, 53.56). Independent of asthma, FLG mutations conferred a substantial risk for atopic eczema. In contrast, when we evaluated the FLG mutations as a risk factor for asthma in the subgroup of children with eczema we couldn't find any correlation with the FLG mutations as a significant, independent risk factor of asthma. Additional analysis revealed a significant effect of allergic sensitization on the complex phenotype eczema plus asthma susceptibility (p = 0.002; OR 3.93 (95% CI 1.52, 10.26) (Table 5).

Further analyses showed that the FLG mutations are a stronger predictor for the development of eczema and eczema plus asthma phenotype with a positive predictive values 85.71% and 66.67%, respectively. Similarly, allergic sensitization was associated with the very high positive predictive values for eczema and eczema plus asthma, 84.0% and 62.5%, respectively. Nonetheless, the presence of both risk factors, FLG mutations and allergic sensitization, provided the best combination of diagnostic specificity (100%) and predicted either eczema or eczema plus asthma phenotype with a positive predictive value of 100% (Table 7 and 8).

Next, we examined the possible interactive effect of FLG mutations and allergic sensitization in relation to complex phenotype eczema plus asthma. We observed that allergic sensitization was associated with 4-fold higher risk for eczema plus asthma phenotype. In contrast, FLG mutations had no additional effect on this phenotype susceptibility in the absence of allergic sensitization. However, the strongest effect was revealed in subjects who carried both risk factors; 7-fold higher risk of having eczema and asthma combination was seen in this high-risk group. As shown in Table 9, relative risks (RRs), odds ratios (ORs) and p values for eczema plus asthma suggested the presence of an interaction, although when modeling interactions, the interaction coefficient was not significant for both risk factors combination (p for interaction > 0.05).

Discussion

This study is, to our knowledge, the first to investigate the association between the FLG mutations R501X, 2282del14, R2447X and S3247X and susceptibility to and severity of eczema in Polish children younger than 2 years of age. Although the potential role of FLG mutations as a major predisposing factor for atopic eczema has received considerable attention in recent years, to date there were only two studies investigating FLG mutations R501X, 2282del4 in Polish adults and older children.[15,16] In our results, we found a significant association between the combined genotype of all 4 FLG mutations and the susceptibility to eczema. The frequency of 2282del14 deletion showed a similar trend, although the statistical significance of this association was borderline. These findings suggest that FLG mutations may contribute to the genetic susceptibility to eczema in Polish children population. However, the frequency of all tested FLG mutations in our population was rather low. In the analysis, heterozygous and homozygous FLG mutation carriers were grouped in one category, as it has been used in the majority of studies. The rationale for statistical analysis using "combined null genotype" is the equivalent effect of FLG null mutations. It has been shown biochemically, both in mice and humans, that distal FLG null mutations are equivalent in term of preventing profilaggrin-to-filaggrin processing and result in a similar reduction or complete absence of filaggrin as well as a similar phenotype and with statistically similar effect.[17,18] Our results are in agreement with previous studies conducted on the Polish adult and older children population[15,16] and confirm the previous findings that FLG mutations are strongly associated with atopic eczema.[3] This highly significant association has been replicated in more than 30 independent studies.[4] Two recent meta-analyses of this data have estimated the odds ratio (OR) of having atopic dermatitis in association with FLG-null genotype to 4.78 and 3.12.[8,9] To date, there have been only 2 published studies that did not shown an association between FLG null mutations and atopic eczema.[19,20]

Our observation that eczema patients carrying FLG mutation were younger at the onset of eczema than those without FLG mutations are in agreement with previous studies indicating that FLG mutations have the effect on the age of onset of atopic eczema and can lead to early-onset that persists well into adulthood.[21,22] Further observations will be helpful in determining the effect of FLG mutations on the persistence of disease in our population.

Unexpectedly, we are not able to find an association between FLG mutations and eczema severity. An explanation for the lack of this association could be the study design using a general population. Previous studies showing a positive association between FLG null mutations and atopic eczema have focused on moderate-or-severe eczema cases recruited from specialist clinics and on children with eczema as part of atopy-related birth cohort studies.[3,10,18,20,22,23] It is possible that an association existed in Polish children with moderate-severe atopic eczema but unfortunately most of our participants have mild (62%) or moderate (38%) eczema. On the other hand, few studies reported that there was no association between FLG mutations and the severity of atopic eczema like our results.[24,25]

Previous studies have shown that FLG gene mutations increase the risk for atopic eczema, but the data for non-atopic eczema has been scarce.[8,10] Therefore, in the present study we investigated the association between FLG mutations and allergic sensitization showing that FLG alleles conferred an increased risk for allergic sensitization, which was strongest in children with eczema. We assessed also the distribution of the FLG gene variants in children with atopic eczema and non-atopic eczema. While the two groups did not differ significantly in case of 2282del14 deletion and the differences was borderline for combined FLG mutations, we demonstrated a positive and significant association between either the combined FLG genotype or 2282del14 deletion and eczema only in allergic group. Our results are consistent with studies in other populations showing that FLG null mutations increased the risk of allergic sensitization.[8,10,12,13,21,26]

Previously established associations between FLG mutations and susceptibility to eczema-associated asthma has been also confirmed in our study. The compound phenotype of asthma in the context of eczema has shown a striking association with FLG null mutations. That is, the combined FLG mutations conferred an approximately 6–fold increased risk for eczema plus asthma phenotype in our population. This is consistent with studies in the European populations, which have reported that variants in the FLG gene are associated with eczema and concomitant asthma.[9–13,26]

It is worth mentioning that either allergic sensitization or asthma was associated with FLG mutations only in the context of eczema. These results suggest that the presence of an FLG loss-of-function mutation might predispose people to the sequential development of these allergic phenotypes instead of acting as an independent risk factor. Thus, the previous onset of eczema appears to be required for subsequent in the context of "the atopic march".[10,12] These findings support the theory that FLG mutations lead to a functional epidermal barrier defect and subsequent allergic sensitization. The mechanism leading from skin barrier dysfunction to allergic sensitization may be increased skin permeability to allergens. Recent animal studies support this mechanism by showing an increased uptake of intact allergen through the skin in FLG-deficient mice and resulting increased IgE sensitization and skin inflammation.[5,27,28] The pathway from FLG defect to airway disease is not yet understood,

as FLG is not expressed in the respiratory epithelium of the nose or the lower airways.[29] It is possible that FLG mutations drive allergic disease at distant mucosal site as a systemic, possibly immunological, response to enhanced penetration by antigens through the impaired skin barrier.[28] Although this molecular pathway is not fully elucidated, former experimental study implicated the Th_{17} response as a key player between epicutaneous antigen exposure and subsequent airway inflammation.[30]

An association between FLG variants and asthma limited only to asthma with the co-expression of eczema, replicating earlier reports and emphasizing the notion that the FLG deficiency might be a predisposing factor to the particular asthma phenotype occurring in the context of eczema in contrast to the form of asthma not linked with eczema. It is conceivable that this distinct endophenotype of both asthma and eczema is initiated by impaired skin barrier function and thus has a unique pathogenic and prognostic implication.[9,31]

Additionally, our analysis revealed that FLG mutations are not an independent risk factor for asthma in children with eczema. The FLG mutations were a strong predictor for eczema-associated asthma, with high positive predictive values of 66.7%. However, we can see that the combination of FLG mutations and allergic sensitization increases significantly the probability of eczema-associated asthma. In our population 100% of children with at least one FLG mutation and allergic sensitization had developed eczema-associated asthma up to the age of 4 years. Similarly, our analysis of interactive effect of FLG mutations and allergic sensitization with respect to eczema plus asthma phenotype revealed that children who carried both risk factors had a significantly higher risk of eczema-associated asthma suggesting some possible synergistic interactions. These findings are in accordance with a study conducted in the German Multicentre Allergy Study (MAS) that found a strong synergistic interaction between the FLG-null alleles and early food sensitization in the disease transition from eczema to asthma.[11]

A potential limitation of our study, typical for all case-control studies, is a relatively small sample size and, as a consequence, rather low statistical power, which may lead to false negative or fortuitous false positive results. Secondly, an interaction analysis requires a large population; hence, our results should be interpreted carefully. Additionally, rather than fully sequencing FLG to identify target mutations, 4 known mutations were examined. Therefore, the effect of other mutations on the development of or severity of atopic dermatitis and eczema-associated asthma could not be determined.

In conclusion, we provide a significant confirmation of the previously reported association of the FLG mutations with eczema and eczema-associated asthma. Furthermore, we demonstrate that these mutations predispose people to the atopic form of eczema. In spite of the significant effect demonstrated in our study, FLG null mutations can only explain a small proportion of the total burden of childhood atopic eczema. Twelve (13.8%) of 87 atopic eczema cases in our population carry heterozygous genotype for at least one FLG mutations and thus can be attributed to FLG deficiency. The residual 86.2% of cases remain to be explained by other genetic and environmental factors. However, FLG is the single most significant genetic factor in atopic eczema that has been identified to date, and our findings support the role of the epidermal barrier and filaggrin insufficiency in the pathogenesis of atopic eczema, allergic sensitization and eczema-associated asthma.

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Differences in echocardiography, blood pressure, stroke volume, maximal power and profile of genes related to cardiac hypertrophy in elite road cyclists

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Abstract

Background. Regular and moderate exercise is beneficial for improving the efficiency of the heart, but high-intensity physical activity may result in cardiac changes.

Objectives. This study focuses on the identification of the differences in echocardiography and blood variables before exercise, as well as the genes associated with cardiac hypertrophy at rest and in response to graded exercise test.

Material and methods. The study group was made up of 28 road cyclists. Echocardiographic parameters and blood pressure were measured before exercise tests (N = 28). Blood samples were collected at rest, at maximal exercise intensity in a graded bicycle test and after 15 min of recovery; afterwards, blood morphology was estimated and RNA was isolated. Analysis of the expression profile of genes was performed for randomly selected road cyclists using the microarray method.

Results. Echocardiographic results and blood parameters divided cyclists into two groups: with and without left ventricular hypertrophy (N = 14). Differences in the structure and function of the left ventricle cyclists with a similar level of training were observed ($p < 0.05$). Diastolic blood pressure and resting heart rate were significantly lower in subjects with left ventricular hypertrophy ($p < 0.05$). The myosin light chain 9 and interleukin-6 signal transducer gene expression were differentially regulated in cyclists with left ventricular hypertrophy compared to athletes with normal heart dimensions in response to intensive exercise.

Conclusions. We have found differences in echocardiography parameters, blood pressure, stroke volume and maximal power in the cyclists examined. These studies indicate the benefits of the recommended echocardiography measurements for professional endurance-athletes. The graded exercise altered the myosin light chain 9 and interleukin-6 signal transducer gene expression in the peripheral blood of road cyclists has also been found.

Key words: gene expression, exercise, echocardiography, left ventricular hypertrophy, road cyclists

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It is well-known that regular and moderate exercise is beneficial for improving the efficiency of the heart. Among others benefits, it reduces the risk of atherosclerosis and improves exercise tolerance in patients with coronary pain and heart failure.[1] High-intensity physical activity may result in cardiac dysfunction and increased incidents of sudden death in athletes. These observations can be found in studies demonstrating that extreme physical load can lead to the transient reduction of the left ventricular function and increases the incidence of arrhythmogenic right ventricular cardiomyopathy.[2,3] That is why the world's sporting and cardiology organizations recommend that medical examinations be performed on competitive endurance athletes for the early detection of possible pathologies.[4,5] Strenuous exercise, such as road cycling, may result in compensatory myocardial hypertrophy, commonly known as athlete's heart.[6] The main morphologic characteristics are left ventricular hypertrophy (LVH), an increase in the coronary reserve and improvement of left ventricular myocardial contractility.[6,7] The primary stimulus for cardiac hypertrophy is mechanical stress, which induces a growth response in the overloaded myocardium.[7] The possible mechanisms of cardiac hypertrophy remain controversial. Several researchers have highlighted the importance of hormonal regulation or an adrenergic nervous system or other factors.[8,9] It has also been suggested that mechanical loading regulates intracellular signals for the gene expression associated with cardiac hypertrophy without participation of humoral or neural factors. Although alterations in the expression of genes implicated in cardiac hypertrophy have been extensively studied in experimental models[10] and clinical studies[1,11] little is known regarding the signaling mechanisms that lead to the gene expression in the peripheral blood of trained cyclists. Literature reviews indicate that all structural and functional changes, which represent physiological adaptation to regular training, increase the functional capacity of the cardiovascular system at rest and during exercise.[9–11] It should be taken into consideration that several mechanisms may be involved in exercise-related cardiac fatigue, including changes in preload conditions, myocardial stunning, β_1-receptor desensitization, the impaired release of cardiac biomarkers and altered autonomic regulation.[9–12] Intriguingly, a remarkably different gene expression pattern was noted between adaptive and maladaptive cardiac hypertrophy.[13,14] Little is known regarding the gene expression which is considered to be related to left ventricular hypertrophy in endurance-trained athletes. Moreover, it has not been clearly defined whether the genes' expression changes in response to maximal physical exertion and/or post-exercise recovery.

Therefore, the goal of this study was to investigate the differences in echocardiographic and other variables before exercise, as well as in the expression profile of genes associated with cardiac hypertrophy at rest and in response to maximal physical exertion and post-exercise recovery in professional road cyclists.

Material and methods

Subjects and study protocol

Twenty-eight male Polish elite road cyclists gave their informed written consent to participate in this study. All subjects were competitive cyclists with the same training status of LVH and non-LVH group (9.9 ± 5.1 and 9.3 ± 4.3 years, respectively). The training status of the subjects is expressed as maximal oxygen consumption (VO_{2max}), the highest value was 71 mL/kg/min, and the mean individual monthly training volume was 655 ± 53 km. They participated in the study during the pre-season period and underwent medical evaluations including clinical history, physical examinations, echocardiography and blood parameters. The study was approved by the local ethics committee and conformed to the standard set by the declaration of Helsinki.

Pre- and post-exercise data collections were carried out in an exercise physiology laboratory in the morning hours between 08.00 and 11.00. None of the subjects had a history of cardiovascular, pulmonary or metabolic diseases. Three days prior to the study all participants were put on a mixed, isocaloric diet (2899 ± 1100 kcal/day) consisting of carbohydrates, proteins and fats. The athletes were asked to refrain from food, juices and caffeine for at least 8 hours and avoid strenuous exercise for 24 hours prior to testing.

Echocardiographic data

All examinations were performed using the Hewlett-Packard Image Point HX ultrasound system with standard imaging transducers. All analyses were made and/or supervised by the same cardiologist. Left ventricular end-diastolic dimensions (LVEDD) were measured at the onset of the QRS complex. LV volumes were derived according to the modified Simpson's method. The left ventricular ejection fraction (LVEF) was calculated in the standard fashion from LV end-diastolic and end–systolic volume.

The intra-observer coefficients of variation were 2.5% for the measurements of LV diameters, and 3.6% for measurements of LVEF. The left ventricular systolic function was determined by measurements of LVEF and stroke volume (SV). LV muscle mass (LVM) was derived according to Devereux.[15] LV mass = 1.04 [(LVEDD + IVSDD + LVPWT) – (LVEDD)], where LVEDD was the left ventricular end-diastolic internal diameter, IVSDD was the intraventricular septum thickness during diastole, and LVPWT was the left ventricular posterior wall thickness.[3] Left ventricular mass was indexed to body surface

area (BSA). Left ventricular hypertrophy was identified when left ventricular mass index (LVM/BSA) was greater than 115 g/m^2 in men.[4]

Maximal graded exercise test

Before each subject performed a maximal graded exercise test using a cycle ergometer (F-Lode Excalibur, Groningen, Netherlands), blood pressure and heart rate were measured with a manual sphygmomanometer. The exercise started with unloaded cycling for 5 min, and then increased by 40 W every 3 min, up to the maximal exercise intensity. The maximal oxygen uptake (VO_{2max}) was achieved if two of the following criteria occurred: a plateau in oxygen uptake (VO_2) with increasing work rate; respiratory quotient greater than 1.15; a heart rate (HR) within 10 beats of age predicted maximum (220 – age); or volitional fatigue. Oxygen uptake was measured continuously from the 6th minute prior to exercise and throughout each stage of the exercise protocol using the Oxycon Apparatus (Jaeger, Germany).

Biochemical data

Following the exercise testing procedures, blood samples were taken using a peripheral catheter inserted into the antecubital vein. Venous blood samples were collected in a sitting position: 15 min before exercise (T_{rest}), at maximal exercise intensity (T_{max}), and after 15 min of recovery (T_{15}). Morphology variables in the whole blood were measured with the ABX MICROS 60 (HORIBA ABX SAS) analyzer, using standard methods and reagents.

Microarray analysis

Immediately after the whole blood sample collection, leukocytes were separated using the standard method. Total RNA was isolated from leukocytes according to the manufacturer's protocol with a ready-to-use TRIZOL® Reagent (Invitrogen Life Technologies, USA). The obtained RNA was purified using RNeasy Mini kit columns with DNase I from Qiagen (Germany). The amount of the obtained RNA and its purity was checked using a GeneQuant II spectrophotometric calculator (Pharmacia Biotech, UK), at wavelengths of l 230/260/280/320 nm. To check the specificity of gene expression changes in cyclists with left ventricular hypertrophy, the microarray data from randomly selected cyclists in both groups was analyzed. The isolated RNA samples were used for cDNA and biotin-labeled cRNA synthesis, which was then fragmented and hybridized with Affymetrix Human Genome U133A expression microarray in accordance with the Gene Expression Analysis Technical Manual (Affymetrix, USA). Fluorescence signals (FS) of the probe set were scanned by the GeneArray Scanner 3000 7G (Affymetrix, USA).

Statistical analysis

The obtained results were analyzed using the Statistica for Windows, v. 12 (StatSoft Inc.). The Shapiro-Wilks W test was used to confirm normal distribution. Values are presented as mean ± standard deviation (SD). Student's t-test was used for comparison between groups (LVH and non-LVH). Using the Affymetrix NetAffx database, 56 mRNAs were selected, which corresponded to cardiac hypertrophy-related genes. The obtained fluorescence signals (FS) were normalized by the RMA Express ($\log_2$ FS) using Matlab software. ANOVA with the Tukey post hoc tests were used to analyze changes in *MYL9* and *IL6ST* over time (T_{rest}, T_{max}, T_{15}). According to the standard method for analyzing the microarray data of gene expression, the results were calculated as a ratio of the normalized FS for the LVH and non-LVH, i.e. as mRNA fold change value (FC).[16] Statistical significance was set at $p \leq 0.05$. The Figure was performed using Microsoft Office Excel 2007 (Microsoft Co.).

Results

Road cyclists characteristics are shown in Table 1. Echocardiography measurements indicated that 14 male athletes (the LVH group) left ventricular hypertrophy was diagnosed. No symptoms of cardiovascular complications were observed in the athletes with and without LVH (Table 1).

We did not find any differences in age, body mass, body mass index, and body surface area as well as the percentage of body fat between athletes without and with LVH (Table 1). The training status of the non-LVH and LVH subjects are expressed as maximal oxygen consumption and aerobic power peak and are presented in Table 1. No significant difference was observed in VO_{2max} between non-LVH and LVH individuals. However, peak aerobic power was significantly higher in LVH than in non-LVH subjects (Table 1). Regardless of anthropometric characteristics, significant differences in echocardiographic parameters were observed between our cyclists.

The results of the echocardiography examinations of the athletes (e.g. left ventricular mass index, intraventricular septum thickness during diastole, left ventricular posterior wall thickness) and function (stroke volume) were significantly higher compared to normal heart dimensions clearly indicating adaptive heart hypertrophy in the LVH group (Table 1).

Resting heart rate (HR) was significantly lower in the LVH group compared to the non-LVH participants. Systolic blood pressure did not differ between the groups; however, diastolic blood pressure was significantly lower in LVH subjects. No significant difference was observed regarding blood morphology variables (Table 1). Maximal oxygen uptake (VO_{2max}) was similar for all subjects;

however, LVH athletes demonstrated significantly higher power output compared to non-LVH athletes.

The most interesting finding was that the gene expression related to cardiac hypertrophy was regulated differently before exercise and/or post exercise and during recovery. The analysis of global gene expression at rest, maximal exercise intensity and after 15 min of recovery revealed that 14 of the 37 genes', associated with cardiac hypertrophy, profile had changed in at least one period of the exercise test. It is interesting to note that there were significant differences only in the Myosin Light chain 9 (*MYL9*) and Interleukin 6 Signal Transducer (*IL6ST*) gene expression when comparing the cyclists with LVH and non-LVH in T_{max} (FC > 1.1; $p < 0.05$; Fig. 1). The statistically significant differences for *MYL9* were observed in T_{max} and T_{15} in LVH cyclists when compared to non-LVH and up-regulated FC values were 1.56 and 1.4, respectively ($p < 0.03$). *IL6ST* was down-regulated in all phases of the experiment and significant decreased expression only at maximal exercise intensity (T_{max}) of the LVH compared to non-LVH subjects was found (FC = -1.17; $p = 0.03$; Fig. 1). We did not find any significant differences in the expression of other cardiac hypertrophy-related genes in LVH cyclists, either at rest or in response to physical exercise.

Discussion

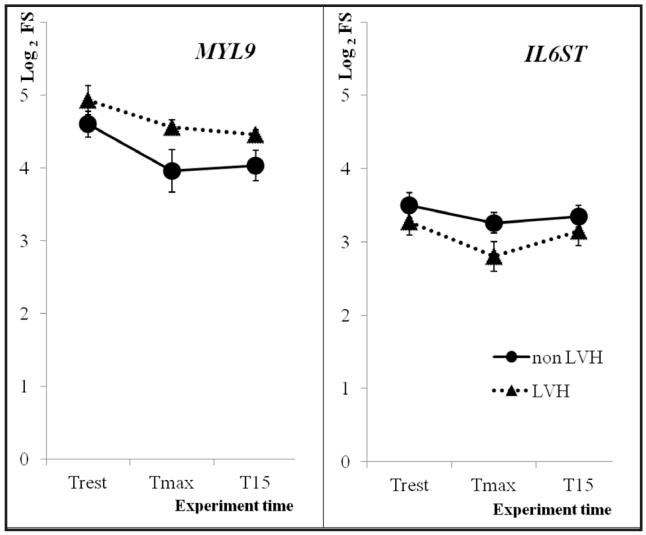
Among the twenty-eight cyclists examined two groups were selected, non-LVH (≤ 115 g/m^2) and LVH (> 115 g/m^2) based on LVMI, where characteristics differed concerning physical capacity, and the function of the left ventricular as well as diastolic pressure and heart rate (Table 1). It is well established in sport physiology that endurance training may improve exercise capacity and cardiac function at rest and during exercise.[5,17] Echocardiographic examinations of all cyclists showed that LVH subjects had an increase in LVMI ($p < 0.001$), as well as, higher LVPWT ($p < 0.05$) and IVSDD ($p < 0.05$). Similar differences were observed in the mass of the left ventricular in other endurance athletes.[18] This is contrary to an Israeli study where cyclists were found to have lower values of LVEDD, even in non-LVH, and 5% of those examined were similar to the LVH group in our study.[19] Cardiac function at rest, as determined by higher SV and lower resting HR, revealed better left ventricular adaptation to endurance training (Table 1). The cyclists did not participate in the echocardiographic study before beginning their sporting activity. Therefore, we can only speculate about the possible impact of endurance training on the cardiac structure and function. The cardiovascular effects of endurance training include decreased heart rate, increased stroke volume of the heart and increased cardiac output as well as total mitochondrial volume in the slow twitch muscle fibers. All these factors have been shown to have beneficial effects on higher efficiency in oxygen transport and distribution. In cardiovascular adaptation maximal aerobic efficacy increases by 75–80% in well-trained endurance athletes. However, LVH athletes demonstrated significantly higher P_{max} compared to non-LVH athletes, we did not observe significant differences in VO_2max between LVH and non-LVH athletes. This

Table 1. Characteristic variables of the examined cyclists measured before exercise test

Variables	Subjects		p-value
	non-LVH (n = 14)	LVH (n = 14)	
Age [years]	25.1 ± 8.8	26.2 ± 6.9	ns
Body mass [kg]	72.0 ± 6.9	72.0 ± 7.1	ns
BMI [kg/m^2]	22.7 ± 2.2	22.4 ± 1.9	ns
BSA [m^2]	1.9 ± 0.1	1.9 ± 0.1	ns
PBF [%]	12.2 ± 3.5	11.1 ± 3.4	ns
HR [bpm]	62.0 ± 13.0	59.0 ± 9.0	0.05
SBP [mm Hg]	123.2 ± 18.3	124.6 ± 13.6	ns
DBP [mm Hg]	81.4 ± 11.7	77.5 ± 10.8	0.05
LVMI [g/m^2]	102.1 ± 16.4	158.0 ± 25.6	0.000
LVEDD [mm]	49.3 ± 5.7	53.9 ± 4.0	0.002
IVSDD [mm]	10.0 ± 1.7	12.1 ± 0.6	0.05
LVPWT [mm]	9.0 ± 1.4	11.0 ± 1.1	0.01
LVEF [%]	60.3 ± 5.3	61.0 ± 4.2	ns
SV [mL]	87.2 ± 12.5	94.4 ± 18.8	0.000
RBC [10^{12}/L]	5.1 ± 0.1	5.2 ± 0.2	ns
HGB [g/dL]	15.5 ± 2.6	15.8 ± 1.9	ns
WBC [10^9/L]	6.6 ± 1.1	6.5 ± 0.8	ns
VO_{2max} [mL/kg/min]	61.5 ± 7.9	62.7 ± 5.5	ns
P_{max} [Watt]	372.0 ± 53.4	400.0 ± 120.0	0.05

BMI – body mass index; BSA – body surface area; PBF – percent of body fat; HR – heart rate; bpm – beats per min; SBP – systolic blood pressure; DBP – diastolic blood pressure; LVMI – left ventricular mass index; IVSDD – intraventricular septum diameter during diastole; LVPWT – left ventricular posterior wall thickness during diastole; LVEF – left ventricular ejection fraction; SV – stroke volume; RBC – red blood cells; HGB – hemoglobin; WBC – white blood cells; VO_{2max} – maximal oxygen consumption; P_{max} – peak aerobic power (maximal power); results were expressed as mean ± standard deviation; p – significant differences between non-LVH and LVH subjects $p \leq 0.05$; ns – no significant differences between non-LVH and LVH subjects $p > 0.05$.

Fig. 1. Expression profiles of MYL9 and IL6ST in consecutive phases of the experiment (T_{rest}, T_{max}, T_{15}); the mean values (n = 3) for cyclists with left ventricular hypertrophy (LVH) and without left ventricular hypertrophy (non-LVH)



finding seems to confirm the hypothesis that aerobic performance depends on changes in the hemodynamics of the circulation system and cardiac remodeling. It has been concluded on the basis of echocardiographic parameters that the response of elite Polish road cyclists, with a similar length of training, age, body mass index, body surface area, maximal oxygen consumption and blood morphology, to regular training is not uniform. Our results demonstrated adaptive hypertrophy in the LVH subjects and normal cardiac dimensions when compared to the non-LVH group; no impairment of cardiac function was observed.[4] Cardiac remodeling is more intensive in cyclists than other athletes, and presents a supernormal pattern of LV diastolic function in relation to non-athletes.[20] Physiological hypertrophy is caused by a proportional increase in myocardium cell length and width. Greater body size is associated with a larger heart. In addition to LV mass, LV volumes at end-diastole and the resultant stroke volume are larger in males. In athletes who participated in endurance training (e.g. cycling), both hemodynamic overload of the heart and high left ventricle wall tension of the heart may lead to eccentric ventricular hypertrophy. Additionally, the type and intensity of the training, as well as age, influence the cardiovascular adaptation and can augment the differences in structure and function of an athlete's heart. A high prevalence of heart hypertrophy was documented in male athletes and was related to their body surface, type, and intensity of training.[6,7,19,20] The relationship between the LVDD and LVMI and age, time and training intensity depend on the sport discipline and individuality of athletes. Therefore, in order to rule out the effect of somatic parameters on metabolic variables, athletes who participated in our study had comparable baseline body weight and body surface. Moreover, the training status and regime was the same. Since the athletes' somatic characteristics and training status did not differ, we might hypothesize that the results of our study most likely reflect the exercise-induced expression of genes related to cardiac hypertrophy in the circulating blood.

Significant changes in the T_{max} were observed regarding *MYL9* and *IL6ST*. Both genes play a crucial role in the physiology and pathology of the cardiovascular system.[10,21] Differential gene expression profiles in response to exercise clearly indicate that not only exercise-related but also post-exercise transcriptional changes may be of importance in adaptive cardiac hypertrophy. This finding is in agreement with those of Gielen et al.[9], who found that molecular regulation in response to endurance exercise differed in athletes with heart hypertrophy, and that these differences might result in greater improvement in cardiac function.

To our knowledge, this is the first analysis of the expression of genes related to cardiac hypertrophy in cyclists, and this could help differentiate between athletes with and without this condition based on their peripheral blood. The up-regulation of *MYL9* observed in the leukocytes of LVH athletes may suggest that exercise training has a beneficial effect on contractile protein content and/or the stimulation of contractile protein isoforms. *MYL9* (*MLC2, MRLC1, MYRL2*) is a gene encoding a muscle and actin-dependent protein called myosin regulatory light polypeptide 9.[22,23] *MYL9* expression was observed in the vascular tissue after injuries, depending on the age of rats used as specimens; upregulation of this gene was associated with increased vascular permeability.[21] The results of in vivo and in vitro experiments on the potential signaling pathways that might regulate cardiac related genes during growth and hypertrophy are not unambiguous.[24–26] Differential regulation of transcripts involved in inflammatory response, signal transduction, energy metabolism and mitochondrial function differentiate maladaptive from adaptive cardiac hypertrophy.[14,25] Additionally, our findings confirm the difference between the expression profile of *IL6ST* in LVH and non-LVH cyclists in T_{max} (Fig. 1). *IL6ST* encodes a transmembrane protein of the interleukin-6 signal transducer known as glycoprotein 130 (gp130; also referred to as IL6-β or CD130).[26,27] Physiologically, this protein serves as a cardioprotective molecule against physiological stress and cardiomyocyte damage; it causes compensatory heart hypertrophy and maintains cardiac function. The study of gp130 knockout mice indicates that gp130 might play a key role in the regulation of cardiac myocyte apoptosis and in cardiomyopathy progression.[26] It has also been suggested that *IL6ST* plays a role in the growth and survival of neonatal rat ventricular cardiomyocytes.[28,29] By stimulating the secretion of circulating IL-6 and, consequently, *IL6ST,* physical exercise has an anti-inflammatory effect.[30]The detection of *IL6ST* in the peripheral blood of athletes suggests

that the gene takes part in the body's defense against the effects of extreme physical effort.

The physiological role of genes estimated for exercise capacity of athletes and their effect on cardiac hypertrophy is not yet known. Both genes in both groups show a similar trend, although their levels in the LVH are different. An increased level of *MYL9* may explain the better performance of the cyclists with LVH (higher P_{max}), while the decreased level of *IL6ST* probably provides good adaptation to physical effort and a good prognosis in terms of hypertrophy. Expression of these genes might have a protective effect on the heart and are more characteristic in the circulating blood during maximum intensities of exercise.

The limitations of this study were its small group of tested cyclists and the high cost of microarrays. However, the conclusion remains valid due to the need for medical care for endurance-trained athletes, who are an increasing part of the patient population. Heart monitoring is obligatory in the prevention of the sudden death of intensively trained athletes. Searching for detecting marker genes or monitoring cardiac hypertrophy can contribute to reducing the costs of medical care. Despite the limitations in the number of athletes surveyed, studies show the importance of this medical problem (heart remodeling in response to long-term exercise training and differences in the gene expression of circulating blood in response to effort) and a possible way for it to be observed.

Intense cycling can lead to eccentric hypertrophy (LV) with differences in heart structure and function. Our studies indicate the benefits of echocardiography measurements for cyclists. Additionally, we speculate that *MYL9* and *IL6ST* may act as important peripheral regulators of myocardial morphology and function. They can show progression in the left ventricular hypertrophy for trained athletes with similar anthropometric parameters; further multicenter studies are needed to confirm these findings.

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Clinical factors in prosthodontic treatment of children with genetic defects

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Prosthodontic treatment of children with genetic disorders is an area that is rarely examined in the current specialist literature. Few prosthodontists will undertake treatment of such patients, who will more often be referred to an orthodontic specialist. After examining the 4 cases of children with genetic disorders described in this paper, it can be concluded that when a prosthodontist includes a few additional procedures in the treatment process, he or she can successfully help such patients.

Objectives. The aim of this paper is to indicate the clinical difficulties faced by prosthodontists who undertake prosthodontic rehabilitation of children with genetic disorders.

Material and methods. The paper is based on data collected during the prosthodontic treatment of 4 children, aged 5–12 years with genetic defects, and analysis of the body of work concerning these defects and their treatment.

Results. Presentation of guidelines for the prosthodontic treatment process and creation of dentures for treated children based on extended procedures.

Conclusions. A prosthodontist is a crucial person in a team of specialists treating disorders within the face among children with a genetic predisposition. A basic knowledge of orthodontics and psychology facilitates the treatment. Prosthetic restoration in the treatment group does not always require complicated operations. Individualization of the tools for downloading orthodontic impressions, designing denture elements and an increased number of checkups are the additional procedures. For the clinician, the emotional aspect of the treatment is the main impediment. Maintaining a good relationship with a patient and his or her caregivers requires interpersonal skills.

Key words: cardio-facio-cutaneous syndrome (CFCs), ectodermal dysplasia (ED), cherubism, prosthodontic treatment of children

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If not met with prompt and effective treatment, the acquired or congenital absence of teeth, originating in the developmental period, can have serious consequences in the form of dental defects, gnathic defects, and even disruption of the proportions of the whole face. Prosthodontic treatment in the early developmental stages is not only meant to rebuild the lost issue, but also to stimulate toothless sections to develop properly, improve speech, aesthetics, and the chewing function, to prevent parafunctional reflexes and occlusive disorders, as well as to eliminate the trauma resulting from the loss of teeth.[1]

Anodontia (total lack of teeth) or hypodontia (reduced number of teeth) often occur as one of the symptoms of the genetic diseases mentioned in this article. ED (ectodermal dysplasia) is a developmental deficit disorder of the tissues derived from ectoderm. Hair growth and sweating disorders, toothing abnormalities and nail dysplasia comprise the 4 clinical symptoms of this disease. The major toothing reduction and teething disorders require earlier prosthetic rehabilitation. Cherubism is a disorder that causes prominence in a lower portion in the face and progressive fibrous bone degeneration, which is the main cause of tooth formation abnormalities and the incorrect positioning of the teeth in the arch. Cardio-facio-cutaneous syndrome (CFCs) is a genetic disorder that effects many parts of the body, particularly the heart, facial features, skin and hair. People with this condition have delayed development and intellectual disability. The most common conditions in the mouth region are high arched palate and maxilla size disorder (micrognathia).

Prosthodontic treatment of patients with congenital dental defects begins in preschool and continues to maturity and therefore coincides with the period of intensive growth of the patient's body. When undertaking the treatment of a young patient with a genetic defect, one must take many factors into account, such as the patient's age, symptoms associated with the genetic disease and the type of dental and gnathic disorders, as well as the relationship with the patient and his caregivers. This paper aims to provide a broad and comprehensive description of the various problems faced by a prosthodontist working to promote better functioning of the stomatognathic system.

Aim

The aim of this publication is to take notice of the need for the prosthetic treatment of children who, by genetic disease, have a reduced number of teeth or have no teeth at all, namely children diagnosed with ED, CFCs and cherubism. The work depicts the specificity of the treatment of pre-school and early-childhood patients with emphasis on the emotional aspects of the checkups. Furthermore, one should also mention, both all the difficulties in the treatment process of children with developmental defects, and an attempt to show how to overcome them.

Material and methods

This paper is based on data collected during the prosthodontic treatment of 4 children with genetic defects and an analysis of the body of work concerning these defects and their treatment.

When discussing the prosthodontic treatment of children, specific aspects of the clinical situation need to be taken into account. When the patient is a young child suffering from a genetic disease who comes to the clinic with his caregivers, this is considerably different than the usual scenario of a prosthodontic patient's visit. The prosthodontist needs to adapt to this situation and analyze a variety of clinical factors, such as the symptoms of the genetic disease (the degree of disorders related to chewing apparatus, disorders of muscular tension, disorders of the masticatory function), the patient's age (his level of intellectual and emotional development) and possible mental retardation which can be associated with the disease. Only a comprehensive approach to the patient can contribute to a proper execution of the prosthodontic restoration process and successful rehabilitation.

A group of 4 children was observed, which was divided into 2 age groups (preschool age and young school age). This division was based on the classification used in developmental psychology. "When researching the psychological development of a child, the following development periods can be distinguished: infancy, postinfancy, preschool age, junior school age and adolescence".[2] According to Joanna de Flassilier-Popławska, "knowledge of subsequent stages, especially developmental changes in cognitive and emotional spheres, allows to better understand a child patient's situation and take it into account in the course of treatment".[2]

Impulsive action and emotional instability are characteristic for preschool age (3–7 years). Children at this age require help from adults, especially emotional support in new and unfamiliar situations. They like to expand their knowledge about the world around them by asking frequent questions. The prosthodontist should be aware of that and therefore should allow the child's caregivers to accompany the child during visits to the dentist's office, fully explain all the procedures that will be used in the course of treatment, answer questions about the tools and instruments used and attempt to minimize the child's fear by speaking in a calm and friendly voice as well as acting in a firm and confident manner. To gain the trust and respect of the child patient, the clinician should communicate both with the child and his caregivers. Subsequent visits should be scheduled during morning hours when the patient is well rested. Particular pro-

cedures that are done during the visit preferably should not last longer than 20 min.

It is recommended that the prosthodontist presents the outline of the treatment plan to caregivers as well as clarify all their doubts during the first visit. This is important as caregivers' attitude, positive or negative, will affect the child's attitude towards the treatment process. "The behavior of people accompanying the child plays an important role in modeling his behavior in the dentist's office. For example, fear, anxiety or a negative attitude to the doctor shown by caregivers is later imitated by the child".[2]

However, it has been observed that there is a different kind of behavior among school children (7–12 years old), who quit relying solely on their parents' opinion. At this stage, the peer group's opinion becomes important to the children. Any deviations are not tolerated, and the importance of physical appearance grows. Patients in this age group cooperate rather well during the dental treatment, as their motivation is higher. They are also able to understand the purpose of the treatment and have better control over their emotions. "The way they function in both cognitive and emotional spheres enables good cooperation with the dentist".[2]

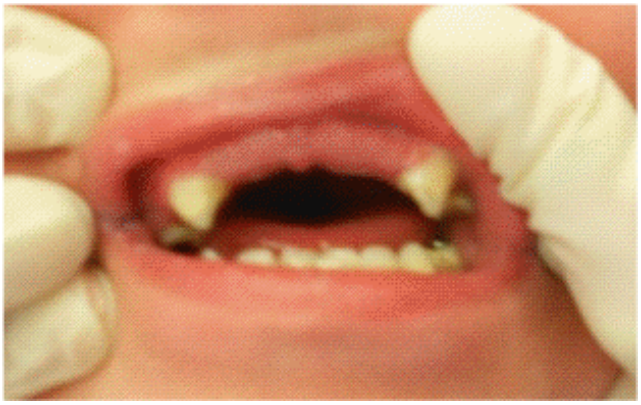
Results

Subject 1

Julia, a 5-year-old girl diagnosed with cardio-facio-cutaneous syndrome, was referred to the Department of Prosthodontics, Wroclaw Medical University, in January 2014. "As with other dysmorphic syndromes the diagnosis of CFC syndrome depends upon a characteristic picture rather than any specific diagnostic test. Demonstration of the underlying genetic abnormality requires sophisticated methodology beyond the scope of a standard clinical or genetic laboratory".[3] "Therefore clinically, CFC syndrome is a RASopathy characterized by craniofacial, dermatologic, gastrointestinal, ocular, cardiac and neurologic anomalies".[4] The disease is a result of de novo mutations, i.e. a new dominant gene being added to the gene pool. Research has identified shared characteristics of craniofacial deformities that included macrocephaly, bitemporal narrowing, convex facial profile and hypoplastic supraorbital ridges.[4] Dental phenotype characteristics for CFC include malocclusion with open bite, posterior crossbite and high-arched palate.[4]

It was necessary to provide Julia with a removable dental prosthesis, since she had prematurely lost deciduous teeth due to their disturbed structure. Intraoral examination revealed the presence of 4 teeth in the maxilla deciduous canines and secondary molars (Fig. 1). Prosthodontic restoration was meant to improve the ability to chew food as well as the aesthetics of the face, which was also significant because Julia attended preschool and had been in contact with other children.

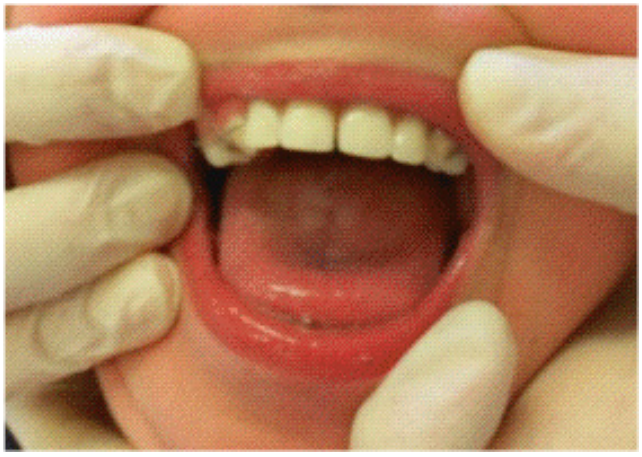
Fig. 1. Patient without artificial replacement



Initial difficulties were encountered while attempting to take a dental impression during the first visit, as contact with Julia was limited due to the medium degree of mental retardation. Despite being 5 years old, the patient behaved as a 1.5 years old child. Under these circumstances, the doctor had limited possibilities to explain the course of treatment to the patient, therefore he needed to gain her trust through acting in a calm and confident manner. During each visit, the patient sat on her mother's lap, which made her feel safe and secure. Cooperation with her parent was an important factor making the treatment process easier and more efficient. Julia's mother was calm, strong-minded and motivated to help her child. She was not afraid to ask questions related to the stages of the treatment process and she acted as a good intermediary between her child and the doctor, making the communication process more efficient. She was a model example of a parent's behavior in the prosthodontist's office. The success of the treatment process was largely dependent on gaining the mother's favor.

Determining the central occlusion and taking all the measurements required for the denture was a big challenge, as the patient manifested excessive nervousness. The doctor had to be patient and composed, but at the same time carry out all the necessary procedures quickly and efficiently. After making a partial denture, the last step which proved to be difficult was checking if it fit correctly as well as convincing Julia that the denture should stay in her mouth and be used as if it were her own teeth. In most cases it is difficult to convince an adult that the denture adaptation period is cumbersome and painful, but only temporary. To convince a child, it's recommended to present treatment as fun and attractive event. Julia's denture required Adams' braces for additional support, because initially the patient tried to remove the denture from her mouth, as she didn't understand the purpose of the treatment (Fig. 2). Additionally, muscular hypotension could have an adverse effect on the way the denture was fixed in the mouth.

Fig. 2. Patient with partial denture



Unfortunately, lack of cooperation from the patient's side made it impossible to make a panoramic X-ray, which would make it possible to plan further treatment. Julia's denture should be checked every 2 months and if the need arises, should be adjusted with a Fischer screw to fit it to bone development dynamics or alternatively should be replaced. During follow up visits, the doctor should always check the oral hygiene and provide the family with detailed instructions concerning hygiene habits, because the patient, suffering from the aforementioned genetic disease, may not be able to take care of that herself.[4] Furthermore, occlusion development should also be monitored and, if necessary, the patient should be referred to an orthodontist for further treatment.

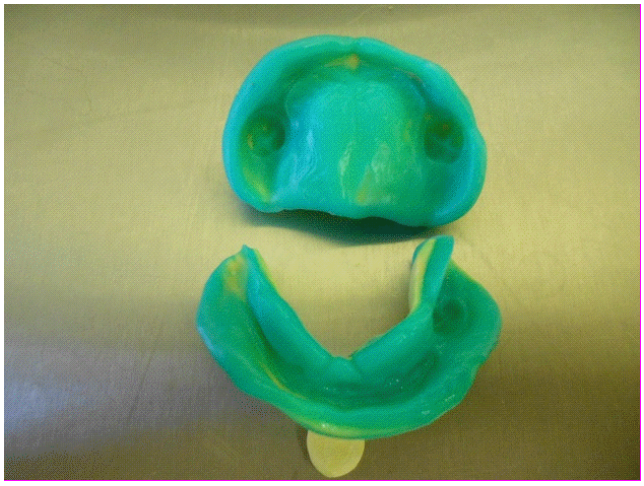
Subject 2

Young patients that visit the Prosthodontics Clinic most frequently are children diagnosed with ectodermal dysplasia. Cwajda describes this disease as "syndromes of disorders caused by anomalies in ectodermal structures". She goes on to say that "154 syndromes related to ectodermal tissues were identified, 120 of which result in symptoms in the mouth area, mainly hypodontia" and that "[s]ymptoms characteristic of ectodermal dysplasia include: frontal bossing, collapsed middle third of the face, saddle nose, thick and everted lips, low-set ears called satyr ears, fair eyes, wide set orbits".[5] Labda et al. have also shown that small children with ED often have problems with speech and eating due to missing teeth or anodontia.[6] They also feel that they are different from their peers.[6] Self-awareness connected with physical appearance can start to form when a child is 4–5 years old, therefore it is important to start aesthetic as well as prosthodontic procedures around that age.[7]

Dawid (aged 5), came to the clinic for a new denture for the maxillae and mandible, because he had lost the previous one. After a meticulous oral examination and careful analysis of anatomic factors, a decision was made to create removable partial acrylic dentures for the maxillae and mandible. "The use of partial and complete acrylic prostheses is an interesting and practical alternative that provides a relatively quick, easy, acceptable and economical solution to the functional aesthetic oral rehabilitation and psychological benefit in young patients with pronounced edentulism".[8] This should only be treated as a temporary solution however, and parents should be informed about the other treatment possibilities given by implant prosthodontics, which can be applied in the future, after the child's developmental stage is completed.[7] Dawid was cooperating well considering his age, however he was also having mood changes.

Taking his dental impression during the first visit was difficult as it was not possible to fit standard children's trays to his anatomy. The small size of the maxillae and mandible caused by missing teeth and underdevelopment of the alveolar process is one of the disease's symptoms. In order to achieve an accurate impression of the prosthetic field as well as prosthetic abutment (2 primary maxillary second molars and 1 primary mandibular second molar), the decision was made to create custom trays (Fig. 3) after taking anatomical impressions using standard children trays. The teeth remaining in the mouth were relatively small sized. Additionally, due to prior lack of full adaption to the denture, Adams' braces were used as supplementary supporting elements.

Fig. 3. Functional impressions of maxilla and mandible on individual trays



Patients with diagnosed ectodermal dysplasia can have denture adaptation problems related to the difficulty in achieving retention and stabilization, as this disease is often accompanied by dry mucosa and underdevelopment of maxillary tuberosity and the alveolar process.[9] It is recommended to widen the base of the denture for such patients in order to make the denture fit the mouth better.[9] Dawid's treatment required performing additional procedures, scheduling visit hours optimal for the child's good mood in order to improve cooperation, explaining the course of treatment and applying additional denture elements – Adams' braces and Fischer's screws

(Fig. 4, 5). Thanks to adjusting the treatment to suit the specific needs of the child, prosthetic rehabilitation was successful at this development stage. "Long term success, however, depends on the patient's regular follow-up visits and maintaining meticulous oral hygiene".[9] Follow-up visits were schedule for Dawid every 2 months.

Fig. 4. Acrylic denture for mandible with Fisher's screw and Adam's braces, lingual view

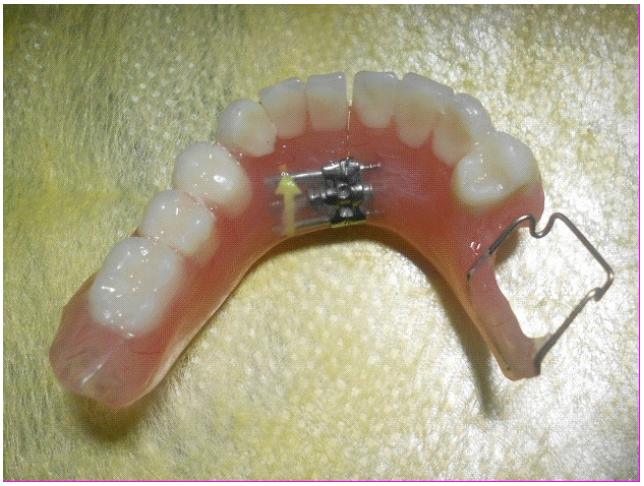
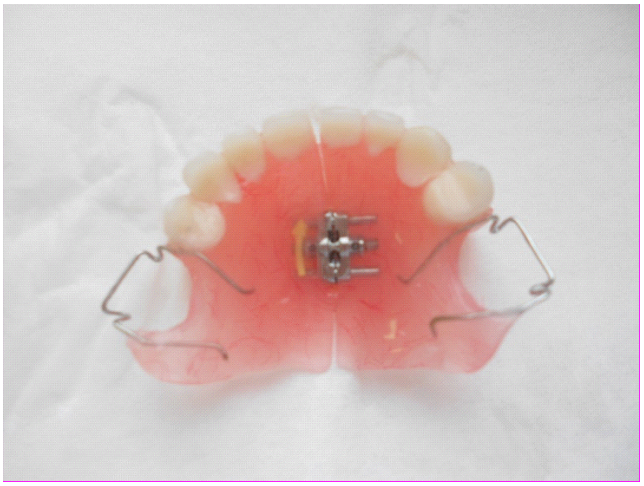


Fig. 5. Denture for maxillae with Fisher's screw and Adam's braces, lingual view



Subject 3

Bartek came to the Prosthodontics Clinic at 12 years old, at rather late age. He had been diagnosed with ectodermal dysplasia a year earlier. The patient came to the Genetics Clinic as his parents were worried about his hypodontia and nail dysplasia (Fig. 7). During the interview, it was established that the deciduous teeth had erupted correctly, just when the patient was 11 years old and after he had lost his lower incisors, there were no permanent teeth. Panoramic X-ray showed that the patient lacked most of the tooth buds for permanent teeth (Fig. 6). The remaining deciduous teeth were not developing at the same rate as the rest of the body, therefore Bartek required overdenture to replace the missing teeth and restore the proper height of the occlusion (Fig. 8).

Fig. 6. Panoramic X-ray showing missing permanent tooth buds

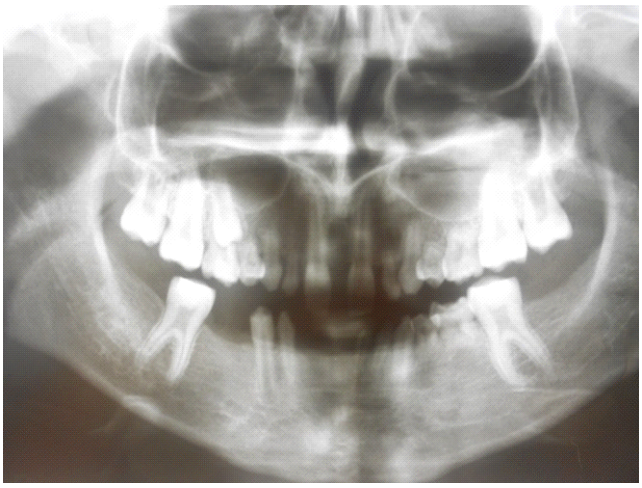


Fig. 7. Distorted structure of the nails - one of the ectodermal dysplasia symptoms

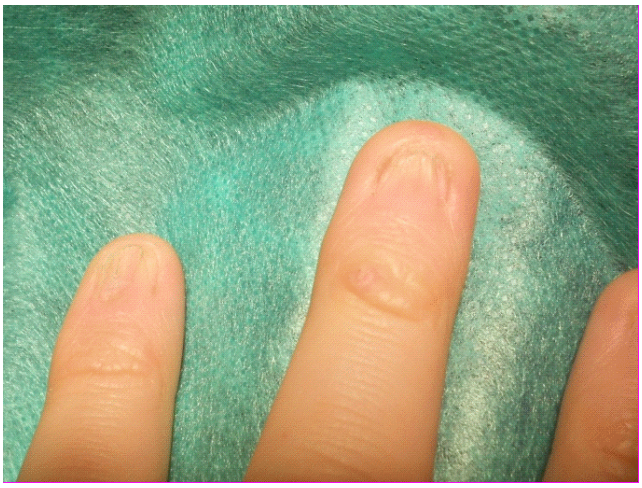
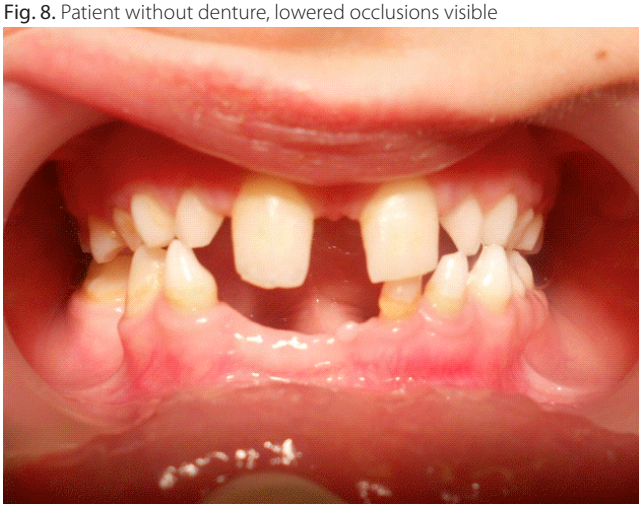
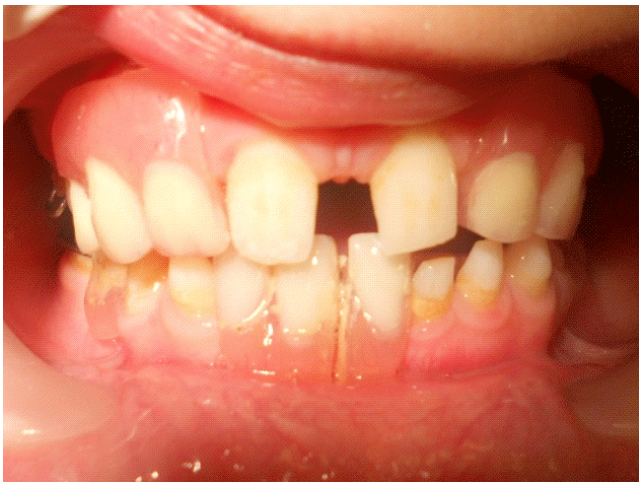


Fig. 8. Patient without denture, lowered occlusions visible



Using Fisher's screw in the denture made a longer use of the restoration possible, as it could be increased along with the facial skeleton. The course of the treatment was good, the patient was motivated and cooperated well. Bartek's treatment was aimed to restore the balance in the stomatognathic system as well as improve the smile's aesthetics, which is an important factor for a patient at that age (Fig. 9). "Well-fitting and functioning prosthesis could be a great help during their schooling years as it will improve appearance and thus boost their self-confidence".[6]

Fig. 9. Patient with overdenture for maxilla and mandible

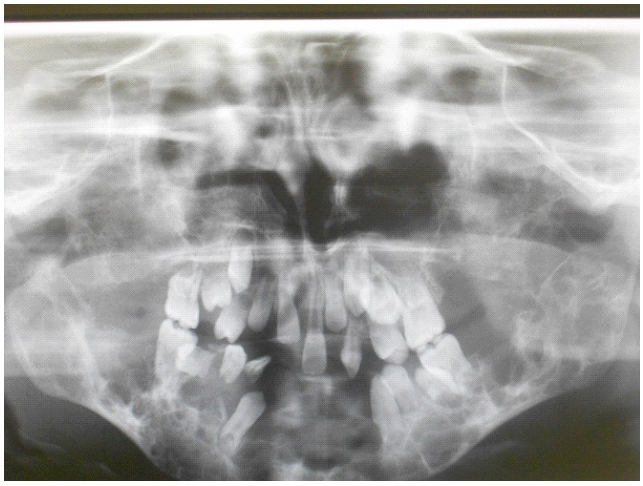


Subject 4

9-year-old Dawid, who had been diagnosed with cherubism, came to the Prosthodontics Clinic in 2014. Yilmaz et al. report that "Cherubism is a rare inherited developmental abnormality that causes bilateral enlargement of the maxilla and/or mandible". Moreover, "[i]t is caused by the autosomal dominant gene located on chromosome 4p16.3 and typically affects men. It is generally accepted that it is a benign disease of bone beginning at the age of 2–3, that progresses in childhood with a peak at the age of 5, and shows spontaneous regression at the end of adolescence".[10] Published sources report that, depending on the severity of the bone changes, the effects the disease has on the mouth can include dislocation of teeth, dental crowding, root resorption, premature loss of deciduous teeth, tooth agenesis and tooth bud involution.[11,12] Dental abnormalities and phonation disorders as well as chewing and swallowing disorders can affect patients with the aggressive form of cherubism.[11,12]

A report from Dawid's craniofacial CT from 2010 indicated the extensive destruction of both maxillae and mandible, which were transformed into a sponge-like structure and expanded. These changes did not affect either mandibular condyles nor zygomatic bones. They had the biggest impact on the body of the mandible, which displayed significant bilateral enlargement. Conducting a thorough interview revealed no family history of the disease in the patient's family. His mother said that the first alarming signals appeared when Dawid was 3.5 years old, when he started to lose deciduous teeth prematurely. A panoramic radiograph of Dawid's mouth confirmed a lack of tooth buds for some of the permanent teeth and also revealed dislocation of the remaining teeth, which was caused by the deformed bone structure of the maxilla and mandible (Fig. 10). It also showed teeth located in radiolucent areas, which is a characteristic image for this anomaly called "floating teeth".[11,12] The disease has not affected the boy's mental development.

Fig. 10. Panoramic X-ray showing distorted structure of mandible bones and dislocation of teeth



During subsequent visits to the clinic, a functionally unstable denture with Fischer's screw and other orthodontic elements helping proper tooth eruption was created (Fig. 12). It was challenging to obtain stabilization of the lower denture due to the sponge-like alveolar process of the mandible with a flat bottom of the vestibule

Fig. 11. Mouth without denture, wide and irregular alveolar process of mandible and elevation of the bone in the sublingual space as well as decreased depth of mouth vestibule visible

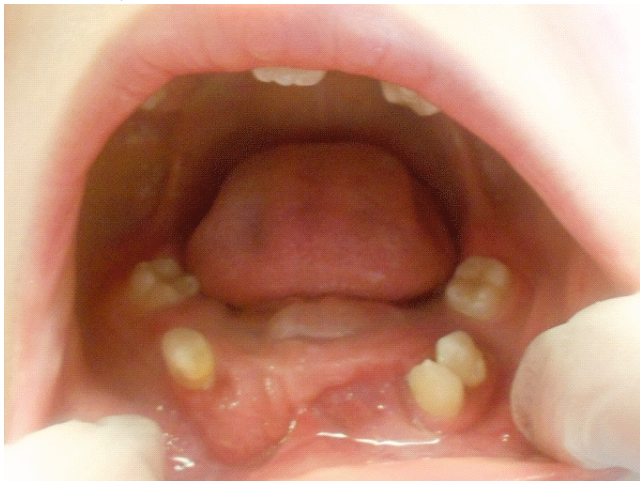
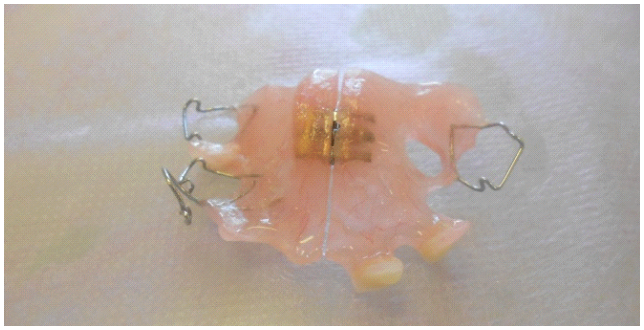
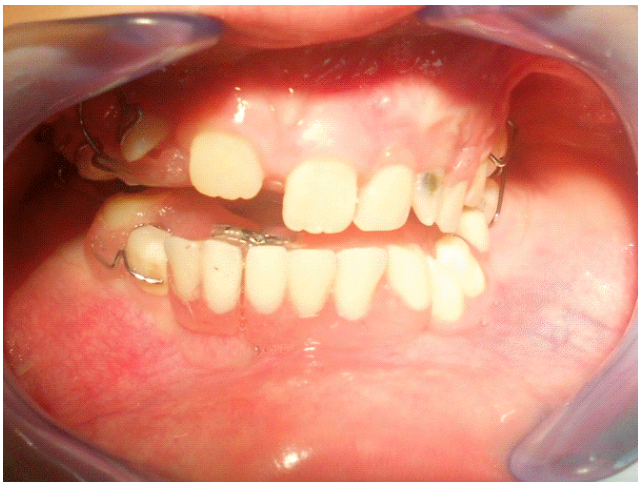


Fig. 12. Denture with orthodontic elements



and sublingual space (Fig. 11). Low, dislocated abutment teeth, caused by the disease, proved to be an additional difficulty. The course of Dawid's prosthodontic treatment will last several years. Currently, it is planned to achieve contact of the teeth and restoring the aesthetics

Fig. 13. Patient wearing maxillary and mandibular dentures



of the smile (Fig. 13). Further steps include removal of the deformed bone tissue along with the retained teeth, transplantation of the removed bone and probably creating an overdenture type prosthesis.

This patient's treatment is a complicated and multistage process, but thanks to the proper motivation, determination and willingness to cooperate from him, satisfactory effects of the treatment can be expected. In the case of patients with such diseases, it is important that the dental prosthetist knows both the clinical and radiological symptoms of the disease, because he may be the first person to diagnose the patient when the patient visits the clinic wanting to improve the esthetics of the teeth. The doctor should recommend further diagnostics in a genetics clinic after the interview with the patient and intraoral examination and X-ray interpretation.

Discussion

The prosthodontic treatment of children is not a well-developed field. Often doctors who are specialized in prosthodontics tend to leave the treatment of such patients to orthodontists. But children suffering from genetic disorders often require medical assistance from a prosthodontist, who can additionally include orthodontic knowledge in his actions. A prosthetist ought to belong to the group of specialists undertaking the rehabilitation of the masticatory system of such patients. After analyzing the treatment of the above-mentioned children, it is worth noting that doctors undertaking the treatment of a young patient should have the necessary qualities. He or she should have proper interpersonal skills, be well prepared and have the right attitude. Interpersonal skills are important in order to establish good contact with the child and his caregiver. Good preparation includes having a basic knowledge regarding orthodontics, pediatric dentistry and psychology. When it comes to the right attitude, we must highlight a comprehensive look at the case and an open mind to new challenges. In the present examples, dentures were made for children taking part in clinical monitoring. This leads to the conclusion that it is not a difficult treatment, but it requires some additional actions and is more time-consuming. Another conclusion that is worth noting is the importance of cooperation between the doctor and patient's guardians. Successful treatment of the patients described in this paper was often possible thanks to a good relationship between the prosthodontist and the child's parents, who presented a positive attitude towards the process. In 1983, Murray stated that "cooperation between the doctor and guardians is a fundamental condition for dental treatment. After all, it's parents that shape hygienic and dietary habits of the child, as well as take care of prevention and bring child to subsequent visits".[13]

Clinical implications

All the children described in this paper accepted their dentures and are using them, despite considerable difficulties with making the restoration and a long adaptation period. They also remain under systematic prosthetic and orthodontic care and report regularly to the designated check-ups.

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The potential role of selected bioactive compounds from spelt and common wheat in glycemic control

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Abstract

Diet is an important lifestyle factor which influences people's health and the prevention of chronic diseases such as type 2 diabetes. Cereal-based foods constitute the main component of the everyday diet worldwide. Old cereal species like spelt (*Triticum spelta* L.) are becoming more and more popular, especially in Europe. This review focuses on the role of bioactive compounds from spelt and their possible biological mechanisms of action in glycemic control. Spelt grain contains a high amount of dietary fiber, which can modulate postprandial glycemia. Other phytochemicals, such as phytic acid and alkylresorcinols, also contribute to controlling blood glucose levels, insulin sensitivity and hiperinsulinemia. Antioxidant compounds present in spelt grain may act as protection from negative outcomes of chronic hyperglycemia. In this paper the composition and beneficial properties of spelt are also compared with those of widely consumed cereals like common wheat (*Triticum aestivum* L.). The health benefits of whole grain as opposed to refined products are also discussed.

Key words: wheat, glycemic control, spelt

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There are several factors that influence diabetes prevention and management. Some of them – such as age 45 or older, or a family history of diabetes and related diseases – cannot be controlled. But there are several risk factors connected to daily habits and lifestyle which can be easily modified. These include physical activity, weight control, alcohol consumption, smoking status, and intake of red meat, fruit and vegetables. Simple changes in the diet can significantly reduce the risk of diabetes or the prediabetic state.

Spelt (*Triticum spelta* L.) is one of the ancient wheat species, along with emmer wheat (*Triticum dicoccum* Schrank ex Schübl) and einkorn wheat (*Triticum monococcum* L.). It has been known since 7000 or 8000 BC. There are several hypotheses with regard to the origin of spelt in Europe; one theory is that it is a hybrid of emmer and common wheat.[1] Spelt has fewer climate requirements than common wheat and can grow even in high mountains.

In recent decades spelt has become more and more popular owing to its composition and its possible protective influence on human health.

Bioactive compounds

Wheat grain is a rich source of dietary fiber and various phytochemicals, which are unevenly distributed throughout the kernel. Endosperm (80–85% of the grain's weight) consists mostly of starch and some proteins, along with bioactive compounds such as β-glucans, arabinoxylans, carotenoids and flavonoids. Bran constitutes 10–14% of the grain's weight, and the list of its bioactive compounds is long. The most important groups are soluble and insoluble fiber, minerals, vitamins, phytic acid, betaine, choline, phenolic acids, lignans (secoisolariciresinol, matairesinol, syringaresinol, lariciresinol, pinoresinol medioresinol and 7-hydroxymatairesinol), alkylresorcinols and phytosterols. As for the germ (3% of the grain's weight), unsaturated fatty acids and phytosterols constitute its greatest nutritional value. Besides those 2 groups, fiber, minerals, enzymes, sulfur-containing amino acids, glutathione, betaine, choline, myoinositol, tocopherols, lignans, flavonoids and benzoxazinoids (benzoxazolinones, lactams and hydroxamic acids) are present in wheat germ.[2,3] Although spelt and common wheat belong to the same botanical genus (*Triticum* spp.), the content of the grains differ. Details of the composition of both species are presented in Table 1 and Table 2.

Glycemic control is associated with dietary fiber and a few groups of the phytochemicals present in cereal grain. According to the American Association of Cereal Chemists (AACC) "dietary fiber is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation and/or blood cholesterol attenuation and/or blood glucose attenuation".[20] Two kinds of dietary fiber can be distinguished: soluble and insoluble, which are further divided into subgroups. Soluble fiber consists of β-glucans, pectins, mucilages, gums and some hemicelluloses; and insoluble consists of celluloses, lignin and

Table 1. Content of macronutrients in spelt (*T. spelta* L.) and common wheat (*T. aestivum* L.) products expressed as g/100 g of DM in grains and g/100 g of sample in food products

Nutrient	Spelt	Reference	Common wheat	Reference
Carbohydrate (total)	69.7	4	71.2	4
	74.20 F	5	75.94 F	5
	51.94 B	5	53.16 B	5
Starch	66.7	4	68.1	4
	72.07 F	5	71.84 F	5
	50.45 B	5	50.28 B	5
Sugars (glucose, sucrose, maltose)	3	4	3.1	4
	2.13 F	5	4.10 F	5
	1.49 B	5	2.87 B	5
Lipids	2.92	6	2.48	6
	1.43 F	5	1.14 F	5
	1 B	5	0.79 B	5
Saturated fatty acids (%)	20.53	4	18	4
	21.01 F, B	5	20.67 F, B	5
Monounsaturated fatty acids (%)	21.45	4	12.10	4
	14.10 F, B	5	7.9 F, B	5
Polyunsaturated fatty acids (%)	58.02	4	69.90	4
	60.49 F, B	5	67.04 F, B	5
Proteins	12.2	4	13.5	4
	11.83 F	5	10.53 F	5
	8.28 B	5	7.37 B	5
Fiber (total)	2.64	4	2.73	4
	2.65 F	5	2.52 F	5
	1.86 B	5	1.76 B	5

F – flour; B – bread; without annotation – in grain.

Table 2. Content of bioactive compounds in spelt (*T. spelta* L.) and common wheat (*T. aestivum* L.) products expressed as mg/100 g

Nutrient	Spelt	Reference	Common wheat	Reference
Phytic acid	437 B	7	300–1500	2
			218B	7
Myoinositol	nd	–	1.9 7.5	2
Alkylresorcinols	39.5	4	47.5 –55.8	4,9
	4.75–69.39 F	8	365.21 Br	8
			2.92–76.33 F	8
Phytosterols	65.15	10	64.11	10
Lignans	3.25 Br	11	0.34–2.3	12
			9.22 Br	11
Benzoxazinoids	nd	–	0.48	13
Tocochromanols	2.31–3.48	14	3.20–3.29	14
Phenolic acids	38.2–72.6	15	32.6–117.1	15
	42.22–125.74	16	45.6–89.2	16
Policosanol	nd	–	0.3–5.62	2
			3.0 Br	17
			0.017 F	17
Betaine	183–277	18	97–294	18
			1500 Br	19
			226.5 B	19
Choline	20–22	18	18–28	18
Lectins	nd	–	nd	–

nd – no data available; F – flour; B – bread; Br – bran; without annotation – in grain.

some hemicelluloses. Although wheat grain is richer in insoluble than soluble fiber[1,2], it is the latter group that mostly affects glycemic control.[20]

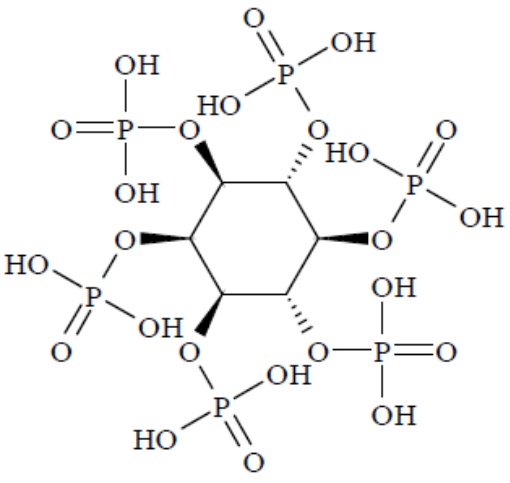
Total arabinoxylans (AX), which consist of water-extractable arabinoxylans (WEAX) and water-unextractable arabinoxylans (WUAX), bind properties of soluble and insoluble fiber.[21] D-xylopyranose residues form the linear backbone of AX. They are connected via β-(1→4) glycosidic linkages with none, 1 or 2 L-arabinofuranose residues in position 2 and/or 3.[21,22] Other compounds which may be tied to the AX structure are short oligomers, uronic acids, acetic acid and hydroxycinnamic acids. The mean content of AX in spelt is 5.74%, including 0.59% WEAX.[22] For spelt flour these values are 1.75% and 0.35% respectively. As for common wheat, the percentage of AX is 1.9%, including 0.50% WAEX.[1] WEAX is capable of creating a gel formation with viscosity proportional to the molecular mass.[21]

β-glucans are another group that has a significant role in controlling blood glucose levels. They are present in smaller amounts than arabinoxylans, with mean percentages of 0.54% (0.23–0.9%) and 0.51% (0.37–0.76%) in spelt and common wheat, respectively.[22] β-glucans are non-branched chains composed of β-D-glucopyranose residues. Usually, 3 or 4 residues linked by β-(1→4) glycosidic bonds create cellulose-like fragments connected to each other via β-(1→3) glycosidic bonds. Sometimes 5–20 residue fragments occur. β-glucans also have gel-forming capacity which depends on the molecular mass, length and ratio of the cellulose-like fragments.[21]

Lectins, also called hemaglutinins, are a large group of proteins with the ability to bind to carbohydrates without changing their structures. They are divided into subgroups based on their highest affinity to the following monosaccharides: mannose, galactose or N-acetylgalactosamine, N-acetylglucosamine, fucose and sialic acid. Among lectins, one specific protein has been distinguished: wheat germ agglutinin (WGA), a small lectin binding to N-acetylgalactosamine. Both adverse and beneficial health outcomes related to this protein have been described. Lectins are not fully degraded during digestion, so they exhibit activity throughout whole gastrointestinal tract.[23] No data is available on lectin content in spelt and wheat.

Phytic acid (Fig. 1), or phytate (the salt form), is a phosphorus storage compound located in the aleurone layer.[6] It is composed of myoinositol hexaphosphate (IP6). Unfortunately, the quantitative phytic acid content in spelt grain has not been evaluated, but bread baked from spelt flour type 1400 contained more phytic acid than bread made from common wheat flour type 1400 (437 mg/100 g and 218 mg/100 g, respectively).[7] For a long time phytic acid was regarded

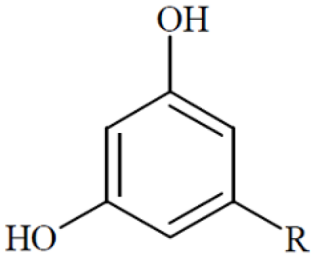
Fig. 1. Phytic acid



as an antinutrient; nowadays, it is described as having health benefits, including glycemic control.[2]

Alkylresorcinols (AR) are phenolic lipids (Fig. 2) restricted strictly to the outer parts of grain. They are 1,3-dihydroxybenzenes with an odd-numbered alkyl chain attached to C5, which can be saturated, mono- or diunsaturated. The length of the side chain is 13–27 carbon atoms.[24] The C17:0/C21:0 homolog ratio is specific for each species. It is 0.1 for both common wheat and spelt, and 1 for rye.[25] AR are absorbed in the small intestine and can be detected as intact homologs in plasma, and as small amounts of intact homologs and their metabolites in urine.[26] The AR content in whole spelt grain is 39.5 mg/100 g; in common wheat it is 47.5–55.8 mg/100 g.[4,9] Refined products contain trace amounts of alkylresorcinols due to a lack of bran.

Fig 2. The basic structure of alkylresorcinols

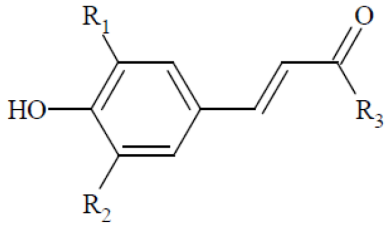


Name		R
5-*n*-Tridecylresorcinol	(C13:0)	$C_{13}H_{27}$
5- *n* -Pentadecylresorcinol	(C15:0)	$C_{15}H_{31}$
5- *n* -Heptadecylresorcinol	(C17:0)	$C_{17}H_{35}$
5- *n* -Nonadecylresorcinol	(C19:0)	$C_{19}H_{39}$
5- *n* -Heneicosylresorcinol	(C21:0)	$C_{21}H_{43}$
5- *n* -Tricosylresorcinol	(C23:0)	$C_{23}H_{47}$
5- *n* -Pentacosylresorcinol	(C25:0)	$C_{25}H_{51}$
5- *n* -Heptacosylresorcinol	(C27:0)	$C_{27}H_{55}$

Phytosterols, also called plant sterols, are a group of secondary metabolites present in particularly high amounts in the germ. They can be found in free or esterified form, rather than as glycosides or acylated glycosides.[3] Quantitative estimates show no significant differences in the phytosterol profiles of common wheat and spelt. However, a higher content of the main compound of this group – β-sitosterol glycoside – has been observed in spelt.[10]

Phenolic acids and tocochromanols (vitamin E forms) create a heterogeneous group. Phenolic acids can be divided into derivatives of hydroxycinnamic acid (Fig. 3) or hydroxybenzoic acid (Fig. 4); tocochromanols can be divided into tocopherols (Fig. 5a) or tocotrienols (Fig. 5b). Examples of hydroxycinnamic derivatives are p-coumaric, ferulic, sinapic and chlorogenic acids. Hydroxybenzoic derivatives include p-hydroxybenzoic, protocatechuic, vannilic and syringic acids.[3,15] All phenolic acids have an aromatic ring in their structure, with at least 1 hydroxyl group. They may appear as free, conjugated or insoluble bound forms (e.g. lignins). The content and composition of phenolic acids differ according to the type of cereal, environmental conditions, time of harvest and morphological fraction, with the biggest portion in bran.[3]

Fig. 3. Derivatives of hydroxycinnamic acid



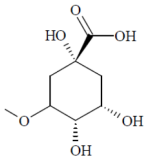
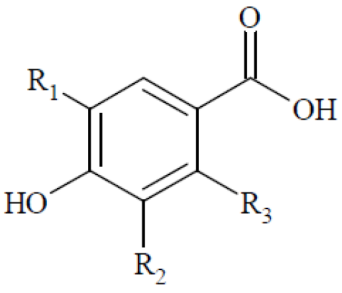
	R_1	R_2	R_3
p-Coumaric acid	-H	-H	-OH
Ferulic acid	$-OCH_3$	-H	-OH
Sinapic acid	$-OCH_3$	$-OCH_3$	-OH
Chlorogenic acid	-OH	-H	

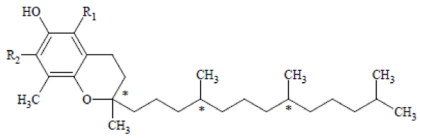
Fig. 4. Derivatives of hydroxybenzoic acid



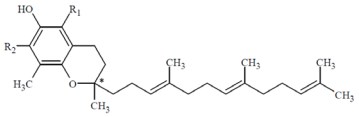
	R_1	R_2	R_3
p-Hydroxybenzoic acid	-H	-H	-OH
Protocatechic acid	-H	-OH	-H
Vannilic acid	$-OCH_3$	-H	-H
Syringic acid	$-OCH_3$	$-OCH_3$	-H

Fig. 5

a) Structure od tocopherols



b) Structure of tocotrienols



	α	β	γ	Δ
R_1	$-CH_3$	$-CH_3$	-H	-H
R_2	$-CH_3$	-H	$-CH_3$	-H

Spelt contains 38.2–125.74 mg/100 g and common wheat 32.6–117.1 mg/100 g.[15,16] Ferulic acid is the most abundant and p-coumaric acid takes the second place.[3]

Tocochromanols are lipid-soluble compounds with vitamin E activity. Their basic structure is a 6-chromanol ring with a 16-carbon hydrophobic side chain attached to C2. Tocotrienol side chains have 3 double bonds, while tocopherol side chains are saturated. Another difference between tocotrientols and tocopherols is the number of asymmetric carbons. Tocotrienols have 1 (at C2 of the chromanol ring), while tocopherols contain 3 (at C2 of chromanol ring, and C4' and C8' of the side chain). The content of tocochromanols in spelt is in the range of 2.31–3.48 mg/100 g, while common wheat contains an average of 3.25 mg/100 g, ranging from 3.20 to 3.29 mg/100 g.[14]

Possible mechanisms of action

Glycemic control is not a simple act; it is a complex process dependent on various factors such as the structure, composition and degree of food processing as well as digestive enzyme activity. Wholegrain products, despite their high carbohydrate content, do not cause such a high and rapid rise in blood glucose level as refined products do. This can be explain by the discrepancy in the content of dietary fiber and bioactive compounds, and different rates of digestion. Glycemia can be controlled on different levels: during food production, by choosing wholegrain and wholemeal raw materials instead of refined ones, and in the body after ingestion. It is difficult to pinpoint specific groups of phytochemicals and corresponding mechanisms of glycemic control. Various groups of compounds act together to produce a synergistic effect. Sometimes the mechanisms of action of different groups mesh together and sometimes they complement each other to achieve the effect.[2]

The macronutrient composition of grain plays a major role in the glycemic response. The content of spelt and common wheat grains differ. The higher protein and fat content of spelt grain favors better glycemic control. Despite that, in a study comparing the glycemic response to spelt bread and common wheat white bread, no difference in the glycemic index (GI) was observed; both breads had a high GI. This might be because refined flour, not wholemeal flour, was used to bake both breads[5]

Lower blood glucose level might be achieved by prolonging the time of digestion. Soluble fiber, arabinoxylans, short-chain fatty acids (SCFA, e.g. propionic acid), which are products of fiber fermentation in the gut, and phytic acid have an ability to delay gastric emptying.[2,27] Phytic acid (or phytate) also hinders digestion by binding with starch and starch-associated protein. The inhibition of enzymes modulates the rate of glucose release from starch. Alkylresorcinols, lectins and phytic acid are able to bind to α-amylase (an enzyme involved in starch digestion), limiting its activity. Phytic acid also influences this enzyme by chelating calcium, which is a cofactor for α-amylase.[2] Another enzyme involved in carbohydrate digestion is α–glucosidase, which can be inhibited by phenolic acids and alkylresorcinols.[28,29] Increased viscosity due to the presence of soluble fiber and arabinoxylans limits access to enzymes and impedes absorption of glucose in the small intestine.[2,27] Thus, changes in glucose level are smoother.[20] Absorption of glucose from the intestines to the bloodstream goes through sodium-dependent glucose transporter SGLT1 localized in the intestinal brush border membrane. Ferulic and chlorogenic acids exhibit an ability to block this transporter, limiting the rate of absorption.[28] All these mechanisms lengthen the time of digestion and prevent rapid changes and high peaks in blood glucose.

Physiologically, the glucose level is under hormonal control. Insulin released by pancreatic β-cells decreases postprandial glycemia. Cell resistance to insulin may be the first step in the development of type 2 diabetes. A study conducted on mice showed that supplementation with arabinoxylans improved insulin resistance.[27] Betaine is another compound with the potential ability to overcome insulin resistance.[2] Various studies have shown that insulin response and postprandial glucose levels were lower in healthy, obese and diabetic subjects when arabinoxylans were added to their diets.[27] β-glucans improve insulin sensitivity as well.[21] In an in vitro study, ferulic acid promoted insulin secretion in cell cultures.[28] Spelt grain is also a rich source of magnesium. A sufficient supply of this mineral is needed to sustain insulin sensitivity. In case of hyperglycemia, pancreatic β-cells are potentially protected from the toxic activity of glucose by tocochromanols.[2]

The blood glucose level is also dependent on the function of the liver. Propionate, one of the SCFAs, reduces hepatic gluconeogenesis and stimulates glycolysis, which

modulates glycemia. Glycolysis is performed by metalloenzymes, most of which contain magnesium in their structure. Thus, an adequate supply of magnesium is necessary.[2]

The last mechanism of glycemic control is modulation of glucose uptake form the bloodstream to muscle cells. This activity is performed by ferulic and chlorogenic acids.[28]

A 9-week intervention study was carried out in animal model with Zucker diabetic fatty (ZDF) rats to investigate the physiological effects of 5 different diets (emmer, einkorn, spelt, rye and refined wheat) on the development and progression of type 2 diabetes. The ancient wheat diets downregulated key regulatory genes expression (hepatic genes PPAR-α, GLUT2, SREBP-1c) engaged in glucose and fat metabolism. Spelt and rye induced a lower acute glycemic response compared to refined wheat. These results provide strong evidence that spelt, in contrast to wheat, plays a role in the prevention or delay of diabetes development.[30]

Hartvigsen et al.[31] checked the impact of arabinoxylans on glycemic control as indicated by postprandial glucose and hormone responses, fermentation and appetite in human beings in an acute randomized crossover study with 15 subjects with metabolic syndrome, which may be associated with type 2 diabetes. A meal of concentrated arabinoxylan combined with rye kernels reduced the acute glucose and insulin responses and the feeling of hunger to a greater extent than the control meal of semolina porridge. Concentrated arabinoxylan and concentrated arabinoxylan combined with rye kernels also stimulated SCFA production, i.e. butyrate and acetate. However, no significant differences were observed for the second meal responses of glucose, insulin, free fatty acids (FFA), glucagon-like peptide-1 (GLP-1) and ghrelin.[31]

Phenolic acids and other polyphenols, such as lignans and flavonoids, are known for their antioxidant properties. Tocochromanols, tocotrienols and phytic acid also contribute to the antioxidant mechanism of action of spelt and wheat because of their chelating properties. Alkylresorcinols possess these properties as well, although they are rather weak.[32] These compounds might be useful in protection against glycooxidation, which takes part in diabetic complications and results in the formation of advanced glycation end products (AGEs) and advanced oxidation protein products (AOPPs). In a study with 52 diabetic patients (18 with type 1 and 34 with type 2), AGEs in serum were elevated only in patients with type 2 diabetes, while AOPPs were elevated in both types of diabetes, with higher levels in type 2.[33] Antioxidants may prevent glucose oxidation and AGE formation, which are increased in hyperglycemia and under the influence of oxidative stress.[34] AOPP formation can also be inhibited by antioxidants, e.g. vitamin E.[35]

Summary

Cereals may play a beneficial role in the prevention of type 2 diabetes. Certain differences between the nutritional and bioactive compound content of spelt and common wheat can endow spelt with a significant protective role.

Phytochemicals such as polyphenols may affect and modify lipid and glucose homeostasis and therefore play an important role in the prevention of type 2 diabetes and related diseases. However, the mechanisms of the protective role of their compounds against diabetes needs to be clarified and requires further study.

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Salivary lipids: A review

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Abstract

Saliva is produced by both large and small salivary glands and may be considered one of the most important factors influencing the behavior of oral cavity homeostasis. Secretion of saliva plays an important role in numerous significant biological processes. Saliva facilitates chewing and bolus formation as well as performs protective functions and determines the buffering and antibacterial prosperities of the oral environment. Salivary lipids appear to be a very important component of saliva, as their qualitative and quantitative composition can be changed in various pathological states and human diseases. It has been shown that disturbances in salivary lipid homeostasis are involved in periodontal diseases as well as various systemic disorders (e.g. cystic fibrosis, diabetes and Sjögren's syndrome). However, little is known about the role and composition of salivary lipids and their interaction with other important ingredients of human saliva, including proteins, glycoproteins and salivary mucins. The purpose of this review paper is to present the latest knowledge on salivary lipids in healthy conditions and in oral and systemic diseases.

Key words: lipids, saliva, salivary glands, salivary lipids

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Salivary glands and saliva

Saliva is produced by 3 pairs of major salivary glands (parotid, submandibular and sublingual) and numerous minor unpaired salivary glands scattered within the oral submucosa.[1–4] Depending on the nature of saliva and its constituent cell types, the following glands can be distunguished: purely serous salivary glands, known as the alveolar salivary glands (e.g. the parotid gland and von Ebner's glands, located at the base of the tongue); purely mucous glands called tubular salivary glands (a plurality of small submucosal glands); and mixed tubulo-alveolar salivary glands.[3,4] The latter glands may occur with a greater number of mucous cells (in the sublingual glands mucosal cells represent approx. 70%) or a predominance of serous cells (in the submandibular glands serous cells represent 80%).

Within a day, the salivary glands produce an average of 1000–1500 mL of saliva when at rest; stimulation may increase the amount of saliva secreted by several times.[5,6] In the absence of stimulation, the parotid, sublingual and minor salivary glands provide about 25%, 7–8% and 7–8%, respectively, of the whole saliva flow. The submandibular gland produces 60% of the unstimulated whole saliva (UWS) flow. When the salivary flow is stimulated, the parotid gland contribution increases by 10–15%, while the remaining salivary glands do not significantly increase saliva secretion.[3,4,6,7] The fluids secreted by the serous parotid glands contribute most of the peroxidase, proline rich glycoproteine (PRG) and amylase, while the mixed, submandibular, sublingual and minor salivary gland secretions are rich in mucin, which is responsible for the viscoelastic properties of saliva and for the blood-group activity of saliva.[8–11,15]

Saliva fulfills a variety of functions in the oral cavity. It is the liquid medium of the oral cavity ecosystem, providing hydration of the teeth and mucous membrane surfaces, thereby allowing articulation and swallowing. Saliva determines the protection of the oral tissues against biological, mechanical and chemical stimuli; allows the perception of taste and temperature; and is responsible for initial food digestion.[16] These functions and properties of saliva are attributed to electrolytes, buffering systems, proteins, glycoproteins, and lipids.[17–22] Salivary proteins, including glycoproteins, and lipids create a "network" which signals the presence of fats in the oral cavity and provides diagnostic and prognostic information.[23–25] Salivary proteins are well characterized, but data for characterizing salivary lipids and their functions is scarce and controversial.[16,21]

A brief review of lipids

There are many definitions of lipids. Previously, lipids were defined as non-polar compounds insoluble in water but easily soluble in organic solvents.[26] Christie describes lipids as "fatty acids and their derivatives, and compounds biosynthetically or functionally related to them".[27]

To facilitate a comprehensive worldwide classification of lipids, the International Lipid Classification and Nomenclature Committee, with the participation of the LIPID MAPS Consortium, divided lipids into 8 main groups: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides.[28,29] The first 5 of these groups of lipids are found in saliva, so the authors decided to present briefly the biochemical characteristics of these lipids. It is worth mentioning that many previous works about salivary lipids use the old division of lipids into neutral lipids, glycolipids and phospholipids, which can be quite confusing.[28,29]

Free fatty acids

Free fatty acids are the primary component of salivary lipids.[30] Free fatty acids are composed of a hydrocarbon chain terminated by a COOH group. The presence of a repeating series of CH_2 determines the hydrophobic nature of the fatty acids, while the COOH group is hydrophilic.[26,31] The proportion of hydrophilic and hydrophobic groups in the molecule accounts for the amphipathic nature of free fatty acids.

Due to the number of carbon (C) atoms, free fatty acids can be divided into short- (< 6 C), medium- (8–14 C), and long-chain (> 16 C) free fatty acids; however, the most common molecules usually contain from 4 to 30 carbon atoms.[32] Furthermore, saturated and unsaturated free fatty acids can be distinguished. In the body only unsaturated free fatty acids can be produced, in which the double bond is at position ω-9. Linoleic acid (ω-6) and α-linolenic acid (ω-3) cannot be synthesized by humans and must be provided by food. They are referred to as essential unsaturated fatty acids.[32]

Glycerolipids

Glycerolipids include all glycerol-containing lipids, among which the largest group are acyloglycerols (esters formed from fatty acids and glycerol). Acylglycerols exist in various physical forms: monoacylglycerols (MGs), diacylglycerols (DAGs), and triacylglycerols (TGs); triacylglycerols are more plentiful than the other 2 groups.[28,29] In contrast to MGs and DAGs, TGs do not form dispersed micelles.[32]

Glyceroglycans are another subclass of glycerolipids, characterized by one or more sugar moieties attached to glycerol via a glycosidic linkage.[29,33]

One of the most numerous subgroups of glycerolipids are glycerophospholipids (phospholipids, PH). The common structural element of this class of compounds is

phosphatidic acid (PA). The phosphate group of PA can be combined through an ester bond with a compound containing a hydroxyl group, for example choline, ethanolamine, serine, inositol or another glycerol, forming a phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, and diphosphatidylglycerol. Among linked fatty acids, acids attached at the C1 carbon atom are typically saturated, while those attached at the C2 atom are unsaturated with one or more double bonds.[29,31,32]

Glycerophospolipids consist of a subset of ether phospholipids, which may contain both ester and ether linkages. This group includes plasmalogens and a platelet activating factor (PAF).[29,31,32] In the plasmalogens, glycerol is attached at carbon atom C1 by an ether linkage to the long chain in the aldehyde enol form. There are 3 classes of plasmalogens: phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine.[29,31,32]

Sphingolipids are lipids that comprise aminoalcohol instead of glycerol: sphingosine or dihydrosphingosine.[29,31,32] The amino group of sphingosine may communicate via an amide bond with fatty acids, forming a ceramide.[34–36] A ceramide is a precursor of sphingomyelin and glycosphingolipids. Glycosphingolipids form a polar sugar chain (consisting of one or more monosaccharides), which is bonded O-glycosidically to the ceramide by a hydroxyl group at the C1 atom of sphingosine. Depending on the type of sugar chain, the following are distinguished: neutral glycosphingolipids, containing neutral saccharides (e.g. galactosylceramide and glucosylceramide); acidic glycosphingolipids (gangliosides containing one or more sialic acid residues); and sulphatides, containing a sulfonic acid group. Another group of glycosphingolipids, which has not yet been demonstrated in saliva, consists of basic glycosphingolipids and amphoteric glycosphingolipids.[28,29]

Cholesterol is a monohydroxy cyclic unsaturated alcohol, which is of exogenous origin or which may be synthesized de novo in the cytoplasm and endoplasmic reticulum of hepatocytes.[37] In body fluids, cholesterol is associated with lipoproteins. It is a major component of cell membranes (with the exception of the mitochondrial membrane) and is present in the cytoplasm in the esterified form with fatty acids.[31,32,38]

Phospholipids, cholesterol and sphingolipids are a structural and functional building material for the cell and intracytoplasmic membranes. Their presence determines the flexibility, fluidity and permeability of these membranes. It should be emphasized that phospholipids may play an important role as second messengers and in the processes of salivation.[39] This group includes inositol phospholipids. Neurotransmitters, such as acetylcholine, norepinephrine and substance P, connecting with their membrane receptors, activate G proteins, in particular protein GP(q). This protein activates phospholipase C, which hydrolyzes membrane phosphoinositol to form phosphatidylinositol-4,5-diphosphate (IP2). Phospholipase C phosphorylates cellular proteins that stimulate the processes of gene expression and cell proliferation, as well as activate membrane channels in the cell. It also increases the synthesis and secretion of proteins by the follicle cells and ducts of the salivary glands. IP3 is released from the membrane into the cytoplasm, where it binds to specific receptors in the membrane of the endoplasmic reticulum, which opens the channels for calcium ions and initiates the production of initial saliva.[6,10]

Salivary lipids in health

Lipids in saliva obtained from the major salivary glands were first detected by Doubleday in 1909.[40] During subsequent years, a quantitative analysis of the lipids in saliva began, focusing on particular classes of lipids.[41–45] Later precise quantitative analyses of lipids focused not only on the major groups of lipids, but also on fatty acids constituting individual groups of lipid. The data on the total lipid concentration in stimulated submandibular and parotid saliva is controversial, since they vary from 0.91 mg/100 mL to 9.52 mg/100 mL and from 0.21 mg/100 mL to 9.24 mg/100 mL, respectively.[46,47] However, early qualitative analyses of the lipids in saliva are similar in all available works in this field. Larsson et al. and Slomiany et al. demonstrated that in parotid and submandibular saliva more than 99% and 98% of lipids, respectively, were non-polar.[46,47] The neutral lipids were represented by cholesterol and its esters, free fatty acids and tri-, di- as well as monoglycerides, which was confirmed by subsequent studies by Tomita et al. and Brasser et al.[48.49] Brasser et al. also identified other neutral lipids, namely squalene and wax esters, and they found that salivary lipids were similar to the profile of skin surface lipids. The only polar lipids identified by Larssen et al. were phosphatidylcholine, phosphatidyletanolamine and sulphatidie, present in both parotid and submandibular fluid; these findings were in agreement with Slomiany et al.[46,50] In another work, Slomiany et al. demonstrated that parotid and submandibular saliva also contain glycolipids: glyceroglicolipidis and glycosphingolipids.[51] The latter were represented by galactosyl ceramide, glucosylceramide and lactosylceramide, and their content varied from 0.5% to 2% of the total salivary lipids. Neutral glyceroglicolipids accounted for approx. 60% of the total glyceroglicolipids, where hexa- and octaglucosylglyceroglucolipids predominated. Sulfated compounds were represented by tri- and tetraglucosylglucolipids.[51] The non-estrified (so-called free) salivary fatty acids – primarily palmitic acid – were the most abundant, followed by stearic, oleic and linoleic acids. Other non-estrified fatty acids were present at concentrations of less than 1μM.[52] Tri- , di-, and monoglycerides were composed mainly of palimitic and

stearic acid, while cholesteryl esters were characterized by the presence of large amounts of residues of palimitic, stearic and oleic acids. Salivary phospholipids included both saturated and unsaturated fatty acids ranging in size from 12 to 26 carbon atoms, wherein palmitic, stearic, oleic and eruic acids were present in the largest quantities.[47] The lipid core of the glyceroglycolipids consisted mainly of glyceryl monodocosyl, monoheneicosyl and monoeicosyl alkyl ethers as well as palimitic, stearic and eruic fatty acids.[53]

While the content and the composition of parotid saliva lipids do not differ significantly from those of submandibular saliva, and stimulation does not significantly change the fatty acid content or the lipid profile of saliva, considerable differences have been found in minor salivary gland lipids.[45] Slomiany et al. showed that 32.4% of the total lipids were neutral lipids, 44.6% were glycolipids and 23% phospholipids. The total amount of lipids in labial saliva was estimated to be 423.8 μg/mL of saliva.[54] Of the total neutral lipids, 43.8% was comprised of free fatty acids, 26.9% was cholesteryl esters, 15.4% triglycerides, 11.6% cholesterol and approx. 3% was made up of mono- and diglycerides. The free fatty acids, cholesteryl esters and triglycerides mainly included palmitic, oleic and erucic acids. Glycolipids were represented by glyceroglicolipids and glycosphingolipids. Glycosphingolipids were represented by glucosylceramide and lactosylceramide; their content in saliva ranged from 0.5% to 1.3% of the total lipids. Sulphated glyceroglycolipids, amounting to 25.4% of the total glycerglycolipids, were primarily tri- and tetrglucosyl compounds. Neutral glyceroglicolipids were demonstrated to be mono-, di-, tetra-, hexa- and octaglucosyl glyceroglucolipids; the last two were predominant, and accounted for 61.6% of the total glyceroglucolipids. The glycolipids were rich in oleic (30.1%) and erucic (19%) acids. Oleic acids (38.9%) were the main lipid component of the phospholipids, which represented 23% of the total labial lipids, (mainly phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine).

The total labial salivary lipids are 4–5 times more numerous than parotid and submandibular salivary lipids, and show a higher percentage of phospholipids and glycolipids. One possibility is that these differences may reflect the different processes in which serous and mucous cells secrete their products, which was explained by Tandler and Poulsen.[55] They suggested that mucous cells of the labial salivary glands secrete their products in a partly apocrine manner in which the release of the contents of the secretory vesicles is accompanied by the loss of part of the cell membrane. Therefore, the discharge of such cells contains lipids as part of the cell membrane (i.e. phospholipids and sphingolipids). It is also possible that the glycoproteins synthesized by cells of the mucous labial gland may combine with lipids of the cell membrane, and these cells are then secreted into the saliva.

On the other hand, the observed differences could be due to the interaction between lipids and proteins suspended in then aqueous solution of saliva. The relationship between salivary lipids and the protein components of saliva and the extent of interaction between them were partly demonstrated by Slomiany et al., who measured the distribution of lipids in the fractions of parotid and submandibular saliva following Bio-Gel A-50 column chromatography.[56] Over 50% of the submandibular salivary lipids, in particular glycolipids, free fatty acids, phospholipids and cholesterol, were detected in the fraction of saliva rich in mucins. In the parotid saliva, free fatty acids were detected in the fraction deprived of carbohydrates, whereas cholesteryl esters and phospholipids were associated with the carbohydrate-abundant fraction.

The physical state of the lipids present in saliva is unknown. Since lipids are practically insoluble in water, they must be complexed with the carrier in order to exist in the saliva. To date, the existence of 2 types of interactions between proteins and lipids has been demonstrated, i.e. hydrophobic and covalent interactions. Hydrophobically bound lipids are represented by neutral lipids, glycolipids and phospholipids; the only covalently attached lipids are fatty acids.[57,58] In a hydrophobic amino acid, side chains participate in the hydrophobic binding. These side chains are located on the protein surface, coming in contact with the hydrophobic parts of the lipids. The covalent bond is created, however, by the commonality of one or more pairs of electron-binding atoms.[31] It has been shown that in the submandibular glands most hydrophobic interactions with lipids are combinations with MUC5B (50%) and MUC7 (30%). These bonds are very strong and resistant to urea and cesium salts.[59,60] Slomiany et al. thought that lipids produced in the parotid glands exist as lipoproteins, and the main glycoprotein taking part in the interactions between proteins and lipids may be a proline-rich glycoprotein (PRG).[47]

The presence of such lipid carriers was recently demonstrated in the saliva of healthy subjects that showed the presence of apolipoprotein B.[61] However, little is known about the fragments of glycoproteins, where hydrophobic interactions with the lipids present in saliva occur. Salivary mucins contain highly glycosylated regions and regions devoid of carbohydrate moiety.[11] The removal of non-glycosylated regions of mucins was associated with a decrease in the phospholipid content as well as an increase in the number of glycolipids (the content of neutral lipids did not change significantly). These observations suggest that phospholipids may interact hydrophobically with non-glycosylated glycoproteins, while glycolipids bind to the glycosylated parts of mucins.[59,60]

Fatty acids are combined through an ester linkage with non-glycosylated regions of salivary glycoproteins, mainly large- and low-molecular mucins. In this bind-

ing, hydroxyl groups of serine, threonine and tyrosine may be involved.[62,63] Most frequently, covalent combinations occur: palmitic, stearic, erucic and oleic acids.[64,65] It has been shown that the attachment of a fatty acid takes place during the biosynthesis of salivary mucin in mucosal cells and occurs at the end of the processing of oligosaccharide chains, just prior to the formation of polymeric high-molecular-weight mucin. In addition, the connection of a fatty acid is catalyzed by a specific fatty acyl transferase and takes place in the region of a non-glycosylated protein chain; however, fatty acids are attached as hydroxy esters rather than thiol esters. Fatty acyl transferase activity was demonstrated not only in the mucus-secreting submandibular, sublingual and minor salivary glands, but also in the serous parotid glands. Because the major proteins synthesized by parotid glands are rich in glycoproteins, it is highly likely that PRG is acylated with fatty acids.[62,63]

Three sources of lipids in human saliva are considered: serum component transudation, exfoliative cells and salivary gland secretion.[66,67] It has been demonstrated that saliva, serum and cell membrane do not differ significantly with respect to neutral lipids and phospholipids, but they differ markedly with respect to glycolipids. The glycolipids occurring in serum and cell membranes are comprised mainly of glycosphingolipids, while mainly glyceroglycolipids are encountered in saliva.[51,68] Moreover, given the fact that serum contains 50–60 times more lipids than saliva, neutral lipids and phospholipids could originate mainly from serum, as has been demonstrated for cholesterol. Salivary cholesterol accounts for 15% of the neutral lipids in saliva, and men show more salivary cholesterol than women, both of which are true of serum; moreover, a positive correlation between cholesterol levels in saliva and in serum suggests that a high percentage of the salivary cholesterol originates from serum, which is in agreement with Slomiany et al.[37,47] A thorough analysis of the contribution of serum and membrane lipids other than cholesterol demonstrates that only a small amount (less than 2%) is plasma-born or is a result of cell exfoliation.[45] Several pieces of evidence point to the fact that salivary lipids mostly originate from the salivary glands. It has been shown that the biosynthesis of triglycerides, phospholipids, sulfated lipids and glyceroglycolipids takes place in the salivary glands.[69,70] Salivary glands contain 2 different sulphonyl transferases, carrying sulfate groups on the sugar chain of glycolipids. The 1st enzyme, present in the microsomal fraction, is transferred by the sulfate groups on the sugar chain of glyceroglycolipids, which are the main glycolipids of human saliva. The 2nd enzyme is present in the cytoplasmic fraction and is responsible for the sulfation of sugar chains of sphingolipids, which represent the main ingredients of the plasma membrane. It is therefore apparent that salivary glands synthesize both secretory and structural lipids.

Salivary lipids and systemic diseases

There is little data on the nature and content of salivary lipids in systemic diseases. Cystic fibrosis is an innate genetically determined human disease involving impaired secretion by the exocrine glands, including the salivary glands. So far, numerous studies have been published on disturbances of the composition and secretion of salivary proteins and glycoproteins in the course of this disease; however, only Slomiany et al. have published a qualitative and quantitative study on neutral lipids, phospholipids and glycolipids in the submandibular saliva of young patients with cystic fibrosis.[50,71–74] They showed that saliva from cystic fibrosis patients differs from the saliva from healthy controls with respect to the lipid content and composition. Submandibular saliva from cystic fibrosis patients contained 66% more lipids than the saliva of the healthy controls. The same neutral lipid composition was found in the saliva of healthy individuals and in the patients with cystic fibrosis, but their percentages were significantly different. The saliva of the cystic fibrosis patients contained significantly more fatty acids (54%), triglycerides (35%) and cholesterol (42%) than the saliva of the healthy subjects. The phospholipid content in the saliva of the cystic fibrosis patients was twice as high as in the saliva of healthy individuals, with no differences in the proportion of individual phospholipid classes in the 2 types of saliva samples. In the cystic fibrosis patients' saliva the presence of 0.2–0.5% glycosphingolipids was demonstrated, in addition to the glycorolipids occurring in the healthy controls' saliva. These patients' saliva contained significantly higher concentrations of di- and octaglucosyl glyceroglucolpids, while the saliva of the healthy individuals presented significantly higher levels of mono- and hexaglucosyl glyceroglucolipids. The acidic tetraglucosyl glyceroglucolipid constituted 80% of the sulfated glyceroglucolipids in the healthy individuals' saliva and 59% of the sulfated glyceroglucolipids in the cystic fibrosis patients' submandibular saliva. No significant differences were observed in the fatty acid composition of the lipid fraction of normal and cystic fibrosis saliva. Other studies by A. Slomiany et al. and B.L. Slomiany et al. demonstrated, however, that submandibular saliva of cystic fibrosis patients was characterized by significantly elevated levels of covalently-bound fatty acids.[57,75] Those authors hypothesized that the elevated levels of neutral lipids in the cystic fibrosis saliva were the result of increased transition of lipids from the blood, since lipids are known to be increased in the serum of cystic fibrosis patients.[76] Another theory was that the increase in neutral lipid levels may be a result of increased synthesis and secretion of salivary proteolipids, since it has been shown that other salivary proteins are also elevated.[72] Elevated concentrations of

Table 1. An overview of major studies about salivary lipids in health and disease

Aim/study design	Results/endpoints	References
The lipid composition of parotid saliva in young men and changes caused by stimulation	There were no significant differences in the composition of neutral and polar lipids in the parotid saliva as a result of stimulation.	45
The lipid content of parotid and submandibular saliva from caries-susceptible and caries-resistant patients	The levels of phospholipids and neutral lipids (i.e. free fatty acids, triglycerides and cholesterol esters) were significantly lower in the parotid and submandibular saliva of caries-resistant patients.	91
The relationship between high-molecular-weight mucus glycoprotein and covalently bound lipids from stimulated submandibular saliva of caries-susceptible and caries-resistant patients with blood type A	The submandibular saliva from caries-susceptible and caries-resistant patients are characterized by a different quantitative composition and different physiochemical characteristics of salivary mucus glycoproteins and covalently bound lipids.	51
The relationship between salivary lipids and proteins and glycoproteins in human saliva	Parotid and submandibular lipids show specific associations with the proteins and glycoproteins in human saliva.	56
The lipid composition of stimulated human labial gland saliva	Of all the salivary lipids, 44.6% were glycolipids, 32.4% neutral lipids, 23% phospholipids.	50
The lipid composition of dental plaque from caries-susceptible and caries-resistant patients	Plaque maturation is associated with elevated levels of neutral lipids and phospholipids, as well as reduced glycolipid content.	92
The quality and quantity of neutral lipids in parotid, submandibular and whole stimulated human saliva	Cholesteryl esters, cholesterol, triglicerydes, diglicerydes, monoglicerydes and free fatty acids accounted for 96-99% of the total salivary lipids.	46
The lipid and protein content in stimulated parotid and whole saliva from caries-susceptible and caries-resistant women	The lipid and protein content was significantly elevated in the parotid saliva from caries-susceptible patients.	48

phospholipids and the presence of glycosphingolipids in cystic fibrosis patients' submandibular saliva may be due to the presence of membrane lipids in an aqueous solution of saliva, and may also be the evidence of the existence of salivary abnormalities associated with the secretion of important proteins by cystic fibrosis patients.

Intracellular lipid accumulation in the salivary glands of diabetic rats has been observed, but it was greater in the parotid than in the submandibular or sublingual glands.[77,78] Morris et al. were the first to investigate the fatty acid profile, the time course of fatty acid accumulation and the effect of insulin treatment on salivary gland lipids in induced diabetes in a rat model.[77] They observed that stearic acid (C18:0) and linoleic acid (C18:2w6) showed a significant increase in all the salivary glands over 4 weeks after the streptozotocin injection to induce diabetes. In contrast, oleic acid (C18:1w9) was significantly down-regulated in the parotid gland after 3 and 4 weeks, which was in agreement with Mahay et al.[79] Decreases in arachidonic acid were significant in the submandibular gland in 3 weeks and in the parotid gland in 2 weeks of experimental diabetes. Insulin treatment decreased the amount of stearic and linoleic acids in the submandibular and parotid glands as compared to the controls. The authors concluded that the decreases in C18:1w9 and C20:4w6 may be a result of inhibition of desaturase enzymes, which are known to be stimulated by insulin.[80] A lack of insulin results in the accumulation of saturated (C18:0) and less unsaturated fatty acids (C18:2w6) as well as an increase in C18:2w6/C18:1w9 ratios. The latter is probably a result of the fact that the amount of linoleic acid is constant, because it comes from the diet, but cannot be desaturated and elongated, while oleic acid cannot be produced from stearic acid. The authors also posited that the accumulation of saturated and less unsaturated fatty acids may be due to a decrease in the consumption of lipids in the synthesis of cell membranes of secretory vesicles. The accumulation of saturated and less unsaturated fatty acids alter membrane structure and fluidity, as well as membrane enzyme function and the secretory function mechanism.[79,81] Treatment with insulin showed that the effects of diabetes on salivary lipids are rapid and totally reversible.

Salivary glands are a major target organ of inflammation in the course of Sjögren's syndrome (SS).[82,83] An immunohistological analysis of the salivary glands of SS patients has disclosed lymphocytic infiltration with a predominance of T lymphocytes, especially CD4 + cells (the ratio between CD4 + and CD8 = 2:1), as well as a reduced number of B lymphocytes and macrophages, which may be the immediate cause of destruction and dysfunction of the salivary glands.[84] Changes in the total amount of saliva secreted are accompanied by changes in the quality of the saliva; however, most researchers have analyzed the protein component of saliva in the course of SS, and data on lipids in the saliva of SS patients is scarce.[85–88] Parotid salivary lipid analyses have demonstrated that patients with SS had twice as many total lipids as healthy controls, 4 times as many glycolipids and 20 times more phospholipids. Neutral lipids in SS patients contain a higher percentage of mono-, di- and triacyloglycerols and cholesterol than healthy controls, and a lower percentage of cholester-

ol esters. A phospholipids profile showed a significant increase in phosphatidylcholine in SS, while a profile of glycolipids revealed an increase in sphingomielin.[89] More recently, Tishler et al. identified eicosanoids in the saliva of SS patients.[90] They demonstrated a significant increase of prostaglandin E_2 and thromboxane E_2 in mixed saliva from SS individuals as compared to healthy controls and xerostomic patients. They stated that elevated concentrations of eicosanoids in the saliva of SS patients could be a good marker of an inflammatory process in the salivary glands in the course of this disease and could help to identify patients suffering from xerostomia, which may result from other diseases.

The results of the main studies about human salivary lipids in health and disease are summarized in Table 1.

Summary

Salivary lipids are among the most essential cellular components of human saliva. In the oral environment they determine the flexibility, fluidity and permeability of cellular membranes and participate in intercellular transport and the signal transduction pathways between the salivary glands and other tissues. Although the qualitative and quantitative content of salivary lipids can change in various pathological states and diseases (e.g. cystic fibrosis, diabetes and Sjögren's syndrome), little is known about the role and composition of salivary lipids, and about their interaction with other important ingredients of human saliva, including proteins, glycoproteins and salivary mucins. Only accurate knowledge of the qualitative and quantitative composition of lipids and their role in the oral cavity will make it possible to understand better the pathological processes occurring in the oral cavity and in the parotid and submandibular salivary glands. There is, therefore, a need for further research on the role of salivary lipids in both health and disease.

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Relationship between toxoplasmosis and schizophrenia: A review

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Abstract

A growing body of evidence suggests a correlation between schizophrenia and exposure to infectious agents. The majority of studied cases concerns the infection caused by *T. gondii*, an obligatory intracellular parasite that infects about 1/3 of the entire human population, according to the available data. The acute stage of the disease, predominantly short-lived and transient, transforms into the latent and chronic phase in which the parasite localizes within tissue cysts, mainly in the central nervous system. The chronic toxoplasmosis, primarily regarded as benign and asymptomatic, might be responsible, in light of current scientific evidence, for a vast array of neuropsychiatric symptoms. Numerous epidemiological case-control studies show a higher prevalence of *T. gondii* infestation in individuals with various psychiatric and behavior disorders, including schizophrenia. This paper tends to review the relevant studies that demonstrate links between schizophrenia and *T. gondii* infestation, of which the latter may be acquired in different developmental phases. Apart from epidemiological correlation studies, some papers on other associations were also presented, describing putative patophysiological mechanisms that might be at least partly responsible for chronic infection-induced neuromediator disturbances, together with morphological and functional alterations, e.g., low-grade neuroinflammation, which are likely to induce psychopathological symptoms. Toxoplasmosis is only one of the putative infectious agents that derange correct brain growth and differentiation, alongside genetic and environmental factors. All of them may lead eventually to schizophrenia. A better knowledge of infection mechanisms and its influence on neurobiochemical and neuropathological pathways may enable more efficient therapy and the prevention of this devastating disease.

Key words: neuropsychiatric symptoms, schizophrenia, *Toxoplasma gondii*

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Toxoplasmosis is an infectious disease caused by a parasitic protozoan *Toxoplasma gondii*, which affects approximately 1/3 of entire human population. It is, therefore, the most common disease of infectious origin, more widespread than malaria, tuberculosis, and other infections and parasitoses, which are commonly regarded as serious global threats.[1] *T. gondii* is able to infect almost all species of mammals and numerous species of birds (warm-blooded animals), while the incidence of infestation in humans varies according to the geographical region, climate, and hygienic and nutritional habits.[1,2] In humans, 2 principal forms of infection occur: inherited – which is transmitted vertically from the mother to the fetus by means of placental tissues; and acquired – mostly via the digestive route by ingesting undercooked meat dishes or accidental contact with cat feces, which is the unique definitive host of *T. gondii*.[2]

A considerable number of health disorders and diseases has been found and studied in correlation with toxoplasmosis. They were presented in more detail elsewhere.[1] It was thought earlier that such a latent form of the disease does not lead to any serious sequelae and it is only the reactivation of infection due to individual disorders of immunity that poses a real threat.[3] However, a growing body of evidence suggests unequivocally that a persistent and dormant infestation might be responsible for various neurologic and psychiatric symptoms.[4] The majority of evidence comes from epidemiological case-control studies which denote a higher incidence of chronic *T. gondii* infection in individuals suffering from various psychiatric disorders in comparison with healthy people (controls).[5–7] The diagnosis of the infection is based on an analysis of specific antibody prevalence.

Schizophrenia is a severe psychiatric disorder with a lifetime prevalence of approximately 1%, regarded as the 9th most common cause of disability all over the world.[4]. The disease manifests itself most often from late teens to early adulthood, although the psychotic episodes can persist throughout the entire life of the patient. Schizophrenia is a heterogeneous disease and its origins are slowly being disclosed. Up to now, no unique causal agent has been detected; therefore, it is legitimate only to describe some factors that were shown to be positively correlated with schizophrenia. The known risk factors include: genetic predisposition, neurodevelopmental disturbances and environmental factors, including infectious agents.[4,5]

The interest in infectious origins of psychiatric disorders can be traced back as early as to the 19th century. In 1896, "Scientific American" published an article with an expressive title: "Is insanity due to a microbe?"[5] It prompted temporary attention in the study of the correlation between infections and psychiatric disorders at the beginning of the previous century. These theories then waned until the closing years of the previous millennium, when the correlation started to be examined again. In the second half of the 20th century, a few dozen papers, studying the correlation between *T. gondii* infestation and psychiatric disorders (especially schizophrenia), were published. Moreover, several reports were issued dealing with other potential infectious factors, acting both at the pre- and perinatal period (influenza A virus, herpes simplex 2, poliomyelitis), and also after delivery – predominantly viruses and bacteria causing meningitis and encephalitis.[6] However, in the majority of cases the attention of scientists was focused on *T. gondii* infection.[6,7]

Note that the very first report was based on studies conducted in Poland (Gdańsk) in 1953 and concerned the investigation of toxoplasmosis prevalence among psychiatric department patients. The rate of infection was significantly higher in patients compared with controls (52% vs 25%; OR 3.19), but the application of now outdated diagnostic tests (skin tests) and the lack of precise inclusion criteria for both groups make this research useless for contemporary meta-analyses; it has only historic value.[8]

Correlation between *T. gondii* infection and schizophrenia

Owing to the high affinity of *T. gondii* to the nervous tissue (predominantly the glia cells – astrocytes) and the established association with inborn cerebral disorders, the interest of researchers was for a long time directed at potential links between exposure to the parasite and the onset of severe psychiatric disorders.[6,9] As for schizophrenia, such correlation was established on base of the following studies.

Higher serological prevalence of *T. gondii* infection in schizophrenic patients

In 2007, Torrey et al. published a review of available research material (also unpublished so far), which included not only English scientific magazines, but also reports from the entire world, written in various national languages.[6] They identified 42 papers, issued over a 50-year span, 23 of which conformed strict inclusion criteria for conducting meta-analysis. The studies covered a total of 3,873 patients and 7,046 people from control groups. The combined odds ratio (OR) was 2.73 (2.10–3.60; $p < 0.000$).[6] Seven of these reports dealt only with patients in the first episode of schizophrenia, but the results of this subgroup did not significantly differ from the entire material (2.54 vs 2.73). Six papers were not previously published and their combined OR was 2.16, compared to OR = 2.97 of the published reports.[6] This is consistent with the common research experience that the works of higher statistical and clinical significance are submitted for publication more readily than reports of lesser significance.[5,6]

The above-mentioned study group published an update of the meta-analysis in 2012. Fifteen additional papers

were included and the new combined odds ratio that was calculated on the basis of all studies, older and new ones, amounted to 2.71 (95% CI: 1.93–3.80).[7]

The results presented above suggest that some individuals with schizophrenia have an increased prevalence of antibodies to *T. gondii*. The odds ratio of 2.73 (2.71) may seem modest, but it exceeds any other genetic or environmental factors found so far, showing without any doubt that *Toxoplasma* is linked in some way to a great number of schizophrenia cases.[7] Moreover, new reports are still being published and they mostly replicate the earlier results.[10] Taking *T. gondii* infestation into account is also crucial, since individuals with higher titers of IgG antibodies often experience more severe symptoms of psychosis and it was observed that infections in schizophrenic patients might be correlated with increased mortality.[6] Dickerson et al. studied the prevalence of anti-*T. gondii* antibodies in 358 patients with schizophrenia who subsequently underwent a follow-up for 5 consecutive years. It was noted that mortality in seropositive patients was 8.6%, while that of seronegative ones was 1.7% ($p < 0.003$).[11]

Increased risk of schizophrenia in the offspring of mothers with serologic signs of infection detected during pregnancy

A growing body of evidence points to maternal infection as a risk factor for schizophrenia in children.[12] These infections include influenza, genitourinary system infections, and also toxoplasmosis.[13] These microbes certainly cause inherited brain malformations and a vast spectrum of cognitive and behavior disorders in childhood.[14] Furthermore, it has been known for many years that people exposed in utero to rubella, measles, toxoplasma, herpes virus 2, and various other infections are more likely to suffer from neurodevelopmental disorders, mental retardation, learning difficulties, sensory dysfunction and structural brain malformations.[14]

As far as toxoplasmosis is concerned, cohort studies of blood samples taken from mothers in the perinatal period revealed an over 2-fold increase (OR 2.61; 1.0–6.82) of IgG antibodies to *T. gondii* in mothers, whose children later developed schizophrenia. It is worth noting that none of the studies detected an acute infection (which is confirmed, e.g., by the presence of specific IgM antibodies), but only serological markers of earlier contact with the parasite (latent infection).[6,15]

Increased prevalence of antibodies to *T. gondii* in newborns who later developed schizophrenia

Research conducted in 2007 in Denmark studied blood samples obtained from earlier neonatal screening tests. The IgG and IgM anti-*T. gondii* antibody levels were measured and the results were attributed to respective adult patients, suffering from schizophrenia-spectrum disorders. Positive IgG antibodies were detected more often in people with schizophrenia (OR 1.79; $p < 0.05$).[16]

Assuming that no IgM antibodies suggesting an acute infection were detected but only those of IgG class that must have been transmitted from mothers via placenta (in children, the ability to secrete IgG antibodies appears no sooner than in the 3rd month after birth), the results of both studies mentioned above suggest one explanation – earlier maternal exposure to *T. gondii* may account for a risk factor of schizophrenia in the offspring.[13,16]

The ability to destroy *T. gondii* cells in vitro by some antipsychotic and normothymic drugs

Three independent studies detected a significant correlation between the first psychotic episode in schizophrenia and serological markers of toxoplasmosis ($p < 0.001$), suggesting that these patients must have acquired the infection earlier.[6] The difference was less marked in patients with a long-standing disease. An assumption was, therefore, made that antipsychotic drugs or other agents used by patients may decrease the titers of circulating antibodies to *T. gondii*.[6] It was discovered even earlier that some psychotropic drugs inhibited in vitro proliferation of the parasite in human fibroblasts. The first relevant study, performed by Jones-Brando et al., investigated the influence of 12 anti-psychotic and normothymic drugs on the proliferation of *T. gondii* cells.[17] Valproic acid in conjunction with haloperidol had the strongest inhibitory effect, while risperidone and trimethoprim were weaker inhibitors.[17] Since that time, several other studies that examined the effect of different psychotropic drugs (including those mentioned above) on the proliferation of the parasite have been published, generating various, sometimes contradictory results. The inhibitory action of the drugs on *T. gondii* growth depends on developmental form of the parasite, stage of infection and duration of illness, but available data suggests that some therapeutic agents used in schizophrenia (e.g. fluphenazine, thioridazine, trifluoperazine, zuclopenthixol, alongside with cyamemazine, olanzapine and loxapine) may exert their effect also in vivo, while their relieving of psychotic symptoms may, at least to some degree, depend also on the inhibition of the parasite metabolism.[18,19] Furthermore, there exists a report in the literature that depicts a case of depression resistant to treatment with standard agents in a patient with *T. gondii* infection. A marked improvement was observed instantly after the parasite had been eradicated with trimethoprim and sulphadiazine.[20]

Studies conducted on rats showed that haloperidol or valproic acid reversed behavioral changes induced by *T. gondii* infection, such as decreased neophobia and transforming the innate aversion to cat odor into unnatu-

ral attraction, on par with standard anti-parasitic regimen. However, those drugs did not prevent acute infection or decrease the number of tissue cysts in the animal brain.[18] What is important, a chronic and occult toxoplasmosis induces specific behavioral changes also in humans, which are well described, especially by Flegr et al.[18,21]

Neurotransmitter secretion disturbances due to *T. gondii* infection which may induce psychotic symptoms

In the natural course of schizophrenia and the manifestation of its symptoms, a pivotal role is attributed to the derangement of secretion or the action of several neurotransmitters, which actually forms the background of pharmacological therapy, directed not only at positive symptoms of the disease, but also at the negative ones.[22] The principal substances involved in pathogenesis of schizophrenia symptoms are: dopamine, serotonin (5-HT), GABA, glutamate, etc.[4,22] Symptoms of the disease, such as hallucinations and delusions, are eliminated or alleviated by dopaminergic D2-receptor-blocking agents; this constitutes the primary, but not exclusive mechanism, of neuroleptic drug action, especially those of the first generation.[22]

Available data suggests that *T. gondii* may elicit or worsen the symptoms of neurodegenerative diseases and psychiatric disorders via the modulation of secretion or the effects of some neurotransmitters, predominantly dopamine.[3,4] The parasite genome includes 2 aromatic acid hydroxylase genes, which may directly influence the biosynthesis of dopamine and/or serotonin.[4] In researches on mice, it has been shown that chronic (but not acute) infection with *T. gondii* elevates the local brain dopamine concentrations, as in patients suffering from schizophrenia.[4,23] Another putative mechanism may be a tryptophan metabolism disruption, which is a typical immunological reaction to a parasite infestation and it leads to accelerated tryptophan depletion by IFN-γ-inducible enzymes: indoleamine-2,3-dioxygenase (IDO) and tryptophan dioxygenase (TDO).[3,4] Disruption of metabolism decreases the levels of tryptophan that is indispensable for *T. gondii* growth and replication, but also generates the accumulation of some harmful metabolites, particularly kynurenic acid (KYNA), an antagonist of N-metyl-D-aspartate- (NMDA) and nicotinic receptors.[4,24] High concentrations of KYNA, detected in cerebrospinal fluid of schizophrenic patients, are one of the potential causes of cognitive disorders in schizophrenia.[4] The principal source of KYNA are astrocytes, the cells preferably chosen by *T. gondii* for replication.[24] To the best of the authors' knowledge, there is a lack of direct evidence so far that parasite-infected cells secrete high amounts of KYNA via IDO-mediated tryptophan degradation, but this seems highly probable, according to some authors.[3,4]

The symptoms of toxoplasmosis in some patients with an acute infection

The symptomatology of acute toxoplasmosis, which is a reactivation of chronic infection in immune-compromised patients, has been well described.[25] Case reports of patients suffering from AIDS with a relapse of latent infection indicate that as much as 60% of them report psychiatric disturbances, including: delusions, auditory hallucinations and thought disorders.[4,25] However, psychopathological symptoms are also relatively frequent in various stages of HIV infection and AIDS, which might be presumably caused by the virus itself, not by opportunistic infections.[26]

Moreover, it has been observed that in some healthy and immune-competent individuals an acute infection may manifest itself with delusions and hallucinations, the symptoms specific mainly but not exclusively of schizophrenia. A study of 114 people with acquired toxoplasmosis detected fairly frequent and serious psychiatric disturbances in 24 of them.[25] In some case reports, the initial symptoms of toxoplasmosis were highly specific of schizophrenia and it was not until the follow-up of patients and the onset of neurological symptoms that the possibility of infection was considered. After anti-parasitic therapy, psychotic symptoms vanished in these subjects.[25] It is suggestive that *T. gondii* infestation may per se induce in some individuals patophysiologic mechanisms that change brain neurotransmitter levels, which eventually leads to psychopathological manifestations.[3,4,21] However, such changes should coincide with other known risk factors, because *Toxoplasma* per se cannot induce schizophrenia, but only influence liability to its development.

Parasite affinity to the brain cells

T. gondii cells show high degree of neurotropism and after an acute stage of infestation, the parasite migrates within the brain tissue (predominantly gray matter), localizing in astrocytes, microglia and neurons.[4,27] The dormant form of *T. gondii* (bradyzoite) can persist in the host brain for many years, presumably until the end of its life.[28,29] Tissue cysts are not constant and passive in nature, but are rather active. They do not cause tangible symptoms in immune-competent individuals, but are subjected to continuous remodeling, vanishing in some brain regions and appearing in others.[30] Available neuroimaging methods cannot visualize single cysts; however, great expectations are put in novel magnetic resonance techniques (with the application of contrast solutions: gadolinium and ultrasmall superparamagnetic iron oxide particles (USPIO)).[29] These obstacles make localizing cyst aggregates in humans particularly challenging (brain biopsy is indispensable), but the precious evidence is supplied by means of studying animal models and histological examination of human brains, made post mortem, which is very rare, unfortunately.[29] Given the results, it can be

concluded that: 1) *T. gondii* is localized within the brain structures responsible for thought processes, emotions and sensorium: amygdalae, hippocampus, striatum, thalamus and cerebellum; 2) latent toxoplasmosis and schizophrenia both induce similar, often discrete changes in brain morphology: gray matter atrophy, ventricle system enlargement and microscopic presence of inflammatory cells in perivascular spaces, around cerebral aqueducts and within the pia mater, features commonly detected also in neurodegenerative diseases.[27,31] Furthermore, considering that chronic and occult *T. gondii* infection causes discrete symptoms of low-grade neuroinflammation, inevitably linked to tissue cyst remodeling and, according to most available studies, additional combined treatment of schizophrenia with anti-inflammatory drugs (e.g. celecoxibe) elicits a better therapeutic response, another putative link can be drawn between the infection and psychiatric disorders.[30,32] As for anti-inflammatory treatment, it is most successful in the beginning stages of the disease (prodromal phase) and applying the therapy thereafter diminishes the percentage of positive reactions.[32]

Other associations

Some less important risk factors for schizophrenia include contact with cats in childhood, which may indeed generate an elevated risk.[28,33] It is true that not all reports confirm the correlation between *T. gondii* infection and the possession of domestic pets (especially cats) but they postulate maintaining a high level of personal hygiene and the appropriate management of cat litters.[1,28] It is worth mentioning that cat bites have been linked to cases of depression, which is a disorder different from schizophrenia but also attributed to disturbances of neuromediator balance.[4,33] Finally, the association between toxoplasmosis and schizophrenia is consistent with infection models in animals, pointing to constant and repetitive behavioral changes in animals with *T. gondii* infestation.[34]

Critical remarks and summary

When creating links between the parasite and schizophrenia, at least some problematic issues should be taken into consideration. First, the majority of available records deals only with the serological markers of infection and *T. gondii*, which are not directly detected in body fluids or tissues.[5,6] However, it must be admitted that the countries with a particularly high rate of infection (France, Ethiopia) do not exhibit a significantly higher prevalence of schizophrenia (however, available data is equivocal and in the Scandinavian countries, where the rate of infection has consistently declined over several decades, a lower incidence of psychoses is noted).[6]

The most serious problem with plausibility is that the majority of patients with schizophrenia do not have measurable levels of antibodies to *T. gondii*.[5,6] Despite the higher rate of infection in schizophrenia patients and their mothers, most people living in areas of low infection prevalence (e.g. USA) do not have detectable antibodies.[1,6] It is highly probable that schizophrenia is of heterogenous origins and infection by the parasite does not play a direct and deciding role in the etiology of most cases within the population.[5,6] However, it cannot be excluded that the circulation of specific antibodies slowly declines over time and exposure in the perinatal period or in early childhood will not result in their detectable levels several decades after.[15,35,36] Moreover, agents used in schizophrenia are able to inhibit the parasite proliferation, which lowers antibody titers in patients undergoing standard therapy.[6,19]

Accumulating scientific evidence suggests that exposure to infectious agents during pregnancy and early childhood constitutes a risk factor of schizophrenia in adult life. The results are particularly meaningful for the parasite *T. gondii*; however, some other microorganisms that share the same pathogenic and biological traits may also be involved.[6,7,37] The mechanisms of the associations are most probably diverse and include direct infection, exposure to some common environmental factors, as well as maternal-fetal transfer of inflammatory mediators.[6,37,38] The individual susceptibility to various pathogens presumably depends on genetic constitution of the host, which is particularly important in schizophrenia, due to the strong correlation between the risk of the disorder onset and genetic factors (highly elevated risk in the offspring and siblings), which have unfortunately remained unidentified up to now.[39] The persistent and chronic inflammatory process in the brain, which most commonly occurs without tangible, specific symptoms (neuroinflammation), is currently being linked to a plethora of neuropsychiatric disorders, e.g., dementia syndromes and Parkinson's disease. Some of those disorders correlate also with *T. gondii* infestation.[1] Moreover, the inflammatory component of schizophrenia might be presumably evoked by activation of brain cells (microglia, astrocytes), due to latent toxoplasmosis.[37,38,40] Lack of universal response to anti-inflammatory and anti-parasitic treatment in schizophrenia has also been regarded by some authors as a proof against its infectious origin.[32,37] It should be noted though that the number of available studies is limited and their results inconclusive, which suggests further studies rather than definitive conclusions.

Summary

Currently proposed mechanisms of schizophrenia pathogenesis assume a simultaneous participation of genetic, infectious and environmental factors that act together and derange the correct growth and differentiation of the brain, i.e. neurodevelopmental theory of schizophrenia.[37,38] Toxoplasmosis is, therefore, not the unique pre-

sumed infectious agent, involved in its etiology; however, it is certainly the best studied and documented one. Better knowledge of neuropathogenic mechanisms might help identify common paths of damage and disruption, similar to other infections, which will enable more efficient therapy and prevention of schizophrenia.

Unfortunately, these ideas are almost universally disregarded within the psychiatric society in Poland. The origin of this almost hostile disregard remains largely unknown, especially in view of a complete lack of such prospective or epidemiological studies in our country. Each association is potentially worth following; therefore, the authors strongly argue that they do not claim to have depicted a universal theory but a mere pathway that probably merits further studies. According to current knowledge, *T. gondii* has been clearly excluded as the causal factor of schizophrenia, although it is able to influence some metabolic and developmental pathways, leading, in consequence, to altered predisposition to the disease.

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