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The Editorial Board kindly informs that since 2014 *Nowiny Lekarskie* has been renamed to *Journal of Medical Science*.

The renaming was caused by using English as the language of publications and by a wide range of other organisational changes. They were necessary to follow dynamic transformations on the publishing market. The Editors also wanted to improve the factual and publishing standard of the journal. We wish to assure our readers that we will continue the good tradition of *Nowiny Lekarskie*.

You are welcome to publish your basic, medical and pharmaceutical science articles in *Journal of Medical Science*.

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The Journal of Medical Science applies the ethical principles and procedures recommended by COPE (Committee on Conduct Ethics), contained in the Code of Conduct and Best Practice Guidelines for Journal Editors, Peer Reviewers and Authors available on the COPE website: https://publicationethics.org/resources/guidelines

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ORIGINAL PAPER

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Ischemia-modified albumin in migraine patients during interictal period

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ABSTRACT

Aim. Ischemia-modified albumin (IMA) is a marker of myocardial ischemia and may be affected by ischemia occurring in other tissues. Migraine has been reported as a risk factor of ischemic stroke or cardiovascular events. Dysfunction of endothelial cells, as well as association with arteriopathies was evidenced in migraine patients. The aim of this study was to evaluate interictal IMA in migraine patients.

Material and Methods. Fifty migraineurs aged 38 ± 9 years were included in the study. The control group consisted of 25 healthy volunteers aged 37 ± 8 years. In all subjects neurological examination was carried on, as well as clinimetric evaluation with the use of: MIDAS, MIGSEV, QVM, VAS and VRS. Ischemia-modified albumin was evaluated by means of spectrophotometric method with the use of cobalt chloride. The concentrations of total cholesterol, HDL- and LDL-cholesterol, triglycerides, homocysteine, C-reactive protein and Lp(a) were analyzed with routine spectrophotometric methods.

Results. IMA was significantly (P = 0.0108) higher in migraine patients (0.101; 0.00–0.327 O.D.) than in controls (0.00; 0.00–0.102 O.D.; median; interquartile range). Migraineurs with aura have also higher IMA than controls. IMA correlated (rS = 0.383, P = 0.0073) with VAS and with homocysteine concentration (rS = 0.430, P = 0.0026). Multiple regression analysis of IMA and atherosclerosis risk factors showed significant correlation (P = 0.0247) with HDL cholesterol (R = 0.2958) and triglycerides concentrations (R = 0.3285).

Conclusions. IMA formation in migraine patients, as a marker of oxidative stress even during interictal period in patients with hyperhomocysteinemia and/or hypertriacylglyceridemia can reflect a milieu of factors which further increases the risk for cardiovascular complications.

Keywords: headache, ischemia-modified albumin, oxidative stress, cerebrovascular diseases.

Introduction

The pathomechanisms considered in migraine involve complex milieu of factors acting both on neural and vascular compartments in the brain. „Stroke-migraine continuum" hypothesis links both sides of those pathomechanisms [1]. Depolarization which propagates across grey matter in the brain, known as cortical spreading depression (CSD) or spreading depolarization (SD), is related to neuronal compartment and contributes to the development of aura symptoms [2, 3]. The relations between CSD and cerebral blood flow (CBF) show quadriphasic pattern: short CBF decrease – marked CBF increase – minor, but sustained CBF elevation – sustained CBF decrease (oligemia) [4]. Oligemia related to CSD, constriction of intracerebral large arter-

ies and endothelium-related hypercoagulability may cause cerebral ischemia in migraine patients [5].

Migraine is considered not only as a risk factor of stroke, but also of other cardiovascular diseases. Meta-analysis showed that the risk of ischemic stroke in migraine patients is more than 2 times higher compared to nonmigraineurs [6], but the results for hemorrhagic stroke are inconsistent [7]. In the HUNT Study (Nord-Trøndelag Health Study in Norway) [8] Framingham risk score, which includes age, sex, total cholesterol and HDL-cholesterol concentrations, smoking status and systolic blood pressure stratified for the use of antihypertensive medication was used as indicator of cardiovascular risk in headache patients [9]. In non-migraine headache patients, migraineurs without aura and migraineurs with aura Framingham risk score was increased compared to controls [8]. The study done in USA also showed that migraine, both with and without aura, is associated with higher risk for cardiovascular diseases including myocardial infarction, stroke, and claudication [10].

Human serum albumin is the main plasma protein. It consists of 585 amino acids formed into a single polypeptide with three homologous helical domains. First three amino acids in N – terminal region form specific binding sites for transition metals like cobalt, copper, nickel and are the most susceptible region for degradation compared to other regions of albumin. The mechanisms involved in ischemia/reperfusion, especially those related to oxidative stress, cause changes to albumin and reduce the transitional metal binding capacity [11]. Such a transformed albumin is called ischemia-modified albumin (IMA). It is proposed to be a biomarker of ischemic heart disease, as it has been noticed that serum albumin of individuals with myocardial ischemia exhibits reduced biding to cobalt in comparison to non-ischemic ones [11]. What is more, this reduced biding of cobalt (Co(II)) to serum albumin was also observed among patients with transient myocardial ischemia after elective percutaneous transluminal coronary angioplasty [12]. IMA was increased in cases of oxidative stress due to ischemia reperfusion injury [13, 14, 15], thus may distinguish between ischemic and non-ischemic patients, which may be clinically important as far as immediate diagnose is concerned.

With this background in mind we have undertaken the study on IMA in migraine patients during interictal period. The aim of the study was to evaluate IMA in migraineurs with and without aura and to correlate it with cardiovascular risk factors determined.

Material and Methods

Patients

We have included in the study 50 migraine patients aged 38 ± 9 and diagnosed basing on the criteria of International Headache Society. The exclusion criteria were: history of cardiovascular disease, hypertension (defined as systolic blood pressure exceeding 140 mm Hg or diastolic blood pressure over 90 mm Hg), diabetes, hyperlipidemia, pregnancy or lactation, inflammation, allergy, and regular use of vasoactive drugs (except hormonal contraceptives).

The control group consisted of 25 healthy volunteers aged 37 ± 8 years.

None of migraine patients and controls showed symptoms of any acute or chronic disease in medical history, during physical examination, and in routine laboratory tests.

All study participants underwent neurological examination and clinimetric evaluation with the use of: MIGSEV (4-item questionnaire which evaluates migraine intensity as mild, moderate or severe basing on pain, nausea, limitation of everyday activity and tolerability of symptoms) [16], MIDAS (Migraine Disability Assessment measures migraine – associated disability basing on 7 questions) [17], QVM (Qualité de Vie et Migraine evaluates quality of life basing on 20 questions) [18], VAS (Visual Analogue Scale: 0 to 10 points, with 10 indicating the most severe pain) and VRS (four-point verbal rating scale: 0 to 3, with 3 for severe pain).

Fasting blood samples were collected in the morning not earlier than 4 days after migraine attack and/or administration of triptans or ergot alkaloids. Routine laboratory tests were performed immediately after blood collection. Samples for IMA evaluation were stored at -80° C until analysis. White blood cells count (WBC) was evaluated with the use of automated hematology analyzer. C-reactive protein (CRP), total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides (TAG) concentrations were analysed with routine methods. Lipoprotein a (Lp(a)) and homocystein concentrations were analyzed by means of enzyme-linked immunosorbent assay (ELISA).

Measurements

Ischemia modified albumin was estimated spectrophotometrically using Sigma-Aldrich (Germany) reagents and spectrophotometer Statfax 1904 Plus (Awareness Technology, USA), according to the manual method developed by Bar-Or et al [19, 20].

The assay is based on the statement that ischemia (first reported for myocardial ischemia) causes some changes in human plasma albumin resulting in reduced binding of exogenous cobalt, Co(II).

The unbound exogenous cobalt (Co) reacts with colored marker (dithiothreitol, DTT) and increased intensity of the reaction reflects a reduced metal binding capacity of albumin, thus increased modification of albumin. The result of the determination is expressed as optical density (O.D.) [20, 21].

The assay procedure

Patients serum (200 µl) was added to 50 µl of a cobalt chloride solution (1 g/l). Next, vigorous mixing and a 10-minute incubation in standard room temperature were performed. After then, 50 µl of 1.5 g/l DTT solution was added, what was followed by mixing and 2-minute incubation in standard room temperature. Finally, 1.0 ml of 9.0 g/l solution of NaCl was added to obtain the assay sample. The procedure of preparing the blank sample required 50 µl of double-distilled water instead of DTT. The absorbance of the assay sample was read at 470 nm against the blank sample, in duplicate.

The intra- and inter-assay coefficients of variation were 4,8% and 6,2%, respectively.

Statistics

Statistical analysis was performed with the use of a licensed version of MedCalc 16.8.4 (64 bit) software. First, the distribution of results was analyzed with the D'Agostino-Pearson test. The variables that had a normal distribution were expressed as mean ± SD and tested with t-Student test; the variables that had a non-gaussian distribution were expressed as median and interquartile range and analyzed with the Mann-Whitney test.

Ethics

All participants signed written informed consent. The study protocol was approved by the Ethic Committee at the Poznan University of Medical Sciences.

Results

Neurological examination of migraine patients did not revealed any deficits. With the use of clinimetric measures we have noticed high intensity of pain reflected by VAS and VRS scores, intermediate to high severity of attacks in MIGSEV score, and severe functional impairment in MIDAS and QVM scale (**Table 1**). There were no differences in clinimetric scores between migraineurs with and without aura (**Table 1**).

Total cholesterol concentrations were higher in migraine patients with and without aura compared to controls. In migraineurs with aura HDL cholesterol levels were higher than in controls. We did not observed differences in results of other routine laboratory tests between migraine subjects and controls (**Table 2**).

IMA was significantly (P = 0.0108) higher in the whole group of migraine patients (0.101; 0.00 to 0.327 O.D.) than in controls (0.00; 0.00 to 0.102 O.D.; median; interquartile range). The subgroup of migraineurs with aura have also higher IMA than controls (**Table 3**).

IMA correlated (rS = 0.383, P = 0.0073) significantly with clinimetric evaluation with the use of VAS (**Figure 1**). Homocysteine was the only result in the panel of routine laboratory tests which correlated with IMA (rS = 0.430, P = 0.0026) (**Figure 2**).

In multiple regression analysis of relation between IMA and plasma lipids in the model including total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides (TAG) and Lp(a) concentrations we have found

Table 1. The severity of migraine symptoms in migraine patients with and without aura

	Migraine with aura N = 23	Migraine without aura N = 27	p
VAS (Visual Analogue scale)	7.96 ± 1.64	8.24 ± 1.33	NS
VRS (four – point Verbal Rating Scale)	2.65 ± 0.57	2.88 ± 0.33	NS
QVM Global Index	26.17 ± 6.68	27.20 ± 5.48	NS
MIDAS	34.48 ± 16.50	42.16 ± 18.87	NS
MIGSEV – Pain	4.0 2.0–4.0	3.0 3.0–4.0	NS
MIGSEV– Nausea	3.3 ± 0.7	3.3 ± 0.5	NS
MIGSEV– Disability in daily activity	3.04 ± 0.82	3.20 ± 0.50	NS
MIGSEV– Tolerability	2.0 1.0–3.0	3.0 1.0–3.0	NS

Variables with gaussian distribution are presented as mean ± SD, variables with non-gaussian distribution as median; interquartile range

Table 2. Results of routine laboratory tests in migraineurs and controls

	Controls	Migraine with aura	Migraine without aura
WBC	6.1 4.9–7.8	5.8 4.1–7.2	5.2 4.7–7.1
CRP	0.64 0.16–1.02	0.46 0.17–0.82	0.42 0.24–1.10
Glucose [mg/dL]	71 62 – 83	66 49 – 68	81 70 – 90
Total cholesterol [mg/dL]	184 ± 35	208 ± 36 *	227 ± 40 +
HDL cholesterol [mg/dL]	59 ± 12,6045	67 ± 12 #	66 ± 14
LDL cholesterol [mg/dL]	114 ± 44	126 ± 40	141 ± 34 $
TAG [mg/dL]	77 55 – 155	90 64 – 118	98 67 – 126
Lp (a) [g/L]	0.08 0.03–0.13	0.09 0.058–0.15	0.06 0.032–0.285
Homocysteine [mmol/L]	12.12 9.24–13.33	12.25 10.58–14.38	12.20 10.06–15.14
Albumin [g/L]	61.7 59.5–65.05	62.0 59.2–63.7	63.0 59.8–64.0

Variables with gaussian distribution are presented as mean ± SD, variables with non-gaussian distribution as median; interquartile range
* – P = 0,0243; + – P = 0,0002 – compared to controls; # – P = 0,0287 – compared to controls; $ – P = 0,0190 – compared to controls

Table 3. IMA in migraine patients and controls

	Controls	Migraineurs total	Migraine with aura	Migraine without aura
IMA [O.D.] median interquartile range	0.0 0.0–0.102	0.101 * 0.0–0.327	0.073 + 0.014–0.346	0.111 0.0–0.235
IMA [O.D.] minimum – maximum	0.0–0.511	0.0–1.932	0.0–1.922	0.0–1.932

*- P = 0.0108; + – P = 0.0103

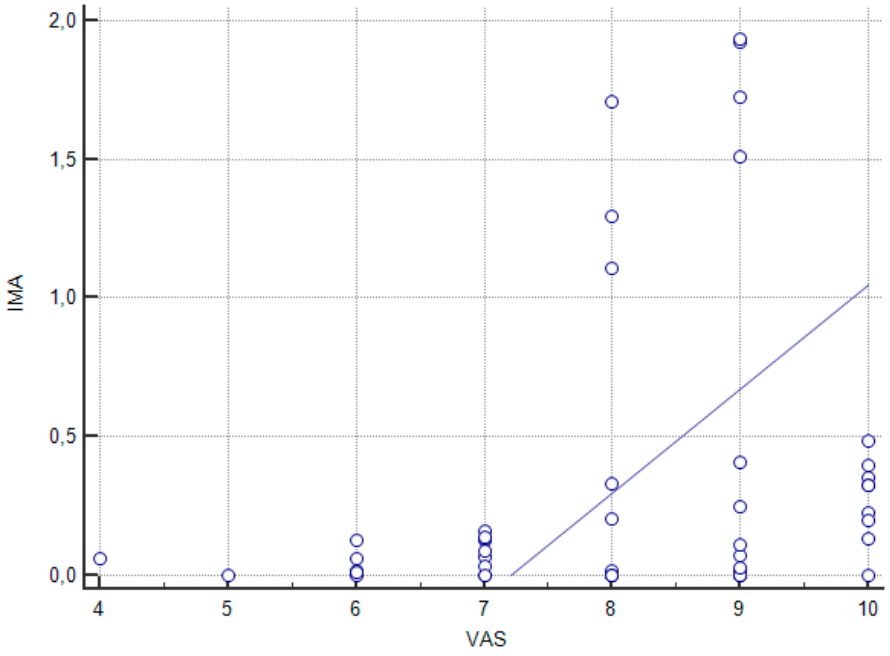

Figure 1. Correlation between IMA and VAS in migraine patients

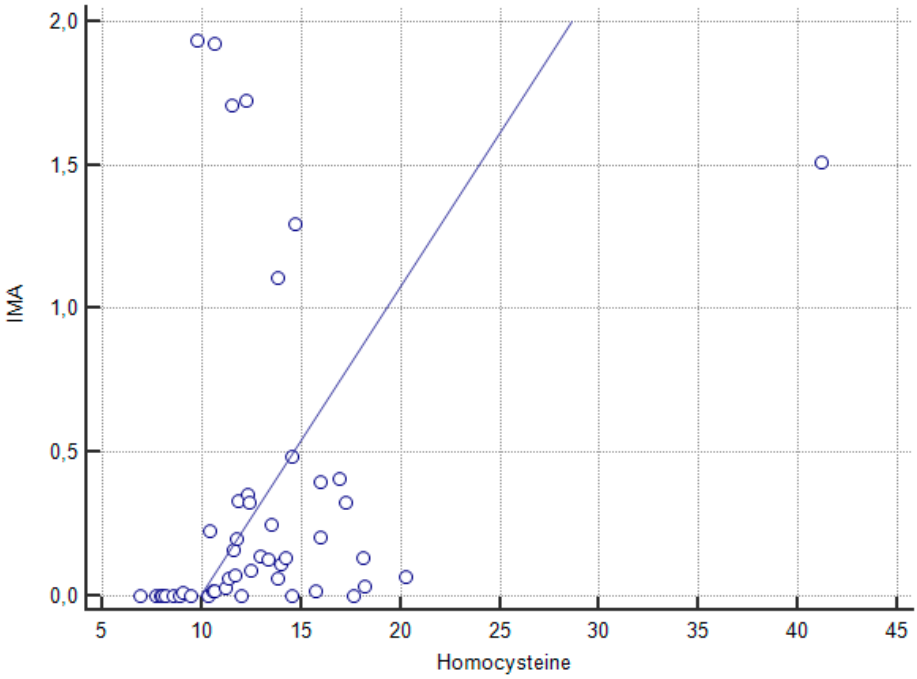

Figure 2. Correlation between IMA and homocysteine concentration in migraine patients

that IMA correlates (P = 0.0247) with HDL cholesterol (R = 0.2958) and TAG concentrations (R = 0.3285).

Discussion

We report the stimulation of ischemia-modified albumin formation in migraine patients during interictal period. In our study IMA correlated both with pain intensity, pro-atherosclerotic risk factors (homocysteine, triglycerides) and with anti-atherosclerotic marker (HDL cholesterol). We are not aware of the studies on IMA in migraine patients, thus it is an initial report on this topic.

IMA as a novel biomarker of oxidative stress and together with low grade systemic inflammation are underlying causes of many diseases. IMA plays a major role in their prediction before the overt onset. It has been reported that IMA is elevated in ischemic heart disease and as it increases within first minutes after ischemia and thus it was considered as the earliest predictor of myocardial infarction [12].

When ischemic changes started with hypoxia, free oxygen radicals lead to peripheral vascular insufficiency, which resulted in ischemia of the limbs and ischemic heart disease. Gunduz et al [22] noticed that there was a significant increase in serum IMA during limb ischemia with sensitivity and specificity over 80% in cases with clinically severe lower limb involvement. In our study we have observed positive correlation between IMA and pain severity measured with the use of VAS. There are very limited data on the correlations between IMA and clinimetric measures of neurological deficits. No correlation between IMA and stroke severity estimated with the use of National Institutes of Health Stroke Scale score (NIHSS) was found in one study [23], but the other [24] reported correlation both with the volume of ischemic lesion evaluated as restriction of diffusion on magnetic resonance imaging (MRI) and with NIHSS score.

According to Falkersammer et al [25] among patients with peripheral arterial occlusive disease (PAOD) IMA was a better predictor of major adverse cardiac events than N-terminal prohormone of brain natriuretic peptide (NT-BNP) or troponin (cTnT).

In diabetic patients oxidative stress and sub-endothelial inflammation resulted in nephropathy, neuropathy and retinopathy. Recently, Chawla et al [26] observed that the higher is elevation of IMA the poorer is glycemic control. Moreover most of the patients with complications showed higher IMA values. In view of the above, it appears that IMA could be related to glycaemic control in type 2 diabetes patients. However, the authors [26] used arbitrary units (ABSU) for the expression of IMA. Thus, the comparison of IMA results between studies requires careful interpretation, which should consider methodological and units

issues. In our study, we expressed IMA in optical density (O.D.) units and we suggest to call such a biomarker as serum IMA-ABS, when cobalt binding is measured spectrophotometrically, contrary to the units (U) used in enzyme-linked immunosorbent assays (ELISA) based on IMA detection by specific antibodies. Further studies are needed on correlations between results of spectrophotometric methods, which estimate „functional" properties of IMA and ELISA, which measures rather the „mass" of protein.

The serum levels of IMA have been reported to rise in many clinical conditions with acute inflammation, such as sepsis. Yin M et al. proved that IMA may be treated as predictor of short-term mortality in patients with severe sepsis [27]. It can be also associated with severity of the chronic inflammatory process [28, 29].

None of patients included in our study showed clinical symptoms or laboratory markers of acute or chronic inflammation. We can claim that the changes in IMA we have observed were not related to the inflammation.

We observed positive correlation between IMA and homocysteine concentration. Such a relations was not found in the study on polycystic ovary syndrome with or without insulin resistance [30]. However, in type 2 diabetic patients with peripheral arterial disease IMA positively correlated with homocysteine concentration [31]. The results of those studies may suggest that IMA is more related to vascular pathology than to metabolic disturbances.

Moreover, we have noticed positive correlations between IMA and triglycerides and HDL-cholesterol. Moderate correlation between IMA and TAG was already reported, as well as weak correlation with total cholesterol [32]. The relations between IMA and HDL cholesterol we have observed may be caused by higher HDL cholesterol concentration in migraine patients with aura, who have also higher IMA.

IMA formation was also related to the oxidative stress. The correlation between IMA and serum malondialdehyde (MDA), a lipid peroxidation marker, was reported during the course of normal pregnancy [33], thyroid gland dysfunction [34] and obstructive sleep apnea hypopnea syndrome [35].

To conclude, stimulated IMA formation in migraine patients, and particularly in migraineurs with aura, can reflect oxidative stress even during interictal period. It can be independent from acute or chronic inflammation, but in patients with additional hyperhomocysteinemia and/or hypertriacylglycerolemia it could reflect the increased cardiovascular risk.

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Conflict of interest statement
The authors declare no conflict of interest.

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Informed consent and ethical approval
Informed consent was obtained from all individual participants included in the study. The study design was positively evaluated and approved by the Bioethics Committee at Poznan University of Medical Sciences.

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A study of ofloxacin and levofloxacin photostability in aqueous solutions

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ABSTRACT

Introduction. The photostability is one of the most important properties of drugs. A comprehensive study of ofloxacin (OFX) and levofloxacin (LVX) photostability in aqueous solutions was performed. Ofloxacin is a chemotherapeutic agent belonging to the second generation fluoroquinolones and is a racemate of (R)-(+)-ofloxacin and (S)-(-)-ofloxacin (LVX).

Material and Methods. Samples of OFX and LVX were subjected to stress conditions of UV irradiation using a mercury-vapor lamp. The study involved development of enantioselective high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) methods for separation of OFX enantiomers and their degradation products. These methods were used to monitor the degradation process of OFX and LVX under irradiation and to determine the kinetics of degradation of these antibacterial agents. Moreover, the identification of photoproducts was also attempted. The structure of the main photoproducts was examined by mass spectrometry (MS).

Results and Conclusions. Using HPLC method it was possible to observe two products of OFX degradation and only one for LVX, while using HPCE method eight products of OFX degradation and six of LVX were observed. Some of the photoproducts retain character of optically active compounds. The trend of the photodegradation of both tested compounds was described by autocatalytic reaction proceeding according to the Prout-Tompkins model. Some of the products of the decomposition catalyze this reaction. The rate of degradation was similar for both enantiomers but t0.5 was slightly longer for LVX than OFX. Based on MS experiments the photodegradation products of the studied fluoroquinolones and possible pathways of UV induced decay were identified.

Keywords: fluoroquinolones, photodegradation, chiral separation.

Introduction

Ofloxacin ((±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1,2,3-de][1,4] benzoxazine-6-carboxylic acid) is a second generation fluoroquinolone. This chemotherapeutic agent is often used for treatment of infections caused by Gram-negative and atypical bacteria [1–3]. Ofloxacin is a racemate of two enantiomers, (R)-(+)-ofloxacin and (S)-(-)-ofloxacin (levofloxacin). Antibacterial activity of levofloxacin is 8–128 times higher than that of R-(+)-ofloxacin and two times higher than that of racemic mixture [4]. The situation that one of the enantiomers (eutomer) is more active than the other (distomer) is relatively common in pharmacology. It is estimated that 40% of available drugs are chiral compounds and 25% are used as pure enantiomers. Administration of drugs in the form of a pure enantiomer is much more beneficial for many reasons. It is possible to use a lower dose, thus reducing the risk of side effects or overdosing. It should be noted that even if distomer is inactive it is still metabolized by the liver. Its elimination from the commercial formulation allows to relieve the organ

[5–8]. Ofloxacin is used therapeutically in its racemate form and, since 1998, additionally as the enantiopure S-(-) isomer [9].

The photostability of the drug is one of the most important properties of the substance which affects not only the stability of the original drug, but also its phototoxicity in the human body. Fluoroquinolones including ofloxacin induce photosensitivity in humans at very low incidence [10, 11]. Photosensitivity includes phototoxicity and photoallergy. The phototoxic reaction is linked to exaggerated sunburn and the action spectrum of the toxicity is thought to be included in the wavelength range of UVA. The photoallergenicity of fluoroquinolones is mainly derived from their photohaptenic moiety, and photomodification of skin epidermal cells with fluoroquinolones is thought to be an initial step for this photoallergy [12–14].

The current research presents a comprehensive study of ofloxacin and levofloxacin photostability in aqueous solutions. The study involved development of enantioselective high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) methods for separation of OFX enantiomers as well as their degradation products. These methods were used to monitor the degradation process of OFX and LVX under UV irradiation and to determine the kinetics of degradation of these antibacterial agents. Moreover, the identification of photoproducts was also attempted.

Experimental

Chemicals and reagents

Ofloxacin, levofloxacin, sparfloxacin (SPX, IS), pipemidic acid (PIP, IS) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) were obtained from Sigma Aldrich Co. (USA), L-isoleucine, $CuSO_4$, $HCOONH_4$, HPLC grade methanol and acetonitrile were obtained from Merck (Germany), H_3PO_4, NaH_2PO_4, NaOH were obtained from POCh S.A. (Poland). All chemicals used in this study were of analytical grade.

HPLC method development

An HPLC system (1200 Series, Agilent Technologies) containing a binary pump G1312A, autosampler HiP-ALS G1367B and diode array detector (DAD) G1315D (294.5 nm) was used. Separation of OFX, LVX and their photoproducts was performed using a reverse-phase column – Zorbax Eclipse XDB-C18 Analytical, 4.6x150 mm, 5-Micron (Agilent Technologies) at 20°C. Mobile phase was a mixture of: chiral mobile phase additives (CMPA):methanol 88:12 (v/v). CMPA consisted of 5 mmol/L L-isoleucine and 4 mmol/L of copper (II) sulfate. The flow rate was maintained at 0.75 mL/min.

Calibration curves were constructed in the range of 5–200 µg/mL for both OFX and LVX with addition of PIP (100 µg/mL) as the internal standard. All calibration curves solutions and mobile phase were filtered through the 0.45 µm nylon-membrane filters (Millipore).

Calibration curves of OFX and LVX were constructed for their area under the peak over IS peak area ratio (A/A_{IS}) as a function of drug concentrations. The results showed good linearity throughout the examined concentration ranges for both racemic mixture and S-(-)-enantiomer. The linear correlation equations were $y = 0.1130 \pm 0.0010x - 0.0625 \pm 0.0086$ ($R^2 = 0.999$) for OFX and $y = 0.1124 \pm 0.0002x - 0.0483 \pm 0.0090$ ($R^2 = 0.999$) for LVX. For validation of the method, parameters such as selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision (repeatability) were investigated (**Table 1**). The proposed RP-HPLC method showed good selectivity that was proved by the fact that the mobile phase chromatogram contained no peaks at the retention times corresponding to OFX and LVX.

HPCE method development

The enantioseparation analysis of ofloxacin and levofloxacin was conducted on G1600 HPCE system (Agilent Technologies), with DAD detection. A fused silica capillary was used with a total length of 64.5 cm and an effective length of 56 cm. New capillary was con-

Table 1. The obtained values of limit of detection, limit of quantification and precision for HPLC and HPCE methods

		LOD [µg/mL]	LOQ [µg/mL]	Precision (RSD, %)
ofloxacin	HPLC	0.01	0.03	0.03
	HPCE	0.07	0.20	0.06
levofloxacin	HPLC	0.01	0.02	0.03
	HPCE	0.07	0.20	0.01

LOD – limit of detection, LOQ – limit of quantification, RSD – relative standard deviation, HPLC – high-performance liquid chromatography, HPCE – high-performance capillary electrophoresis

ditioned by rinsing with 0.1 mol/L NaOH for 15 min, methanol and deionized water for 10 minutes. Before each analysis the capillary was preconditioned according to the following schedule: 1.0 mol/L NaOH for 1.5 min., water for 1 min and finally background electrolyte (BGE) for 1.5 min. Moreover, after each run the capillary post conditioning procedure was also involved: 0.1 mol/L HCl, methanol and water for 1 min. As a background electrolyte the phosphate buffer (pH = 2.5) with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) as a chiral separator was used. Phosphate buffer (pH = 2.5) was prepared by mixing an appropriate aliquots of orthophosphoric acid solution (50 mmol/L) with sodium dihydrogen phosphate solution (50 mmol/L). The pH of the buffer was adjusted to 2.5 by addition of orthophosphoric acid (50 mmol/L) or sodium hydroxide (0.1 mol/L) solutions. Afterwards, the amount of 2-hydroxypropyl-β-cyclodextrin was dissolved in phosphate buffer to obtain the final concentration (HP-β-CD) of 40 mmol/L. Finally the BGE solution was filtered through the 0.65µm nylon-membrane filters (Millipore) and degassed in ultrasonic bath for two minutes.

The samples of OFX and LVX were injected in hydrodynamic mode directly onto capillary under the pressure of 50 mbar for 7 seconds. The enantioseparation was performed in positive mode, in electric field of 25kV for 30 minutes and detection was set at 296.4 nm, at the maximum absorption of fluoroquinolone molecule. The HPCE studies were performed in the range of 10-150 µg/mL for both OFX and LVX in the presence of sparfloxacin (SPX) (25 µg/mL) as the internal standard. The calibration curves of OFX and LVX were plotted as peak areas ratio (A/A_{IS}) as a function of their concentration (µg/mL). It was stated that the HPCE enantioseparation method, used in presented studies exhibits good linearity in the analyzed range of concentrations and good coefficient of determination (R^2). The calibration curves were described by following equations: $y = 0.03425(\pm0.00071)x+0.0702(\pm0.0587)$, $R^2 = 0.999$ for OFX and $y = 0.03287(\pm0.0007)x-0.0917(\pm0.0593)$ $R^2 = 0.999$ for LVX. The method was validated and the parameters of the validation are presented in **Table 1**.

MS conditions

Hybrid triple quadrupole/linear ion trap mass spectrometer 4000 QTRAP (Sciex) with electrospray ion source (TurboIonSpray source) was used. The MS measurements were carried out in positive ionization mode. Ion spray voltage, entrance potential, declustering potential were set at 5500 V, 10 V and 30 V, respectively. Nitrogen was used as a curtain gas (10 psig). In MS/MS measurements nitrogen was used as a collision gas (medium).

Photostability study

Photodegradation study of OFX and LVX was performed following the recommendations of the ICH (1QB Photostability Testing of New Drug Substances and Products). The concentration of analyzed solutions of both OFX and LVX was 100 µg/mL. The pH = 4.5 was obtained by dissolution of amounts of OFX and LVX in 2.5 mL of 0.1 mol/L HCl solution. Solutions were filtered through the 0.45µm nylon-membrane filters (Millipore) and irradiated for 210 min in a quartz cylindrical cuvette placed at a distance of 15.5 cm from a high pressure mercury lamp (HBO-50 NARVA, emission of UVB-UVA = 280–400 nm, intensity 0.119–0.125 mW/cm^2). The irradiation process was performed in triplicate.

During HPLC study of the photodegradation process of OFX and LVX a sample of 60 µL was taken every 15 min, mixed with 10 µl of PIP (714.51 µg/ml) as an IS and introduced onto the column in triplicate. For HPCE study of the degradation of OFX and LVX aqueous solutions a sample of 60 µL was taken every 15 min, mixed with 10 µl of SPX (316.4 µg/ml) as an IS and introduced into the HPCE system in triplicate. For MS analysis irradiated solutions (0, 60, 120, 180 min) were evaporated to dryness (vacuum concentrator miVac Duo, Genevac). Dry residue was dissolved in 1 mL of ammonium formate solution (0.005 mol/L in water:methanol 50:50 v/v). Before MS analysis solutions were filtered through the 0.45 µm nylon-membrane filters (Millipore). Solutions were introduced to MS system directly from a syringe pump.

Results and Disscusion

The enantioseparation of OFX and LVX and their photodegradation products were achieved by HPLC and HPCE methods.

A chiral ligand-exchange reversed-phase HPLC method for the determination of OFX- racemic mixture, LVX and monitoring of their photodegradation processes was developed. Before the irradiation three peaks were observed on the OFX chromatogram (1– pipemidic acid 10.68 min; 2– (S)-ofloxacin 15.85 min; 3– (R)-ofloxacin 19.64 min) and two on the LVX chromatogram (1– pipemidic acid 10.68 min; 2– (S)-ofloxacin 15.85 min) (**Figure 1**). The complete decomposition of both drugs occurred after 150 min of irradiation (no peaks corresponding to OFX or LVX occurred). Peaks corre-

sponding to the products of degradation (FP) on chromatograms were observed. The number of photoproducts was different for OFX and LVX. LVX decomposed with formation of only one FP (3.81 min, appeared after 90 min of irradiation) while two FPs formed in case of OFX (3.81 and 4.62 min, appeared after 120 min of irradiation) (**Figure 2**). It was found that the compounds which were formed by the decomposition of OFX were the pair of enantiomers, while in the case of LVX a single peak was observed probably with the S-(-)- configuration. Moreover, it is supposed that after 90 min of the light exposure some part of LVX molecules changed spatial configuration and peak with t_r = 19.64 min appeared on the chromatogram.

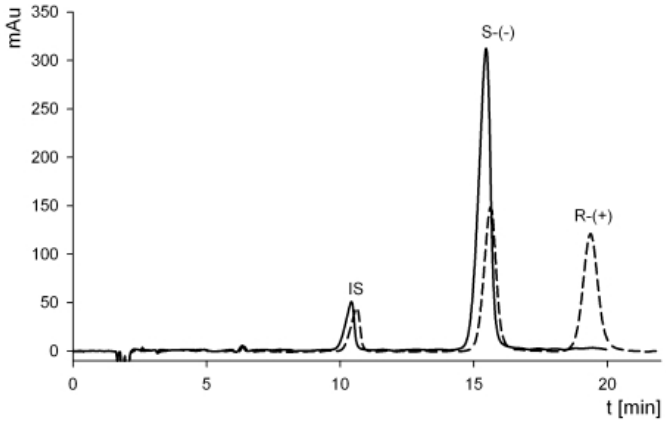

Figure 1. Chromatogram of ofloxacin (---) and levofloxacin () before the irradiation. IS – internal standard, S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin

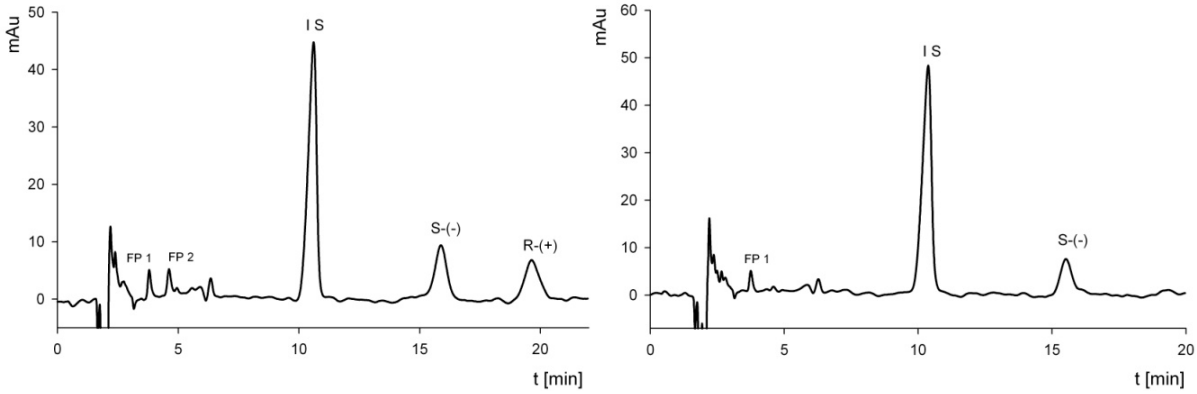

Figure 2. Chromatograms of ofloxacin (left) and levofloxacin (right) after 120 min of irradiation. FP 1 – product of degradation 1, FP 2 – product of degradation 2, IS – internal standard, S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin

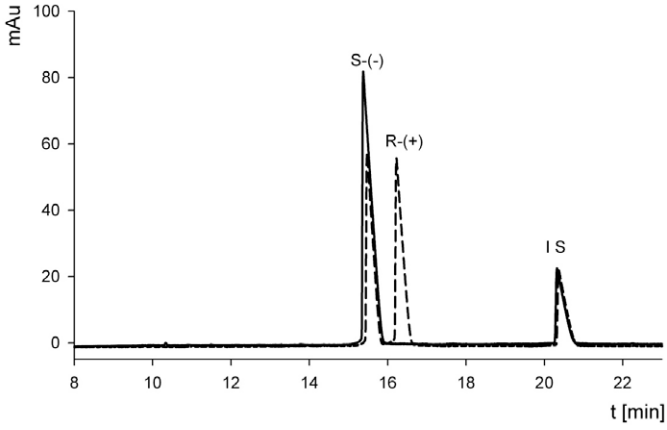

Figure 3. Electropherograms of ofloxacin (---) and levofloxacin () before the irradiation. S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin, IS – internal standard

In HPCE method HP-β-cyclodextrin was used as a chiral selector, and allowed to separate S-(-) and R-(+) enantiomers from the racemic ofloxacin. Before the irradiation three peaks were observed on the OFX electropherogram (1– S-(-)-ofloxacin 15.25 min; 2– R-(+)-ofloxacin 15.98 min; 3– sparfloxacin as IS 20.36 min) and two on the LVX electropherogram (1– (S)-ofloxacin 15.29 min; 2- sparfloxacin as IS 20.34 min) (**Figure 3**). It was noticed that concentration of OFX and LVX decreases over the time of irradiation, while new peaks photoproducts appeared on electropherograms. Concentration of photoproducts increased with the light exposure time (**Table 2**, **Figure 4**).

The presence of six main degradation products of LVX (A-F) and eight of OFX (B, C, C', D, D', E, E', F) was observed. Six of OFX photoproducts formed three pairs of peaks with slight differences in their migration times (C and C', D and D', E and E'). On the LVX electropherogram there were observed only single peaks (C, D, E). In addition, in case of OFX each pair of peaks appeared after the same time of exposure. This means that the photodegradation products still retained properties of original chiral substances. It was also likely that during the photodegradation of LVX certain percentage of S-(-)-ofloxacin was converted into R-(+)-ofloxacin (FP A). It can be proved by the same migration times of FP A and R-(+)-ofloxacin.

Based on the obtained results (HPLC and HPCE) of decrease in the concentrations of examined compounds after increase of the irradiation time, the kinetics of the decomposition of OFX and LVX in aqueous solutions was determined. The rates of photodegradation reaction was time and product formation dependent – autocatalyzing reaction. The curves of concentration changes vs time were characterized by sigmoidal shape. For both of the tested compounds increasing of the reaction rate was observed in the initial stage of the reaction (15–30 min) and decreasing in the final stage of exposure to light (195–210 min). The course of photodegradation of the tested compounds was best described by the second-order autocatalytic reaction equation according to the Prout-Tompkins model.

The second-order autocatalytic reaction follows the model:

$$A \xrightarrow{C} C$$

where: A – substrat, C – product and autocatalyst.

The concentration changes of the OFX and LVX during irradiation were described by the equation:

$$ln\frac{[C]}{[A]} = ln\frac{[C]_0}{[A]_0} + ([C]_0 + [A]_0)kt$$

where: $[C]$, $[A]$ – concentration of the product and substrate respectively, $[C]_0$, $[A]_0$, – the initial concentrations of the product and substrate respectively, k – the rate constant, t – time.

Table 2. Migration times [min] of ofloxacin and levofloxacin degradation products

Degradation product	A	B	C	D	E	F
Irradiation time [min]	90	90	120	135	165	165
ofloxacin	-	14.50	12.87 (C); 13.02 (C')	13.64 (D); 13.80 (D')	13.32 (E); 13.38 (E')	16.33
levofloxacin	15.98	14.53	12.86	13.63	13.29	16.38

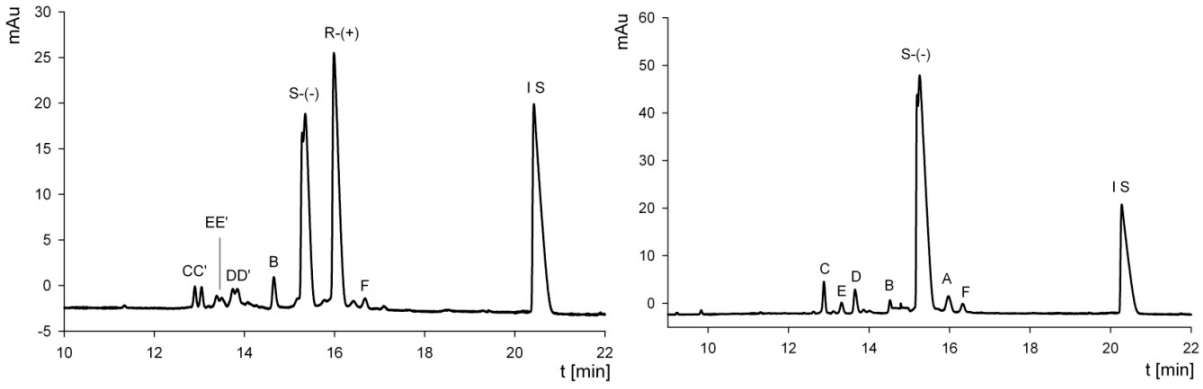

Figure 4. Electropherograms of ofloxacin (left) and levofloxacin (right) after 165 min of irradiation. S-(-) – (S)-(-)-ofloxacin (levofloxacin), R-(+) – (R)-(+)-ofloxacin, IS – internal standard, A, B, C, C', D, D', E, E', F – degradation products

Table 3. Kinetic parameters of ofloxacin and levofloxacin photodegradation reactions determined by HPLC and HPCE methods

	k [s^{-1}]	$t_{0.1}$ [min]	$t_{0.5}$ [min]
HPLC			
ofloxacin	5.43×10^{-4}	39.28	88.11
levofloxacin	6.05×10^{-4}	58.86	105.61
HPCE			
ofloxacin	5.34×10^{-6}	65.28	130.07
levofloxacin	5.76×10^{-6}	72.93	135.70

HPLC – high-performance liquid chromatography, HPCE – high-performance capillary electrophoresis

Based on the theory of the second-order autocatalytic reaction the Prout-Tompkins model was used to describe the decomposition of the substance, according to the following equation:

$$ln\frac{c_0 - c}{c} = \gamma t + const$$

where: c – percentage concentration of substrate, $(c_0 - c)$ – the difference between the initial concentration of substrate and the concentration at time t, γ – slope, $const$ – integration constant.

Calculated kinetic parameters of OFX and LVX photodegradation processes are presented in **Table 3**.

The analysis of the photodegradation products of ofloxacin and levofloxacin was also performed by mass spectrometry. The spectra of fluoroquinolones before the irradiation were compared with those after 60, 120 and 180 min of exposure to UV light. Peaks corresponding to the photoproducts were selected by comparing their intensity on the spectra of the solutions before and after irradiation and then an attempt was made to identify them. After 60 min of irradiation only one photoproduct of *m/z* 336.0 appeared. After 120 min of irradiation more degradation products were observed in the mass spectrum: *m/z* 348.0; *m/z* 346.0; *m/z* 336.0; *m/z* 322.0; *m/z* 279.0; *m/z* 103.0 and *m/z* 101.0. The number of the photoproducts decreased after 180 min of irradiation and the following peaks were detected in the spectrum: *m/z* 346.0; *m/z* 294.0; *m/z* 279.0; *m/z* 103.0 and *m/z* 101.0. It should be noted that m/z values of some FPs differed by 2 (348 and 346, 103 and 101) indicating the possibility of loss of 2 hydrogens leading to the formation of double bond. Our observations were consistent with previous literature data, which pointed formation of double bonds as characteristic for UV induced degradation of fluoroquinolones [15].

In the next step, major photoproducts of the examined fluoroquinolones were fragmented using MS/MS experiments. Fragmentation made it possible to better define the structure of photoproducts. Based on the fragmentation mass spectra an attempt was made to determine the putative degradation pathways and the chemical structure of

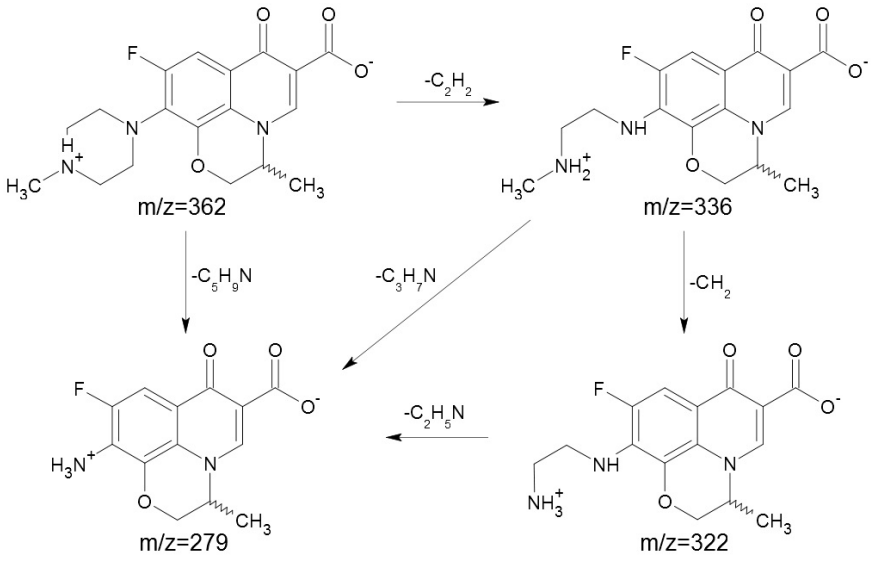

Figure 5. The diagram of ofloxacin and levofloxacin photodegradation

the formed compounds (**Figure 5**). In aqueous solutions, under the influence of UV light, the piperazine ring decomposes first. On the basis of *m/z* values of FPs it can be concluded, that disintegration proceeds by the removal of small fragments or the whole ring. In examined conditions (low pH = 4.5) there is neither fluorine atom nor carboxylic group separation observed.

Conclusions

The purpose of this study was to separate and identify products created in the photodegradation process of ofloxacin and levofloxacin and to determine the kinetics of the decomposition reactions. In order to achieve that, solutions of ofloxacin and levofloxacin were prepared and irradiated using a mercury-vapor lamp for various time periods up to 210 minutes. Photoproducts separation was carried out using two analytical techniques: a chiral ligand-exchange reversed-phase HPLC and HPCE with 2-hydroxypropylo-β-cyclodextrin as a chiral selector. It was found that numerous products appeared as a result of photodegradation. Using HPLC method it was possible to observe two products of OFX degradation and only one for LVX, while using an HPCE method eight FPs of OFX and six of LVX were observed. It was concluded that some of the photoproducts still retain character of optically active compounds. It is supposed that during irradiation some amount of (S)-ofloxacin enantiomer is converted into (R)-ofloxacin. The course of photodegradation of the tested compounds is best described by the autocatalytic reaction proceeding according to the Prout-Tompkins model. Some of the products of the decomposition are also catalyzing this reaction. Based on MS experiments it was possible to identify the photodegradation products of the studied fluoroquinolones and possible pathways of UV induced decay.

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Conflict of interest statement
The authors declare no conflict of interest.

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In vitro biofilm formation and antibiotic susceptibility of *Pseudomonas aeruginosa* isolated from airways of patients with cystic fibrosis

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ABSTRACT

Introduction. *Pseudomonas aeruginosa* is the predominant cause of airway infections in patients with cystic fibrosis (CF) as a result of its ability to form biofilm. Resistance to antimicrobial agents is the most important feature of biofilm infection. The aim of this study was to evaluate biofilm formation and to compare antibiotic susceptibility of *P. aeruginosa* living in two modes of growth: planktonic and biofilm, isolated from respiratory tract of CF patients.

Material and Methods. Biofilm formation and biofilm susceptibility to antibiotics were determined using modified microtitere plate method. For susceptibility testing of planktonic culture to antibiotics serial microdilution broth method were used.

Results. More than 95% of isolates were capable to form biofilm. Isolates grown as biofilms were more resistant to tested antibiotics compared to those grown planktonically. Ciprofloxacin showed the highest activity against *P. aeruginosa* biofilm. In contrast, no bacteriostatic activity was obtain for the highest concentration of piperacillin tested against most of *P. aeruginosa* strains growing in a biofilm (BIC > 4096 mg/L).

Conclusions. Our study indicates the need to develop a standardized susceptibility testing method for biofilm mode of growth of pathogens. It appears that it is appropriate to introduce a biofilm susceptibility testing to routinely performed tests, as the effect of antibiotics on biofilm eradication may be variable and unpredictable.

Keywords: biofilm resistance, chronic infections, susceptibility testing.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that grows in soil, water, as well as on plant and animal tissues. Because of ability of this bacterium to product multiple virulence factors facilitating invasion and colonization, *P. aeruginosa* is a major opportunistic human pathogen responsible for bacteremia in burn patients, urinary-tract infections in catheterized patients, and pneumonia in mechanically ventilated patients [1–3]. *P. aeruginosa* is also the predominant cause of lung infections in cystic fibrosis patients [4]. The pathogenesis of *P. aeruginosa* lung infections is multifactorial and depends on numerous virulence factors, including secretion of extracellular enzymes (e.g. elastase, phospholipase C, alkaline protease, exotoxin A, pyoverdine, neuraminidase) and the presence of cell associated factors, such as flagella, pili and lipopolysaccharide (LPS) [5, 6]. Another important factor contributing to the pathogenesis of *P. aeruginosa* severe infections is its tendency to form organized communities, known as a biofilm, on biotic and abiotic surfaces [7]. Natural resistance of *P. aeruginosa* to several group of antibiotics, and the resistance to disinfectants together with the ability to biofilm for-

mation make this bacterium responsible for high rates of morbidity and mortality [8, 9].

The discovery of bacteria existing in a biofilm form has led many researches to revisit the pathogenesis of chronic infections. Biofilm is three-dimensional structured, specialized community of adherent microorganisms enclosed in a self-produced extracellular polymeric substance (EPS). Resistance of biofilm towards component of host immune system as well as antimicrobial agents appears to be risk factor for persistent infections and makes them difficult to eradicate [8, 10].

Bacteria living in biofilm undergo a phenotypic shift in behavior, and large groups of bacterial genes are differentially regulated. In this way, biofilm community obtains numerous advantages, such as passive resistance, metabolic cooperation, byproduct influence, quorum sensing systems, an enlarged gene pool with more efficient DNA sharing, and many other features which help bacteria to adapt to environmental condition and protect them from effects of external factors [11–13].

Cystic fibrosis (CF) is a genetic disorder due to recessive mutations in the CF transmembrane regulator gene which regulates chloride transport across epithelia cells. These mutations lead to dysfunction of the CF transmembrane regulator protein which constitutes a part of a chloride channel. This can result in a built-up of thick, sticky mucus, among others, in the respiratory tract, pancreas, and reproductive organs leading to multiple organ disorders. The span life and quality of life are most often dependent on changes in respiratory tract. As a result of hyperinflammation and a reduced ability to remove bacteria by mucociliary action, bacteria colonize the lungs [14]. Chronic airway infections in CF patients differ significantly from acute pulmonary infection in non-CF patients, bloodstream or urinary tract infections. *P. aeruginosa* grows in the CF lung very slowly as chronic biofilm infections and despite the use of a range of antipseudomonal antibiotics, eradication of the infection is quite rare [15–17].

This study was performed to compare antibiotic susceptibility of *P. aeruginosa* isolated from respiratory tract CF patients living in two modes of growth: planktonic and biofilm.

Material and Methods

Investigated bacterial strains

Pseudomonas aeruginosa strains were obtained from Microbiological Laboratory of Transfiguration of the Lord Clinical Hospital. The strains originally isolated from respiratory tract of CF patient were frozen and stored in Microbank cryogenic vials (ProLabDiagnostics, Canada) at -70 ± 10°C. Before each experiment subcultures were prepared on Tryptic soy agar (bioMérieux, Poland).

Staphylococcus epidermidis ATCC 35984, with a proven biofilm-forming ability and *Staphylococcus* epidermidis ATCC 35983 – a non-biofilm producer were used as positive and negative controls for biofilm production, respectively. *Pseudomonas aeruginosa* ATCC 27853 was applied as the quality control strain to verify the test procedures for determination of MIC.

Antibiotics applied in MBC and BIC determination

Piperacillin (PIP), ceftazidime (CAZ), ciprofloxacin (CIP) and amikacin (AN) were obtained from Sigma-Aldrich (Poland). Stock solution from dry powders were prepared at a concentration of 4096 mg/L for piperacillin and 1024 mg/L for ceftazidime, amikacin and ciprofloxacin. The stock solutions were stored at -70 ± 10°C before experiments.

Biofilm formation assay

Biofilm formation was determined by the microtiter plate assay, as previously reported [18]. Briefly, 200 µL of an overnight cultures grown on tryptic soy agar (bioMérieux, France) suspended in a tryptic soy broth (bioMérieux, France) and adjusted to a turbidity of 0.5 MacFarland in tryptic soy broth (TSB) were inoculated into 96-well flat-bottom microtiter plates (Medlab-Products Ltd., Poland) and incubated. Following incubation at 37°C for 20 hours the cultures were removed and the wells were washed three times with 200 µL of phosphate buffered saline (PBS, pH = 7.4; Sigma-Aldrich, Poland) and dried at room temperature. Biofilms were stained with 0.1% crystal violet (Merck, Poland) for 15 minutes, washed with water and air dried overnight. The crystal violet from stained biofilm was resuspended in 250 µL of 95% ethanol. The optical density (OD) of adherent biofilm was measured using an Infinite M200 (Tecan) plate reader at a wavelength of 590 nm. Wells containing uninoculated TSB media served as a negative control. Tests were repeated three times. The interpretation of biofilm formation was done according to the Stepanovic criteria presented in **Table 1** [19].

Susceptibility testing of planktonic cells

The stock solution of each antibiotic was two-fold serially diluted in a Mueller Hinton II broth (MHB II; bioMérieux, Poland) to concentrations ranging from 256

Table 1. Classification of biofilm formation

OD values	Biofilm formation
≤ ODc	None
2 x ODc ≥ OD > ODc	Weak
4 x ODc ≥ OD > 2 x ODc	Moderate
> 4 x ODc	Strong

ODc = mean OD of control probes + 3SD, OD – optical density, SD – standard deviation

Table 2. Detection of biofilm formation by the microtiter plate method

Biofilm formation	No of isolates [%]	Isolate	Absorbance at 590 nm
None	1 [4.5]	51	0.200
Weak	1 [4.5]	14	0.419
Moderate	2 [9.0]	23, 26	0.517 – 0.694
Strong	18 [82.0]	4, 5, 9, 13, 16, 20, 24, 28, 29, 30, 31, 32, 33, 37, 38, 39, 40, 52	1.069 – > 3.00

to 0.5 mg/L. Aliquot of 100µL of each dilution were distributed into the wells of a sterile 96-well microtiter plate. An overnight bacterial culture was suspended in MHB II, adjusted to a 0.5 McFarland standard, corresponding to a concentration of 10^8 CFU/mL and diluted 1:100 in MHB II. Bacterial suspensions were added to all wells except the wells, which were used as sterility controls. Growth wells (with bacteria and without antibiotics) were also included. The final concentration of bacteria was c.a. $5x10^5$ CFU/mL and final concentrations of antibiotics ranged from 0.25 mg/L to 128 mg/mL. The plates were prepared in triplicate and then incubated at 37°C for 20 h. The MIC was determined as the lowest concentration of antibiotic that inhibited the visible growth of the tested microorganism.

Biofilm susceptibility assay

Antimicrobial susceptibility of *P. aeruginosa* biofilm assay was performed according to Moscowitz [20] with modifications. Briefly, bacterial biofilm was formed by immersing the wells of flat-bottom microtiter plates as described above. Negative control wells were filled with sterile medium. The 24-hour biofilms were washed three times with PBS solutions and air-dried. Serial two-fold dilutions of antibiotics, ranging from 4 mg/L to 4096 mg/L, were prepared in MHB II. Next, 100 µL of each concentration was added to each corresponding well and plates were incubated 18 h at 37°C. Antibiotics were aspirated gently after incubation and plates were washed three times with sterile PBS solution. To each well 100 µl of MHB II was added and the plates were sonicated using sonicating water bath for 5 minutes to disrupt the biofilm. The optical density at 650 nm (OD_{0h}) was measured on a microtiter plate reader (Infinite M200,Tecan) before and after incubation at 37°C for 6 h. Adequate biofilm growth for the positive control wells was defined as a mean OD_{650} difference (OD_{650} at 6 h minus the OD_{650} at 0 h). The biofilm inhibitory concentrations (BICs) were defined as the lowest concentrations of drug that resulted in an OD_{650} difference at or below 10% of the mean of two positive control well readings. The 10% cut-off represents a 1-log10 difference in growth after 6 h of incubation.

Statistical analyses

The results of antimicrobial activity were analyzed using Kruskal-Wallis test. *P* value <0,05 was considered as significant. The STATISTICA software was used in the statistical analyzes.

Results

Biofilm formation

The established cut-off values of ODc for assessment of biofilm formation for all strains were 0.157. Final OD value of tested strains was calculated as an average OD value of the strain reduced by ODc value. The interpretation of biofilm formation was performed according to the following criteria: OD ≤ 0.157 – none producer; 0.157 > OD ≤ 0.313 – weak producer, 0.313 > OD ≤ 0.626 – moderate producer and OD > 0.626 – strong producer (**Table 2**). Of the 22 isolates, 21 formed biofilm, of which only one was weak producer, two strains were moderate and 18 strong producers with an average OD_{650} value of 0.418 ± 0.078, 0.516 ± 0.027 – 0.694 ± 0.042 and 1.069 ± 0.540 – > 3.000 respectively. For the six strains OD value were above upper limit of measurement range of plate reader (OD > 3.000).

Effect of PIP on biofilm and planktonic P. aeruginosa mode of growth

Significant difference was observed between the inhibitory effect of piperacillin on biofilm and planktonic culture of *P. aeruginosa* CF isolates (**Figure 1**). The MIC value ranged from 4 mg/L to 64 mg/L and according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoints (>16 mg/L) only three strains were resistant to PIP. In contrast, no bacteriostatic activity was obtain for PIP at the highest concentration tested against most of *P. aeruginosa* strains growing in biofilm (BIC > 4096 mg/L) and the BIC was from 64 to > 1024 fold higher than MIC.

Effect of CAZ on biofilm and planktonic P. aeruginosa mode of growth

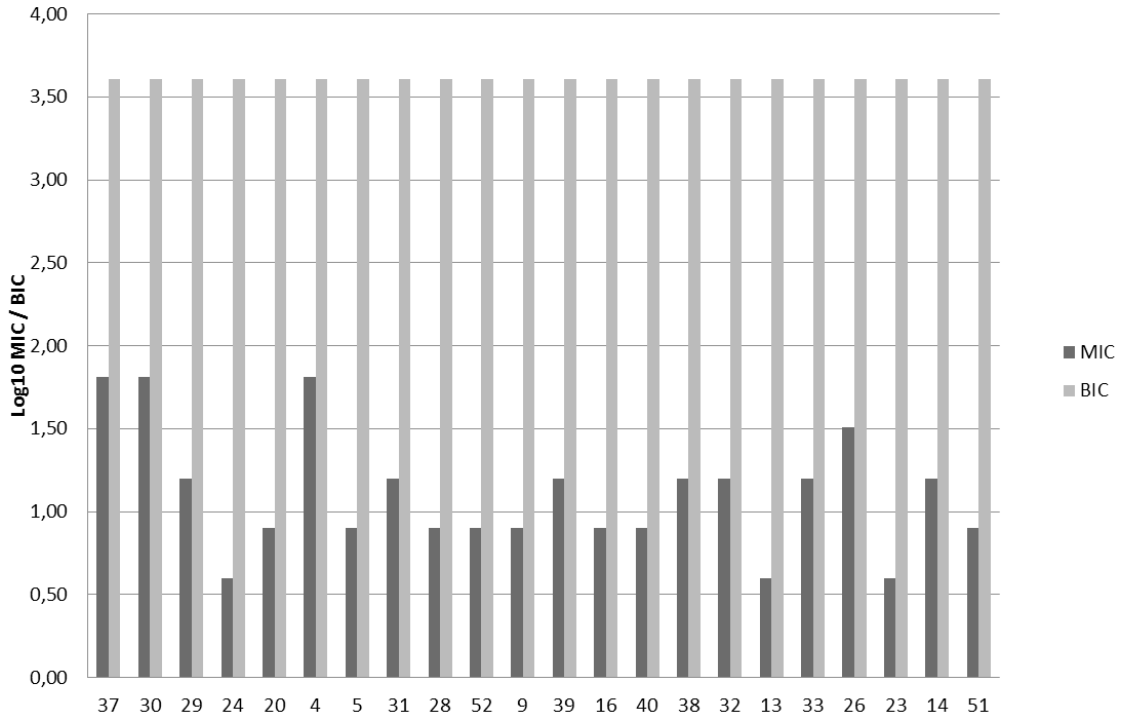
Figure 2 presents the MIC and BIC of ceftazidime for *P. aeruginosa* isolates. The obtained BIC values, ranged from 64 mg/L to 512 mg/L, and were 8 to 64 fold higher than MICs. There were no correlation in differences between planktonic and biofilm inhibition concentrations and susceptibility assessed according EUCAST guidelines.

Effect of AN on biofilm and planktonic P. aeruginosa mode of growth

Determination of MIC for planktonic culture of *P. aeruginosa* showed that the majority of tested strains were susceptible to amikacin (MIC ranged 4–8 mg/L), six were intermediate susceptible (MIC 16 mg/L) and only one was resistant (MIC 64 mg/L) (**Figure 3**). Antibiofilm activity of amikacin for all tested isolates decreased and BIC value increased from 2 to 8-fold in relation to MIC.

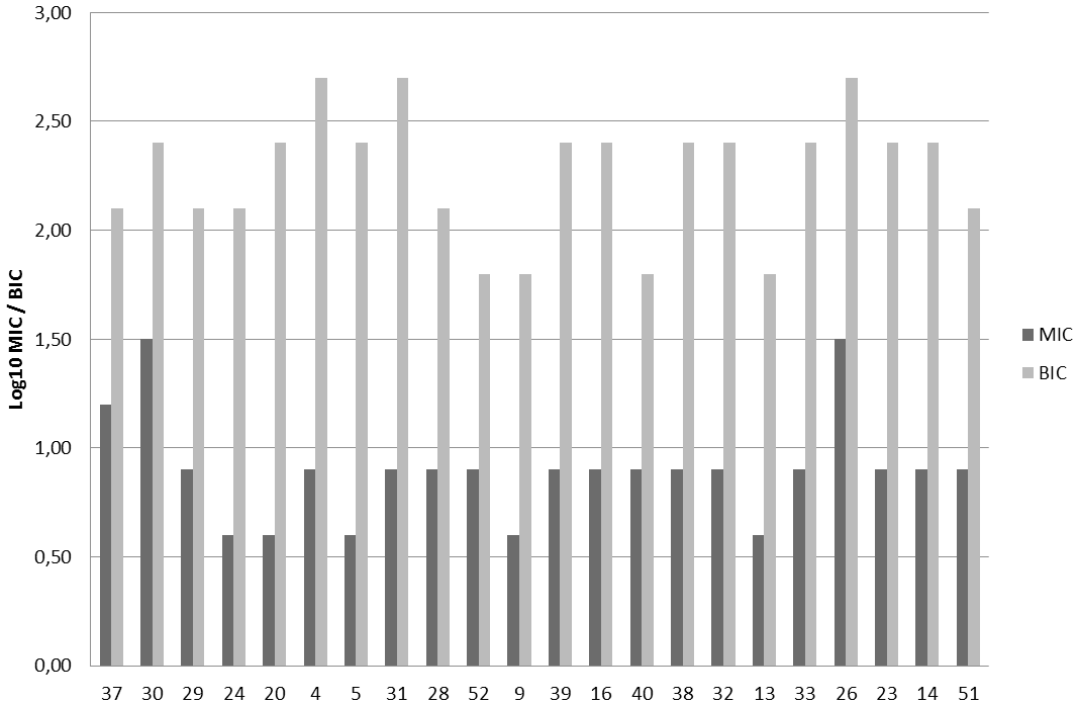
Effect of CIP on biofilm and planktonic P. aeruginosa mode of growth

Planktonic cultures of *P. aeruginosa* isolates were inhibited at ciprofloxacin concentration of 4 mg/L to 16 mg/L (**Figure 4**). Despite the fact that *P. aeruginosa* isolates cultured in floating form were resistant to CIP (according to EUCAST breakpoints), the concentration inhibiting the growth of *P. aeruginosa* biofilm (BICs) were equal or two to four-fold higher than MICs.



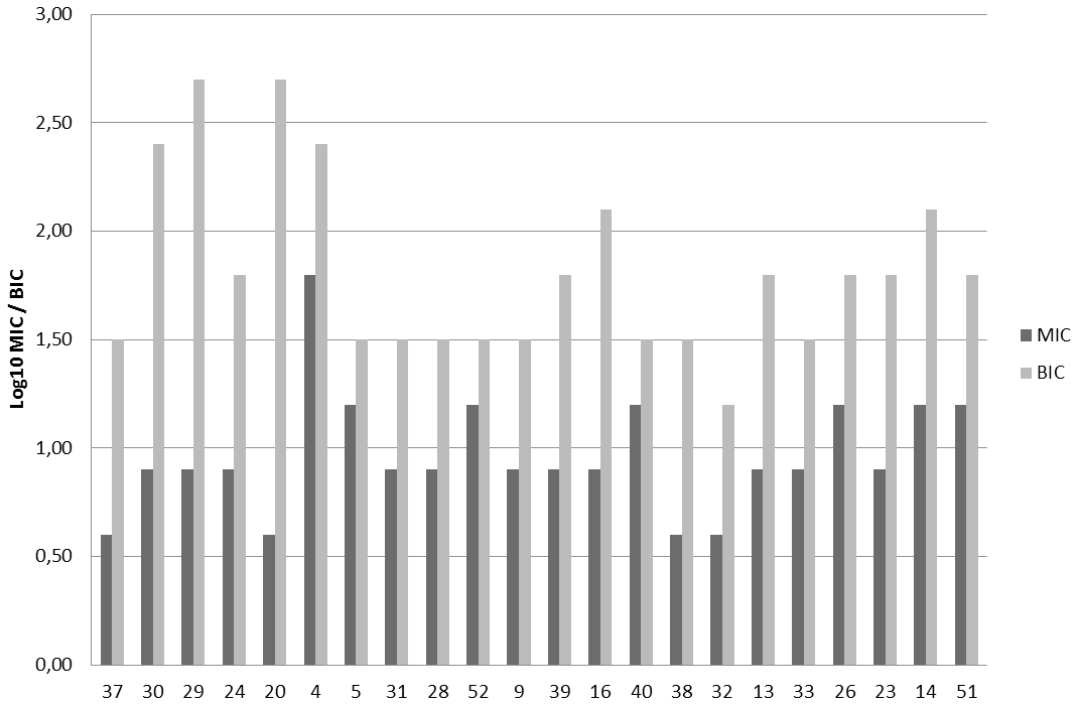
Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	64	64	16	4	8	64	8	16	8	8	8	16	8	8	16	16	4	16	32	4	16	8
BIC [mg/L]	>4096	>4096	>4096	4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	4096	>4096	>4096	4096	>4096	>4096
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 1. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to piperacillin obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to piperacillin was not correlated to the intensity of biofilm formation by the strains tested



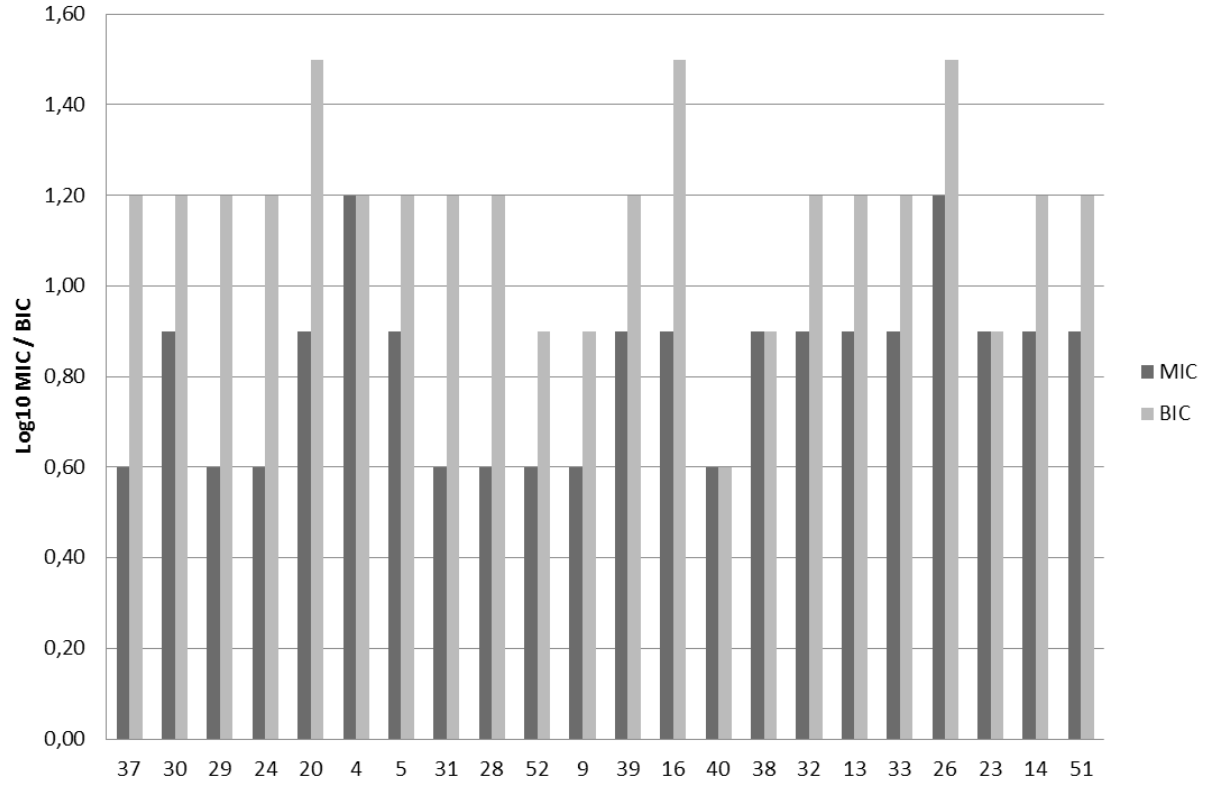
Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	16	32	8	4	4	8	4	8	8	8	4	8	8	8	8	8	4	8	32	8	8	8
BIC [mg/L]	128	256	128	128	256	512	256	512	128	64	64	256	256	64	256	256	64	256	512	256	256	128
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 2. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to ceftazidime obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to ceftazidime was not correlated to the intensity of biofilm formation by the strains tested



Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	4	8	8	8	4	64	16	8	8	16	8	8	8	16	4	4	8	8	16	8	16	16
BIC [mg/L]	32	256	512	64	512	256	32	32	32	32	32	64	128	32	32	16	64	32	64	64	128	64
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 3. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to amikacin obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to amikacin was not correlated to the intensity of biofilm formation by the strains tested



Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	4	8	4	4	8	16	8	8	4	4	4	8	8	4	8	8	8	8	16	8	8	8
BIC [mg/L]	16	16	16	16	32	16	16	16	16	8	8	16	32	4	8	16	16	16	32	8	16	16
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 4. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to ciprofloxacin obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to ciprofloxacin was not correlated to the intensity of biofilm formation by the strains tested

Correlation between biofilm formation and biofilm resistance

No significant correlation was observed between biofilm formation and BICs value for all tested antibiotics (**Figures 1–4**). No biofilm producer (strain No 51), weak (strain No 14) and moderate biofilm producers (strains No 23, 26) were equal to or more resistant to antibiotics than strong producers (e.g. strains No 13, 40, 9, 52, 28, 37).

Discussion

As bacteria were usually considered as free-living, unicellular organisms, we are aware now that they exist predominantly as adherent multicellular biofilms in diverse environmental niches. Most of the bacteria have the ability to form a biofilm on different surfaces and in various organs, such as implants, urinary catheters, teeth or lung tissue [21–23]. According to the data obtained from Centers for Disease Control and Prevention (CDC), biofilms are the background of at least 65% of all human bacterial infectious [24, 25]. Understanding bacterial physiology and the mechanisms of bacterial resistance to lethal concentrations of antibiotics it is crucial to elaborate on the effective eradication of resistant strains [26–29].

P. aeruginosa is a prime example of bacteria known to grown in a biofilm form [30–32]. Recent evidence indicating a biofilm mode of growth in the respiratory tract and the presence of biofilm quorum-sensing signals in the sputum of CF patients support the contestation that *P. aeruginosa* biofilms are present in an airway of cystic fibrosis patients [33, 34]. Many reports provided strong evidence for potential role of *P. aeruginosa* biofilm in pathogenesis of lung infections in CF patients. Bjarnsholt et al [35] detected both biofilm forming microcolonies and non adhered planktonic bacteria in samples of sputum from 77 chronic *P. aeruginosa* infected CF patients in that study and no other bacteria were isolated. In our study *in vitro* biofilm-forming capacity of *P. aeruginosa* isolated from airway CF patients were detected in more than 95% strains but with diverse intensity. The variability in biofilm formation amongst *P. aeruginosa* isolates was supported by others [7, 9, 13, 26, 36]. The reason for the variety in biofilm formation seems to be multifactorial [26, 35, 37].

Comparison of MICs and BICs demonstrated differences in activity of tested antibiotics. In general, sessile bacteria were inhibited by much higher concentrations of antibiotics than floating cells.

The huge increase in biofilm resistance to antibiotics occurred with piperacillin. Most isolates grown in planktonic culture were susceptible to piperacillin, however, we observed a loss of activity of PIP (BICs ≥ 4096 mg/L) when these isolates were grown as biofilms. For ceftazidime the BIC values for all strains were much more lower than for piperacillin, but still high (8 to 64 x MIC). The reason why these β-lactam antibiotics are not as active against biofilm as it is on planktonic cell is that β-lactam antibiotic required rapid cells growth to kill the bacteria.

Amikacin, the aminoglycoside antibiotic was slightly more effective against *P. aeruginosa* biofilm than ceftazidime. Fluoroquinolone – ciprofloxacin showed the lack of therapeutic useful activity (MIC higher than EUCAST clinical breakpoint) against planktonic cell, but against the biofilm-forming cells, compared with the other antibiotics, the inhibiting concentrations were lower.This is supported by some investigators [38, 39, 40] which indicated that β-lactam were less active antibiotics against sessile *P. aeruginosa,* in contrast to fluoroquinolones which were most active.

These results are in accordance with commonly accepted statement that biofilms are more resistant to antibiotics than planktonic cells [38, 41]. Currently we are aware that the resistance of bacteria living in the biofilm is not associated directly with mutations characteristic to specific strain. Several factors have been suggested to explain the biofilms resistance to antibiotics for example presence of exopolysaccharide substance that can slow the diffusion of antimicrobials. One hypothesis is that reduction in antibiotics penetration through the biofilm is owing to an alginate, synthesized by *P. aeruginosa* an exopolysaccharide, which acts as a barrier for biocides. The permeability studies of the alginate indicated that this factor was not the most important barrier for azithromycin, erythromycin and ceftazidime as their penetration rates were respectively 100%, 100% and 95%, and the bactericidal activity was low (bactericidal concentration ≥ 2560 mg/L) [42].

Biofilm resistance to antibiotics must thus be considered as a combination of the transfer limitation and other factors such as slow growth and decreased metabolic activity, neutralization of the antibiotics by biofilm matrix components (e.g. ability of negatively charged biofilm components to bind cationic compounds), and modifications in gene expression and cell physiology [7, 39, 43, 44].

Results of our study indicate that the effects of antibiotics on biofilm eradication may be variable and unpredictable. This makes it difficult for clinicians to choose the most active antibiotic. Antibiotics should be selected on individual bases, and the assessment of their effectiveness on both bacterial forms, planktonic and in a biofilm should be performed. Then an antibiotic therapy will have a chance of success. Our study suggest the need to develop and to introduce a standardized susceptibility testing method for biofilm mode of growth of pathogens into routinely performed tests as a part of clinical care, especially for patients suffered from CF.

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Conflict of interest statement
The authors declare no conflict of interest.

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Intravenous paracetamol vs. ketoprofen for pain management after the abdominal aortic surgery – pharmacokinetics and therapeutics

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ABSTRACT

Introduction. Acute postoperative pain continues to be a dilemma to patients and clinicians.
Aim. To define the efficacy, tolerability and pharmacokinetics of paracetamol and ketoprofen in patients after the abdominal aortic surgery. Setting and design in University hospital – intensive therapy unit (clinical part), clinical pharmacy and biopharmacy unit (biochemical part), and pharmaceutical company (statistical part). Prospective randomized study.
Material and Methods. 40 adult patients (50–84 years) undergoing abdominal aortic surgery were randomized equally into two groups. After extubation the patients in group 1 (G1) were administered a 1 g paracetamol infusion, and in group 2 (G2) – a 100 mg ketoprofen infusion, both within 15 minutes. All the patients received an epidural infusion of bupivacaine with fentanyl. The following parameters were recorded: mean arterial pressure (MAP), heart rate (HR), central venous pressure (CVP), plasma concentration of paracetamol and ketoprofen. Postoperative pain was assessed with the visual analogue scale (VAS).
Results. The mean values of the MAP, HR and CVP were within normal limits in the both groups. No significant differences were noticed in the assessment of postoperative pain and total use of an opioid. The mean therapeutic plasma concentration of paracetamol and ketoprofen remained up to 180 minutes and up to 120 minutes, respecively.
Conclusions. The study enabled us to conclude that intravenous paracetamol as well as ketoprofen have good effectiveness and tolerability. There is no need to modify dosage of these drugs to elderly patients. After paracetamol infusion the therapeutic plasma concentration remains longer than after the ketoprofen infusion.

Keywords: paracetamol, ketoprofen, postoperative pain, pharmacokinetics.

Introduction

In spite of considerable progress in pain therapy the effective treatment of acute postoperative pain continues to be a dilemma to patients and clinicians. It is estimated that in about two thirds of patients the alleviation of postoperative pain is insufficient and pain becomes the cause of unnecessary suffering [1].

The intensive development of pharmacology enabled the introduction of multimodal analgesia. This is a method of analgesic treatment which consists of connecting different techniques of local anaesthesia

with combination pharmacotherapy. It enables both the use of the additive and synergistic effects of individual drugs, considerable reduction in the dosage of those drugs as well as reduction in the frequency of adverse reactions occurrence [2].

In view of those facts, apart from opioids the clinical practice of postoperative pain treatment also applies non-steroidal anti-inflammatory drugs and paracetamol, the latter of which is widely used in outpatient medical practice. At present, thanks to the new intravenous formula it can also be applied to patients after surgeries. The recommendations for postoperative pain treatment after the surgeries with considerable tissue trauma include intravenous patient controlled analgesia (PCA) and the techniques of regional analgesia, such as continuous epidural analgesia [3].

Intravenous paracetamol (also known as acetaminophen) is an analgesic and antipyretic substance, recommended worldwide as a first-line agent for the treatment of pain and fever in adults and children [4] The availability of intravenous paracetamol (Perfalgan®, Ofirmev®) has greatly extended the use of this drug in the intensive care settings [5].

Ketoprofen is a non-steroidal anti-inflammatory drug with a strong anti-inflammatory, analgesic and antipyretic effect. In chemical terms it is a 2-(3-benzoylphenyl)-propionic acid, available in the intravenous, intramuscular, oral, rectal and percutaneous form [6] The intravenous form is the most suitable and practical for administration in the postoperative period. Ketoprofen was synthesised by the chemists from Rhone-Poulenc company in 1967, 3 years after its prototype – ibuprofen [7] Intravenous ketoprofen is chiefly used for short-term treatment of postoperative pain.

In spite of the fact that intravenous paracetamol is more and more widely applied in clinical practice, the data comparing the clinical efficacy, safety and clinical pharmacokinetics of this drug with other analgesics are limited [8] Vascular surgery patients present a formidable challenge to the practising intensivist. These patients are often at an advanced age and carry significant cardiac, respiratory, and renal co-morbidities [9] Among different types of non-cardiac surgery, peripheral vascular surgery is likely to have the highest cardiac morbidity and overall mortality.

The purpose of this study was to define the clinical tolerability of paracetamol and ketoprofen in patients after the abdominal aortic surgery, the dosage profile of these drugs to this population of patients and the clinical pharmacokinetics with influence on the postoperative analgesic effect.

Material and Methods

After obtaining institutional Bioethics Committee approval, this research was conducted in the intensive care unit (ICU) of the University Hospital. Written informed consent was obtained from all included patients. Forty patients (50–84 years old, 7 females, 33 males, ASA 3–4) qualified for reconstruction of the abdominal part of the aorta due to aortic aneurysms or chronic aortoiliac occlusive disease were included into the study. The patients were randomly divided into two groups. After the extubation group I (G1) received an intravenous infusion of paracetamol (Perfalgan®, Bristol-Myers Squibb, Anagni, Italy) and group II (G2) received ketoprofen (Ketonal®, Lek, Ljubljana, Slovenia). The patients with liver and renal dysfunction or with a documented allergy to the medication were excluded from the survey. All the patients received 10–20 mg of temazepam 60 minutes before the surgery. Anaesthesia was induced intravenously by infusion of etomidate 0.1 mg/kg and fentanyl 3 μg/kg, with muscle relaxation induced by pancuronium 0.1 mg/kg. Then the patients were intubated and received one dose of fentanyl 0.1 mg in 10 ml 0.9% NaCl and constant infusion of 0.125% bupivacaine 5 ml/h into the lumbar epidural space (L2-L3 or L3-L4) through a catheter (16G) inserted to all the patients the day before anaesthesia.

Anaesthesia was maintained with up to 1.5 MAC of volatile anaesthetic isoflurane in a mixture of oxygen and air (FiO_2 0.4) in a low-flow circuit (fresh gas flow of 1 l/min), with fentanyl in boluses of 0.1 mg and pancuronium 0.03 mg/kg and with a constant infusion of bupivacaine into the epidural space.

Just after the operation the patients were admitted into the ICU. After the extubation G1 (20 patients) received an intravenous infusion of paracetamol (1 g within 15 minutes) and G2 received an intravenous infusion of ketoprofen (100 mg in 100 ml of 0.9% NaCl within 15 minutes). Apart from the above-mentioned medications the patients in both groups were applied a constant infusion of 0.125% bupivacaine with fentanyl 2 μg/ml into the epidural space at a rate of 5–8 ml/h. An opioid (pethidine) was also applied in the patient-controlled anaesthesia (PCA) system. This protocol has been applicable according to therapeutic standard in the department.

All the patients were constantly monitored for the mean arterial pressure (MAP), heart rate (HR) and central venous pressure (CVP). In G1 the concentration of paracetamol and in G2 the concentration of ketoprofen were measured. All the measurements listed above

were made before the infusion of the medications under study (paracetamol and ketoprofen) after extubation – T0, immediately after the end of the infusion – T1, and 5 – T2, 15 – T3, 30 – T4, 60 – T5, 120 – T6, 180 – T7, 240 – T8, 300 – T9 and 360 minutes – T10 after the end of the infusion. The pharmacokinetic parameters of the medications were assessed. The total dose of an opioid used in the PCA system was also measured during the study.

Apart from that, the side effects of the early postoperative period were also monitored, such as the haemodynamic changes (with a cardiac monitor IntelliVue MP60, Phillips), allergic reactions and others.

Arterial blood (3 ml) was taken from an arterial cannula in the radial artery. After centrifuging the plasma was frozen and stored at -20°C until all the material from a particular cycle of the research was collected.

At each point of time (T0–T10) the mean, minimum and maximum concentrations of the medications were analysed. The correlations between the main concentration of paracetamol and the visual analogue scale (VAS) median in G1 and between ketoprofen and the VAS median in G2 were estimated. The values of the pharmacokinetic parameters of paracetamol and ketoprofen were calculated on the basis of a model-independent pharmacokinetic approach. The multifactor analysis of covariance based on the linear model of coexisting variables was used to estimate the influence of body weight and age on the pharmacokinetic parameters of the medications.

The paracetamol plasma concentrations were measured with a TDx apparatus (Abbott Diagnostic Division USA, 1996; Abbott/Shaw Lifecare Infusion Pump, Model 3) by means of the fluorescence polarisation immunoassay (FPIA).

The ketoprofen concentration in the plasma was measured by means of high-performance liquid chromatography with an ultraviolet detector [10] The quantification limit was estimated at 0.05 mg/l. The within-day and between-day coefficients of variation were lower than 10%.

Both ketoprofen and paracetamol pharmacokinetic parameters were calculated by means of the non-compartmental (NCA) model with Phoenix™ WinNonlin® 6.3 (Certara L.P.). The area under the plasma concentration-time curve (AUC) from time 0 to the last sampling point was calculated by means of the linear trapezoidal linear interpolation method. The elimination half-life ($t_{1/2}$) was estimated from the last four plasma concentration time points. The NCA model was used to calculate the following pharmacokinetic parameters for paracetamol and ketoprofen: area under the plasma concentration-time curve from time zero to infinity (AUC_{∞}), elimination half-life ($t_{1/2}$), clearance (CL), volume of distribution (Vd), and mean residence time (MRT_{∞}).

Statistical analysis

Age, body weight, height, MAP, HR, CVP and the total consumption of an opioid were described as the mean value with the standard deviation (**Tables 1** and **2**). The Shapiro-Wilk test was used to check the consistence with the normal distribution. The t-Student test was used to compare the two groups of measurements (paracetamol vs. ketoprofen) for independent trials

Table 1. The demographic data, classification of physical state, indications for surgery and total dose of an opioid as means with standard deviations

Parameters	G1 (paracetamol)	G2 (ketoprofen)
Age (years)	63.9 ± 7.08	64.7 ± 8.96
Sex (M/F)	15/5	18/2
Body weight (kg)	75.55 ± 16,89	76.8 ± 15.97
Height (cm)	170.94 ± 7.15	171.88 ± 10.47
Body mass index (BMI)	25.6 ± 5.66	25.81 ± 4.96
Classification of physical state (ASA) – III – IV	 14 6	 15 5
Diagnosis: – aneurysm – Lerich syndrom – aneurysm and Lerich syndrom	 12 7 1	 11 7 2
Total consumption of pethidine dose (mg) – number of patients (n) – mean ± SD	 14 33.1 ± 27.9	 16 30.5 ± 26.8

None p correlations were found between the groups

Table 2. The results of hemodynamic parameters as means and standard deviations

Parameters	MAP [mmHg]		HR [beats/min]		CVP [cmH_2O]	
Group Time points	G1 (paracetamol)	G2 (ketoprofen)	G1 (paracetamol)	G2 (ketoprofen)	G1 (paracetamol)	G2 (ketoprofen)
T0	105.1 ± 14.6	95.7 ± 11.6#	84.7 ± 14.0	87.7 ± 14.8	6.6 ± 3.1	7.4 ± 2.1
T1	97.7 ± 13.6*	88.6 ± 13.4#	82.6 ± 11.2	84.0 ± 14.2	6.4 ± 3.1	7.2 ± 2.1
T2	94.8 ± 13.0*	86.8 ± 14.4	82.4 ± 11.1	84.8 ± 14.6	6.2 ± 2.8	6.7 ± 2.0
T3	94.1 ± 13.9*	84.8 ± 14.3#	82.7 ± 12.7	82.3 ± 14.1*	5.7 ± 2.5	6.2 ± 2.3
T4	94.3 ± 13.7*	82.3 ± 13.9#	82.9 ± 11.0	83.8 ± 14.1	5.7 ± 2.5	6.1 ± 2.4
T5	90.9 ± 13.1*	83.2 ± 11.7	83.0 ± 11.6	82.3 ± 13.8*	5.8 ± 2.1	6.7 ± 2.4
T6	92.5 ± 14.3*	82.7 ± 11.8#	80.1 ± 11.2	82.7 ± 13.9	5.2 ± 2.7	7.2 ± 2.4#
T7	91.1 ± 14.3*	81.2 ± 13.0#	81.8 ± 11.1	82.2 ± 13.4*	5.5 ± 2.5	7.4 ± 3.2#
T8	93.8 ± 15.4*	83.3 ± 13.7#	83.8 ± 10.1	81.9 ± 13.3*	4.9 ± 2.4	7.6 ± 2.9#
T9	95.2 ± 14.0*	82.3 ± 14.8#	81.5 ± 12.0	81.0 ± 12.8*	5.6 ± 2.4	7.7 ± 3.4#
T10	94.2 ± 14.6*	83.2 ± 12.3#	80.8 ± 10.8	81.5 ± 13.3*	5.2 ± 2.5	8.2 ± 3.7#

MAP – mean arterial pressure, HR – heart rate, CVP – central venous pressure
* The statistically significant difference within one group ($p < 0.05$)
# The statistically significant difference between both groups ($p < 0.05$)

when consistence with the normal distribution was present. If not, the Mann-Whitney test was chosen.

The analysis of variance (ANOVA) was used for repeatable measurements at the 11 time periods with the Tukey post-hoc test for the distribution of data compatible with the normal distribution.

The parameters presented on a serial scale as VAS were described with the median, minimum and maximum values. For confirmation of the two groups the Student t-test or Mann-Whitney test were used for independent trials.

For the assessment of consistence between the concentration of the drug and the VAS the Spearman factor of non-parametric correlation was used.

The statistical analysis was made with specific software (Statistica, version 8.0.), p-values <0.05 were considered statistically significant.

The influence of body weight and age on the pharmacokinetic parameters of the drugs was estimated by means of the multifactor analysis of covariance based on a linear model including the patient's body weight and age as coexisting variables. The measurements were made with the PROC GLM procedure of the statistical package SAS (SAS Institute Inc. 2002-2003. The SAS System for Windows v. 9.1.3, Service Pack 4, Cary, NC, USA).

Results

No one of 40 patients resigned or was excluded from the study. The assessed groups were homogeneous. There were no differences in the demographic parameters or the risk of operation. The majority of patients was categorized as the class III of ASA scale. The mean of total PCA pethidine consumption in G1 and G2 was 33.1 mg and 30.5 mg respectively, and did not significantly differ between both groups (**Table 1**).

The mean values of hemodynamic parameters (MAP, HR and CVP) are presented in (**Table 2**). The distinguish changes between both groups were observed for MAP (throughout the whole sampling time) and CVP (from T6 to T10 sampling time). With respect to HR, the only few results in the ketoprofen group were recognized as statistically significant within that group.

The values of VAS score obtained from patients' interview are described in (**Table 3**) as median, minimum and maximum measurements, for both group and at each sampling time (T0 – T10). The median of VAS values decreased in similar way in both groups throughout the whole sampling time. It reduced from 4.5 to 2.0 and 5.5 to 1.0 for G1 and G2, respectively. The minimum values were almost the same in both groups at corresponding sampling points. Of note, the maximum results were comparable only from T0 to T4. Maximum values at T5, T6, T9 and T10 were lower for paracetamol group, whereas results at T7 and T8 slightly favored ketoprofen. Increase of maximum VAS results in both groups at the last sampling time points (T9 and T10) may be related with the end of therapeutic concentrations estimated for both drugs (**Figures 1, 2** and **3**).

Basic pharmacokinetic parameters for both drugs were investigated (**Table 4**). With respect to paracetamol, of note, much higher values of AUC_{∞}, Vd, CL and MRT_{∞}

Table 3. Values of VAS score in the paracetamol group (G1) and ketoprofen group (G2)

Parameter	VAS score					
	Median		Minimum		Maximum	
Group Time points	G1 (paracetamol)	G2 (ketoprofen)	G1 (paracetamol)	G2 (ketoprofen)	G1 (paracetamol)	G2 (ketoprofen)
T0	4.5	5.5	1.0	1.0	10.0	9.0
T1	4.0	5.0	1.0	1.0	10.0	9.0
T2	3.5	5.0	1.0	1.0	9.0	9.0
T3	3.0	4.5	1.0	1.0	9.0	8.0
T4	3.0	3.0	1.0	1.0	8.0	8.0
T5	3.0	3.0	0.0	1.0	6.0	8.0
T6	2.0*	2.0*	0.0	0.0	5.0	8.0
T7	2.0*	1.5*	0.0	0.0	5.0	4.0
T8	2.0*	1.0*	0.0	0.0	5.0	4.0
T9	2.0*	1.0*	0.0	0.0	7.0	8.0
T10	2.0*	1.0*	0.0	0.0	6.0	8.0

* The statistically significant difference within one group ($p < 0.05$)

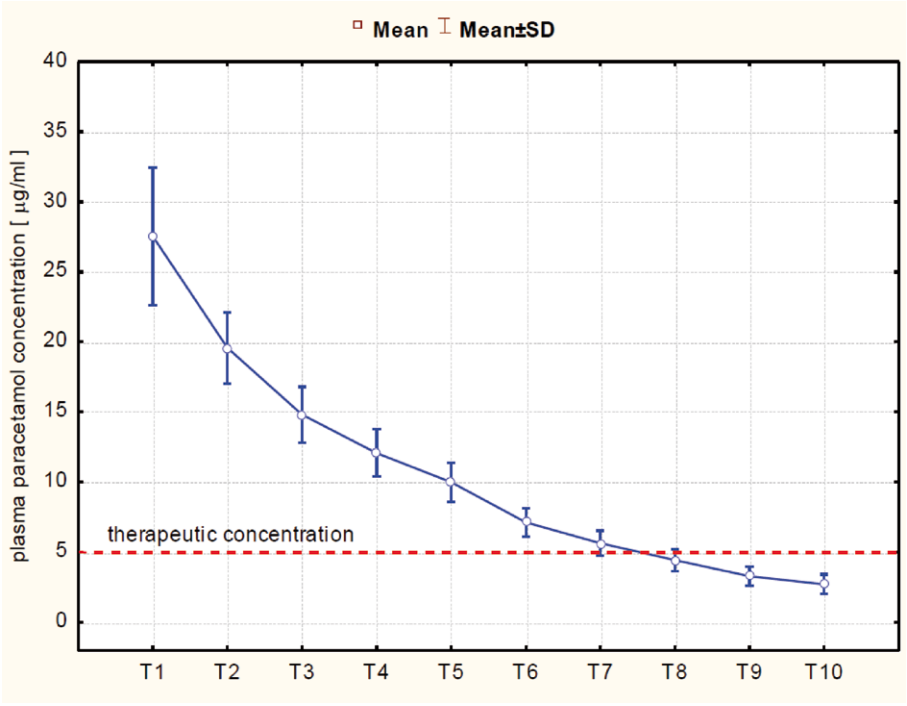

Figure 1. The mean values of paracetamol concentration

were observed for paracetamol than ketoprofen, whereas elimination phase $t_{1/2}$ was similar for both drugs.

The statistic analysis (ANOVA variation) of an influence of the age and body weight of patients to the above-mentioned parameters were calculated (**Table 5**). The only significant relation in the paracetamol group was found between body weight and MRT. In contrast, the statistically important dependence were shown between age and AUC_{∞} or MRT_{∞}, body weight and CL, Vd or MRT_{∞} in the ketoprofen group.

Mean values of pharmacokinetic parameters obtained in our study were similar to those presented in the Flouvat survey (**Table 6**).

The mean concentration values of paracetamol and ketoprofen at each sampling time, and their direct comparison are presented in **Figures 1, 2** and **3**.

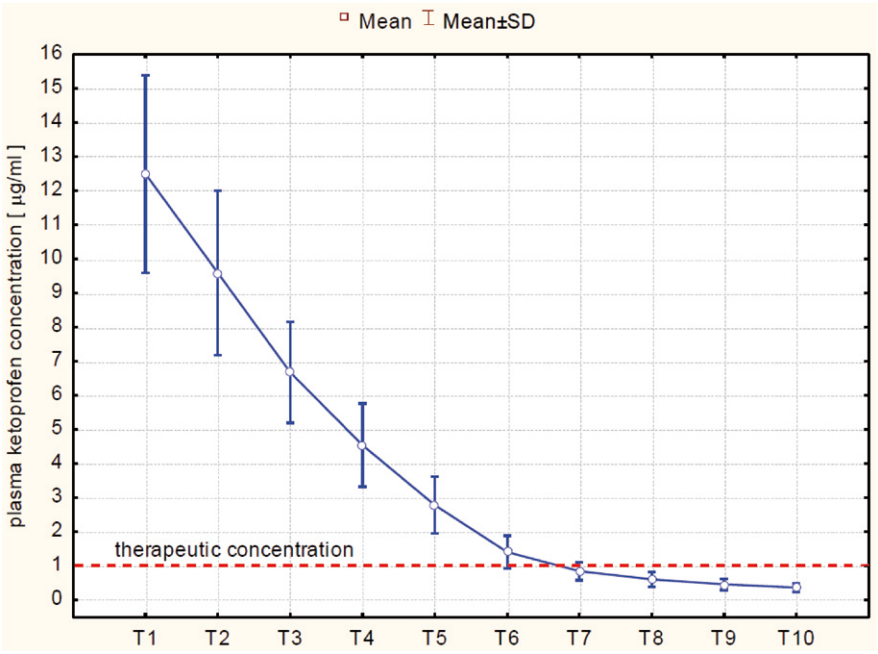

Figure 2. The mean values of ketoprofen concentration

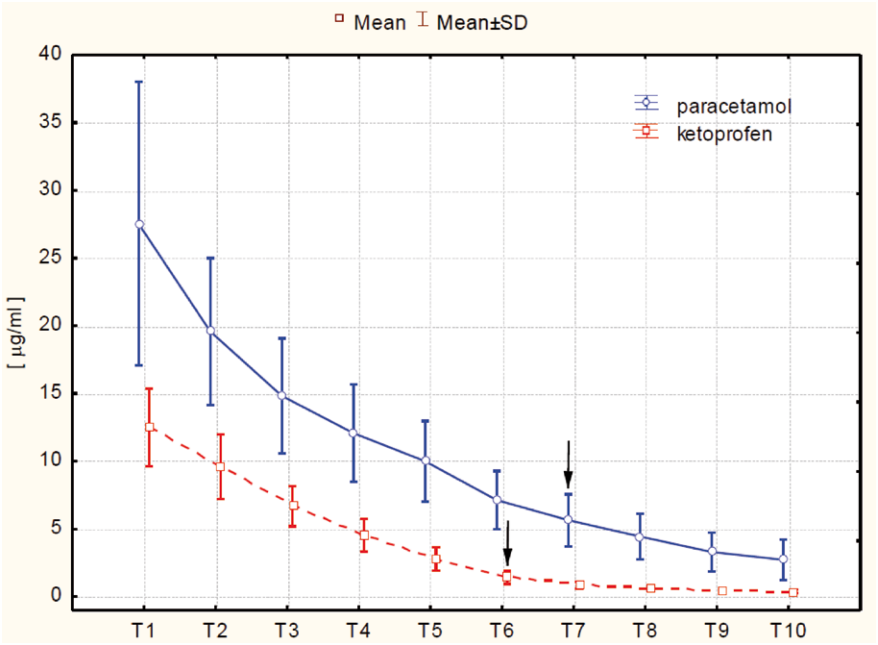

Figure 3. The mean concentration of paracetamol and ketoprofen (the pointers indicate the end of therapeutic concentration)

Table 4. The mean values of pharmacokinetic parameters of paracetamol and ketoprofen

	paracetamol			ketoprofen		
Parameter	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum
AUC_{∞} [mg·h/l]	55.01 ± 25.88	23.78	135.91	13.11 ± 3.25	8.25	23.78
Vd [l]	84.88 ± 33.15	26.02	186.93	27.76 ± 11.13	12.90	54.68
CL [l/h]	21.74 ± 9.46	7.36	60.89	8.02 ± 1.81	4.20	12.13
$t_{1/2}$ [h]	2.85 ± 1.36	1.42	6.45	2.36 ± 0.07	1.44	3.64
MRT_{∞} [h]	4.04 ± 1.84	2.06	9.27	2.23 ± 0.45	1.64	3.05

AUCoo – the area under the plasma concentration-time curve, Vd – volume of distribution,
CL – the apparent total clearance, t1/2 – terminal phase half-life, MRToo – mean residence time

Table 5. The statistic assessment ANOVA – the influence of body weight and patient's age on pharmacokinetic parameters of paracetamol and ketoprofen

	paracetamol		ketoprofen	
Parameter	Age (p-value)	Body weight (p-value)	Age (p-value)	Body weight (p-value)
AUC_{∞}	> 0.05	> 0.05	0.0104	> 0.05
CL	> 0.05	> 0.05	> 0.05	0.0124
Vd	> 0.05	> 0.05	> 0.05	< 0.0001
$t_{1/2}$	> 0.05	> 0.05	> 0.05	> 0.05
MRT_{∞}	> 0.05	< 0.05	< 0.05	< 0.05

AUC∞ – the area under the plasma concentration-time curve, Vd – volume of distribution
CL – the apparent total clearance, t1/2 – terminal phase half-life, MRT∞ – mean residence time
The p-values <0.05 indicate statistical significance

Table 6. The mean values of chosen pharmacokinetic parameters of paracetamol in our research in comparison to Flouvat survey

Parameter	Minimum Our research	Maximum Our research	Mean ± SD Our research	Mean ± SD Flouvat survey
AUC_{∞} [mg·h/l]	23.78	135.91	55.01 ± 25.88	57.6 ± 10.4
Vd [l]	26.02	186.93	84.88 ± 33.15	69.2 ± 8.6 l
CL [l/h]	7.36	60.89	21.74 ± 9.46	17.9 ± 3.4 l
$t_{1/2}$ [h]	1.42	6.45	2.85 ± 1.36	2.72 ± 0.35

AUC∞ – the area under the plasma concentration-time curve, Vd – volume of distribution,
CL – the apparent total clearance, t1/2 – terminal phase half-life

Postoperative period

The groups did not differ significantly in the number of perioperative side effects and complications. In G1 two patients (10%) suffered from postoperative complications, i.e. haemorrhagic shock and iatrogenic pneumothorax. In G2 three patients (15%) had postoperative side effects or complications, i.e. supraventricular arrhythmia, iatrogenic pneumothorax, acute ischemia of the lower extremity. None of local side effects or complications caused by the use of analgesic medications was noted in either group.

Discussion

Our study was comparing intravenous paracetamol with ketoprofen, an NSAID, in terms of clinical pharmacokinetics. Postoperative analgesia is an important factor relieving pain and decreasing complications. The additional use of non-steroidal analgesics decreases pain and it may also reduce the side effects caused by the use of opioids [11, 12]. Sinatra and other authors documented the fact that the intensity of pain decreases significantly in the patients receiving an intravenous infusion of paracetamol or non-steroidal analgesics as a supplement to morphin in the PCA system, as compared with the use of morphine only in a monotherapy [13–15]. However, non-steroidal analgesics, such as ketoprofen, increase the effectiveness of opioid analgesia, but they cause numerous side effects. Ketoprofen increases the risk of perioperative bleeding and the risk of renal dysfunction in patients with renal insufficiency [16–18].

Both Moller et al. and Sinatra et al. stated in their reports that the infusion of paracetamol did not have any clinically significant influence on the patients' haemodynamic parameters [13, 19, 20]. However, Peduto et al. in their research assessing the drug efficacy in orthopaedic surgery documented the fact that the heart rate in the group of patients receiving propacetamol was lower than in the group receiving a placebo, but the difference was not statistically significant [21]. In our study in the group of patients receiving paracetamol the heart rate ranged within the normal values during all periods of the research and it did not differ significantly between one another either after finishing the infusion or later. The average values of the mean arterial pressure ranged within the normal values during the whole study period, but they decreased after the end of infusion of the drug. However, these values were significantly greater than in the group receiving ketoprofen. Cusson et al. assessed the influence of ketoprofen on the blood pressure of patients suffering from arterial hypertension, who were treated with captopril and they found that it is safe to apply the drug to the patients only in a short-term therapy. The values of the patients' blood pressure were similar to those found in the patients receiving a placebo [22, 23].

Intravenous paracetamol is well tolerated by elderly people, including patients with high perioperative risk

[24]. Our research proves this fact, because the average age in the group of patients who received paracetamol was 63.9 ± 7.08 years, III and IV class according to the ASA scale. Sinatra et al., whose findings were mentioned above, arrived at similar conclusions [13].

In our research the efficacy of paracetamol and ketoprofen as well as other methods of multimodal analgesia (PCA and epidural) was proved by low VAS values. According to the Visual Analogue Scale (VAS), after the infusion of the drug in G1 79.5% of the results reached lower values than 4, which proves the appropriate effectiveness of analgesia. Low VAS values remained until the end of the investigation in spite of the fact that the mean paracetamol concentration from the 240th minute to the end of the infusion (T8) was lower than 5 µg/ml (**Figure 1**). The authors are of the opinion that it is the lower limit of the therapeutic concentration [25] On the other hand, in G2 after the infusion of the drug 73.5% of the results reached lower values than 4, according to the Visual Analogue Scale (VAS). The mean concentration of ketoprofen remained within the therapeutic range only until the 120th minute after the end of the infusion (T6) and amounted to 1.41 ± 0.48 µg/ml. Główka et al. estimate the therapeutic concentration of ketoprofen at 1–5 µg/ml (**Figure 3**) [26].

The paracetamol and ketoprofen groups did not differ significantly in the total dose of an opioid the patients applied during the period under investigation. Fletcher et al. arrived at similar conclusions in their study, which was mentioned above [27]. However, our research cannot assess the opioid-sparing effect of those analgesics, because there was no control group with a placebo available. The data from reference books prove the fact that in comparison with a placebo both paracetamol and ketoprofen decrease the demand for opioids [27–29]. Previous studies suggested that action of paracetamol might involve the opioidergic system but Pickering et al. in their pilot trial did not prove that yet [30].

The plasma paracetamol concentration which is required to achieve the necessary analgesia has not been fully investigated. It is thought that the therapeutic antipyretic concentration is 5–20 µg/ml [25]. Probably the plasma concentration which is necessary to achieve the analgesic effect needs to higher, although both higher and lower values are suggested [31]. In the article by Gibb and Anderson, published in March 2008, it is suggested that the necessary concentration to achieve the antipyretic effect is 5 µg/ml, whereas it is 10 µg/ml for the analgesic effect [32].

The mean maximum values of plasma paracetamol concentrations in the patients in this research were comparable with the results obtained by Flouvat et al., Murat et al. and with the values given by the drug manufacturer [33, 34]. After the end of the infusion the mean maximum concentration of the drug was 27.53 µg/ml in our research, 29.9 µg/ml in Flouvat's and 30 µg/ml in Murat's. The latter value is the same as the one given by the manufacturer. Also, such pharmacokinetic parameters as: the total area under curve for time-dependent variations in the drug concentration (AUC_{∞}), the mean volume of distribution (Vd), the mean total clearance (CL) or the half-life at the elimination stage ($t_{1/2}$) did not differ significantly from the values obtained by Flouvat (**Table 6**). Flouvat researched a group of young healthy volunteers (aged 19–37 years), who neither received other drugs nor were anaesthetised immediately before the investigation. Hence the conclusion that the pharmacokinetic parameters and metabolism of paracetamol in elderly patients (the mean age of the patients in group I was 63.9 ± 70.8 years) with numerous preoperative burdens do not change and it is not necessary to modify the drug dosage to those patients.

Immediately after the end of the infusion the plasma paracetamol concentration was higher than 40 µg/ml (40.84 µg/ml, 48.3 µg/ml and 53.08 µg/ml) in three patients from group I. Prins et al. suggest that if the values of paracetamol concentration reach such a high level, this may potentially result in the hepatotoxic effect from the increased production of the toxic metabolite NAPQI involving the cytochrome P450 isoenzyme CYP2E1. However, Jackson et al. think that the risk of damage to the liver appears only when the plasma concentration exceeds 150 µg/ml, which is much higher than the concentration from therapeutic doses [25].

Debruyne et al. studied the pharmacokinetics of ketoprofen after the intravenous administration of 100 mg of the drug and they obtained the following values of pharmacokinetic parameters: AUC_{∞} – about 14 mg·h/l, $t_{1/2}$ – about 2.5 h and CL – about 5.1 l/h [36]. These results are similar to the values obtained in this research, which may indicate that after the reconstructive surgery of the abdominal aorta the elimination of ketoprofen is not impaired. The statistical analysis proved the influence of body weight on the Vd parameter value. When the volume of distribution per kg of body weight value is calculated, a decrease in the inter-individual variation can be observed. The correlation between the AUC_{∞} parameter and the patient's age was also proved. The bioavailability of the drug

increases by 0.21 along with each consecutive year of life in the age group under investigation. On the other hand, the clearance value and the elimination half-life were not observed to decrease as the age increased.

Advenier et al. compared the pharmacokinetics of ketoprofen after the oral administration to younger and elderly people. They proved a significant increase in the values of the total area under the curve of variations in the time-dependent concentration of the drug (AUC_{∞}) and $t_{1/2}$, but there was a decrease in CL. The patients' age span was much larger in that study, i.e. on average 24 ± 1.3 years in the group of younger patients and 86 ± 2.4 in the group of geriatric patients [37].

Our research findings do not point to the correlation between the patient's age and AUC_{∞}, Vd, CL or $t_{1/2}$ parameters for paracetamol. This is in agreement with the earlier data from reference books, which do not indicate the need to modify the dosage of the drug to elderly people [38].

Further clinical investigations are necessary to specify the place of intravenous paracetamol in pain therapy in different groups of patients. The drug has a wide range of advantages, which are particularly useful in the postoperative period. Our research findings also confirm the fact that after an intravenous administration the effect begins as soon as 5–10 minutes [8, 13, 39].

To sum up, intravenous paracetamol and ketoprofen administered to patients with moderate or severe postoperative pain after the reconstructive surgery of the abdominal aorta are effective, safe and well tolerated procedure.

The investigations in this study point to the fact that intravenous paracetamol and ketoprofen-are useful components of multimodal analgesia in the treatment of postoperative pain in patients after the reconstructive surgery of the abdominal aorta.

Conclusion

The study enabled the following conclusions: intravenous paracetamol as well as ketoprofen has good tolerability; there is no need to modify dosage to elderly patients and the therapeutic drug plasma concentration remains longer after a paracetamol infusion than after a ketoprofen infusion.

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Physical activity and the risk of malignant breast cancer development in women

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ABSTRACT

Introduction. The role of physical activity in preventive healthcare constitutes a subject matter of numerous research. In fact, it was proven that physical effort has an impact on lowering the risk of some neoplasms.
Aim. The aim of the paper was to assess the influence of physical activity on the increase or a decrease of odds ratio for developing malignant breast cancer in women.
Material and Methods. The research included healthy women and women diagnosed with malignant breast cancer on the basis of biopsy material or surgical intervention. The research involved 850 women, aged 21–84.
Results. Increased physical effort, both in terms of household duties and physical activity, in patients presented as follows: 1102.61 MET for passive rest at home, 3803.47 MET for household chores, and 1971.54 MET for sports activities. On the other hand, in subjects without malignant lesions in the breasts the study indicated the following results: 1024.05 MET for passive rest, 4150.97 MET for domestic activities and 1651.46 MET for sports activities.
Conclusions. Medium and high physical activity associated with household duties decreases the risk of breast cancer development. In order to lower the risk of developing breast cancer in women, active lifestyle should be promoted in terms of physical effort within medium physical activity, i.e. 600–1500 MET.

Keywords: breast cancer, physical activity, professional work.

Introduction

The role of physical activity in malignant tumour aetiology has been the topic of many research studies. In fact, the research indicates that physical effort may contribute to the decrease in the development of breast, colon, prostate and endometrial cancer. What is more, the benefits stemming from an active lifestyle involve lowering the risk of chronic diseases, such as cardiovascular diseases, diabetes, osteoporosis and hypertension [1–4].

Additionally, minimizing the risk of malignant tumours is directly proportional to the intensity of physical activity, although intensive form of exercise is not indicated for patients with cardiovascular disorders [5].

Furthermore, regular and moderate physical activity influences proper weight and BMI within 18.5–25 kg/m^2. In fact, it is recommended to involve in physical exercise 3 times a week for 30 minutes [5].

The advantages of a healthy lifestyle involving a balanced diet, appropriate physical activity and maintaining proper body weight may contribute to a decrease in the incidence of malignant cancer.

Aim

The aim of the paper was to assess the influence of physical activity associated with domestic duties, professional work, as well as with the recreational activities on an increase or a decrease in malignant breast tumour odds ratio in women.

Material and Methods

The research was conducted among the patients of the Gynaecology and Maternity Teaching Hospital at Poznan University of Medical Sciences between 2011 and 2013. It involved healthy subjects (n = 683), not diagnosed with malignant breast cancer, as well as patients with breast cancer (n = 167) diagnosed on the basis of the histopathological examination. The research in total included 850 women aged 21–84.

The questionnaire was based on questions assessing physical activity in professional work and leisure time. The patients were asked to choose forms of physical activity which they had been involved in prior to the malignant breast cancer diagnosis. A given unit of physical effort was assigned to a physical activity form, whereas in order to assess the intensity of the activity, a metabolic equivalent in MET units (Metabolic Equivalent of Task) was attributed to it.

Estimated physical activity was presented in MET units, as a value of the following parameters: MET value, number of days in a week when the activity was performed, and the activity duration in minutes per day. Additionally, MET coefficient facilitated the division of patients into 3 groups in terms of physical activity: low (under 600 MET), moderate (600–1500 MET) and high (more than 1500–3000 MET) [6].

The assessment of physical activity in professional work was attempted on the basis of a modified Freidenreich's questionnaire [7]:

$$\sum \frac{\left[(final\ age - initial\ age) * \left(\frac{months}{year}\right) * (4{,}33) * \left(\frac{number\ of\ days}{week}\right) * \left(\frac{hours}{day}\right)\right] / 52}{age}$$

The intensity of the professional activity was defined as follows:

1. Profession involving only sedentary work with minimal walking
2. Profession involving little physical effort, without increased breathing rate and without slightly increased heart rate
3. Profession involving carrying light load (2.2–4.5 kg) with increased heart rate
4. Profession involving carrying heavy load above 4.5 kg, quick pace walk, mainly in the fresh air, with increased heart and breathing rate.

The odds for developing breast cancer were calculated when the risk factor was present:

$$Odds\ ratio_{positive} = \frac{\frac{a}{a+c}}{1 - \frac{b}{b+d}}$$

In addition, it was also calculated when it was absent:

$$Odds\ ratio_{negative} = \frac{\frac{b}{b+d}}{1 - \frac{b}{b+d}}$$

By means of logistic regression model, odds ratio (OR) as a relative risk was calculated (**Table 1**) withits confidence intervals (CI) at 95%.

$$OR = \frac{a * d}{c * b}$$

Statistical analysis

The calculations were performed using StatSoft, Inc. STATISTICA Version 10.

Odds ratio (OR) with confidence intervals at 95% was established by means of logistic regression model. The odds ratio relevance was verified with a test where statistical hypotheses were the following H_0: $OR_i = 1$, H_1: $OR_i \neq 1$. Moreover, Wald test statistics was estab-

Table 1. Odds ratio was calculated for each risk factor

Risk factor	Present	Absent	TOTAL
Research group	a	b	a + b
Controls	c	d	c + d
Total	a + c	b + d	a + b + c + d

lished which is characterized by asymptotic distribution χ^2 with first degree freedom. On the basis of p value compared with relevance level α = 0,05 the following decision was made: if p ≤ α,H_0 was rejected, whereas H_1 was accepted. On the other hand, if p > α there was no ground to reject H_0.

The research was approved by the Poznan University of Medical Sciences Ethical Board.

Results

35.4% of the subjects diagnosed with breast cancer went for a walk daily, 11.4% took a stroll once a week, and 17.7% did not undertake it at all. The majority of the patients (81%) did not go to the swimming pool, 11.4% went to the swimming pool less frequently than once a month, whereas 3.8% went for a swim once a week. More than a half of subjects (50.6%) did not ride a bicycle at all, 16.4% rode a bike 3 times a week, and 10.1% participated in this activity every day.

Nearly half of the subjects without malignant lesion in the reproductive organs (40.6%) did not ride a bicycle, 11.2% participated in this activity once a week, and 9.3% took part in it six and more times a week. However, 15.8% of the patients went for a walk every day, 13.4% did so once a week, whereas 10.4% went for a walk less frequently than once a month. 23.8% of patients did not take part in such an activity at all.

Increased physical effort during household duties and physical activity in patients with breast cancer presented as follows: 1102.61 MET for passive rest, 3803.47 MET for household duties, and 1971.54 MET for physical activity. However, in the patients without malignant lesions in breasts the results were: 1024.05 MET for passive rest at home, 4150.97 MET for household duties, and 1651.46 MET for sports activities.

Professional work analysis in the studied groups, revealed the following results: the average number of hours per week in the breast cancer patients was estimated at 19.9 hours. On the other hand, in subjects without malignant breast lesions it was 31.9 hours.

The average MET value during household duties was the following: the highest value of 1297.5 MET was attributed to patients without malignant breast lesions in the course of preparing meals, whereas in subjects diagnosed with breast cancer this value was 799.4 MET. Detailed data is presented in **Figure 1**.

What is more, the influence of physical activity on an increase or a decrease in developing breast cancer odds ratio was also analysed.

Subjects assessing their sports activities between 600–1500 MET daily have 1.29 times higher odds ratio for developing breast cancer, where OR = 1.29; 95% CI 0.68–2.44. On the other hand, participating in sports activities above 1500 MET daily indicated a 1.72 increase in the risk of developing cancer, where OR = 1,72; 95% CI 0,99–2,98, as compared to patients undertaking little physical activity. The results are presented in **Table 2**.

Moderate physical effort during household duties decreases the risk of breast cancer development. The odds ratio equals to OR= 0.52; 95% CI 0.06–4.53 in comparison with low physical effort.

On the other hand, in subjects participating in passive rest of 600–1500 MET daily the risk is increased. Odds ratio for developing breast cancer is OR = 1.51;

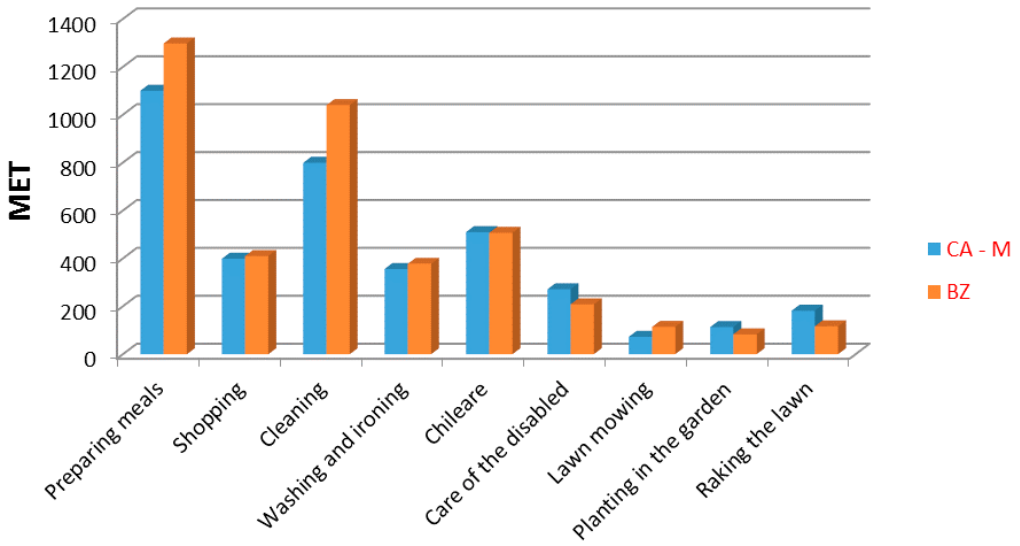

Figure 1. Average MET value during household duties in the research group

Table 2. Odds ratio for breast cancer development on the basis of physical activity

Sports activities	Odds Ratio OR	Confidence Intervals 95%
600–1500 MET	1.29	0.68–2.44
≥ 1500 MET	1.72	0.99–2.98

Table 3. Odds Ratio for the breast cancer development on the basis of the declared passive rest

Passive rest	Odds Ratio OR	Confidence Intervals 95%
600–1500 MET	1.51	0.81–2.81
≥ 1500 MET	1.33	0.65–2.72

95% Cl 0.81–2.81, whereas in patients characterized by passive rest higher than 1500 MET the odd ratio was elevated to OR = 1.33; 95% Cl 0.65–2.72. The data is shown in **Table 3**.

What is more, the influence of physical effort associated with professional works on the odds ratio increase was also analysed. In these calculations the following time spans were established: up to 10 hours of physical effort a week, 20–30 hours per week, and more than 30 hours per week.

Discussion

The role of physical activity in the malignant cancer aetiology has been a subject of numerous research. In fact, it was proven in a number of analyses that regular participation in physical exercise has a substantial influence on lowering morbidity rates due to chronic diseases and malignant cancer [8–14].

Furthermore, there are more data suggesting that in order to lower the risk of breast and colon cancer development, physical effort is optimal when it is performed 45–60 minutes at least 5 times a week. In addition, physical activity may reduce the risk of breast cancer by decreasing the time endogenous steroids affect breast gland epithelial cells, as well as by controlling a woman's weight throughout her life [8].

What is more, Henderson et al. suggest that physical activity presents beneficial influence on breast cancer development also in terms of decreasing insulin and insulin-like growth factor (IGF-1) concentration level. It is the IGF which stimulates cell division, slows cell death and decreases glucose level, at the same time increasing hormone binding globulin concentration. Another physical effort defensive mechanism type is enhancing the immune system where regular and moderate physical activity may decrease the risk ofbreast cancer development by active enzyme regulation, which possess the properties of free radicals inhibitors, as well as by an increase in biogenic antioxidants [9].

The majority of research papers indicates a decrease in the risk of breast cancer development reaching 10–60% in women who are physically active as compared to those who rarely participate in physical effort [15–17].

In our research, moderate physical effort during household duties decreased the risk of developing breast cancer. The odds ratio was OR = 0.52; 95% Cl 0.06–4.53 as compared to low physical activity. In patients who were physically active in their professional work for 11–20 hours, odds ratio was equal to OR = 0.58, 95% CL 0.32–1.07.

Similar results were obtained by Kruk J. who observed a decrease in breast cancer development in women declaring moderate and high physical effort associated with household duties and work in the garden. Additionally, the research indicated that a50% decrease in developing of breast cancer was presented in women participating in high physical activity in comparison to those who remained inactive [16].

In the course of analysis, it is clear that not all of the authors present the protective influence of physical activity on the development of malignant tumours. Research by Dosemeci et al. is a suitable example where the protective influence of increased physical effort on the relative risk of breast cancer development was not observed. In the group of women with high activity, the relative risk was estimated at 1.4 as compared with patients characterised by low physical activity which was confirmed in our study. An increase in breast cancer development is visible in patients with physical activity established at 1500 MET when compared to subjects with low physical activity [18, 19].

Regular physical effort contributes to a decrease in the risk of breast cancer development by means of hormonal regulations, and an increase in the immune system function. However, intense physical activity may contribute to a delayed first menstruation, as well as pri-

mary or secondary amenorrhoea. Furthermore, the production of steroid hormone binding globulin increases, thus decreasing oestrogen function [11, 20–22].

As far as prevention is concerned, three 30-minute intensive units of training are sufficient to reduce the risk of breast cancer development by half [12].

Therefore, physical effort should be one of the basic elements of a healthy lifestyle. What is more, in the course of health education, the importance of positive health behaviours should be stressed, particularly in terms of a proper diet, stimulants avoidance, as well as participation in regular physical activity.

Conclusion

1. In order to decrease the risk of breast cancer development in women, active lifestyle should be emphasised which can be expressed by participating in physical effort within moderate physical activity of 600–1500 MET.
2. Moderate and high physical effort associated with household duties decreases the risk of breast cancer development.
3. The promotion of increasing physical activity should be aimed at women presenting low physical activity, i.e. below 600 MET, especially in their spare time.

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Purpose in life and quality of life of patients with rheumatoid arthritis based on Purpose In Life scale and Arthritis Impact Measurement Scales 2

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ABSTRACT

Introduction. The definition of quality of life conditioned by health condition became the basis for studies on the assessment of the quality of life of patients with rheumatoid arthritis in terms of patients purpose of life.
Aim. The aim of the study is to determine the purpose of life and the quality of life of patients with rheumatoid arthritis. Research questions:
1. What is the quality of life of patients with rheumatoid arthritis?
2. Are patients with rheumatoid arthritis have goals in life?
3. Is there a correlation between the result of the analysis of the purpose of life and the areas of quality of life?
Material and Methods. The study involved 60 patients with diagnosed rheumatoid arthritis based on the ARA and EULAR criteria. 84% of the respondents were women. The average age of patients was 59.5 ± 2.3. To determine the life goals of respondents Polish version of the Purpose In Life scale was used. The scale contains 20 questions. Maximal number of points that could be received is 100, while the lowest number of points is 20. Patients who achieved less than 50 points do not have specified life goals for that particular moment. To evaluate the quality of life Polish version of the Arthritis Impact Measurement Scales 2 was used. The scale consists of 72 questions concerning the following areas: mobility, walking and bending, hand and finger function, satisfaction, arm function, self-care tasks, household tasks, social activity, support, arthritis pain, work, level of emotional tension, mood, satisfaction, health perception, disease impact. The range of scores varies between 0–10, wherein the higher the score, the lower the quality of life.
Results. The highest mean values concerned: health perception (6.96), disease impact on quality of life (6.22), hand and finger junctions function (6.17), arthritis pain (6.04), walking and bending (5.05), emotional tension (4.36), satisfaction (3.61), mobility (3.24). Whereas the lowest mean values were characteristic for support from family and friends (1.25). The statistically significant relation was indicated between social activity (mean value: 4.72) and the sum of life purposes (mean value: 75.54), support from family and friends (mean value: 1.25) and the sum of life purposes (mean value: 75.54), as well as the mood (mean value: 3.01) and the sum of life purposes (mean value: 75.54)
Conclusions. The researches indicate that the concept of the purpose of life in patients with RA provides a basis for further studies. Knowledge of life purposes may reflect the other emotional problems of patients. Evaluation of the quality of life and recognition of the life purposes of patients with RA gives important information for nursing care and psychological planning.

Keywords: purpose of life, quality of life, rheumatoid arthritis, Purpose In Life Scale, Arthritis Impact Measurement Scales 2.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic, progressive inflammatory process of unknown etiology, which begins in the synovium of joints and leads to the destruction of joints, joint tissues, distortions, and finally to the impairment of the joints function. The disease may result in changes in many and organs, such as vasculitis, uveitis, inflammation of the lungs [1–3].

Evaluation of quality of life of patients with rheumatic diseases began to be interested in in the sixties of the twentieth century, the development of research did not occur until the late eighties and early nineties, while the decade 2000–2010 was named by the WHO the Decade of Bone and Joint including an evaluation of the quality of life [4]. In practice, the studies over the evaluation of quality of life usually refer to physical disability, pain, and sometimes general health [5–10]. Some of the researchers focus on the education of patients with RA and the ways of patients knowledge measurement [5, 6–11]. New trend appear to be the life purposes and their measurement [12].

Life goals have are related with mental dimension of quality of life. Verduin et al [12] show the importance of life purposes in patints with RA and define tchem as a feature, inner strength and stress that the life purposes reflect the inner condition, patient's own „I", as well as give the reason and life's motivation. They are also defined as stable and the general intentions to which one strives for in order to achieve something [12].

Aim

The aim of the study is to determine the purpose of life and the quality of life of patients with rheumatoid arthritis. Research questions involved:

1. What is the quality of life of patients with rheumatoid arthritis?
2. Are patients with RA have goals in life?
3. Is there a correlation between the result of the analysis of the purpose of life and the areas of quality of life?

Material

The study involved 60 patients with diagnosed rheumatoid arthritis, based on the ARA [13] and EULAR [1] criteria, treated in rheumatological outpatient practice. 84% of respondents were women. The average age of the patients was 62.25 (12.63). The age range between 20–29 years of age included 7 subjects, the age range: 30–39 years of age included 6 patients, 11 patients were in the age range between 40–49 years of age, while 36 patients were above 50 years of age. 25% of respondents lived in the countryside, the subjects suffering from the disease for more than 10 years represented 52% of the respondents, in 30% of the respondents duration of the disease was from 5 to 10 years, while 18% of the respondents suffered from the disease for less than 5 years. All of the patients were taking non-steroidal anti-inflammatory drugs, corticosteroids and Methotrexate, patients did not were treated with biological agents. The level of pain (in VAS scale) in the range between 4 and 6 included 55% of the patients. While the level of pain in the range from 7–10 was declared by 17% of subjects, 28% of the patients identified the level of pain in the range between 0–3 (**Table 1**).

Methods

To determine the life goals of respondents Polish version of the scale Purpose In Life was used [12]. The scale includes 20 questions; patient was asked to select a number from 1 to 5, which at the time seemed the truest to him. For example: "my life" – with no a specified goals and objectives (resp. 1 point) or – my goals are clear (resp. 5 points). The maximal number of points was 100, while the lowest number of points were 20.

Table 1. Sociodemographic characteristics of the total sample (N = 60)

Variables	Total (n = 60)	Women (n = 50)	Men (n = 10)
Age (mean years, SD)	62.25(12.63)	59.88 (11.91)	61(9.12)
Marital status (n,%):			
Married	41 (69)	35(70)	6(60)
Single	19 (31)	15(30)	4(40)
Education (n,%):			
Primary	4(7)	1(2)	3(30)
Secondary vocational	14(23)	10(20)	4(40)
Secondary and	42(70)	39(78)	3(30)
University-level			
Paid work (n,%)	26(43.3)	24(48)	2(20)

Patients who achieved less than 50 points do not had specified life goals for that particular moment. Purpose In Life scale was translated from German to Polish in accordance with the guidelines contained in the guide concerning correct procedure for linguistic and cultural validation [14].

To evaluate the quality of life Polish version of the Arthritis Impact Measurement Scales 2 was used [15]. The scale consists of 72 questions concerning the following areas: mobility, walking and bending, hand and finger function, level of satisfaction, arm function, self-care tasks, household tasks, social activity, support, arthritis pain, work, level of emotional tension, mood, satisfaction, health perception, disease impact. The range of scores varies between 0–10, where in the higher the score, the lower the quality of life in this particular area of quality of life.

The AIMS 2 scale was translated from English to Polish in accordance with the guidelines contained in the guide concerning correct procedure for linguistic and cultural validation [14].

The level of pain was investigated with VAS scale, where indicates lack of pain and 10 unbearable pain [16].

Statistical methods

For the needs of study groups and variables characteristics the following measures of descriptive statistics were used: mean, median, standard deviation and fractions rates. To examine the co-variableness of the examined variables the r Spearman linear correlation coefficient was used.

Results

Life purposes were evaluated n the basis of mean values and median value in accordance to Purpose In Life scale

The overall average value for the purposes in life in the whole group was 75.54, the median was 76. The minimal value: 36 points, and the maximal value: 100 points (**Table 2**).

The quality of patients' life was evaluated on the basis of mean values and median values in accordance with AIMS2 scale (**Table 3**). The highest mean values concerned: health perception (6.96), disease impact on quality of life (6.22), hand and finger joints function (6.17), arthritis pain (6.04), walking and bending (5.05), emotional tension (4.36), satisfaction (3.61), mobility (3.24). Whereas the lowest mean values were characteristic for support from family and friends (1.25) (**Table 3**).

The statistically significant relation was indicated between social activity (mean value: 4.72) and the sum of life purposes (mean value: 75.54), $p = 0.002$, support from family and friends (mean value:1.25) and the sum of life purposes (mean value: 75.54), $p = 0.002$, as well as the mood (mean value: 3.01) and the sum of life purposes (mean value: 75.54), $p = 0.022$ ($p < 0.05$) (**Table 4**).

Discussion

One of the main investigated aspects within patients with rheumatoid arthritis is the quality of life [4–10, 17, 18]. However, more and more often, articles about the purpose of life can be found [12]. Quality of life is defined by the authors as mental, physical, social and environmental well-being, as well as life satisfaction [19, 20]. It is extremely important to consider all dimensions of life during the quality of life survey so that the assessment is as comprehensive as possible, and to select proper research tool [12]. In our study a standardized Polish version of the AIMS 2 scale was used to evaluate the quality of life, while the Polish version of the Purpose In Life scale was used to identify life purposes.

The authors increasingly investigate particular quality of life areas, such as mobility, self-care, satisfaction, support, professional activity, level of pain, interpersonal relationships, functional disability, spirituality or material status of patients with rheumatoid arthritis. Our studies evaluated 15 areas of quality of life, analyzing psychical, physical and social dimension in accordance with quality of life definition [4–10, 17, 18]. The authors emphasize the decrease in the quality of life in the area of mobility, hence the functional disability is one of the most important factors in the study of the quality of life of patients with RA. In the researches of the other authors the most frequent way of spending the free time among these patients was watching TV, which probable cause may be the problems with mobility among these patients [8]. The authors stress that functional disability is also affected by the disease activity. The CRP levels, inflammatory process activity, advanced radiological changes in the youngest patients, age of the patient at the time of illness or pain levels are important determinants of disability in these patients. The same authors divide the determinants of disability process into disease-related and disease-unrelated. Within disease-related factors they include disease activity, level of pain, joints and periarticular tissues damage and extra-articular

Table 2. Purpose in life of the patients according to Purpose In Life scale (n = 60)

Life purposes according to Purpose In Life scale	Mean	SD	Median	Min	Max
1. Usually I am: bored / enthusiastic	3.68	0.84	4.0	2.0	5.0
2. Life to me seems: completely routine / always exciting	3.62	0.74	4.0	2.0	5.0
3. In my life: no goals or aims / clear goals and aims	3.86	0.99	4.0	1.0	5.0
4. My personal existence is: utterly meaningless without purpose / purposeful and meaningful	3.81	0.91	4.0	1.0	5.0
5. Every day is: exactly the same / constantly new and different	3.63	1.08	4.0	1.0	5.0
6. If I could choose, I would: prefer never to have been born / want more lives just like this one	3.73	1.01	4.0	1.0	5.0
7. After retiring, I would: loaf completely the rest of my life / do some of the exciting things I`ve always wanted to	4.05	1.03	4.0	1.0	5.0
8. In achieving life goals, I have: made no progress whatever / progressed to complete fulfillment	3.53	0.95	3.5	1.0	5.0
9. My life is: empty, filled only with despair / running over with exciting things	3.5	0.68	4.0	1.0	5.0
10. If I should die today I would feel that my life was: completely worthless / very worthwhile	4.6	4.02	4.0	1.0	34.0
11. When I think of my life: often wonder why I exist / always see reasons for being here	4.1	0.90	4.0	1.0	5.0
12. As I view the world in relation to my life the world: completely confuses me / fits meaningfully with my life	3.64	0.93	4.0	1.0	5.0
13. I am a: very irresponsible person / very responsible person	4.32	0.71	4.0	3.0	5.0
14. Concerning freedom to choose, I believe humans are: completely bound by limitations of heredity and environment / totally free to make all life choices	3.57	0.88	3.0	1.0	5.0
15. With regard to death, I am: unprepared and frightened / prepared and unafraid	2.87	1.27	3.0	1.0	5.0
16. Regarding suicide: thought of it seriously as a way out / never given it a second thought	3.94	1.24	4.0	1.0	5.0
17. I regard my ability to find a purpose in life: practically none / very great	3.74	0.91	4.0	1.0	5.0
18. My life is: out of my hands and controlled by / in my hands and I`m in control of it	3.75	0.88	4.0	1.0	5.0
19. My daily tasks are: a painful and boring experience / a source of pleasure and satisfaction	3.68	0.90	4.0	1.0	5.0
20. I have discovered: no mission or purpose in life / a satisfying life purpose	3.75	0.80	4.0	1.0	5.0
The sum of life purposes	75.54	11.33	76.0	36.0	100.0

Patient was asked to select a number from 1 to 5, which at the time seemed the truest to him. For example: "my life" – with no a specified goals and objectives -1point; „ my goals are clear" – 5 points.
The maximal number of points is 100, the lowest number of points is 20, while obtaining less than 50 points reflects lack of specified targets in life

symptoms, whereas factors disease-unrelated factors were divided into demographic and social factors- eg. age, sex, psychological – eg. Dealing with illness, co-morbidities, environmental factors – eg. architectural barriers, and psychical factors – eg. depression [21–23]. However, in our research, the patients most often complained about performance difficulties in in the subjects of quality of life related to the hand function as well as walking and bending. They obtained an average of 6–10 points in AIMS 2 scale in the area of hand function and 4–6 points in the field of walking and bending. They evaluated the areas of the arm function, mobility and household management on the average level.

In the researches concerning purposes in life the authors believe that defined goals provide greater motivation for life in patients with RA and are conditioned by functional status and performed social role [12].

Table 3. The quality of patients' life in accordance with AIMS 2 scale (n = 60)

Areas of AIMS 2	Mean	SD	Median	Min.	Max.
Mobility	3.27	2.45	2.5	0.0	9.5
Walking and bending	5.05	2.74	5.0	0.0	10.0
Hand and finger joints function	6.17	2.77	6.5	0.5	10.5
Arm function	2.84	2.40	2.5	0.0	8.5
Self-care	2.21	1.86	1.8	0.0	5.6
Household tasks	2.45	2.44	1.8	0.0	10.0
Social activity	4.72	1.55	4.5	0.0	8.0
Support from family and friends	1.25	1.62	0.6	0.0	6.8
Arthritis pain	6.04	2.21	6.0	1.5	10.0
Work	3.02	2.54	2.5	0.0	10.0
Emotional tension	4.36	1.60	4.0	0.0	9.5
Mood	3.01	1.55	3.0	0.0	9.0
Satisfaction	3.61	1.90	3.4	0.6	10.0
Health perception	6.96	2.36	6.6	0.0	13.3
RA impact on quality of life	6.22	2.24	7.5	2.5	10.0

Score range 0–10; 0-high quality of life, 10 – poor quality of life

Table 4. The relationship between the areas of quality of life and the sum of life purposes

Areas of quality of life / sum of life purposes	R	t (N-2)	p
Mobility and the sum of life purposes	-0.162	-1.219	0.227
Walking and bending and the sum of life purposes	-0.241	-1.84	0.071
Hand and finger joints function and the sum of life purposes	-0.145	-1.087	0.281
Arm function and the sum of life purposes	-0.033	-0.25	0.803
Self-care and the sum of life purposes	-0.101	-0.755	0.453
Household and the sum of life purposes	-0.021	-0.16	0.873
Social activity and the sum of life purposes	-0.391	-3.128	0.002
Support and the sum of life purposes	-0.398	-3.196	0.002
Pain and the sum of life purposes	-0.165	-1.232	0.223
Work and the sum of life purposes	-0.023	-0.108	0.914
Emotional tension and the sum of life purposes	-0.101	-0.743	0.461
Mood and the sum of life purposes	-0.304	-2.347	0.022
Satisfaction and the sum of life purposes	-0.121	-0.893	0.375
Perception and the sum of life purposes	-0.009	-0.066	0.946

Patients with greater social performance gain higher scores in the scale of life purposes. Life goals are also affected by education, level of pain, active religious affiliation, as well as the ability to cope with illness [12]. Our research confirmed that the patients with better social performance obtain higher numbers of points in the purposes in life scale, meaning they have their life goals defined. In the conducted analysis a statistically significant difference was found between the number of points obtained in the life purposes scale and the following areas: social activity, support, mood.

The literature is not very rich when it comes to publications on life purposes with respect to condition of health and quality of life. Plach et al [24] found that life purposes are conditioned by the functional state, related with social role of the patient as well as significantly correlated with pain. Whereas, Mangelli et al [25] reported that significant correlations between two variables – anxiety, depression and life goals do not contribute to clarification of life goals. In the researches of Verduin et al [12] the younger aged patients with rheumatoid arthritis in good mental state, with the optimal style of coping (participation in recreation or social activities) were characterized by more explicit life purposes.

In spite of many limitations authors believe [12] that these research indicates that the concept of purposes of life in patients with RA provides a basis for further research and should be investigated. The authors also emphasize that the question of life goals may require

time and skill of the interviewer. The problem of not specified life goals may reflect other emotional problems, hence it is a kind of appeal to the psychological intervention and it is worth consideration. Moreover, consideration over the impact of psychological interventions on care for patients with RA improvement [12].

The authors suggest that life purposes require further specification in future researches and focusing on psychological and social performance, quality of life and psychological interventions in the care of patients with RA.

Conclusions

1. The quality of life in the areas of pain, impact of the disease, hand and fingers function and health perception were evaluated the lowest by the respondents. Within the areas of social activity, emotional tension and walking and bending the patients are performing at the average level. The highest scores were obtained in the field of social support, self-care, mood and satisfaction of running a household.
2. Patients with RA specify their life purposes at the average level; obtaining 75 points in Purpose In Life scale. Whereas the strongest influence on the clarification of the purposes of life has the good performance in such quality of life areas as social activity, support from family and friends, and mood.
3. The researches and analysis of the literature indicate that the concept of the purposes of life in patients with RA provides a basis for further studies. Problems with specifying life purposes with patients suffering from RA may reflect other emotional problems of these patients, which recognition would contribute to improvement of specialized care over RA patients.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPER

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Lower diastolic blood pressure in healthy subjects with vitamin K deficiency: a preliminary cross-sectional study

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ABSTRACT

Introduction. There is a growing body of evidence for the role of vitamin K in cardiovascular health. As a cofactor of carboxylation of the matrix Gla protein it prevents arterial calcification. However, the data on the relationship between vitamin K status and the blood pressure are scarce, and particularly so in persons without the burden of cardiovascular risk factors.

Material and Methods. We performed a pilot cross-sectional study, in which we hypothesized that vitamin K deficiency is associated with a higher blood pressure in young, healthy people. The concentration of protein induced by vitamin K absence-II (PIVKA-II) larger than 2 ng/mL was chosen as a proxy for vitamin K deficiency; it was assessed in serum using ELISA. Blood pressure was measured using a validated, automated oscillometric monitor in triplicate.

Results. Twenty-three healthy subjects were enrolled (16 female; mean age 21.3 ± 1.6 years; body mass index 20.6 ± 2.4 kg/m^2). The diastolic blood pressure (DBP) was lower in vitamin K-deficient subjects (58 ± 9 vs. 67 ± 5 mmHg, $p = 0.01$). The mean arterial blood pressure also differed (75 ± 9 vs. 83 ± 6, $p = 0.02$). PIVKA-II levels correlated with DBP only (Pearson's $R = -0.41$, $p < 0.05$; Spearman's ρ ns.). Stepwise regression identified PIVKA-II concentrations as the only independent parameter associated with DBP (adjusted $R^2 = 13.1\%$; PIVKA-II: $\beta = -0.41$; 95%CI -1.87-(-0.00098), $t = -2.08$, $p < 0.05$).

Conclusions. The relationship between vitamin K deficiency and low DBP in young adults should be investigated further.

Keywords: menaquinone, hypertension, K2, arterial stiffness, osteocalcin.

Introduction

Vitamin K is a group of enzyme cofactors that enable carboxylation of proteins containing gamma-carboxyglutamate (Gla) domains. Apart from the constituents of the coagulation cascade other proteins belong to this group, including the matrix Gla protein (MGP, present in the vasculature). Importantly, MGP carboxylation is affected by vitamin K insufficiency in the first line, long before clotting processes become disturbed. The resulting larger fraction of uncarboxylated MGP (ucMGP) favorizes arterial calcification.

This link between vitamin K and the cardiovascular health has been confirmed by a number of studies in humans and animal models. For instance, in 1001 participants the levels of ucMGP were shown to correlate

with vascular stiffness measured by pulse wave velocity calculated on the basis of applanation tonometry and this effect sustained after adjustment for potential confounders [1]. On a populational level, the insufficient supply of vitamin K2 in the diet was recently predicted to be responsible for 6.9% of cardiovascular mortality before the age of 65 years – more than the male gender (6.1%) [2].

However, the data on the relationship between vitamin K status and the blood pressure are scarce, and particularly so in persons without the burden of cardiovascular risk factors. We performed a pilot study, in which we hypothesized that vitamin K deficiency is associated with a higher blood pressure in young, healthy people.

Material and Methods

Healthy subjects were recruited in the year 2014 in the city of Poznan, Poland, as a control group for a study concerning atherosclerosis in cystic fibrosis [3]. The inclusion criterion was no acute or chronic disease. Exclusion criteria were: hypercholesterolemia or hypertriglyceridemia and early coronary disease or vascular brain diseases in the family (< 65 years in women and < 55 years in men).

As a proxy of vitamin K status we employed the level of protein induced by vitamin K absence-II (PIVKA-II). PIVKA-II concentrations above 2 ng/mL were considered to indicate moderate vitamin K deficiency; they were determined in serum using enzyme-linked immunosorbent assay (MyBioSource, San Diego, USA).

Non-high density lipoprotein (non-HDL) cholesterol concentration was chosen and calculated as the most relevant supplementary cardiovascular risk factor. Total cholesterol was assessed with the use of an enzymatic method with esterase, cholesterol oxidase, and Trinder reaction. HDL cholesterol concentrations were measured employing glycerophosphate oxidase and Trinder reaction (Advia 1800 Chemistry, Siemens Healthcare, Erlangen, Germany).

Blood pressure was measured using a validated, automated oscillometric monitor Omron M5-I (Omron, Kyoto, Japan) [4, 5] in the Department of Cardiology-Intensive Therapy of Poznan University of Medical Sciences, Poznan, Poland. An average of three measurements was obtained for systolic (SBP) and diastolic (DBP) pressures and subsequently the mean arterial pressure (MAP) was calculated.

Statistical analyses were performed using Statistica 12 (Statsoft Inc., Tulsa, USA). The Shapiro-Wilk test was used to check for the normality of distribution in subgroups. The F-test was used to verify that there were no statistically significant differences in variances between the compared subgroups. The hypothesis that mean values of parameters in subgroups are equal was tested using the Student's t-test. Correlations were checked by computing both Pearson's r and Spearman's ρ. Forward stepwise multivariable regression models were built to compensate for confounding, which included the following parameters: age, sex, BMI, non-HDL cholesterol, and PIVKA-II concentration. In additional explorative analyses the Mann-Whitney U-test was applied.

The study respected the rules and values set forth in the Declaration of Helsinki and was approved by the bioethical committee at Poznan University of Medical Sciences (decision no. 250/10). All the volunteers provided written, informed consent for their participation in the research.

Results

Twenty-three subjects were recruited for the study, of whom 16 were female (70%). The mean age was 21.3 ± 1.6 years. The average mass, height and body mass index (BMI) were: 60.2 ± 9.2 kg, 171 ± 9 cm, 20.6 ± 2.4 kg/m^2. The PIVKA concentration in the group was varied: mean 3.85 ± 3.58 ng/mL (minimum 0.22, maximum 10.13 ng/mL). The average pressures were: SBP 113 ± 9 mmHg, DBP 63 ± 8 mmHg, and MAP 79 ± 8 mmHg. Non-HDL cholesterol concentration was 104 ± 32 mg/dL.

The comparison of the above parameters in vitamin K-deficient and vitamin K-sufficient healthy persons is presented in **Table 1**. A lower average diastolic and mean blood pressure was found in vitamin K-deficient subjects (**Figure 1**).

PIVKA-II levels correlated with DBP only (Pearson's R = -0.41, $p < 0.05$; Spearman's ρ ns.; **Figure 2**). Of the three regression models built to predict blood pressure values, only the one explaining DBP was valid. PIVKA-II alone was found to independently associate with DBP (adjusted R^2 = 13.1%, model $p < 0.05$; PIVKA-II: β = -0.41; 95%CI -1.87-(-0.00098), t = -2.08, $p < 0.05$).

We performed explorative analyses to characterize the five vitamin K-deficient participants in whom DBP < 55 mHg was found (Figure 2). The five subjects (4 female, 1 male) did not differ from the rest of the group nor from the other vitamin K-deficient persons in age, weight, height, BMI, or non-HDL cholesterol concentration (all p values > 0.40).

Table 1. Comparison of blood pressure and other characteristics of vitamin K-deficient and vitamin K-sufficient healthy persons

Parameter	Vitamin K-deficient	Vitamin K-sufficient	P
Number of persons	11	12	
Age, years	20.9 ± 1.7	21.7 ± 1.5	0.21
Sex	7 female (64%)	9 female (75%)	0.67[a]
Weight, kg	61.2 ± 6.6	59 ± 11	0.62
Height, cm	170 ± 9	171 ± 10	0.81
BMI, kg/m^2	21.1 ± 2.0	20.1 ± 2.7	0.30
Non-HDL cholesterol, mg/dL	108 ± 31	100 ± 34	0.55
SBP, mmHg	110 ± 9	115 ± 8	0.23
DBP, mmHg	58 ± 9	67 ± 5	0.01
MAP, mmHg	75 ± 9	83 ± 6	0.02
PIVKA-II, ng/mL	7.01 ± 2.62	0.95 ± 0.47	< 10^{-6}

[a] Fisher's exact test, two-tailed p-value.
BMI – body mass index, DBP – diastolic blood pressure, MAP – mean arterial pressure, non-HDL – non-high density lipoprotein, SBP – systolic blood pressure

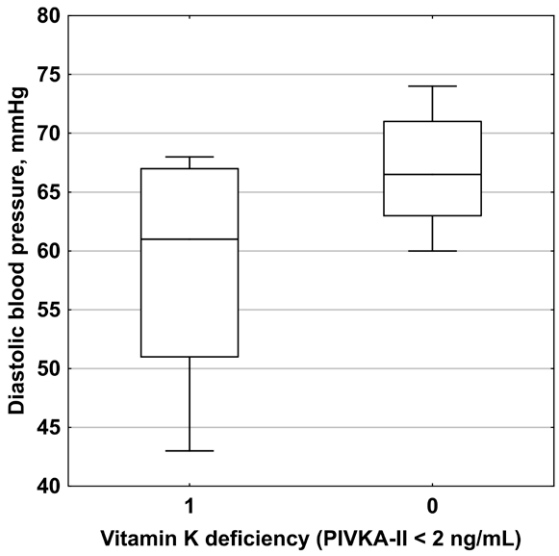

Figure 1. Medians, 1st–3rd quartiles and 5th–95th percentiles of diastolic blood pressure depending on vitamin K status assessed using protein induced by vitamin K absence-II

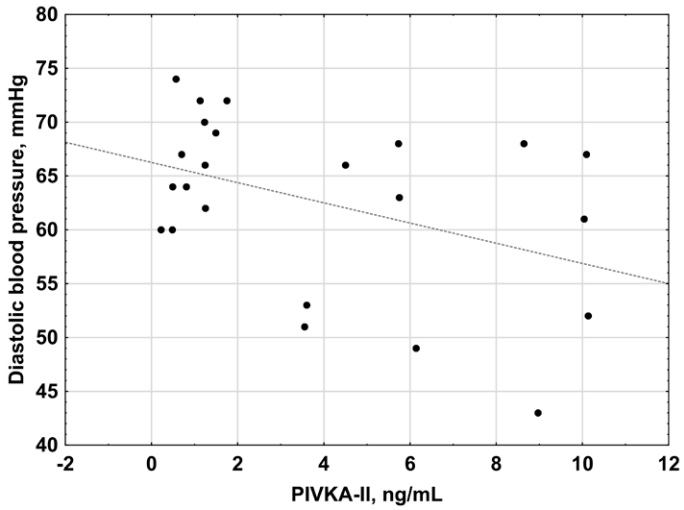

Figure 2. Scatterplot illustrating the relationship between the diastolic blood pressure and protein induced by vitamin K absence-II in 23 healthy subjects. A regression line is shown

Discussion

This pilot study reveals an unexpected association between vitamin K deficiency and a lower DBP in healthy subjects. This goes against the common knowledge, which focuses on the role of MGP and arterial calcification. However, some indirect support for our findings is provided by the study of Streit et al., who analyzed data regarding 4412 patients with hypertension, of whom 569 received phenprocoumon, a vitamin K antagonist. Even after the adjustment for potential confounding, the patients on phenprocoumon had lower mean SBP (-8.4 mmHg) and DBP (-1.5 mmHg). The authors proposed that this could be explained by better compliance to antihypertensive pharmacotherapy – our study adds that there might indeed be a mechanistic explanation to their observation.

Other research regarding vitamin antagonists also yielded important information on the role of Gla carboxylation in cardiovascular health. The Stroke Prevention in Nonrheumatic Atrial Fibrillation (SPINAF) trial, which investigated the impact of warfarin and placebo in 284 patients with atrial fibrillation, did not find any changes in DBP after a mean follow-up of 24 months. A cohort study by Lim et al. followed 116 diabetes patients, half of whom received warfarin, over a period of 36 months. No increases in SBP in the warfarin group were found [6]. Unfortunately, blood pressure data are missing from many other vitamin K antagonists trials [7].

Treatment with warfarin was proposed as an animal model for isolated systolic hypertension [8] and vitamin K was shown to rescue warfarin-induced increase in SBP [9]. Furthermore, a striking warfarin-induced vascular calcification was found in rats with adenine-induced chronic kidney disease. In this case the effect was also mitigated by vitamin K [10]. In humans, total arterial calcium associated with a high osteocalcin ratio, which reflects vitamin K insufficiency [11]. A randomized study in postmenopausal women (n = 244) showed that supplementation of vitamin K2 (180 µg of menaquinone 7 – MK7) during 3 years led to a decrease in arterial stiffness [12]. A large trial investigating high-dose vitamin K2 (360 µg of MK7) in coronary calcification is ongoing in the Netherlands (VitaK-CAC) [13].

In a randomized controlled study by Fulton et al. 80 older people were given either 100 mg of vitamin K2 (MK7) or placebo daily over 6 months. The blood pressure did not change [14]. It should be added that the dose used by Fulton et al. was moderate [15]. In a small randomized study comparing vitamin K2 (1.5 mg daily of menaquinone 4 – MK4) and placebo a reduction in both SBP and DBP was observed, but the results remained inconclusive in this respect since a decrease in blood pressure was observed in the control group as well [16]. Menaquinones of various length are naturally produced by the intestinal microbiome and may have different biological functions. This is an area of ongoing research; one of its fruits is the latterly introduced concept of menaquinotype [17].

In a cross-sectional analysis of the National Health and Nutrition Examination Survey (NHANES) study vitamin K1 (phylloquinone) intake inversely correlated with elevated blood pressure [18]. There are also other strong data indicating that a simultaneous supplementation with vitamins K and D could lower blood pressure [19]. Interestingly, in a group of 1035 patients ucMGP positively associated with the renal resitive index [20].

A major limitation of this preliminary observational, cross-sectional study is the small sample size, which both reduces the chance of finding a true effect and increases the probability of a false positive finding [21]. In order to address this problem, we used various approaches to the data, one of which (Spearman's rank-sum correlation) did not replicate the main finding. On the one hand, the forward stepwise regression, which is a useful tool for identifying correlations, is known to inflate the significance of findings. On the other hand, the t test, which is robust, indicated a highly significant finding and a linear correlation between the raw (non-categorized) PIVKA-II levels and DBP was also confirmed. As a consequence, the thought-provoking finding that we present herein should be interpreted with due caution.

Conclusion

The relationship between vitamin K deficiency and low DBP in young adults should be investigated further.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPER

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Influence of ghrelin on rat pituitary GH3 cell line proliferation

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ABSTRACT

Introduction. Human ghrelin is the endogenous ligand of the growth hormone secretagogue receptor type 1a (GHSR1a). It is suggested that ghrelin is involved in pituitary adenomas pathogenesis. There are inconsistent data regarding the effect of ghrelin on cell proliferation. In this study the outcome of ghrelin in the rat pituitary adenoma GH3 cell line on morphology and proliferation ratio was evaluated. The ghrelin receptor (*Ghsr*) mRNA expression in GH3 cell line was established as well, because it was found that heterogeneous expression pattern characterized physiological and pathological conditions of tissues of different origin.

Material and Methods. Suitable experimental model pituitary tumor (rat GH3 cell line) was stimulated with ghrelin in the final concentrations 10^{-12} M, 10^{-9} M and 10^{-6} M. Reverse transcription followed by real time polymerase chain reaction was used for ghrelin receptor gene transcript detection. The morphology as well as cell cycle of those cells were analyzed using Axio Vert.A1 Microscope (Zeiss) and BD FACSCalibur™ flow cytometer (Beckton Dickinson), respectively. The percentages of cells in the G_0/G_1, S, G_2/M cycle phases were evaluated using the ModFit™ software (Verity Software, Inc., USA).

Results. *Ghsr* mRNA presence was confirmed in GH3 cells. Ghrelin did not affect conspicuously GH3 cells morphology, however the ghrelin-induced proliferation index increase was caused by both decline of G_0/G_1 phases cells count and increase those being in $S+G_2/M$ ($p < 0.05$).

Conclusions. In conclusion, this study indicates that ghrelin stimulates GH3 cells proliferation and may play role in pituitary tumorigenesis *via* an autocrine/paracrine pathway.

Keywords: ghrelin; ghrelin receptor; proliferation, pituitary adenoma.

Introduction

The role of ghrelin (GHRL) in pituitary tumorigenesis is unexplained so far. Expression of the receptor through which ghrelin mediate its effects was identified in different types of pituitary tumors, including the majority of somatotropinomas, in which the highest expression of growth hormone (GH) secretagogues receptor (GHSR) was detected [1]. There are reports suggesting that ghrelin may be an antiproliferative factor. Its inhibitory impact on proliferation was confirmed in studies conducted on cancer cell lines of the thyroid gland, breast, pituitary and lungs [2]. Other studies suggest that it may also stimulate cells proliferation [3, 4].

GH3 cell line derived from rat's anterior pituitary tumor is an *in vitro* model to study pituitary adenoma

development. In the previous studies GH3 cells were also used as a suitable cell line model regarding human somatotropinoma. There are only few reports concerning the proliferative effect of ghrelin on somatotropic cells of above mentioned cancer cell line [5–7]. Nanzer *et al*. found that both the acylated and non-acylated ghrelin stimulated proliferation of cells of GH3 cancer cell line [5]. Stevanovic *et al*. and Milosević *et al*. after administration of ghrelin into rats cerebral ventricles, found that the weight of the pituitary, the volume of both, GH-producing cells and their nuclei increased. This suggests enhanced potency of ghrelin, which may contribute to its transcriptional activity [6, 7].

In this study we examined the *Ghsr* gene expression in GH3 cell line, and whether ghrelin affects somatotroph pituitary rat adenoma GH3 cell line morphology and proliferation ratio.

Material and Methods

Cell culture and stimulation

All experiments were performed on rat pituitary adenoma GH_3 cell line obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Ham's F-10 medium (Cytogen, Germany) supplemented with 2.5% fetal bovine serum (FBS, Biowest, USA), 15% horse serum (Sigma Aldrich, USA), 100 µg/mL penicillin, 100 µg/mL streptomycin (Cytogen, Germany) and 2 mM L-glutamine (Cytogen, Germany). Cell line was maintained in aseptic conditions at 37°C, 5% CO_2 in a humidified incubator and confirmed free of mycoplasma contamination through regular testing (Mycoplasma PCR Test Kit, AppliChem, Germany). Cells were cultured until 90% confluence. At this point they were washed with phosphate buffered saline (PBS, Biowest, USA) and detached from culture dishes with 0.25% trypsin solution (Biowest, USA). After 3 minutes of incubation trypsin was removed, complete growth medium was added and resuspended cells were transferred into sterile 6-well plates at a density of 100,000 cells per well.

Ghsr gene expression analysis

GH3 cells prepared as described before were used for *Ghsr* expression analysis using reverse transcription followed by real time polymerase chain reaction. This method was performed to analyze the expression in qualitative and not quantitative way, because of its sensitivity and specificity (provided with the TaqMan® probes).

RNA isolation and reverse transcription

Total RNA was extracted from GH3 cells using ready-to-use RNA 3-zone reagent (Novazym, Poland) according to manufacturer's protocol with modification in RNA precipitation step performed in -80°C instead of room temperature. The quality of total RNA and its concentration were analyzed with the use of NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity was evaluated by ribosomal RNA bands analysis after electrophoretic separation of 1µg RNA in 0.8% agarose gel in 1xFA buffer (20 mM 3-[N-morpholino]-propanesulfonic acid (MOPS) (free acid), 5 mM sodium acetate, 1 mM EDTA, pH 7.0, Sigma-Aldrich, USA) and presence of ethidium bromide and 0.8% paraformaldehyde (Avantor, Poland) providing denaturing conditions.

RNA was reversely transcribed to cDNA in three-step reaction conducted in accordance with Transcriptor Reverse Transcriptase manufacturer's protocol (Roche, Germany) in the total volume of 10 µL. In the first step mixture of: 5 mM oligo(d)T_{10} (Genomed, Poland), RNA (0.5 µg) and RNase-, DNase- and pyrogen-free water (Life Science) was denatured 10 min at 65°C. Subsequently, the samples were cooled on ice. In the second step of incubation 10U/µL ribonuclease inhibitor (RNasin, Roche), 10 U/µL of Transcriptor reverse transcriptase (Roche), 100 mM dNTPs (Novayzm) and 1x reaction buffer (Roche) were added. Thermal profile was as follows: 10 minutes at 25°C (binding of primers to the template), 60 minutes at 55°C (cDNA synthesis step) and 5 minutes at 85°C (enzyme denaturation). Until real time PCR was performed cDNA was stored in -20°C.

TaqMan® real time polymerase chain reaction

RNA expression pattern analysis was performed using the LightCycler 2.0 carousel-based system. Real time PCR for the Rat (*Rattus Norvegicus*) *Ghsr* (GeneBank: NM_032075.3) was conducted with TaqMan® hydrolysis probes (Roche) and primers (Genomed, Poland) designed with the Universal ProbeLibrary assay design on-line software (Roche). Sense and antisense primers and the fluorescent probes numbers were: 5'-AGGAAGCTATGGCGGAGAC-3' and 5'-GAAAGCAAACACCACCACAGC-3', probe #112 (Roche cat. N° 04693469001). Rat ready to use *Actb* (β-actin) Reference Gene Assay (Roche assay N° 5046203001) was used as internal control and for relative concentration ratio evaluation. Reactions were performed in the total volume of 20 µL reagent mix containing: 1x LightCycler® FastStart™ TaqMan® Probe Master mix (Roche), 5 µL of cDNA, 0.5

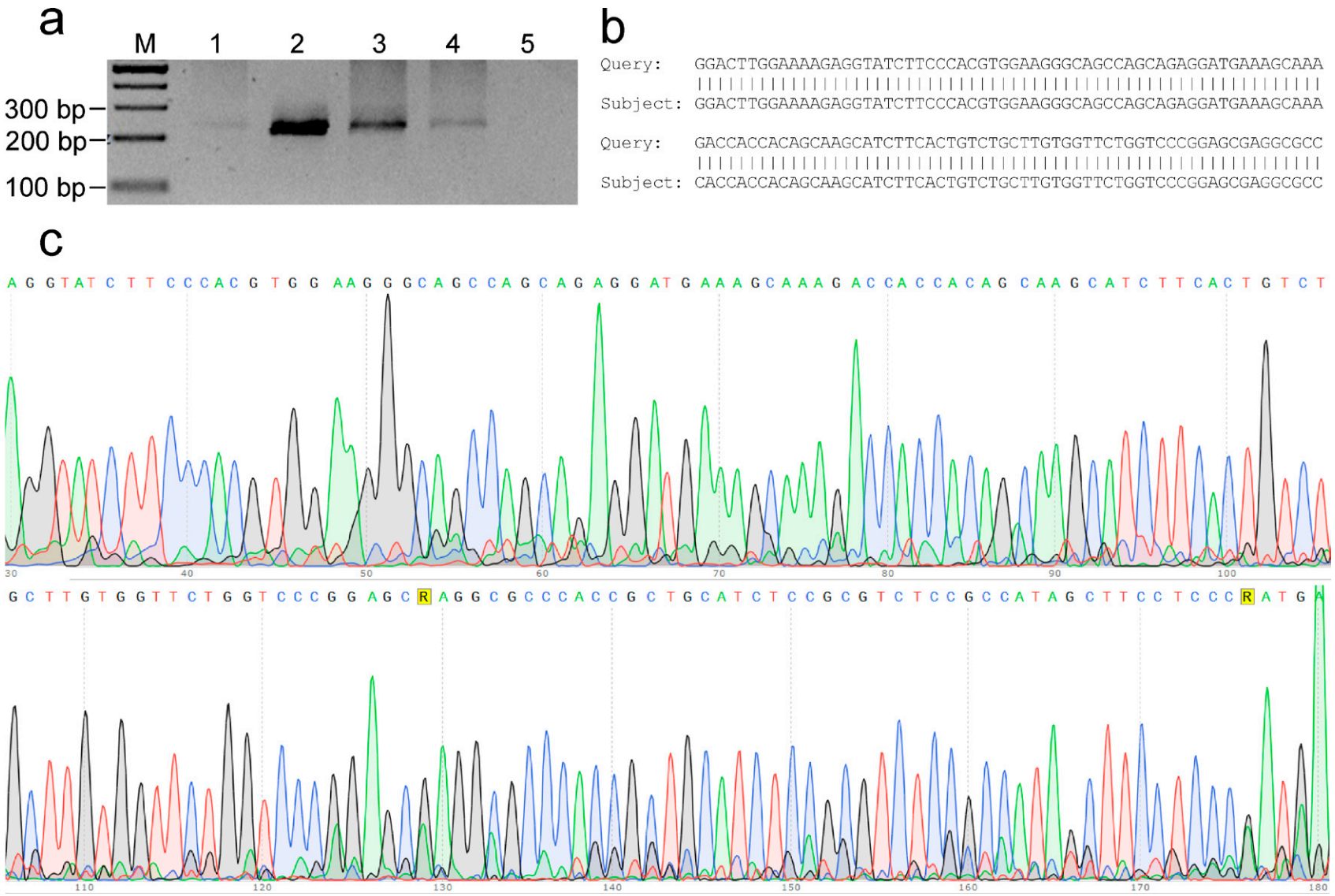

Figure 1. Expression of *Ghsr* mRNA in GH3 pituitary tumor cell line. (a) Gel electrophoretic separation of real time PCR products: M – Nova 100 mass marker, 1-4 qPCR bands representing experiments derived from mRNA isolated from GH3 cell line, 5 – negative control; (b) alignment of the sequencing reaction result with NCBI reference sequence and (c) example of Sanger sequencing of PCR product analysis

mM of each primer (Genomed) and 0.1 mM hydrolysis probe (Roche).

5 µl of qPCR product was analyzed and compared with the Nova 100 molecular mass marker (Novazym) after electrophoretic separation in 2% agarose gel (FMC BioProducts, Rockland, ME USA) containing 1x Tris/Boric Acid/EDTA (TBE) buffer (Bio-Rad) at presence of 500ng/ml ethidium bromide (Sigma-Aldrich).

The presence of TaqMan® hydrolysis probes ensured the specifity of the reaction. ZymoClean™ gel DNA recovery kit (Zymo Research, USA) was used for PCR products gel recovery and purification followed by Sanger sequencing analysis (Genomed).

Cell morphology

Cells morphology was evaluated using using Axio Vert. A1 microscope (Zeiss, Germany) after 24 and 48 hours of incubation with different ghrelin concentrations: 10^{-12} M, 10^{-9} M and 10^{-6} M.

Cell cycle analysis

Cells were treated with ghrelin for cell cycle analysis in different final concentrations: 10^{-12} M, 10^{-9} M and 10^{-6} M. All analyses were made in triplicates. Control contained non-stimulated cells. Cells prepared due to this procedure were used for further investigations.

After 24 and 48 hours of incubation cells were harvested as described above, washed in PBS and fixed in 70% ethanol (Avantor, Poland) at 4°C for 30 minutes. Next, cells were pelleted by centrifugation, resuspended in 1 mL of PBS containing RNase A (10 mg/mL) and incubated at room temperature for 30 minutes. After that, cells were centrifuged and the pellet was suspended in 500 µL propidium iodide staining solution (50 µg/mL) for 1 hour at room temperature in the dark. Subsequently, cell samples were analyzed with the use of the BD FACSCalibur™ flow cytometer (Becton Dickinson, USA). For each experiment 10,000 cells were examined. The fluorescence of propidium iodide was excited using an argon laser (488 nm) and emission of red fluorescence was detected in the FL3 channel (>650 nm). Data were collected and analyzed using CellQuest Pro software (v.5.2.1) (Becton-Dickinson). The percentages of cells in the sub G_0, G_0/G_1, S, G_2/M cycle phases were evaluated using the ModFit™ software (Verity Software, Inc., USA). After flow cytometric analysis, Modfit software was used to calculate the proliferation index (PI) ($S+G_2M/G_0G_1x100$) by dividing the percentages of proliferating cells (cells in S and G_2/M phases) by non-proliferating cells (cells in G_0/G_1 phase).

Statistical analysis

The data were analyzed using the Statistica 10 software (StatSoft Inc., USA). The distributions of the data were assessed by the Shapiro-Wilk test. Due to nonparametric distributions Kruskal-Wallis test with Dunn's post-hoc test were applied. The data were calculated for three separate experiments and shown as mean ± standard deviation (SD). Statistical significance was taken at $p < 0.05$.

Results

Ghsr gene expression analysis

Presence of *Ghsr* gene was confirmed after reverse transcription real tie polymerase chain reaction with the use of TaqMan® hydrolysis probes by gel electrophoresis (**Figure 1a**) and Sanger sequencing (**Figure 1c**). The size of the electrophoretic separated DNA band as well as alignment (**Figure 1b**) of the sequencing reaction result scored entirely with this deposited in NCBI database and confirmed the identity with *Ghsr* GeneBank N° NM_032075.3

Ghrelin and GH3 cells morphology

GH3 cells were loosely adherent with floating clusters and exhibited differences in their morphology was culture time-dependent. After the attachment to the Petri dish, the cells were characterized by a spherical shape which has changed to more polygonal with increasing cell confluence (**Figure 2**). Ghrelin did not affect GH3 cells morphology regardless of ligand concentration and incubation time.

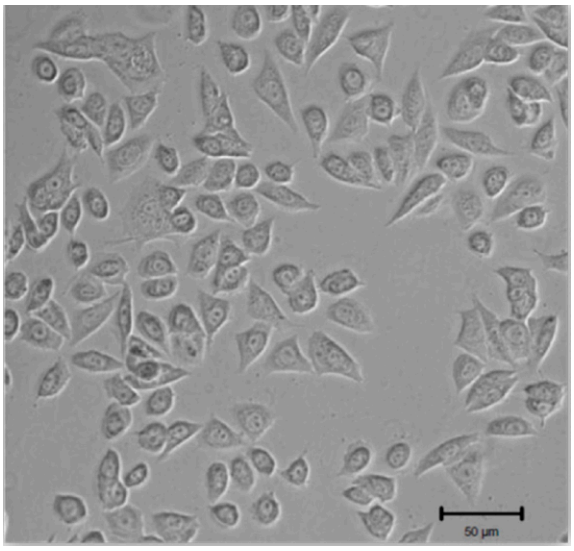

Figure 2. GH3 pituitary tumor cell line morphology. Representing example of non-stimulated cells

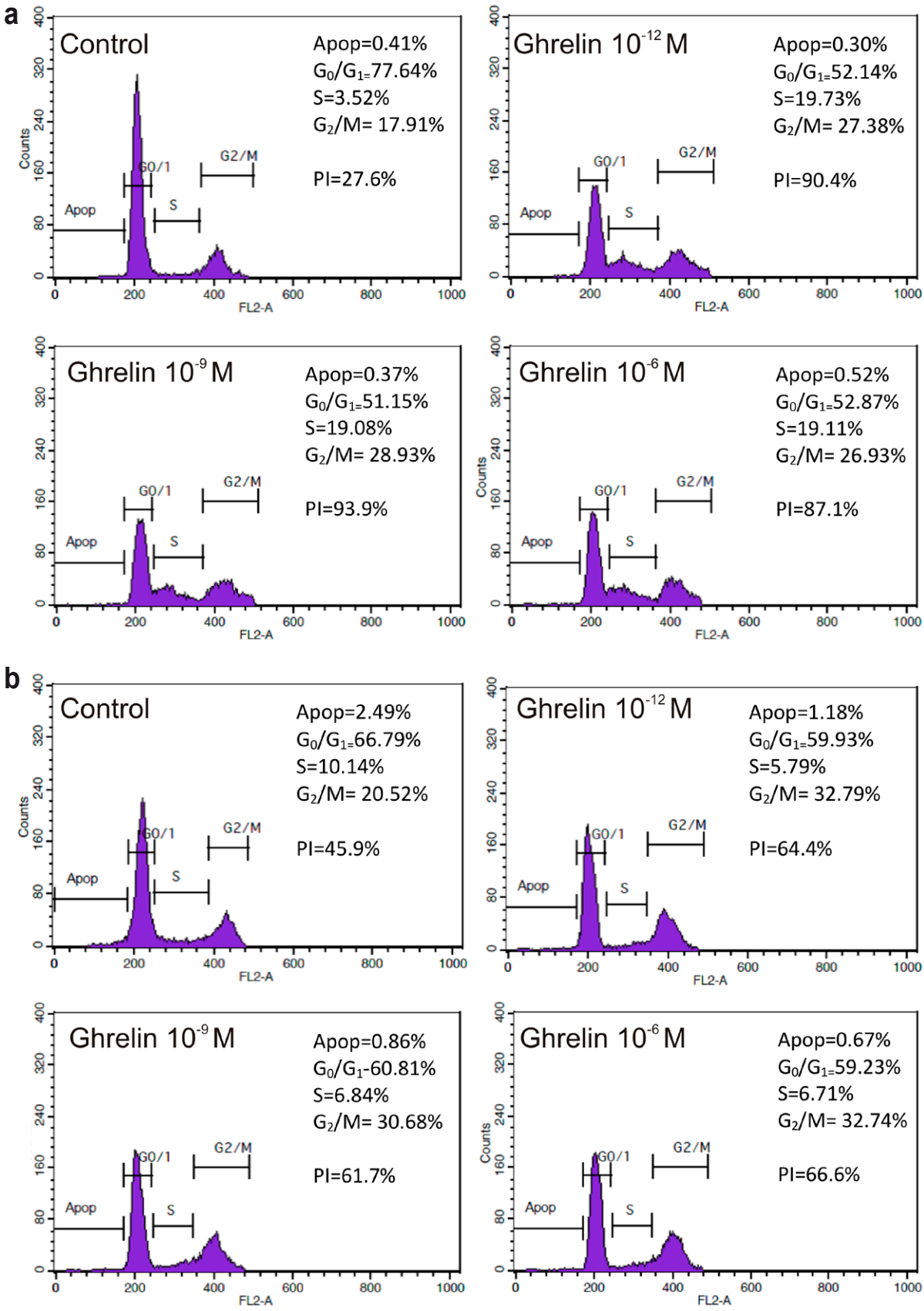

Figure 3. Representative example of cell cycle redistribution of GH3 cells after ghrelin stimulation. GH3 cells ware incubated with ghrelin in different doses (10^{-12}, 10^{-9} or 10^{-6} M) for 24 or 48 h. The cell cycle was analyzed using FACSCalibur™ flow cytometer. The percentages of cells in the sub G_0, G_0/G_1, S, G_2/M cycle phases were evaluated using the Modfit software. PI – proliferation index ($S+G_2M/G_0G_1x100$), Apop – apoptotic cells percentage

Ghrelin and GH3 cell cycle

In the control group no changes in the cell cycle of GH3 cells were observed during 48 hours observation period (p = NS, **Figure 3**). The proliferation index (PI) ranged from 26% to 44% (29.3 ± 3.5%) after 24 h and 25% to 44% (31.2 ± 6.4%) after 48 h. Compared to control group, an increase of PI was observed 24 h after stimulation with different ghrelin concentrations: 29.3 ± 3.5 *vs* 79.6 ± 9.5% ($p < 0.0001$) at a concentration 10^{-12} M, 29.3 ± 3.5 *vs* 90.5± 2.3% ($p < 0.0001$) at 10^{-9} M and 29.3 ± 3.5 *vs* 88.0 ± 1.8 % ($p < 0.001$) at 10^{-6} M. Within the used range of ghrelin concentrations, we did not observe dose-dependent changes. An increase of PI was also observed 48 h after ghrelin stimulation and was as follows: 31.2 ± 6.1 *vs* 59.2 ± 4.9% ($p < 0.0001$) at a concentration 10^{-12} M, 31.2 ± 6.1 *vs* 57.0± 7.3% ($p < 0.0001$) at 10^{-9} M and 31.2 ± 6.1 *vs* 59.2 ± 8.27 % ($p < 0.001$) at 10^{-6} M, however, as well as in case of 24 h observation, there were not visible dose-dependent changes (**Figure 3**). Observed increase in the proliferation index was due to both a decrease in G_0/G_1 cell count and the increase in the $S+G_2/M$ cells' population (**Figure 4**).

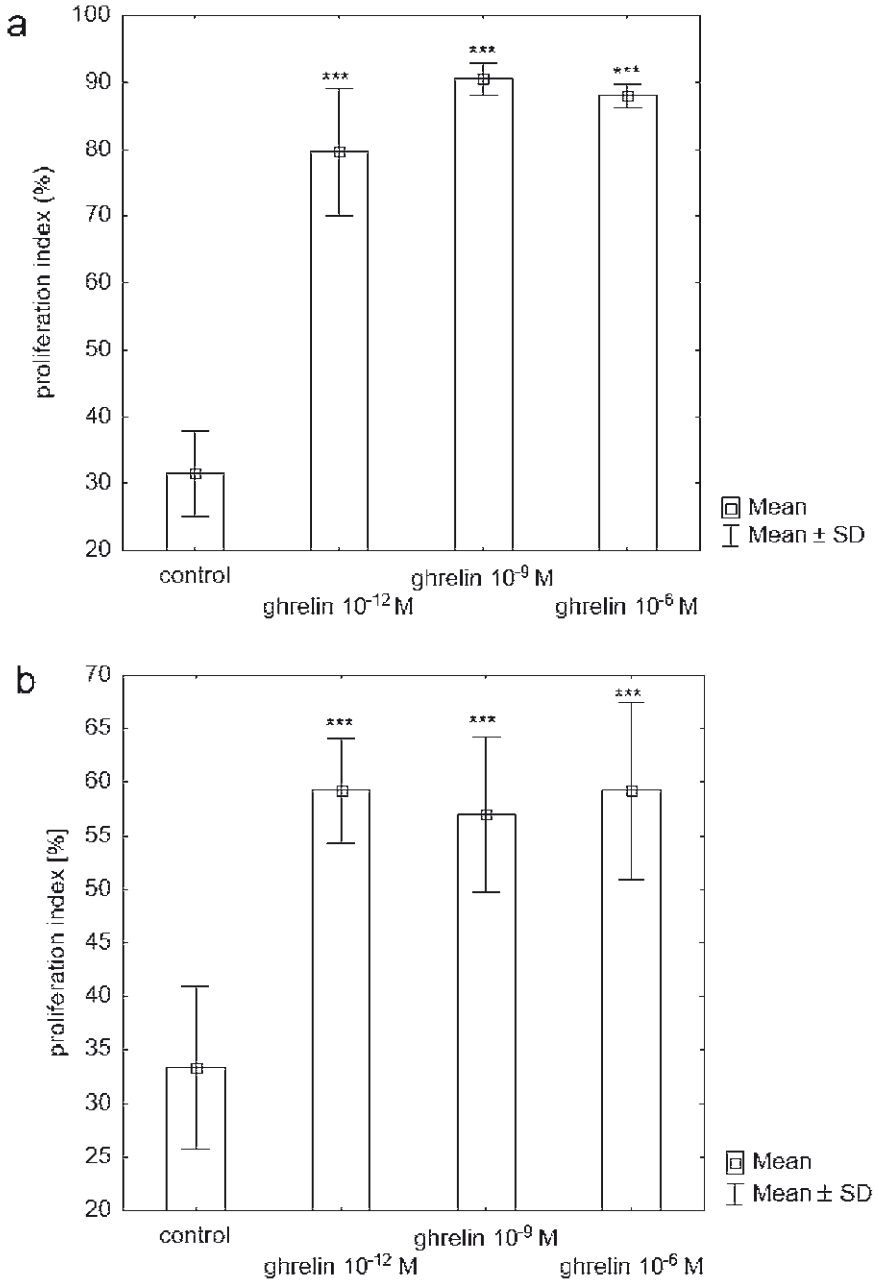

Figure 4. Dose-dependent effect of ghrelin stimulation in GH3 cells. Proliferation index (a) 24 hours and (b) 48 hours after stimulation. Results are shown as means ± standard deviation. Significance is referred to the control (***P<0.001)

Discussion

Several studies have demonstrated that ghrelin and its receptors are expressed in pituitary adenomas [8–10]. In this study, we aimed to establish ghrelin influence on somatotropic cells morphology and proliferation. Ghrelin did not affect GH3 cells morphology regardless of ligand concentration and incubation time. However, ghrelin significantly increased GH3 cells proliferation index, as a result of decreased cells count population in G_0/G_1 phase and increased $S+G_2/M$. In human cells, these effects may be mediated through the GHSR1a, corresponding to this shown to be expressed in GH3 pituitary somatotroph tumor cell line (Ghsr).

Published data regarding ghrelin impact on cells proliferation revealed either stimulation or inhibition. Our results are in line with previously published data concerning GH3 cell line [5, 11] as well as data concerning different cell types of: adrenocortical tumor [12], hepatoma [4], prostate tumors [11], neural [13], pre-adipocytes [14], osteoblasts [12] or cardiomyocytes [15]. Nanzer *et al*. in a study applying ^{3}H-thymidine incorporation assay demonstrated that ghrelin showed stimulating impact on rat somatotroph pituitary tumor cells proliferation. It was suggested that extracellular signal-regulated kinase (ERK) was involved in this mechanism. Desoctanoyl ghrelin showed a similar effect as octanoylated form [5]. Baldanzi *et al*. suggest that ghrelin and des-acyl ghrelin inhibit cell death through ERK-1/2 an PI3-kinase/AKR. Baldanzi shown that ghrelin stimulates tyrosine phosphorylation and activates ERK-1/2 and Akt [15]. Activation of ERK-1/2 by ghrelin was observed previously by Murata et al. [4]. The authors postulated that, independent of its acylation, ghrelin gen products may act as a survival factor [15]. Tian *et al*. demonstrated that nitric oxide (NO) blocked ghrelin-activated GH3 cells proliferation. The mechanism of NO action was mediated by inhibition of extracellular signal-regulated kinase 1/2 [16].

It is postulated that ghrelin impact on GH3 cells proliferation involves Ghsr receptor [5]. The GHSR is encoded by a single-copy gen located on chromosome 3 in humans, whose alternative splicing can generate two mRNA splice variants, *GHSR1a* and *GHSR1b*. The functional activity of GHSR1b remains to be fully elucidated. GHSR1a is the receptor responsible for intracellular acylated-ghrelin transduction signal pathway [17]. However, some authors postulate that ghrelin impact on cells proliferation involves other systems of signal transduction [9, 18–20]. Volante *et al*. described the presence of ghrelin binding sites in cellular membrane of human thyroid neoplastic cells, however the GHSR1a and GHSR1b presence was not observed [19]. Nanzer *et al*. suggested that ghrelin stimulate cell proliferation directly *via* the MAPK pathway involving the GHSR1a [5].

Anti-proliferative ghrelin effect was described in thyroid [19], breast [2] or pituitary [21] neoplastic cell lines. Either acylated or non-acylated ghrelin was studied [2, 22]. This indicates that non-acylated ghrelin, previously considered inactive due to lack of GH secretion stimulation, manifested its biological activity [12, 15, 21].

We observed that ghrelin stimulated GH3 cell proliferation in a way that was not directly dose-dependent. In fact, after 24h incubation, the maximal stimulatory effect of ghrelin was observed at the 10^{-9} M but not at the highest concentration of 10^{-6} M. After 48h incubation, the reaction was not also dose-dependent. Maccarinelli *et al*. studied the effect of ghrelin (10^{-11} – 10^{-8} M) on proliferation and differentiation of osteoblastic cells and observed maximal stimulatory effect at 10^{-10} M but not at higher concentrations. Those authors speculate that ghrelin dose increase leads to another receptor subtype representing inhibitory proliferative activity recognition [23]. On the other hand, Tian *et al*. found that ghrelin induced the GH3 cells proliferation in a dose-dependent manner after 48h incubation. They used bromodeoxyuridine (BrdU) assay to determine the proliferation of GH3 cell. Their results revealed that after 48h incubation, ghrelin increased the incorporation of BrdU into GH3 cells in a dose-dependent manner (with significant responses to concentration ranging from 10^{-9} M to 10^{-6} M) [26].

The proliferation index was assessed to evaluate cell population ratio in each phase of cell cycle. The observed increase of the proliferation index was due to both: decrease of G_0/G_1 cell count and increase of the $S+G_2/M$ cells population. In the BrdU method, an analog of the DNA precursor thymidine is incorporated into newly synthesized DNA by the cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. The difference between the techniques may be responsible for the small discrepancy of the results. However, both techniques confirmed stimulating effect of ghrelin on GH3 cells proliferation.

In conclusion, we used GH3 cells as a model of GH-releasing adenoma *in vitro*. We demonstrated that GH3 cell line express *Ghsr* receptor. We showed that ghrelin significantly stimulated GH3 cells proliferation and may play a role in pituitary tumorigenesis *via* an autocrine/paracrine pathway.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPERS

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The association of the IL-1β-31 polymorphism and development of neuroinfections

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ABSTRACT

Introduction. Inflammation of the meninges can have various clinical courses, from mild, self-limiting in some viral neuroinfections to severe, sometimes ending in death. The pro-inflammatory cascade and defects in the inhibitors of the inflammatory response play an important prognostic role. Single nucleotide polymorphisms (SNPs) of the genes encoding cytokines, influence the severity of the inflammatory response.
Aim. The aim of this study was to evaluate the effect of selected polymorphisms of proinflammatory cytokines IL-1β, TNF-α and IL-8 on the development of neuroinfections.
Material and Methods. We evaluated the laboratory results of 30 patients treated for bacterial and viral meningitis and compared those to 30 healthy volunteers. The following 4 variants were analyzed for occurrence of genetic polymorphism in patients with meningitis versus the control group: IL-1β 3953, IL-1β -31, TNF-α -308, and IL-8 781. Then, we assessed the association between these genetic polymorphisms and the inflammatory response during the course of meningitis.
Results and Conclusions. We observed that polymorphism of the IL-1β-31 significantly differs between patients and healthy subjects, the IL-1β -31AA polymorphism existed only in healthy individuals ($p < 0.001$). The WBC count was dependent on the TNF-α -308 polymorphism with a statistically significant difference ($p = 0.021$) occurring among persons with variants AA and AG. In conclusion the study showed that the presence of the AA genotype of IL-1β-31polymorphism may have a protective effect on the development of meningitis. This polymorphism was not observed in any patient with meningitis.

Keywords: polymorphisms of genes, neuroinfection, IL-1β, TNF-α, IL-8.

Introduction

The pathophysiology of meningitis is a complex process which consists of factors dependent on the micro-organisms (eg. endotoxins, adhesive ability, the presence of the areola), and host factors [1, 2]. Proinflammatory cytokines, activated by bacterial or viral antigens, play a very important role in causing damage to the blood-brain barrier, and the result is the penetration of bacteria into the subarachnoid space and the development of the classic symptoms of the disease [3, 4]. Various courses of disease from mild to very severe in the case of the same causative pathogens have been observed, indicating that the proinflammatory pathway and defects in inhibitors of the inflammatory pathway play a very important predictive role. Single nucleotide polymorphisms (SNPs), genes that encode cytokines, influence the severity of the inflammatory response. Identifying these will allow an accurate assessment of the patient's prognosis. Classification of meningitis depends on the type of etiologic

agent, there are two main groups: bacterial meningitis (BM) and viral meningitis (VM) [5, 6]. BM, due to the type of pathogens that cause inflammation are divided into purulent and non-purulent [5, 7]. VM are usually mild, with a self-limited course and rarely cause neurological sequelae, and have a low mortality rate [6, 8, 9]. However, there are cases of VM described with quite dramatic courses [5, 8–10].

Aim

The aim of our study was to evaluate the influence of interleukin-1β (IL-1β), tumor necrosis factor (TNF-α) and interleukin 8 (IL-8) gene polymorphisms on the development and intensity of inflammation markers during the course of neurologic infections in patients treated in the Department of Infectious and Tropical Diseases, University Hospital in Krakow, Poland.

Material and Methods

We evaluated the results of 30 patients, 12 men and 18 women treated for BM and VM in the Department of Infectious and Tropical Diseases at the University Hospital in Krakow [mean age: 39.9 years]. The control group consisted of 30 healthy volunteers, 19 men and 11 women [mean age 44.6]. The study included 11 patients with BM and 19 patients with VM. Exclusion criteria included: other acute and chronic inflammatory states of immunosuppression and immunosuppressive therapy. In the test group, we analyzed blood morphology, the concentration of C reactive protein (CRP) in the blood, and tested the cerebrospinal fluid (CSF) for the number of cells, and concentrations of glucose and protein using standard methods. Patients and control subjects were evaluated for polymorphisms of IL-1β + 3953AG (rs1143634), -31AG (rs 1143627), TNF-α -308AG (rs1800629) and IL-8 + 781AG (rs2227306). For this purpose, DNA was isolated from blood using a DNA Qiamp DNA Mini Kit in accordance with manufacturer recommendations (Qiagen, Germany). After quantitative and qualitative assessments, the DNA samples were normalized to a concentration of 9 ng/ul. Genotyping was performed using a TaqMan SNP Genotyping Kit (Life Technologies, USA) and CFX384 Touch Real Time PCR Detection System (Bio-Rad, USA).

Statistical methods

We used nonparametric tests for statistical analysis. For comparison of the two groups, we used the Mann-Whitney test. Interdependence between selected parameters was determined by the Pearson correlation coefficient. Effects of polymorphisms on the level of the selected parameters were analyzed using ANOVA-Kruskal-Wallis. We also used the Chi^2 test of independence. A P value <0.05 was considered statistically significant. Calculations were performed using Statistica 10 (StatSoft® Inc., U.S.).

The study was conducted in Accordance with the Declaration of Helsinki (1975) and approved by the Jagiellonian University Ethics Committee. All participants of the study signed an informed consent form.

Results

In the test and control groups, there was no statistically significant difference in gender (p = 0.07) or age (p = 0.21). The 19 patients with VM had an average CRP concentration of 7.40 mg/l (1.00–37.10 mg/l) whereas in the group of 11 patients with BM the average CRP concentration was 74.02 mg/l (34.00–135.62 mg/l); the difference between the groups was statistically significant (p = 0.005). The WBC count (x10'3 /ml) was 7.83 (5.43–10.00) in the VM and 16.54 (6.37–22.12) in the BM, with a statistically significant (< 0.001) difference. CSF analysis of the VM group showed 83.00 cells/ml (37.00–272.00), 0.72 g/L of protein (0.43–0.83) and 2.79 mmol/L of glucose (2.49–3.20). The BM group had an average of 398 cells/ml (37.00–1150.00), 1.45 g/L of protein (1.00–2.89), and 1.51 mmol/L of glucose (1.10–2.05). Statistically significant differences were observed between the two groups in the blood glucose (p = 0.001) and protein (p = 0.003).

There was a statistically significant difference in the distribution of AA genotype of the IL-1β -31 polymorphism (p < 0.001) between the test and control groups. The IL-1β -31AA genotype was present in the healthy group but not in the test group. In addition, the GG polymorphism was more frequently observed in the test group than in the control group. In terms of the other assessed polymorphisms, IL-1β 3953, TNF-α -308, IL-8 781, there was no statistically significant difference in the distribution of these polymorphisms between the test and control groups (**Table 1**).

We assessed for the association between the examined gene polymorphisms and inflammatory parameters of blood and CSF. We observed a relationship between the number of WBC and the TNF-α -308 polymorphism, wherein a statistically significant difference existed between the AA and AG (p = 0.02). In terms of the other assessed polymorphisms, IL-1β 3953, TNF-α -308, and IL-8 781, there was no statisti-

Table 1. Genotype frequencies of IL-1β, TNF-α and IL-8 genes in patients with meningitis and controls

SNP	Test group		Control group		Chi2 test
	n	GF (%)	n	GF (%)	p
IL-1β +3953					
GG	20	66.67	18	60	p = 0.56
AG	10	33.33	11	36.67	
AA	0	0.00	1	3.33	
IL-1β -31					
GG	18	60.00	3	10.00	p < 0.001
AG	12	40.00	16	53.33	
AA	0	0.00	11	36.67	
TNF-α -308					
GG	19	63.33	20	66.67	p = 0.84
AG	9	30.00	9	30.00	
AA	2	6.67	1	3.33	
IL-8 +781					
GG	3	10.00	7	23.33	p = 0.20
AG	8	26.67	7	23.33	
AA	16	53.33	16	53.33	

GF, genotype frequencies; IL-1β, interleukin 1β; IL-8, interleukin 8; SNP, single nucleotide polymorphism, TNF-α, tumor necrosis factor α

cally significant relationship between them and WBC, CRP, TNF-α IL-1β in CSF, or inflammatory parameters of CSF: white cells, protein, and glucose.

Discussion

Our study for the first time evaluated the IL-8 + 781 polymorphism in infection, and polymorphisms of the IL-1β: -31 and +3953 in neurological infections. A limitation of our study is that due to the small sample size, the selected gene polymorphisms of IL-1β, TNF-α and IL-8 were analyzed together in both types of meningitis.

We have shown that the IL-1β -31AA genotype demonstrates a statistically significant ($p < 0.001$) difference between the test and control groups. Patients in the test group did not have the IL-1β -31AA genotype, while this genotype was present in the control group. It is possible that this genotype protects against the development of meningitis. This issue requires further research on a larger group of patients. In addition, more patients in the test group were found to have the GG genotype.

Thus far, the incidence of the IL-1β -31 polymorphism in neurologic infections has not been evaluated. The effects of IL-1β polymorphisms have been demonstrated in sepsis. Wen et al. observed that a polymorphism at position 1470 GG, AG and 51 31GA increases the risk of severe sepsis in patients after trauma [11]. Polymorphisms at positions -31 (G/A) and -511 (A/G) are also associated with more severe infections of *Plasmodium falciparum* [12]. Liu et al., demonstrated a correlation between the presence of a G allele at position 31, and susceptibility to infection with influenza virus AH1N1pdm09 [13]. Allele 511G, the GG genotype, and haplotype 511G/3953G can be considered one of the factors responsible for susceptibility to the development of visceral leishmaniasis, as opposed to the A allele or AA genotype at position 511 and haplotype 511A/3953, which can be considered as factors promoting immunity against the disease [14]. Polymorphisms at position 3953 (G/A) was also observed in chronic hepatitis caused by HCV genotype 4, where the presence of the A allele was associated with poorer clinical response and more severe fibrosis [15]. Sa-Ngasang et al., showed that carriers of IL1β -31G have a higher risk of developing shock during Dengue fever (Dengue Shock Syndrome), which suggests a connection with production of interleukins in the pathogenesis of the disease [16].

The most widely investigated polymorphism of the gene encoding TNF-α is a polymorphism in the promoter region at position 308. Depending on the purine presence, two TNF-α alleles may be present: guanine at position -308 is associated with TNF-α 1 and adenine at this position is associated with TNF-α 2. Allele TNF-α 2 is less frequent, and it is associated with higher production of TNF-α as compared to TNF-α 1 [17]. However, the occurrence of a polymorphism at position -863 (G/A) is associated with a lower expression of the gene and lower levels of this cytokine [18]. Studies on

the effects of the presence of TNF-α polymorphisms on the course neurologic infections are few. Titmarsh et al., evaluated the polymorphism of TNF-α -308 in groups of patients with VM, specifically invasive meningococcal disease (IMD) compared to a control group. They showed significant differences between the groups, with the genotype GG of TNF-α -308 polymorphism associated with a lower production of TNF-α which correlated with a higher risk of developing IMD [19]. Pujikhari et al. showed that people with -308 alleles and -863G allele were more likely to develop severe Japanese encephalitis [20]. Fontes et al., in comparing the distribution of TNF-α -308 genotypes demonstrated that the TNF-α -308 is present more often in BM patients than in healthy people [21].

Polymorphisms in the region of -308 have been demonstrated in other acute infections, bacterial, viral, or parasitic. Thus far a correlation between the occurrence of allele TNF-α 2 and more severe malaria has been shown. McGuire et al. observed that children who are homozygous for TNF-α 2/2 were at seven times greater risk of developing the cerebral form of malaria or death [22]. Similarly, Cabrera et al. observed a correlation between the presence of the allele of TNF-α 2 and susceptibility to mucocutaneous leishmaniasis infection [23]. With respect to the role of TNF-α in the development of septic shock, polymorphisms of the gene coding for TNF-α have been demonstrated to have an effect on the course of sepsis. Song et al. have demonstrated the relationship between the presence of the TNF-α 2 allele and the risk of severe sepsis, but no correlation was observed with the occurrence of this polymorphism and death [24]. Teuffel et al., came to similar conclusions in their meta-analysis [25]. Polymorphisms in the region of 308 were also demonstrated in the course of viral infections. The presence of the TNF-α 2 allele, and thus increased production of TNF-α, was associated with an increased risk of haemorrhagic dengue fever when re-developing the disease [26]. In the course of infection with influenza virus AH1N1pdm09 the presence of -308G allele was associated with more severe disease [27].

In terms of the analyzed polymorphisms of TNF-α -308 in our study there was no statistically significant difference in the distribution between the test and control groups. The WBC count was dependent on the TNF-α-308 polymorphism, but statistical significance was only reached between AA and AG. TNF -308 polymorphisms did not affect other analyzed parameters: CRP in the peripheral blood and CSF studies: TNF-α, IL-1β, cell count, protein, or glucose.

Studies on the effect of SNP IL-8 in the course of infection are few, and primarily surround infections of the gastrointestinal tract. Jiang et al. demonstrated in 2 separate studies that the AA genotype at the -251 position of the IL-8 is a significant risk factor for primary CDI [28, 29]. Jiang et al. further demonstrated the influence of the same polymorphism on the development of the enteroaggregative forms of Escherichia coli (EAEC) [30]. In terms of neurologic infection, Titmarsh et al., compared the prevalence of polymorphisms of IL-8 -251 in groups of patients with VM, IMD, and healthy controls. Significant difference between groups was noted; for the IL-8 -251 polymorphism, IL-8 -251AA was associated with a higher risk of VM [19].

In our study we chose the IL-8 SNP +781, which has not been analyzed in the progression of any infection. No statistically significant difference in IL-8 +781 polymorphisms were noted between the test and control groups. Polymorphisms of IL-8 +781 had no impact on peripheral blood WBC, CRP, TNF-α, IL-1β or CSF studies, specifically: cell count, protein and glucose.

As a conclusion, we observed that the IL-1β -31AA genotype may play a protective role in the course of neurologic infection – it was not observed in patients who presented with this disease. The WBC count in peripheral blood correlated with the TNF-α -308 polymorphism, and there was a statistically significant difference between the AA and AG subsets.

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Conflict of interest statement
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Abbreviations
BM, bacterial meningitis; CRP, C reactive protein; CSF, cerebrospinal fluid; EAEC, enteroaggregative forms of Escherichia coli; IL-1β, interleukin-1β; IL-8, interleukin 8; IMD, invasive meningococcal disease; SNPs, single nucleotide polymorphisms; TNF-α, tumor necrosis factor, VM, viral meningitis

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Evaluation of oral hygiene in school children from the eastern region of Wielkopolska

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ABSTRACT

Aim. The aim of the study was to assess oral health in school-age children on the basis of subjective and objective judgement, based on selected indicators.
Material and Methods. The study included students aged 10–13 years of primary and junior-high schools in the Mid Eastern region of Wielkopolska. Oral hygiene assessment was based on a prepared subjective questionnaireas well as on the basis of indicators: API P1.I, GI, including the sex of children.
Results. Of the 161 children of school age, most of them, as many as 145 showed attention to oral health, brushing teeth twice a day; some of them apply additional measures for oral hygiene – 39 children. In both sexes there were no deviations indicators examined. Between gender showed no difference, as confirmed statistically.
Conclusions. Oral hygiene in children with high health awareness rated positively, it should be emphasized that despite noninvasiveness the research group of respondents accounted for a small percentage of the children invited to the study, which points the need to undertake educational activities on a larger scale.

Keywords: girls and boys, indicators of bacterial plaque, oral hygiene, survey.

Admission

On the basis of the definition of the World Health Organization hygiene (Hygeinos – medicinal) is a branch of medicine, studying the environmental impacts on physical and mental health of men. Oral hygiene is an important part of the quality of life of every human being. The oral cavity is a different environment for every man hence the specific bacterial flora differs slightly between individuals. Plaque that lives in the mouth is a cohesive mass of yellowish-white color, exhibits cohesive structure, which can be removed by mechanical means. The clinical evaluation of visual supragingival and subgingival dental plaquemis performed during each dental visits, as in adolescent orthodontic patients [1–4]. Objective assessment is carried out using a variety of indicators such as the rate of platelet.: Pl.I (Plague Index), API (Aproximal Plaque Index) indicators bleeding BI (Bleeding Index), SBI (Sulcus Bleeding Index), BOP (bleeding On Probing) and papillary bleeding index PBI (papillary bleeding Index), gingival index GI (gingival Index). Properly done oral treatments has positive influence on maintaining good health. Ailments associated with improper hygiene, left behind plaque and leftover food can cause tooth decay, gingivitis manifested by swelling, redness, bleeding, the same pain, which often discourage the patient to brush teeth. Fifteen years ago, literature reviews have shown that as a result of neglect oral hygiene comes to the deterioration of oral health [5, 6].

For many years, there has been conducted research on the ways and forms of patient education, effective learning hygienic habits and the use of additional gear

oral hygiene, as a good oral hygiene contributes use additional gear [7–9]. We carried out research related to the use of various additional measures to enhance the cleanability of the oral cavity during orthodontic treatment, such as lotion: 0.2% chlorhexidine solution [10, 11].

Aim

The aim is to show the state of oral health of children based on subjective survey conducted using questionnaires and objective based on selected indicators.

Material and Methods

The subject studied were children of both sexes, of school age. The study invited students aged 10–13 years attending primary and secondary schools in the district of Koło, with over 1000 children. Schools' administration all primary and secondary schools have agreed to the announcement about the planned study, during meetings with parents. The parent/legal guardian of each student, having regard to the written information on the method and purpose of the study has given his written consent to the study. The study included only those students who participated in the study, that is, 161 children (boys and girls) in similar numbers four age groups: 10 years (group I – 43 people), 11 years (group II – 42 persons), 12 years old (group III – 37 people), 13 years (group IV – 39 people). As a criterion to qualify taken into account the child's age and attending school in the district of Koło – eastern region of Wielkopolska. The study involved 89 girls and 72 boys – **Table 1**. For this project, a written consent was issued by the Bioethics Committee of the Medical University them in Poznan (Resolution No. 907/12).

During the illnesses' history interview, information was collected on the past or existing general diseases. They were asked about a history of injuries and treatments within the craniofacial, medications, dietary habits, type of diet. The survey questionnaires were analyzed only questions about the age and place of residence of the test and the related oral hygiene. The responses were marked in the check boxes "yes"or "no" or supplemented eg.: additional funds for oral care. In a clinical study was conducted intraoral where drawn diagram and permanent dentition, and also rated oral health of children using selected indicators:

- Approximal plaque index (API) – used to assess the presence (denoted by a "+") or absence (denoted by "-") plaque in the interdental spaces. The survey was carried out in the upper quadrant of the arc buccal and III quadrant of the lower arch from the language. In total, we examined 10–12 interdental spaces for every child. The value of the calculated ratio API is given in percent: API = sum of interdental plaque divided by all test interdental spaces and the value multiplied times 100%. The criterion for the assessment rate: 100 – 70% – poor/poor oral hygiene, 70 – 40% – average oral hygiene, 39 – 25% – good oral hygiene, < 25% – optimal oral hygiene.
- Indicator of bacterial plaque (Pl.I) – used to evaluate the thickness of plaque localized around the neck of the tooth on the 4 surfaces (buccal / labial, linguistic, mesial, distal). The thickness of the plates was assessed in 4-point scale: 0 – no plates, 1 – thin layer of tiles adjacent to the cervical invisible to the naked eye, 2 – moderate accumulation of tiles visible to the naked eye, at the gingival margin, and (or) on the surface of the tooth and gingival pocket, 3 – Extensive accumulation of deposits in the pocket, the gingiva and the tooth surface. The thickness of the plate was tested on six teeth 16, 12, 24, 36, 32, 45. The values obtained from the four tooth surface were summed up and then divided by 4, then Pl.I all teeth were added together and then divided by 6. At values 0 – 0 6 oral hygiene was determined as a good, 0.7 – 1.8 as the average, 1.9 – 3.0 as bad.
- Gingival index (GI) – is used to evaluate inflammation gums. In the absence of visual signs of inflammation were labeled 0. A value of 1 was determined by redness without bleeding during probing 2 – to mark redness, swelling or hypertrophy of the gums while bleeding, 3 – with a pronounced inflammation of the trend to spontaneous bleeding. The assay was performed in four quadrants with a periodontal probe inserted into the interdental spaces, and the index value was calculated as follows: GI = number obtained from the survey divided by the number of spaces. The indicator values were interpreted as follows: 0 – healthy gums, 0.1 – 1.0 – mild inflammation, 1.1 – 2.0 – moderate inflammation, 2.1 – 3.0 – severe gingivitis.

Table 1. Test

Age	Girls	Boys	Together
10 years	28 (65.2%)	15 (34.8%)	43 (100%)
11 years	27 (64.3%)	15 (35.7%)	42 (100%)
12 years	18 (48.6%)	19 (51.4%)	37 (100%)
13 years	16 (41.1%)	23 (58.9%)	39 (100%)
A total of	89 (55.3%)	72 (44.7%)	161 (100%)

The study was conducted in the dental office with a sterile dental mirror, probe and periodontal probe.

From the data obtained a computerized database, which were statistically analyzed using statistical tests programs Statistica 10 and StatXact 8.

As the level of statistical significance adopted $\alpha = 0.05$ when $p < \alpha$.

Results

The resulting slight differences in the rates of oral hygiene in girls and the boys proved to be statistically insignificant (**Table 2**). Analysis of the survey showed that the majority of children brushing teeth one or two times a day (81 girls and 64 boys), also did not reveal statistically significant differences with regard to gender. Out of 89 respondents, only 23 girls to apply additional measures for oral hygiene including 14 students mouthwash, 72 boys 16 apply additional measures for oral hygiene, including 12 students mouthwash, the result was also statistically insignificant.

Discussion

After conducting studies, it should be pointed good performance indicators of oral hygiene, also in both sexes. However, the fact that a small number of children from a very large group of invitees who have declared their willingness to participate in a non-invasive and painless tests, which strongly emphasized is disturbing. Given the age range further shows that the awareness of the benefits of this study did not increase with the age of the child, because the frequencies in age groups were similar. Regardless of this fact should be emphasized that every parent / legal guardian, taking into account the will of the child, has the right, at any stage of the research project, submitted in writing to withdraw consent to the study, and this decision can not be questioned [12]. Important, therefore, it seems to health promotion and health education, the ability to use offered to the public and research projects diagnosis, during which professional assessment of oral health is an aspect of prevention [13]. It is an opportunity to obtain information on the proper hygienization, the correction in terms of bad eating habits and others.

From the literature review carried out for many years it showed that patients treated with orthodontic who are under constant supervision of a physician or a dentist regularly, have better hygiene than patients not treated with orthodontic [14, 15]. Many years ago Glans et al [16] demonstrated the dependence of the state of the gums of the type and severity of crowding teeth; in his observations they pointed out that hygiene instruction has a influence on gingival bleeding index GBI. Assuming that dental abnormalities such as too crowded teeth are a common disorder of the dental arches in all populations, it is important that not only pedodonta first contact dentist with the child, but also a qualified dental hygienists or nurses pediatric, which is also highlighted in the foreign literature [17, 18]. Orthodontic treatment usually lasts a few years, it is also a good time to educate the patient regarding hygienisation. Słomkowska and Jabłońska-Zrobek, [19] evaluated the impact of teaching on the level of hygiene plaque. They observed 30 patients (aged 10 to 14 years old) during the active phase of orthodontic treatment. The research preceded the professional instruction of oral hygiene OHI (Oral Hygiene Instruction). Pl.I plaque index was used to measure the state of oral hygiene before test and after one month of treatment. The results clearly indicated a beneficial effect OHI instruction on oral hygiene, plaque index decreased in all patients. Constant oral hygiene instruction bring positive results in decrease of oral cavity Presented above results are encouraging; however we must stress that the whole project enveloped children who come from higher than average health awareness families.

Table 2. Summary of results indicators API Pl.I and GI by gender

Population sex variable median	Mediana N♀; N♂ 89 72	Minimum N♀; N♂ 89 72	Maximum N♀; N♂ 89 72	Lower quartile N♀; N♂ 89 72	The upper quartile N♀; N♂ 89 72
Indicator API%	92 100	18,2 17,0	100 100	75 81,7	100 100
Indicator Pl.I	0,88 1,0	0,08 0,13	0,6 1,75	0,7 0,75	1,04 1,2
Indicator GI	0,08 0,05	0,0 0,0	1,2 1,21	0,0 0,0	0,25 0,2

Conclusions

Oral hygiene in school children, with a large pro-health awareness is good, but the study group accounted for about 16% of the invited students from the eastern region of Wielkopolska for non-invasive preventive examinations, which indicates a large need for education in this area.

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Conflict of interest statement
The authors declare no conflict of interest.

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Impact of sex and body mass index on cecal intubation time. Is it a myth that colonoscopy is easier to perform in obese than lean people?

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ABSTRACT

Introduction. Colonoscopy is the gold standard for prevention and early diagnosis of colorectal cancer. Procedure quality is an important issue. Current quality indicators, such as cecal intubation rate, adenoma detection rate, and withdrawal time, are important, but cecum intubation time influences all of them. Factors that determine cecal intubation time (CIT) include body mass index (BMI), age, sex, history of abdominal surgery, quality of bowel preparation, and visceral adipose tissue. Among those who perform colonoscopy, it is believed that the procedure is easier to perform in obese people.
Aim. To determine whether cecal intubation time depends on body mass index and sex of patients undergoing colonoscopy.
Material and Methods. An analysis of the technical aspects of colonoscopy, such as the time required to intubate the cecum, with respect to BMI and sex in 100 patients.
Results. The average time taken to reach the cecum or ileum was slightly longer in obese people than in people with normal weight. Average CIT was almost one minute longer in men than women. Average CIT in obese men was slightly longer than in normal weight men. There was no difference in average CIT in obese and normal weight women. The differences were not statistically significant.
Conclusions. This study demonstrates that the claim that endoscopic examination of the lower gastrointestinal tract is easier to perform in obese people cannot be objectively confirmed.

Keywords: colonoscopy, cecal intubation time, quality in colonoscopy.

Introduction

Colonoscopy is the gold standard for the prevention and early detection of colon cancer and rectal cancer [1–6]. It is important to remember that the effectiveness of colonoscopy depends on the quality of the procedure, which is assessed using a variety of quality indicators. Historically, the most commonly applied and recognized indicators include availability of the cecum and frequency of detection of adenomas [1, 3, 4], but these are not the only quality indicators. Others indicators include time of retraction of the apparatus [1, 4–7], degree of bowel preparation for examination [2, 6, 7], time required to reach the cecum until the retraction of the apparatus [1, 8, 9], endoscopist experience [2, 6], patient tolerance to examination [5, 6], suitable periodicity of examinations [4, 7], and use of diastolic drugs and analgosedation [6].

The availability of the cecum and many of the above-mentioned quality indicators are significantly affected by the time needed to reach the cecum/ileum or cecal intubation time (CIT). CIT reflects the endoscopist's level of experience and technical difficulties

and affects tolerance and time of the examination. Some authors argue that CIT is just as important as the time to retract the apparatus, because while entering, the apparatus detects more polyps greater than 1 cm than in the process of retraction (75% vs. 25%) [10]. Among the many factors affecting time needed to reach the cecum are body mass index (BMI), gender, age, previous abdominal surgeries, degree of bowel cleansing, amount of visceral fat, and number of pregnancies and births in women [11–16]. The available literature does not offer much in the way of evaluating the effect of patient BMI and gender on CIT and the available texts present differing results although anecdotal evidence is commonly presented. It was therefore decided to investigate the impact of patient BMI and gender.

Material and Methods

The analysis included 100 diagnostic colonoscopy examinations performed by a single experienced endoscopist during the period from November 2014 to July 2015. The analysis excluded surgical colonoscopies and those performed under analgosedation. The following parameters were evaluated: BMI, gender, and time to reach the cecum or ileum. There were 58 women and 42 men examined. The average age of women was 63.0 and for men 59.3 years. Patients were divided into groups by gender and BMI (below and above 25 kg/m^2). The group of patients with BMI below 25 kg/m^2 numbered 34 people.

The group of patients with a BMI greater than 25 kg/m^2 numbered 66 people. Among the women, 39 had a BMI over 25 kg/m^2 and 19 had a BMI less than 25 kg/m^2. Among men, 27 had a BMI greater than 25 kg/m^2 and 15 had a BMI less than 25 kg/m^2. The research was carried out both on an outpatient basis and in a hospital. All patients were prepared using the same laxative preparation. The quality of cleansing was rated with the Lieberman scale. The tests were commenced typically in a left-sided position and, if necessary, the position was switched to the back; additional help from an endoscopy nurse was available. All tests were performed under sedation with midazolam or pethidine. Statistical analyses included calculation of arithmetic mean and standard deviation. Comparisons between groups were performed using Student's t test. P-values < 0.05 were considered statistically significant.

Results

Patients were divided into two main groups according to gender and BMI and four subgroups by BMI within gender groups. Group 1 consisted of patients with a BMI of less than 25 kg/m^2, normal body weight; group 2 – patients with a BMI greater than 25 kg/m^2, overweight/obese. Subgroups 1A and 1B were women and men of normal weight, respectively. Subgroups 2A and 2B were overweight/obese women and men, respectively. The average time to reach the cecum in patients with a BMI of less than 25 kg/m^2 was 7.88 ± 3.6 minutes, and in patients with a BMI of over 25 kg/m^2 was 8.12 ± 4.4 minutes (p = 0.440). Average CIT, in the case of overweight/obese patients was slightly longer than the average CIT in people with normal body weight, but the demonstrated difference was not statistically significant.

In women, the average CIT was 8.33 ± 4.2 minutes and in men 7.47 ± 3.9 minutes (p = 0.766). The average CIT for men was shorter by less than a minute, but this difference was not statistically significant. In the subgroup of women with normal body weight, CIT was 8.3 ± 4.2 minutes and in the subgroup of overweight/obese women was 8.6 ± 4.5 minutes (p = 0.411). There was no difference in the CIT in these subgroups of women. In the subgroup of men with normal weight, CIT was 7.7 ± 4.7 minutes and in the subgroup of overweight/obese men was 6.9 ± 1.9 minutes (p = 0.677).

Table 1. Mean time required to intubate the cecum by gender (mean ± standard deviation)

Group	Women	Men	Total
Normal Weight (BMI < 25 kg/m^2)			
Number of patients	19	15	34
Average time of cecum intubation (minutes)	8,6 ± 4,5	6,9 ± 1,9	7,8 ± 3,6
Overweight/Obese (BMI > 25 kg/m^2)			
Number of patients	39	27	66
Average time of cecum intubation (minutes)	8,3 ± 4,2	7,7 ± 4,7	8,1 ± 4,4
All Patients			
Number of patients	58	42	100
Average time of cecum intubation (minutes)	8,3 ± 4,2	7,4 ± 3,9	8,0 ± 4,1

Differences between groups were tested using Student's t test

CIT was a bit lower for overweight and obese men, but this difference was also not statistically significant. The results are presented in table form in **Table 1**.

Analysis of the obtained values shows that the time to reach the cecum in obese people differed slightly between groups, but the difference between groups was not statistically significant. No significant difference between obese and lean people was found with respect to CIT.

Discussion

In order to be effective, efficient, and safe for the patient, every medical procedure must be performed carefully. In assessing the quality of colonoscopy, of key importance is the viewing by the endoscopist of the entire colon and detection of any pathological changes. Viewing the cecum is particularly important as there has recently been an increasing incidence of cancers located in the right half of the colon. Many presently recognized quality parameters, such as availability of the cecum, adenoma detection rate, and time of endoscope retraction, amongst others, influence time to reach the cecum and therefore alter the degree of difficulty of the examination and patient tolerance.

It is known that better tolerated examinations favor an increase in the number of detected adenomas [1]. A few studies have analyzed the impact of various factors on colonoscopy quality. Of particular interest is the impact of patient BMI and gender on CIT.

Chung et al. analyzed 1386 patients who underwent colonoscopy and a computed tomography (CT) scan of the abdomen on the same day. They evaluated age, gender, BMI, height, diameter of the waist and hips, previous operations in the abdomen region, irritable bowels syndrome symptoms, quality of bowel cleansing, endoscopist experience, and amount of visceral fat in the abdomen as assessed by CT. The authors found that female gender, older age, lower height, lower BMI, previous operations in the abdomen region, as well as a smaller amount of body fat, were associated with longer time required to reach the cecum [12]. Park et al. assessed the factors influencing the successful intubation the cecum. The authors analyzed 2050 people who had undergone colonoscopy. A complete colonoscopy was achieved in 83.9%. Failure was associated with factors such as female gender, BMI <18.5 kg/m^2, poor bowel cleansing, and previous surgical procedures in the abdomen [13]. Uddin et al. found that obesity (BMI > 30 kg/m^2) is a factor inhibiting the execution of colonoscopy and reaching the cecum. They compared average time to reach the cecum in two groups of obese patients depending on the position in which the examination was performed. The first group was tested laying on their stomach and the other in the traditional left-sided position. A statistically significant reduction in the time to reach the cecum was found in the first group [14]. Bernstein et al. examined factors related to the time required for intubation of the cecum. They found that the extra time needed to reach the cecum is associated with factors such as older age, female gender, lower BMI, poor bowel preparation, and a less experienced endoscopist [15]. Andreson et al. studied the effects of BMI on the effectiveness of reaching the cecum. They analyzed 2000 examinations carried out over two years in one center, dividing patients into groups according to gender and BMI. They found that a failure of examination was associated with female gender and low BMI, especially < 22 kg/m^2 [16]. Review of the literature and finding presented here suggest that there is no conclusive evidence that high BMI is a factor that facilitates the endoscopist in the performance of an effective colonoscopy. However, in many studies BMI is mentioned as one of the factors influencing the time of examination. Such opinions were also analyzed in this study and it was found that it cannot be concluded that there is a significant association between patient BMI and/or gender and cecal intubation time during endoscopic examination of the colon.

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BRIEF REPORT

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CXCL13 CSF level inversely correlates with duration of disease in primary progressive multiple sclerosis

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ABSTRACT

Chemokines are important factors in the immunopathogenesis of multiple sclerosis. The objective of the study was to examine the CSF and serum levels of CXCL13 and CCL5 chemokines in primary progressive MS, compare results with relapsing remitting MS and control group with other noninflammatory neurological disorders. The levels of chemokines were measured by ELISA method. The CXCL13 CSF levels in PP and RR MS were higher in comparison with control group, without significant differences between these podgroups. Additionally CXCL13 level in PP MS inversely correlated with duration of the disease. CCL5 CSF level was also significantly higher in PP MS in comparison with control group. The results demonstrate involvement of CXCL13 and CCL5 chemokines in the immunopathogenetic mechanisms of primary progressive MS.

Keywords: chemokines, multiple sclerosis, immunopathology.

Introduction

About 10–15% of patients with multiple sclerosis represent primary progressive (PP) form with gradually increasing neurological disability. Patients with PP are older at onset as well as immunopathogenetic mechanisms show differences in comparison with relapsing remitting MS [1]. Although neuroaxonal degeneration underlines progressive disability in PPMS, inflammatory reactions are also present with evident microglial activation in healthy looking white matter additionally to cortical demyelination [2]. The extent to which inflammatory-immune reactions are involved in the pathogenesis of PP MS and its nature is not clear. Chemokines are important mediators in MS, in migration of inflammatory cells through blood brain barrier. The chemokine CXCL13 plays an important role in humoral immunity through recruitment of B cells to the CNS and also T cell subsets expressing chemokine receptor CXCR5 [3]. RANTES (CCL5) mainly acts as a chemoattractant for activated T cells and monocytes [4].

The objective of our study was to examine the CSF and serum levels of CXCL13 and CCL5 chemokines in PP MS, compare the results with RR MS and control group with other noninflammatory neurological disorders.

Additional purpose was to evaluate correlation between CSF and serum levels of studied chemokines with age of patients, duration of the disease, IgG index and EDSS level.

Material and Methods

Patients

10 patients with PP MS (median age 42.3 ± 9.6 years, EDSS 3.5 ± 1.2), 15 patients with RR MS not on immunomodulatory treatment (median age 31.6 ± 7.5 years,

Table 1. CXCL13 and CCL5 concentrations in patients with PP MS, RR MS and controls

Mean ± SD	PP MS	RR MS	Controls
CXCL13			
CSF (pg/ml)	54.6 ± 63.1	44.6 ± 48.6	3.03 ± 3.2
Serum (pg/ml)	36.4 ± 20.2	55.1 ± 35.6	58.4 ± 27.5
CCL5			
CSF (pg/ml)	13.8 ± 8.9	7.9 ±2.9	8.3 ± 7.14
Serum (ng/ml)	50.1 ± 14.7	48.4 ± 19.0	72.8 ± 46.5

EDSS 1.8 ± 0.9) and 13 control patients (median age 36.2 ± 7.4 years) were included in the study. All patients with MS have fulfilled diagnostic criteria for RR and PP MS [5] control group included patients with noinnflammatory neurological disorders (tension headache n = 10, vertigo n = 3). Duration of the disease was 3.0 ± 2.8 years for PP MS group and 3.1 ± 2.9 years for RR MS group. The study was approved by the Ethics Committee of Poznan University School of Medicine. All patients involved in the study gave written informed consent.

CXCL13 and CCL5 measurements in the CSF and sera

CSF and serum samples were frozen and stored at -80ºC until the analysis was performed. The levels of CXCL13 and CCL5 were measured by ELISA method according to the manufacturer's protocol (CXCL13/BLC/BCA-1 Quantikine Human Colorimetric Sandwich ELISA, CCL5/RANTES Quantikine Human Colorimetric Sandwich ELISA (R&D Systems). Plates were analyzed at OD 450 nm for CXC13 and for CCL5 using Microplate Reader Elx 800 (Biotec Instruments Inc.). All assays were performed in duplicate. The intra- assay and inter- assay coeffifficients of variation were 2.5% and 10% respectively.

Statistical analysis

The nonparametric Mann-Whitney U test for comparison of two different groups and Spearmans´ s rank test for correlations of the values were used for the statistical analysis. In all studied groups CSF and serum CXCL13 and CCL5 levels have been evaluated and correlated with IgG index, age, duration of the disease and EDSS.

The work has been approved by the ethics committee and all human participants gave informed consent to the work

Results

In PP MS patients CSF CXCL13 level 54.6± 63.1 pg/ml was significantly higher ($p < 0.005$) in comparison with control group (3.03 ± 3.2 pg/ml).In RR MS patients CSF CXCL13 level (44.6 ± 48.6 pg/ml) was also significantly higher in comparison with control group ($p < 0.005$). No significant differences in the CSF CXCL13 levels have been shown between PP and RR MS patients. CSF CXCL13 level in PP MS inversely correlated with duration of the disease, but not with age, IgG index or EDSS values.

In PP MS patients CSF CCL5 level (13.8 ± 8.9 pg/ml) was significantly higher ($p < 0.05$) in comparison with control group (8.3 ± 7.14 pg/ml.. No significant differences in the CSF CCL5 levels have been observed between PP and RR patients.

No correlations of CSF CCL5 levels with age, duration of the disease, IgG index and EDSS values have been observed.

The serum CXCL13 and CCL5 levels did not differ significantly between studied groups and their values are presented in **Table 1**.

Discussion

Our results demonstrate involvement of two important chemokines: CXCL13 and CCL5 in imunopathogenetic mechanisms in PP multiple sclerosis. CXCL13 chemokine is crucial for migration of B cells [6]. The CXCL13 CSF level was higher in PP MS patients suggesting involvement of humoral immunity in this form of MS. Increased CSF CXCL13 chemokine levels were also observed in PP MS patients by Sellebjerg et al [3]. The CXCL13 CSF level inversely correlated with duration of the disease in PP MS patients, but not in RR MS, showing differences between these two forms of MS. This is the first such observation and should be confirmed on larger group of patients.

CXCL13 CSF level was also increased in RR MS patients. This confirms previous results by Krumbholtz et al [7] who has found also a significant correlation between CSF CXCL13 level in RR MS patients and number of B cells and T cells in the CSF.

The CCL5 chemokine (RANTES, regulated upon activation, normal T cell expressed and secreted) is an agonist of CCR1, CCR3 and CCR5 receptors, which

are expressed on target cells in MS plaques. We have not found increased CCL5 levels in the CSF of RR MS patients. Some previous studies revealed an increased CCL5 level in the serum and CSF of RR MS patients [4] while others failed to find any changes [8]. The inverse correlation between CSF CXCL13 levels and duration of the disease reflect decreasing immunological reactions with time course of the disease in PP MS patients.

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BRIEF REPORT

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The level of knowledge on dietary supplements among patients of pharmacies in the Greater Poland region

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ABSTRACT

Introduction. An appropriately balanced and varied diet should cover the body's demand for energy and all necessary nutrients. In case of health disorders caused by malnutrition, a change of eating patterns or introduction of temporary dietary supplements containing deficient nutrients should be considered.
Aim. Assessment of the level of knowledge among patients of pharmacies in Poznań/Greater Poland on nutritional supplements and factors which influence or decide about their use in everyday life.
Material and Methods. A study concerning the use of dietary supplements was conducted on the basis of an original survey in a group of 401 persons, both men and women. The survey consists of 17 single choice questions and one multiple choice question. In order to assess preferences within the scope of use of a chosen group of supplements, a five-grade hedonic scale was used.
Results. The study showed that most patients considered their knowledge to be at least at a good level. Those results were not confirmed by an objective assessment of their knowledge. The most preferred supplements were products including Omega-3 and Omega-6 acids, vitamin D, probiotics and prebiotics, calcium and vitamin and mineral complexes. The preferences among women and men in relation to the choice of specific groups of dietary supplements were similar.
Conclusion. The study showed a varied level of knowledge on dietary supplements – especially on the legal aspects of their introduction on the market. The general level of knowledge on supplements was higher in the group of women.

Keywords: pharmacy patients, dietary supplements, assessment of the level of knowledge.

Introduction

Appropriate nutrition should be based a balanced and varied diet, covering the body's demand for energy and all necessary nutrients – proteins, fats, carbohydrates, vitamins and minerals [1, 2]. Non-compliance with the aforementioned requirements reflected in over or undersupply of nutrients leads to nutritional disorders and, as a consequence, to development of diet-related diseases [3, 4, 5]. In a situation, where it is impossible to balance the daily food ration using components naturally occurring in food, compensation of deficiencies by introduction of a dietary supplement should be considered [6]. Supplementation of a deficient nutrient/deficient nutrients should take the form of a short-term intervention carried out under the supervision of a physician or a pharmacist [7]. According to the definition, a dietary supplement is a product intendent for ingestion, whose aim is to complement a regular diet and provide a concentrated source of vitamins and minerals or other substances with a nutritional or physiological effect [8]. It is launched onto the market in a form "allowing for its dosing – capsules, tablets, pellets or

other, similar ones, as well as powder sachets, ampules with liquid, bottles with droppers or other similar forms of liquids or powders allowing for ingestion of its small and carefully measured quantities with the exception of products with properties of a medicinal products within the understanding of the pharmaceutical law" [8, 9]. The basic difference between a medicinal product and a dietary supplement is visible in the definition itself. While medicines are intended for people suffering from diseases and are supposed to improve the state of health pharmacologically or metabolically, dietary supplements are always recommended to healthy persons suffering from temporary deficiencies of some nutrients, serve a nutritional function and support correct functioning of the body [10]. A dietary supplement (for example, vitamin C) is subject to food regulations, and a medicine – to pharmaceutical regulations, which means that these are different products of the same commercial presentation [11, 12]. The issue related to the maximum content of selected vitamins and minerals in dietary supplements have not been solved yet, not to mention the problem with "third components" which are – according to the definition – other substances with a single or complex nutritional or physiological effect. They pose a threat of exceeding the recommended intake of vitamins and minerals, not to mention other active pharmacological compounds, whose content is not standardised [13]. In addition, pushy advertising in the press, radio and TV assures us that dietary supplements are a necessary component of our diet, guaranteeing physical and psychological health [14, 15].

Aim

The aforementioned premises became an incentive to study the level of knowledge of patients of pharmacies in Poznan/Greater Poland on the subject of dietary supplements and factors which influence or are decisive for their use in everyday life.

Material and Methods

The assessment of the level of knowledge on dietary supplements was carried out using a survey – a direct, face to face interview conducted among the patient of pharmacies in Poznan and Wielkopolskie Voivodeship. The study was conducted between 2015 and 2016 on a group of 401 persons – 296 women (age average: 45 years old) and 105 men (age average: 39 years old). The research tool was an original survey created on the basis of the literature available and own observations of the authors concerning the sales of dietary supplements in the pharmacy. The survey consisted of 17 single choice questions, among which two were matrix questions with the answer ranging from 1 (insignificant) to 5 (very significant) and one multiple choice question. Preferences relating to the choice of a selected group of supplements were tested using a 5-point scale marked with designations from 1 "insignificant" to 5 "very significant" and a neutral field – 3 "neither significant nor insignificant". In order to rank the preferences, the average values of preferences (x) were classified to one of the three ranges of values: $x < 2.34$ – low preferences, $2.34 <= x < 3.67$ – medium preferences and $x >= 3.67$ – high preferences. The results obtained from the survey were inputted into a MS Excel spreadsheet, and then – for the needs of their further analysis, to a MS Access relational database. The results were statistically processed using an arithmetic average calculated for all respondents' responses to a specific question, or a variant average in case of an analysis of relationships between gender, age and education. To assess the relationship between the significance of choice of a specific group of supplements among men and women studied, a nonparametric Kendall's tau rank correlation test at the significance level of $\alpha = 0.05$ was conducted. The statistical analysis was carried out using the Statistica 12.0 PL statistical software created by StatSoft – Tulsa, USA.

Results

The socio-economic specification of the patient group studied is showed in **Table 1**, whose analysis shows that in terms of the place of residence, the respondents of the study were men and women from cities of the number of residents of over 100 thousand (39.9% of women and 45.7% of men of the total number of respondents) and cities of up to 50 thousand residents (43.2% of women and 38.1% of men of the total number of respondents). Considering the level of education, 96% of women and 100% of men taking part in the study belonged to the group of those with secondary, incomplete higher (Bachelor degree) or higher education. The financial situation of the patients studied was good or average (82.4% of women and 81.9% of men). A small percentage of the respondents (about 3.0%) declared that their financial situation was bad, while 15.0% described their situation as good. **Table 2** shows the results of the self-assessment of the patients studied concerning the level of their knowledge on dietary

Table 1. Socio-economic specification of the patients' group

Parameter analysed	Parameter characteristics	Gender	
		Women n(%)	Men n(%)
Place of residence	City of over 100 thousand residents	118 (39.9%)*	48 (45.7%)
	City of 50–100 thousand residents	20 (6.8%)	6 (5.7%)
	City of < 50 thousand residents	128 (43.2%)	40 (38.1%)
	Rural areas	30 (10.1%)	11 (10.5%)
Education	Higher	118 (39.8%)	66 (62.9%)
	Incomplete higher education	39 (13.2%)	22 (20.9%)
	Secondary	128 (43.3%)	17 (16.2%)
	Vocational	3 (1.0%)	0 (0%)
	Primary	8 (2.7%)	0 (0%)
Financial situation	Very good	43 (14.5%)	16 (15.2%)
	Good	114 (38.5%)	37 (35.2%)
	Average	130 (43.9%)	49 (46.7%)
	Bad	9 (3.1%)	3 (2.9%)

* – the number of respondents and its percentage in comparison to the total number of respondents

Table 2. The respondents' level of knowledge on dietary supplements according to themselves – the percentage of all respondents

Level of knowledge	Women	Men
Very good	5.7%	1.9%
Good	29.4%	34.3%
Sufficient	43.6%	25.7%
Low	16.9%	27.6%
Very low	4.4%	10.5%

supplements. The analysis of the answers obtained showed that 73.0% of women and 60.0% of men evaluated their knowledge on dietary supplements as good or satisfactory, while only a small percentage (5.7% of women and 1.9% of men) declared that their level of knowledge was "very good". Insufficient knowledge on dietary supplements was declared by about 20% of women and 40% of men. An analysis of the actual level of knowledge of the respondents is shown in **Table 3**. Let us analyse answers to the questions asked in a chronological order (from 1 do 7). The first question concerning equivalence of the terms "medicine" and "dietary supplement" was answered correctly by as many as 98% of the respondents. In case of the second question, only 37.7% of the respondents answered correctly that a leaflet is not required in a packaging of a dietary supplement. Over half of the respondents (52.8% of the total number of respondents) did not know that clinical research is not required in the process of registration of a dietary supplement, and only 38.2% of the persons surveyed were aware that before launching a supplement on the market, no control of its active substances content is done. An appropriate answer concerning the lack reimbursement for dietary supplements from the National Health Fund (NFZ – Narodowy Fundusz Zdrowia) was given by 75.6% of the respondents. Of all the persons surveyed, 88.3% answered correctly that the process of a supplement registration is different than that for a medicine, and almost everyone (93.3%) stated correctly that the packaging of a product must include information if it is a medicine or a supplement. The assessment of preferences and significance of use of a specific group of dietary supplements is shown in **Figure 1** and was completed by the results related to the sex of the respondents, indicated in **Table 4**. As it can be seen in the figure, the surveyed patients deemed that taking dietary supplements with Omega-3 and Omega-6 acids is the most important (an average evaluation of significance: 3.31). Men evaluated this group of preparations slightly higher (3.41) than women (3.27). Then, there were Vitamin D supplements, assessed only slightly lower (average: 3.29), with a grade of 3.35 among women and 3.12 among men. According to the respondents, important dietary supplement include also calcium (women – 3.03; men – 3.08), and then vitamin and mineral complexes as well as probiotics and prebiotics (average: around 3.0). The first of the groups was ranked higher by men (3.41) than by women (2.85), just as it was the case with the latter one (3.15 vs 2.95). An average of

Table 3. The patients' level of knowledge on the legal aspects of the use of dietary supplements – the percentage of all respondents

Question No.	Question	Women		Men	
		YES	NO	YES	NO
1	The terms "medicine" and "dietary supplement" mean the same.	1.0%	99.9%	4.8%	95.2%
2	A leaflet must be included in the packaging of a dietary supplement.	62.2%	37.8%	62.9%	37.1%
3	Clinical research is not required during the process of registration of a dietary supplement.	49.7%	50.3%	40.0%	60.0%
4	The declared content of active substances is controlled before launching the product on the market.	58.4%	41.6%	71.4%	28.6%
5	The National Health Fund does not fund a vast majority of dietary supplements.	70.9%	29.1%	88.6%	11.4%
6	The process of registration of a dietary supplement is the same as for a medicine.	12.2%	87.8%	10.5%	89.5%
7	The packaging of a preparation must state if it is a dietary supplement or a medicine.	91.2%	8.8%	99.0%	1.0%

Table 4. The average assessment of importance of use of a chosen group of dietary supplements in the group of the patients studied, according to the sex of the respondents.

Type of a dietary supplement	Women (x)	Men (x)
Mineral and vitamin complexes	2.85	3.41
Vitamin D (D3)	3.35	3.12
Omega-3, Omega-6 acids	3.27	3.41
Calcium	3.03	3.08
"Weight-loss" products	1.55	1.59
Hair, skin and nails preparations	2.55	2.31
Fish oils	2.89	2.51
Lutein preparations (for good sight)	2.48	2.70
Probiotics, prebiotics	2.95	3.15
Digestion or liver preparations	2.60	2.50
Menopause products	2.53	2.30
Preparations for immune system (ginseng, guarana)	2.43	2.90
Preparations for venous circulation s (diosmin etc.)	2.93	2.70
Kendall rank correlation coefficient	0.5195; $p < 0.05$	

x – value of the arithmetic mean of the frequency

the assessments of significance of menopause as well as hair, skin and nails products was similar and ranged between 2.55 and 2.30. A slightly higher position was assigned to supplements supporting the cardio-vascular system, the immune system (men) and fish liver oils, which are a source of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids – the significance level was between 2.51 and 2.93. As the least important group of supplements the respondents indicated "weight-loss" preparations, giving them an average grade of 1.56. The calculated value of Kendall tau coefficient indicated that there is a statistically significant rank correlation concerning the choice of chosen supplements by women and men (0.5195; $p < 0.05$).

Discussion

The studies carried out on a representative group of patients of pharmacies from Greater Poland allowed for initial assessment of their level of knowledge of on the use of dietary supplements. Residents of Poznan and Greater Poland cities of up to 50 thousand residents were the main group taking part in the study. A great majority of them were persons with a secondary or higher education, describing their economic status as good or quite good. The results presented below concern only a part of the study conducted on focus only on the results concerning the knowledge on dietary supplements according to the participant's

self-assessment (subjective assessment), and an objective assessment based on answers to the set of 7 questions. The studies showed that the majority of respondents (38.9%) assessed their level of knowledge on dietary supplements as sufficient, while 30.7% declared that they were informed at a good level. More woman than men classified their level of knowledge as very good, and less women than men considered it to be low or very low, which can be a result of, among others, greater interest of women in health issues. Krejpcio et al. obtained similar results with as much as 88% of respondents declaring their level of knowledge to be average [16]. A great majority of respondents was aware of the basic differences between a medicine and a dietary supplement – a vast majority (98%) differentiated between the terms "medicine" and "dietary supplement", however, more than a half of respondents did not know the legal aspects concerning the safety and efficiency of supplements, such as the lack of a requirement for clinical studies or control of the content of active substances before placing the supplement on a market. The results obtained are compliant with the study conducted by Wierzejska et al [17], according to which 71% of respondents knew the term "dietary supplement", however, over a half of them did not deem dietary supplements as foodstuffs, just like 19.5% of persons surveyed in the study by Tyrakowska et al [18]. According to Wierzejska et al., such results are a consequence of misleading suggestions in advertisements concerning the healing effect of supplements and the similarity between their form and packaging to those of medicines [17]. In order to characterise the group of patients further by taking into account the type of supplements they prefer and the 5-point scale of preferences, the value of the arithmetic mean of the frequency the proposed answers were chosen was calculated. The answers were as follows: "insignificant", "slightly significant", "neither significant nor insignificant", "significant" and "very significant" and numeric values on a scale from 1 to 5 were assigned to them. During such a procedure, it can be seen that both men and women did not deem the evaluated groups of supplements as very significant (average >3.67). In most cases, the significance of their use/preferences was within the range of medium values (from 2.34 to 3.67). In the group, the respondents deemed that it is the most important to take dietary supplements with Omega-3 and Omega-6 acids, then, in descending order, vitamin D preparations, calcium, vitamin and minerals complexes and probiotics and prebiotics. This data slightly varies from the results of studies of Quato et al., where the most frequently used supplements were vitamin and mineral complexes, and then calcium, while Omega-3 and Omega-6 acids and vitamin D were not ranked so highly [19]. Similar results were obtained in the study of Khoura et al, in which the highest percentage of respondents also indicated vitamin and mineral supplements as the most frequently used ones, which, according to the authors, can be caused by an attempt to protect themselves against deficiencies, as well as their willingness to increase the ration of selected nutrients in the diet [20]. The studies conducted showed that there are marginal differences between men and women in terms of preferences of chosen dietary supplements, but the value of the rank correlation coefficient indicates its statistical significance, which suggest that men and women have similar preferences concerning supplements.

Conclusion

The study showed a varied level of knowledge on dietary supplements – especially in terms of legal aspects related to their placement on the market. The general level of knowledge on supplements was higher in the group of women, while the preferences concerning the choice of a chosen group of supplements were similar between men and women.

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REVIEW PAPER

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Genetic variants and magnetic resonance imaging measures in multiple sclerosis: a systematic review

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ABSTRACT

Introduction. Although environmental factors play the major role in the etiopathogenesis of multiple sclerosis (MS), genetic factors are implicated as well. We aimed to summarize the current knowledge on the relationship between genetic variants and magnetic resonance (MR) imaging measures in MS.
Material and Methods. A systematic review. In December 2016, Scopus (since the year 1980; including MEDLINE) was searched for studies meeting predefined criteria designed to identify articles regarding: multiple sclerosis, genetic variants, and MR imaging. These were then analyzed to identify publications linking polymorphisms and MR findings.
Results. The search yielded 290 items; 26 were included in the final analysis. Two genome-wide association studies (GWAS) and two projects employing panels of a few dozen of genes of interest provided most of the data. The other publications concerned no more than 5 genes at a time. Twenty studies reported positive findings. The relationship between *HLA-DRB1*15:01* or *BDNF* rs6265 (Val66Met) and the radiologic course of MS was not consistent across the studies. An intersection of the results of the two GWAS yielded: *OPCML* (rs11223055), *PTPRD* (rs1953594), and *WWOX* (rs11150140, rs1116525) (brain atrophy) as well as *CDH13* (rs692612) and *PLCB1* (rs6118257) (lesion load).
Conclusions. Genetic variants were shown to correlate with MS-related brain atrophy and lesion load. Further research in the field is required.

Keywords: brain, spinal cord, cortical, atrophy, lesion, polymorphism, snp, haplotype, imaging.

Introduction

Although environmental factors play the major role in the development of multiple sclerosis (MS; OMIM: 126200), genetic factors are implicated as well. Firstly, variants in human leukocyte antigen (HLA) complex genes are known to confer susceptibility to MS. The strongest evidence in this respect exists for the *HLA-DRB1*15:01* haplotype. Secondly, over a hundred single-nucleotide polymorphisms not related to HLA system are also known to influence the risk and/or course of this disease [1]. A number of studies specifically investigated the potential associations

between genetic variants and measures of central nervous system involvement in magnetic resonance (MR) imaging. We aimed to systematically review the literature on this topic and present the main data in a legible format.

Material and Methods

On December 8th, 2016, Scopus (Elsevier, Amsterdam, Netherlands; includes MEDLINE [2]) was queried with the following term: "TITLE (multiple sclerosis) AND TITLE-ABS-KEY (lesion OR lesions OR hyperintensity OR hyperintensities OR hyperintense OR hypointensity OR hypointensities OR hypointense OR enhancing OR enhanced OR enhance) AND TITLE-ABS-KEY (rs* OR variant OR variants OR polymorphism OR polymorphisms)." All types of documents were thus searched without a time limit. The 290 results were exported and further analyzed after excluding one item published before the year 1980, when magnetic resonance was first used in a clinical setting; although the first studies of genetic polymorphisms in MS were performed later, we did not filter our results further on the basis of the year published. All the entries were written in English. After screening titles and abstracts for information confirming that the studies investigated genetic variants, 91 of them were selected for further assessment. Among these, 26 reported investigating a possible link between genetic variation and radiological findings in the abstract; these were chosen for the final analysis (there were no duplicates). One of the articles was not included since the full text could not be obtained and imaging-related results in the abstract were unclear [3]. Another article was identified as relevant in references of the chosen studies [4]. We followed the approach proposed in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [5].

Results

Out of 26 studies, 20 found relationships between genetic variants and radiological findings in MR imaging of the central nervous system (**Table 1**).

The two genome-wide association studies (GWAS) provided a wealth of data [8, 19]. Of special interest are also the works by Sombekke et al., in which variants found in 44 MS-related genes were analyzed in the context of MR findings [14], and by Inkster et al., who focused on genes involved in epigenetic regulation [12]. The relationships between one or more HLA haplotypes and MR measures were searched for by seven non-GWAS studies. The remaining studies focused on particular genes of interest, of which most commonly researched were *BDNF* and *CCR5*. The methods of MR data acquisition and image method analysis varied between the studies, as did characteristics of patient groups.

None of the SNPs that were top-rated by the study of 44 genes by Sombekke et al. was found on the list of MR parameter covariates by Baranzini et al. *BTNL2* rs2076530 associated with MS susceptibility, but not MR measures. None of the findings from studies of individual genes (*GRIN1*, *BDNF*, *IRF5*, *PCK1, CCL5, CCR5, SIRT4, HDAC11, HDAC9*) was replicated by Baranzini et al. The above-listed genes were also missing from the list of 67 genes correlating with MR measures in all cerebral regions of interest in the two recruitment centers of GWAS by Matsushita et al. An intersection of the list by Matsushita et al. with the regression correlate list by Baranzini et al. yielded: *OPCML* (rs11223055), *PTPRD* (rs1953594), and *WWOX* (rs11150140, rs1116525) (Baranzini et al.: brain atrophy) as well as *CDH13* (rs692612) and *PLCB1* (rs6118257) (lesion load). The relationship between BDNF rs6265 (Val66Met) and the radiologic course of MS was not consistently replicated across the studies. While this was also true for *HLA-DRB1*15:01*, the recent evidence is convincing [6]. Overall, few positive findings of the reported studies were consistent.

Discussion

This brief systematic review gathered the data relating genetic variants to MR correlates of neurological lesions in MS. Any comparison of the included studies should consider the fact that MS diagnostic criteria constantly evolve [32]. MR imaging was featured in the clinical criteria in the year 2001, then reviewed in 2005 and 2010. For instance, the latest revision of McDonald criteria permit for an earlier MS diagnosis, but at the cost of the specificity. The work to further improve the guidelines is ongoing [33].

The association of MR measures in MS patients and *CDH13*, *PLCB1*, *PTPRD*, *OPCML*, and *WWOX* polymorphisms listed above warrants additional study. In conclusion, genetic variants were shown to correlate with MS-related brain atrophy and lesion load.

Table 1. Summary of the evidence regarding the relationship between genetic variants and magnetic resonance (MR) measures in multiple sclerosis (MS) patients

Study	n_{MS}	Gene	Variant	Evidence
Isobe et al. 2016 [6]	586	HLA-A, HLA-B, HLA-DRB1, HLA-DQB1	HLA-DRB1*15:01	In women, higher HLA genetic burden associated with lower volume of subcortical grey matter. *HLA-DRB1*15:01* was the haplotype most strongly linked to the finding and HLA-B*4402 had a protective role. No relationship between *HLA-A*02:01* and MR findings.
Yaldizli et al. 2016 [7]	85	HLA-DRB1	rs3135388 (*HLA-DRB1*15:01*)	No association of *HLA-DRB1*15:01* haplotype with cortical grey matter volume or magnetization transfer ratios in lesion or healthy grey matter.
Matsushita et al. 2015 [8]	464 211	Genome-wide association study	550,067 SNPs	*RYR2* and *CDH13* consistently associated with cortical thickness in 9 predefined regions in both MS cohorts. Additionally, 194 genes associated with one or more regions in both MS populations. No single SNP reached the significance threshold.
Huang et al. 2013 [9]	123	HLA-DRB1 HLA-DPB1 NOTCH4 IL7R	genotyping genotyping rs422951 rs6897932	In MS not meeting the Barkhof criteria *HLA-DRB1*04:05* was more frequent. In MS meeting the Barkhof criteria: *HLA-DPB1*03:01* and rs6897932-CC were more frequent; *HLA-DRB1*09:01* and *HLA-DPB1*04:01* less frequent.
Rossi et al. 2013 [10]	691	GRIN1	rs4880213	No association with lesion load. (Association of rs4880213-TT with thinning of the retinal nerve fiber thickness on optical coherence tomography in PPMS.)
Fera et al. 2013 [11]	26	BDNF	rs6265 (Val66Met)	Brain response greater than in HS while encoding and retrieving information in Val66 homozygous MS. Lower connectivity between the hippocampus and the posterior cingulate cortex on retrieval in Val66 homozygous MS. Other specific findings.
Inkster et al. 2013 [12]	326	Epigenetic regulatory genes	467 SNPs rs3135388 (HLA-DRB1*15:01) 3997 supplementary SNPs	Associations of *SIRT4* rs2522129; *HDAC11* rs2675231; *HDAC9* rs2389963 with various of 7 performed MR brain measurements, which included normalized brain volume and brain volume change in a year. No association between HLA-DRB1*15:01 and the volume of T2 lesions.
Vosslamber et al. 2011 [13]	75	IRF5	rs2004640 rs47281420	More new T2 lesions on MR during interferon-beta therapy in patients with *IRF5* rs2004640-TT. Association with MR non-responder status.
Sombekke et al. 2011 [14]	208	A selection of SNPs associated with MS	69 SNPs in 44 genes	An increased probability of lesions: *CCL5* rs2107538-CC, *IFNGR2* rs9808753-AA, *BTNL2* rs2076530-AG, *PNMT* rs876493-AG, MHC class II (*HLA-DRA*) region rs2227139-CT (most consistently associating); the major alleles of *CCL5* rs2107538 and *IFNGR2* rs9808753. A decreased probability of lesions: *FAS* rs3781202-CT, rs2234978-TT, *BTNL2* rs2076530-GG, *CRYAB* rs762550-AA, *NDUFS7* rs2074897-GG, *UCP2* rs659366-CC. Association with total T2 lesion volume: *UCP2* rs659366-CC, *BTNL2* rs2076530-GG and AG.
Ramasamy et al. 2011 [15]	188	BDNF	rs6265 (Val66Met)	Higher cingulate grey matter volume patients carrying at least one copy of Met66.
Weinstock-Guttman et al. 2011 [16]	209	BDNF	rs2030324	TT genotype associated with lower left thalamic volume, but not with total lesion measures or brain volume.
Xia et al. 2010 [17]	641	PICALM CR1 CLU PCK1 ZNF224	rs3851179 rs6656401 rs11136000 rs8192708 rs3746319	*PCK1* rs8192708-G associated with a smaller brain volume (brain parenchymal fraction) and a higher hyperintense T2 lesion load.
Sombekke et al. 2009 [18]	150	A selection of SNPs associated with MS	68 SNPs in 44 genes	Correlation with the count of lesions in the spinal cord: *MHC2* rs3135388, rs2395182, rs2239802, rs2227139, rs2213584. *CCL5* rs2107538 associated with T2 lesion load (false discovery rate-corrected p = 0.07).

Table 1. (continued)

Study	n_{MS}	Gene	Variant	Evidence
Okuda et al. 2009 [4]	505	HLA-DRB1	HLA-DRB1*15:01	Association of *HLA-DRB1*15:01* with increased white matter lesion volume and decreased normalized brain parenchymal volume.
Baranzini et al. 2009 [19]	794	Genome-wide association study	551,642 SNPs	Associated with brain parenchymal volume: *IRX1* rs4866550, *CDH10* rs10078091, *C20orf133* (*MACROD2*) rs368380, *MORF4* rs4473631, *SOX11* rs1869410, *BICD1* rs261902, *CAST1* rs11719646, *CHORDC1* rs1354913, *NLGN1* rs13067869, *PPP3CA* rs9307252, *FOXO3A* rs9480865 and rs9486902, *SVIL* rs1927457, *MXI1* rs716595, *KCNIP1* rs11957313, *SLITRK6* rs9319189, *CDCA1* rs10917727. Associated with the load of T2 lesions: *PLD5* rs12097667, *KIAA1706* rs1806468, *GPR126* rs146250, *HIVEP2* rs263153, *NPHP3* rs6794496, *CHRND* rs2602397, *FUT9* rs6899560, *NUBPL* rs2039485, *HIP2* rs305124, *IGF2R* rs6917747, *CPAMD8* rs11666377 and rs6512158, *IGF2R* rs12202350.
Zivadinov et al. 2007 [20]	209	BDNF	rs6265 (Val66Met)	The presence of Met66 associated with larger normalized grey matter volume and smaller T2 lesion volume. No link to whole brain or white matter volume.
van Veen et al. 2007 [21]	192	CCL5 CCR5	rs2107538 rs1799987 rs333 (CCR5Δ32)	A smaller risk of severe axonal loss with *CCL5* rs2107538-G. Lower T1 and T2 lesion volumes in MS with CCR5 rs1799987-G. Lower T2 lesion volume and black hole ratio when CCR5Δ32 present.
Kaimen-Maciel 2007 [22]	124	CCR5	rs333 (CCR5Δ32)	Associated with a lower frequency of at least one gadolinium-enhancing lesions.
Liguori et al. 2007 [23]	50	BDNF	rs6265 (Val66Met)	Lower cerebral grey matter volume in RRMS carriers of Met66.
Wergeland et al. 2005 [24]	63	IL10	rs1800896 rs3021097 rs1800872	More T1 contrast-enhancing lesions in patients with GCC phenotype during first 6 months of treatment with interferon.
Schrijver et al. 2004 [25]	96	TGFB1	rs1800470 (rs1800473) rs1800471	MS homozygous for *TGFB1* rs1800470-C (Leu10Pro) had greater annual increases in ventricular fraction and hypointense T1 lesions.
Zwemmer et al. 2004 [26]	408	APOE	ε4 (rs429358-C; rs7412-C) ε2 (rs429358-T; rs7412-T)	No link between ε2 or ε4 genotype and lesion volume or brain atrophy.
van Veen et al. 2004 [27]	514	CTLA4 CD28	rs5742909 rs231775 rs3116496	No link to lesion volume or brain atrophy.
van Veen et al. 2002 [28]	382	FAS	rs1800682	No link to lesion volume or brain atrophy.
Schreiber et al. 2002 [29]	70	DRB1 CCR5 APOE	*HLA-DRB1*15:01* rs333 (CCR5Δ32) ε4	No association of *HLA-DRB1*15:01* and *APOE* ε4 to total lesion area divided by MS duration. A non-significant trend for a lower value of this measure in CCR5Δ32 carriers.
Weatherby et al. 2000 [30]	50	GSTM1, GSTM3, GSTP1, GSTT1	genotyping	*GSTT1* null genotype associated with more gadolinium-enhancing lesions and more frequent occurrence of ≥ 3 lesions.
Nishimura et al. 1997 [31]	57	HLA-DRB1, HLA-DRB3, HLA-DRB5	genotyping	*HLA-DRB1*15:01* associated with the Western (more brain lesions, less enhancing spinal cord lesions), as opposed to the Asian type of MS.

PPMS – primary progressive MS; RRMS – relapsing-remitting MS; SNP – single-nucleotide polymorphism

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REVIEW PAPER

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Perspectives for gallotannins neuroprotective potential – current experimental evidences

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ABSTRACT

Gallotannins are class of hydrolyzable tannins consisting of gallic acid and a sugar moiety. Currently, there is growing interest around a possible neuroprotective effect of this class of phytochemicals, which is suggested to be a result of their active metabolites. Evidence from experimental studies has suggested that tannin-rich plant preparations might be effective at reversing neurodegenerative pathology and age-related declines in neurocognitive performance. This mini-review summarizes, based on experimental studies, current knowledge about diverse neuroprotective abilities of gallotannins, mostly via antioxidant properties and some mechanisms of the effect are proposed including blocking accumulation of nitrites, inhibiting expression and activity of heme oxygenase 1(HO-1), and decreasing degradation of poly(ADP-ribose) glycohydrolase (PARP).

Keywords: neuroprotection, plant extract, gallotannins, galloylated cyanogenic glycosides, poly(ADP-ribose) glycohydrolase, 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose.

Introduction

In the last decades, numerous studies on a protective role of plant polyphenols against several chronic diseases, including neurodegeneration, have been published [1, 2]. Currently, substantial interest in health benefits of tannins is emerging, especially regarding their neuroprotective potential [3, 4].

Tannins are a subclass of naturally occurring polyphenols found in both condensed and hydrolyzable forms. Hydrolysable tannins are multiple esters of a sugar moiety and organic acids such as gallic acid in gallotannins or ellagic acid in ellagitannins. Instead, condensed tannins occur as oligomerics or polymerics of flavan-3-ols, mainly derivatives of epicatechin and catechin. Tannins have been reported to exert several biological effects, including antioxidant and free radical scavenging activity as well as antimicrobial, anti-cancer, and cardio-protective properties [5]. Evidence from epidemiological and human intervention studies and animal studies have suggested that tannin-rich plants might be effective at reversing neurodegenerative pathology and age-related declines in neurocognitive performance [3–5]. Despite a great volume of literature data showing various biological effects of polyphenols, including tannins, it is still a matter of debate because of their questionable bioavailability. Most of them are poorly absorbed through the gastrointestinal tract, highly metabolised, and rapidly eliminated [6]. There is also contradictory evidence as to whether polyphenolics may cross the blood–brain barrier or not. How-

ever, recent research on the metabolism, and pharmacokinetics of phenolic compounds have revealed that they can act mainly through metabolites and catabolites formed after their intake [7]. Furthermore, various mechanisms underlying the neuroprotective effects of tannins are proposed. Since the goal of the neuroprotection approach is to limit pathological mechanisms leading to neuronal dysfunction at the molecular level such as: oxidative stress, neuroinflamation, protein aggregation, mitochondrial dysfunction and aberrant cellular signalling [8], herein, this mini-review outlines current knowledge about potential diverse neuroprotective abilities of gallotannins present in different plant preparations (mostly extracts) that are relevant to the mechanisms.

Evidences for neuroprotective properties of gallotannin and its derivatives

Since oxidative damage to neuronal micro-organelles or cell bodies, accumulation of iron ion species and a decrease in the cellular antioxidant pool in the brain play a pivotal role in pathophysiology of neurodegeneration, antioxidant properties of tannins appears to be beneficial for neuroprotection [6; 9]. However, it is suggested that due to low, physiological concentrations polyphenols in brain, including tannins, act rather as "indirect" antioxidants by modulating the activity of antioxidant enzymes [6; 10]. Results of several studies gave directions to the coming to that thesis. Polyphenols have been shown to interact with critical neuronal/glial intracellular signalling pathways involved in memory, neuronal differentiation and neuronal resistance to neurotoxins, including oxidants and inflammatory mediators [10]. Therefore, several experiments have been conducted aimed at demonstrating these neuroprotective properties for tannins [6, 10, 11]. A protective effect of water extract of *Uncaria sinensis* (OLIV.) HAVIL., a main medicinal plant composing Choto-san – a Kampo (traditional medicine of Japan) formula (Diao-Teng-San in Chinese) (consisting of: Atractylodes lancea rhizome, *Poria* sclerotium, *Cnidium* rhizome, *Uncaria* Hook, Japanese *Angelica* root, *Bupleurum* root, and *Glycyrrhiza*) [12–15], on glutamate-induced neuronal death in cultured cerebellar granule cells through the inhibition of Ca2+ influx was presented by Itoh et al [16]. The *Uncaria sinensis* (US) extract in a dose-dependent manner (at concentrations of 10(-5) to 10(-4) g/ml) caused a significant protective effect against glutamate-induced cell death of cultured cerebellar granule cells compared to exposure to glutamate only. In a dose-dependent manner it blocked of the Ca2+ influx into cells by glutamate [16]. These results prompted researchers to investigate whether identified in *U. sinensis* extract tannin compound possess neuroprotective activities. Furthermore, an evidence of neuroprotective property of epicatechin, catechin, procyanidin B-1, procyanidin B-2, hyperin and caffeic acid isolated from the hooks and stems of *Uncaria sinensis* (HSUS) via protection against glutamate-induced neuronal death in cultured cerebellar granule cells by inhibition of Ca2+ influx was provided by Shimada et al. It was shown that the treatment with epicatechin (100–300 uM), catechin (300 uM), procyanidin B-1 (30–300 uM) and procyanidin B-2 (100–300 uM) caused a significant increase of cells viability and inhibition of Ca2+ influx into cells induced by glutamate [17].

Early *in vitro* cell-free studies and cellular assays have revealed that gallotannin, a complex mixture of tannins purified from oak gall, has been shown to inhibit PARG (PARP (poly(ADP-ribose) glycohydrolase – a key enzyme degrading ADP-ribose polymers) activity [18;19]. It was also shown that it significantly reduced oxidative (H2O2)-induced cell death (after 24 and 72 h of H2O2 exposure; 100 nM of gallotannin) in murine astrocytes cell culture with 10-fold more potent activity than the PARG inhibitor benzamide in preventing such process [20]. Another study by Ying et al [21] revealed that gallotannin and nobotanin B (another gallotannin) equally decreased PARG (PARP1) proteins in mouse and astrocytes cell cultures exposed to hydrogen peroxide or N-methyl-d-aspartate (NMDA), the DNA alkylating agent, and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) compared to reference benzamide causing their marked reduction. Gallotannin and benzamide both prevented the NAD(+) depletion resulting from PARP1 activation by MNNG or H(2)O(2), with opposite effects on protein poly(ADP-ribosyl)ation. In this case benzamide decreased, while the gallotannin showed tendency to increase of poly(ADP-ribose) proteins accumulation during MNNG exposure in neuron cultures. Thus, results obtained by Ying et al. suggest that PARG inhibitors do not inhibit PARP1 directly, but instead prevent PARP1-mediated cell death by slowing the turnover of poly(ADP-ribose) and thus slowing NAD(+) consumption. One possible explanation for why gallotannin treatment leads to an apparent accumulation of PARG proteins may be it unspecific binding to biomolecules and protein staining [21].

Another, a complex experiment giving the evidence that gallotannin is an inhibitor of PARG was made by Falsig et al., however the result showed that such activ-

ity is not specific, and that it rather does not work in cells [22]. For this purpose a comparison of the PARG inhibitory activities between tannic acid, gallotannin and compound N-bis-(3-phenyl-propyl)-9-oxo-fluorene-2,7-diamide (GPI 16552 – a potentially specific PARG inhibitor) was made in an *in vitro* cell-free experiment and in three PARP-1-dependent cell death (murine fetal astrocytes) models plus one inflammation model in primary astrocytes [22]. Moreover, an ability of gallotannin to inhibit recombinant human PARG was also examined. It was found that it was indeed an inhibitor of PARG as previously shown. However, results from these experiments showed that gallotannin (neither the reference inhibitor – GPI 16552) in conducted experimental cellular death models not fully effectively inhibited PARG activity. The IC50 of gallotannin in a PARG assay based on a cellular lysate was increased to about 150 uM. In primary astrocytes studied gallotannin dose dependently (10 – 50uM; 30 min exposition) blocked almost completely all cell death in the H2O2 and SIN-1 models, but in the MNNG model did not rescue cells – it enhanced cell death in a concentration-dependent fashion. Its action in this case was different from that observed in the case of GPI 16552 which did not gave any effect in MNNG model (in the concentration range 20, 40, 60, 80, 100, 120, 140 uM), even at highest concentrations (in SIN-1-induced cell death a small effect was seen at high GPI concentrations). This effect could be due to strong unspecific protein binding. Unspecific protein binding by gallotannin possibly also inhibited NOs as described in cell-free systems [23–25]. Authors indicated that this molecule might apparently "inhibit PARG", because it causes DNA strand breaks that activate PARP [26]. Such quite strong antioxidant effects of gallotannin against H2O2 could also be due to its strong iron chelating effects [27]. Further analysis revealed that two other gallotannin-similar, polyphenolic compounds – quercetin (five phenol groups and one resonating oxo group) and catechin (five phenol groups) blocked cell death in the hydrogen peroxide model at concentrations similar to gallotannin, suggesting that PARG inhibition is not necessary for cytoprotection by this tannin only. Authors statement that its ability to effectively penetrate the cell membrane may be questionable should be here highlighted [22].

In H2O2-activated cells a decrease of PAR staining was observed and was in opposite of what was previously reported and inconsistent with PARG inhibition [28]. If gallotannin acted as a PARG inhibitor, a delay in the decay of PAR, and thus a prolonged PAR accumulation would be expected, which was not observed. Studied gallotannin (similarly to GPI-16552) after exposure of astrocytes with 1mM of H2O2 did not enhanced the staining for PAR at any time point of experiment duration (0 – 30 min) which may suggest its unselective PARG inhibition due to the fact that, according to authors, a selective PARG inhibitor is expected to enhance PAR accumulation and/or delayed the time-dependent loss PAR presence. A possible inhibition of PARP was also visible in HeLa cells at concentration of gallotannin above 100 uM which caused a significant increase of PAR accumulation [28]. And this effect from experiment in HeLA cells was opposite to results from Ying et al [21]. According to the authors, it is not excluded that the activity of other cellular PAR-hydrolysing enzymes could be responsible for the lack of a PAR accumulation in cells treated with specific PARG inhibitors [22, 28]. In sum, observed protective effects from H2O2 at 5–10 uM, i.e., 10- to 15-fold below the IC50 values of gallotannin in cell extracts again suggested that gallotannin does not protect due to PARG inhibition [28].

A significant anti-inflammatory potential of gallotannin correlated with its antioxidant properties was also emphasized in cell-free conditions (concentrations: 0; 1; 5; 10; 20 uM) and in a validated model of primary astrocyte inflammation (stimulated with a complex mixture of proinflammatory cytokines (CCM) containing TNF-a, interleukin-1h, and IFN-g) involving the production of NO [22]. In this experiment gallotannin blocked the accumulation of nitrite (concentrations: 0; 2.5; 5; 7.5; 10 uM) to a similar extent and with a concentration–dependent manner, thus it acted as a scavenger of NO without ever interfering with cellular processes. Interestingly, this inhibition was not due to transcriptional or translational inhibition, because gallotannin rather increased the levels of iNOS protein [22]. Further *in vitro* (non-cellular) experiments also indicated the ability of studied gallotannin to inhibition of PARG (PAR degradation) (almost completely at 5–10 uM concentration) which suggest that this compound was indeed a potent *in vitro* inhibitor of PARG. This effect was even stronger than observed in the case of reference GPI-16552 [19]. Based on above studies it was concluded that gallotannin is a PARG inhibitor in cell-free assays and acts as a strong antioxidant that can protect cells from oxidative stress. Moreover, high concentrations of gallotannin may be required to inhibit PARG in complex biological system. However, the concentration used in the cell lysates-based PAR accumulation assays would cause massive cell death in other cellular system. Authors hypothesized also

that due to the fact that no effect in intact cells and no penetration in studied monolayer was detected it could be suggested that gallotannin is cell impermeable and mediates its effect extracellularly by lowering the concentrations of reactive oxygen species, and therefore its activity can be linked to the extracellular oxidative system. However, so far, no evidence suggesting its penetration through the cellular monolayer were found [22]. Hence, the biological activity of gallotannin in cells is still the subject of scientific inquiry.

It is also suggested that biological activity of tannins is strongly dependent on number of gallated rings. Choi et al [29] revealed that the 1,2,3,4,6-Penta-O-galloyl-beta-D-glucose (PGG) (10–50 uM), a major component of the crude *Paeonia suffruticosa* ANDREWS root (*Ranunculaceae*), was able to protect neuronal Neuro 2A cells from oxidative stress via the significant, concentration- and time-dependent induction of HO-1 (heme oxygenase 1; an inducible stress protein that degrades heme to the neuroactive molecule, carbon monoxide and the anti-oxidant, biliverdin) gene expression and its activity. Pretreatment of these cells with PGG resulted in enhanced cellular resistance to hydrogen peroxide [29]. Tan et al [30] demonstrated that several gallotannins (1,2,3-tri-O-galloyl-β-D-glucose; 1,4,6-tri-O-galloyl-β-D-glucose; 3,4,6-tri-O-galloyl-D-glucose; 1,2,3,6-tetra-O-galloyl-β-D-glucose; 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG); 3,6-di-O-galloyl-D-glucose), among others bio-active compounds like galloylated cyanogenic glycosides, isolated from the leaves (ethyl acetate extract or aqueous extracts, respectively) of *Phyllagathis rotundifolia* (*Melastomataceae*) exhibited remarkable neuroprotective activities against oxidative damage *in vitro* in neuroblastoma-glioma hybrid NG108–15 cells as compared to galloylated cyanogenic glucosides and ellagic acid derivatives in a dose-dependent manner. Analyzed gallotannins also increased the neuroblastoma-glioma hybrid cell viability in a dose dependent manner. The compound PGG and 1,2,3,6- tetra-O-galloyl-β-D-glucose significantly inhibited H2O2-induced neuron cells damage in a dose-dependent manner at concentrations of 6.25–100 μM. The inhibitory activity of 1,2,3,6-tetra-O-galloyl-β-D-glucose was comparable to that of catechin. However, the neuroprotective activity of PGG was more potent than that of catechin [30]. This compound has also been reported to not only increase the cellular resistance to H2O2 but also highly protected neuronal cells from H2O2-induction damage via induction of HO-1 gene expression [29, 30]. According to Warden et al., the level of galloylated catechins in human urine after black tea consumption was 10-fold lower than that of non-galloylated catechins, which strongly indicates that galloylation increases resorption of catechins [31].

Among many different polyphenol subgroups, gallotannins have been relatively poorly examined in the context of the anti-amyloidogenic activities. Only few publications have appeared in recent years documenting their strong neuroprotective abilities. It has been shown, for example, that PGG exhibited a strong anti-aggregation effect on β-amyloid in Alzheimer disease [32]. An in vitro SK-N-SH cell line experiment demonstrated that PGG isolated from *Paeonia suffruticosa* at the 3 μM concentration inhibits Aβ1–42 fibrils formation of over 50%, while the 100 μM concentration completely inhibits formation of Aβ1–42 fibrils. Fujiwara and co-workers have also demonstrated that PGG oral administration to mice Tg2576 APPswe race 8 mg/kg/day strongly decreased level of Aβ1–40 aggregates (from 4000 pmol/g brain to about 2500 pmol/g brain). In the case of aggregates of Aβ1–42 the dose was approximately 100 pmol/g brain in the control and about 50 pmol/g brain in animals used in studies [32]. Another compound – tannic acid – turned out to be a natural β-secretase inhibitor that prevented cognitive impairment and mitigates AD pathology in PS/APP transgenic mice. In addition, it reduced the effects of Alzheimer's like neuropathology in mice overproducing Aβ1–40 and Aβ 1–42 [33]. Another in vivo studies by Hartman et al. showed that in other transgenic mice (strain APP_{SW} / Tg2576) who drank the pomegranate juice (containing 115 ppm of ellagic acid, 5 ppm of gallic acid, 1880 ppm of hydrolysable tannins; among them: gallotannins, ellagitannins, punicalagin and 369 ppm of anthocyanins and their glucosides) the level of Aβ plaques and fibrils in both the hippocampus and cortex dorsal has been decreased [34]. Determining which of these compounds showed these properties and with which intensity requires further complex research.

Conclusions

Summarizing, data presented in this manuscript provide some evidences about the neuroprotective potential of gallotannins. However, their mechanism of actions remains still not fully understood. Therefore, it is highly justified to further explore the mechanism of this class of natural-origin protective agents against neurodegeneration. The complex knowledge about their polypharmacological activities may be of significance for neuroprotection.

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Conflict of interest statement
The authors declare no conflict of interest.

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Characteristics of Regulatory T cells

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ABSTRACT

Regulatory T cells (Tregs) is heterogenic subpopulation of T cells that is able to suppress function of effector cells during the immune response. Among them are natural (nTreg) and induced Treg (Tr1, Th3, $CD4^+CD25^-$). CD25, CD45Ro, CD152, GITR, LAG-3, several adhesion molecules, chemokine receptors as well as Toll-like receptors are present on the surface of Treg. Mechanism of suppression used by nTreg is not completely understood.

Keywords: Tregs, regulatory T cells, autoimmunity, cancer.

Introduction

The subset of suppressor T cells loomed from the work of Gerdhon and Kondo in the early 1970s. Although not that widely noted the existence of T cells with suppressive function was also suggested by Nishizuka and Sakakura [1, 2].

This distinct T-cell population was originally characterized in mice, representing 5–10% of peripheral $CD4^+$ T cells.

In humans, Treg cell population is a small subset of $CD4^+$ T lymphocytes (about 5%), with the expression of high-intensity CD25 ($CD25^{high}$) and they control immunity by interfering with the generation of effector function *in vivo* [3, 4].

Immunophenotype

Treg population is heterogeneous and markers for specific subtypes are now the major objective.

nTreg lymphocytes were identified as $CD4^+$ T cells expressing high levels of IL-2Rα (CD25), along with low expression of the IL-7Ra chain (CD127) and a unique transcription factor FoxP3, which acts as a master regulator gene for inducing Treg phenotype and lineage [5].

Treg cells express several molecules such as CTLA-4, CD122, GITR, Galectin 10, LAP, ICOS, PD-1 and GARP and Toll-like receptors [6, 7].

Treg cells show elevated levels of adhesion molecules such as CD11a, CD44, CD54, and CD103 [7, 8].

There is some evidence suggesting that Treg cells might also exhibit a characteristic chemokine receptor profile: CXCR3, CXCR4, CCR4, CCR3, CCR5, CCR6 and CCR8 [7, 9, 10].

Others Tregs markers are LAG-3, an MHC class II binding CD4 homologue and neutropilin (Nrp1), which is involved in axon guidance, angiogenesis and T lymphocyte activation [11].

A relatively new marker HELIOS, from the Ikaros family transcription factors, defines Treg subsets with distinct phenotypic and functional characteristics [12].

It has been recently reported that nTregs express CD39 and CD73 antigens. Expression of CD39 and CD73 on Tregs was first described by Borsellino *et al.* and Deaglio *et al.* in 2007 [6, 12].

The FoxP3 gene was identified in 2001 as the disease – causative in Scurfy mice, which spontaneously develop severe autoimmunity/inflammation as a result of a single gene mutation on the X chromosome [11, 13].

FoxP3 seems to activate or repress hundreds of genes directly or indirectly through forming a transcription complex with other key transcription factors such as NFAT [14].

FoxP3 probably controls cell-contact dependent inhibition of the activation and proliferation of T cells,

killing or inactivating APC and/or T cells, and/or via suppression of cytokines such as IL-10 and TGF-β [14–16].

Mechanism of action and role of Tregs

Presently, a role of Tregs is not restricted to maintaining self-tolerance. Tregs are believed to regulate immune response against self-antigens, infectious agents, tumor antigens and transplantation antigens.

Tregs maintain immunological tolerance by inhibiting helper T cell, cytotoxic T lymphocytes, dendritic cells, and NK cells function [17–19].

Tregs can secrete blocking cytokines like IL-10, TGF-β, IL-35 and use them as the main immunosuppression inducing factors [20].

Tregs can also use the cytotoxicity involving perforin and granzyme as a mechanism of suppression. It has been proven that human Tregs activated by anti-CD3 and CD46 express granzyme A and B and can kill their own immune cells. Killing involving Tregs is perforin-dependent and FasL-independent [20].

Tregs may also limit the immune response by affecting the APCs. In studies involving Treg, Tef and DC interactions in lymph nodes using intravital microscopy, it has been shown that Tregs are capable of direct interaction with antigen-binding dendritic cells. Contact between Tregs and DC can lead to Tef activation blocking [20].

Tregs in pregnancy

Tregs induced during pregnancy are involved in immune tolerance induction of mother organism on fetus. On the periphery a double increase in the number of Tregs in pregnancy is observed, and the maximum number of these cells falls on the period from the second trimester and lasts for 6–8 weeks after birth. In women with recurrent, spontaneous miscarriages, the number of Tregs drops suddenly in both decidua as well as in peripheral blood compared with women whose pregnancy is proceeding correctly [21, 22].

Autoimmunity

The Treg function impairment can lead to the development of autoimmune diseases.

In humans, the quantitative and qualitative disorders of Treg populations is said to be one of the courses of this type of diseases [17].

For instance, in both newly diagnosed and chronic type I diabetes patients a reduced percentage of Tregs has been stated. In addition, these cells less inhibited T cell proliferation *in vitro* [23–25].

Treg cells in rheumatoid arthritis patients were in anergy, inhibited the proliferation of effector cells, but did not inhibit the secretion of inflammatory cytokines by effector lymphocytes and monocytes. In patients with multiple sclerosis, there was no decrease of $CD4^{+}CD25^{high}FoxP3^{+}$ cells, however the ability to inhibit the effector lymphocyte proliferation, and their cytokine secretion was reduced, compared to the control group [26, 27].

Neoplasms

Numerous studies show connection of T regulatory cells with the induction of tolerance to cancer. Cancer antigens derived from the host and many cancer-associated antigens are also self-antigens, which Treg cells recognize as their own and promote tolerance. Tregs are also capable of inducing suppression of NK cells, which control tumor growth *in vivo*. It has been found that the regulatory T-lymphocytes can induce suppression of both innate and adaptive immune response [28–30].

An increased number of Tregs in the circulation of patients with different types of cancer has been shown (including lung, breast, ovarian, colorectal, esophageal, renal and gastric cancer, as well as hepatocellular carcinomas, leukemias, lymphomas and melanomas). The increased infiltration of Tregs in tumors and neoplastic exudates is associated with poor prognosis in multiple cancers. It is known that infiltration of CD8+ cells is a preferred prognostic factor, but increased ratio of Tregs to CD8 + cells is a negative factor. It seems that the relationship between Tregs and effector cells in the cancer microenvironment creates the balance between immunity and tolerance. As a result cancer could reduce the immune response by promoting the recruitment, expansion and activation of Tregs [28–32].

Conclusion

Tregs regulatory functions are critically important for maintaining balanced immune responses. In healthy individuals, this balance is controlled by nTregs. In patients with cancer and viral infections, the rules for nTregs are changed. In recent years there has been tremendous progress in understanding the function

of Tregs in cancer, autoimmunity, graft rejection and other reactions depending on immune response. These interdependencies, however, require further clarification.

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Clinical trials in complementary and alternative medicine – the myth of limitations

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ABSTRACT

The paper aims to dispute common arguments put forward by practitioners of complementary and alternative medicine (CAM) in discussions against conducting clinical trials in CAM treatment protocols. It is argued that CAM therapies cannot be evaluated by the same criteria as those applied in conventional medicine due to specificity of CAM. This paper suggests that this line of thought undermines not only the validity of CAM therapies, but, importantly, is delaying understanding their therapeutical value. We also argue that despite apparent differences in approach both conventional medicine and CAM aim to improve human well being therefore CAM should be validated with well established and widely accepted process of balancing of risks and benefits of individual therapies as in conventional medicine clinical trials.

Keywords: clinical trial, CAM, prognosis, decision making.

Introduction

Introducing the evidence-based medicine (EBM) into medical practice has changed significantly our understanding of benefits of medical therapies. The EBM principles define rules how to design good clinical study in order to evaluate efficacy and adverse effects of treatment so that the conclusions can be trusted and applied in clinical practice. Despite of long history of modern medical sciences, since Renaissance, it is only recently that the principle of rigorous evaluation of clinical practices has been established in conventional medicine via clinical trial. It seemed natural that the same process, possibly delayed would occur in CAM therapies. Nevertheless, introducing the EBM principles into CAM is not entirely a smooth transition. One of obstacles is that numerous CAM providers believe that evidence-based concepts are not valid for of CAM [1]. Conducting clinical trials, and randomized control trials in particular, is certainly the major issue under dispute between the proponents and opponents of the evidence-based complementary and alternative medicine (EBCAM). In this dispute, it is the individual practitioners of CAM who very often turn out to be the strongest opponents of EBCAM. When putting forward their argument, they point to certain limitations, according to which, it is impossible to plan and carry out clinical trials in CAM. There are two major arguments presented. One is that CAM therapies concern ailments that are not treated by conventional medicine and that, therefore, we do not have any precise criteria for including patients in clinical trials. Establishing such a criterion is, on the other hand, a prerequisite for carrying out an appropriate trial. The other argument is that the majority of CAM therapies cannot be standardized, because they seek to meet the individual needs of patients. Both of these arguments will be referred to as *the arguments from the specificity of CAM*. The present paper argues that these arguments not only question the validity of CAM therapy, but, first and foremost, delay the process of establishing scientific knowledge within the scope of this discipline.

Discussion

Let us discuss the first argument that in CAM we do not have precise admission criteria. It is true that CAM has some limitation of scope as compared to conventional medicine. Conventional medicine relies heavily on understanding causative relationship and therefore employs many tests. Therefore admission criteria are formulated in such a way as to be useful for the study. Good illustration would be evaluation of the prognostic value of certain size of lung nodule as detected by chest CT. Thus, it seems obvious that conventional medicine manifests its efficacy not only via the therapies employed, but also includes the possibility of predicting the future conditions of the body on the basis of previously gathered data. The argument that points to the difficulties in including patients in clinical trials in CAM, has two negative implications that are of crucial importance for the development of CAM. Firstly, it leads to the conclusion that we are completely incapable of diagnosing the patients that CAM therapies should be employed to. Secondly and most importantly, it rules out the possibility of making any prognoses.

The tenet guiding modern medicine is first to understand the causal mechanisms that are responsible for the occurrence of various bodily processes (physiology), then to understand what goes wrong (pathophysiology) so that we can make prognoses, intelligently design and validate treatments [2]. Until the development of EBM, the knowledge of the causal mechanisms was assumed to be a prerequisite for any prognosis [3]. Now, we know that data obtained from clinical trials are of greater value than results predicted from preclinical tests. As it is paramount that for given therapeutical approach the evaluation of benefits over risks is much more important than understanding of mechanisms then there are no limitation of CAM to be tested the same way as conventional medicine.

The other argument, so often advocated by individual CAM practitioners, is even more dubious. It says that no standardization of therapeutic techniques in CAM is possible due to the necessity to adjust these techniques to the individual needs of patients. First and foremost, it has to be noted that, contrary to the widespread opinion, the problem is encountered not only in CAM but also in conventional medicine. When treating a single patient we need to appreciate if he/she 'falls within' the group for which the clinical trial has been conducted. Generally, it can be said that the problem of standardization of therapeutic techniques is related to modeling therapeutic interventions. Models of therapeutic interventions are group of algorithms which specify what actions need to be taken with regard to people who fall within the group that has been chosen on the basis of the diagnosis. The same process occurs both in conventional medicine and in CAM. In conventional medicine, models of therapeutic interventions can be made using theories of a basic science (biochemistry, pharmacology etc.). In this case, the model of therapeutic intervention, which is based on scientific argument, specifies how the therapy works and what result can be expected. The very same theories specify the contraindications for its use with regard to both the patient's general condition and other pharmaceutical agents that the patient is taking. Thus, the therapist's knowledge allows them to determine how a given group of patients should be treated. The problem is that we cannot use one universal model to all patients within a given group. Various factors of social, economic, and medical nature need to be taken into account when choosing a particular therapeutic model. The effects of the therapy are as important as the patient's expectations and preferences: the quality of life and medical costs in particular [4].

Models of therapeutic interventions can also be established using clinical trials. In this case, they are far more reliable, since previous experience shows that scientific theories do not fails to predict all the effects of the therapy employed. Suffice it to remind the results of the CAST trial, which revealed the harmfulness of certain antiarrhytmic agents (encainide and flecainide) administered to MI (myocardial ischemia) patients. Moreover clinical trials make it possible to correlate the information about short- and long-term effects of treatment with knowledge about the patient's preferences. That is precisely why the information obtained from clinical trials makes it possible to adjust clinical treatment to the patient's expectations in the most desirable way [5].

In CAM, models of therapeutic interventions are based on various sources, ranging from oral folk traditions and common religious beliefs to the 'theories' of alternative medical systems (e.g. Chinese medical system). It is desired in both conventional medicine as well as in CAM that models of therapeutic interventions should be characterized by the greatest accuracy possible, i.e. they should, in the most precise way, assign particular therapeutic actions to given groups of people, taking into consideration the individual preferences of the patients. Thus, maintaining that 'CAM techniques are not subject to standardizations' is tantamount to not specifying what actions need to be

taken with respect to a group of people diagnosed in a particular way, or, more specifically, those who have been classified to particular groups in accordance with their ailments, age, sex, life style and patients expectations. It is therefore surprising, that such a statement raises serious objections by CAM practitioners. It can be explained by the fact, that majority of CAM therapists make models of therapeutic interventions quite arbitrarily. However, one should expect that the choice of the therapeutic model would be entirely rational. The rationality of the choice presupposes that the therapists who have particular knowledge about the patient's expectations and the available methods of treatment make the same conclusions that result in the choice of similar models of therapeutic interventions [6].

A confounding issue in CAM is that there is a large number of equally justified therapeutic actions. The problem also is that without conducting any clinical trials in CAM, we have no instrument for eliminating false therapeutic models that forestall the implementation of therapeutic goals. Yet, the possibility of falsification is an essential criterion for distinguishing a science from non-scientific beliefs [7].

A therapy evaluation requires – as Lundberg and Fontanarosa have observed – answering a few important questions [8]. The question whether or not a given therapy is effective seems to be of secondary importance. First of all, it has to be established: (i) *what does the therapy consist in?* and (ii) *when should it be employed?* The inability to answer these questions means that the actions undertaken cannot at all be regarded as therapeutic. The problem is that *the arguments from the specificity of CAM*, which individual practitioners put forward so often, challenge the validity of raising these questions. Consequently, the arguments delay the process of establishing standards of treatment, which would make unification of therapeutic procedures in CAM possible. Establishing standards of treatment, that would answer the questions (i) and (ii) appears, at the moment, to be the most important factor determining the future development of CAM, as it is the starting point for the process of establishing scientific knowledge in complementary and alternative medicine.

Conclusions

It appears that the appeal to *the arguments from the specificity of CAM*, which is so common among individual practitioners, poses, in fact, a threat to the status of this discipline. These arguments create a myth of limitations for clinical trials in CAM. In fact these limitations are not a consequence of the specificity of CAM, but they are rather temporal in nature, as they are due to the problem that this discipline is evolving rapidly and its relations to conventional medicine are in flux. Whether it becomes 'magic' or a reliable scientific discipline is being determined now in the process of establishing the standards of scientific accuracy. As it is now, putting forward the *arguments from the specificity of CAM* is dangerous, since it disqualifies complementary and alternative medicine to be taken seriously and jeopardizes a chance to take potentially important place in broadly understood medicine.

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Proteomic and metabolomic strategy of searching for biomarkers of genital cancer diseases using mass spectrometry methods

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ABSTRACT

The project entitled "Proteomic and metabolomic strategy of searching for biomarkers of genital cancer diseases using mass spectrometry methods" is a study based on novel omics techniques. The main assumption of this research is the development of innovative model of searching of biomarkers in ovarian and prostate cancers using proteomics and metabolomics methodologies supported by bioinformatics analysis. The innovatory strategy based on the latest achievements in the field of mass spectrometry will allow for the implementation of the unique studies for discovery new biomarkers, which are useful in prediction, diagnosis and treatment of the genital cancers. To date, there is no comprehensive data including set of proteins and other endogenous compounds involved in the development and differentiation of these diseases. Therefore, the proposed approach may contribute to the discovery of biomarkers with high sensitivity and specificity, which will provide new information about genital cancers characterization.

Keywords: ovarian cancer, prostate cancer, proteomics, metabolomics.

General information

The project entitled "Proteomic and metabolomic strategy of searching for biomarkers of genital cancer diseases using mass spectrometry methods" was awarded a grant in the OPUS8 Competition organized by the Polish National Science Center (grant number: 2014/15/B/NZ7/00964). The project is planned for 36 months and it is run by the Department of Inorganic and Analytical Chemistry and the Gynecologic Oncology Department, at Poznan University of Medical Sciences, Poland. The principal investigator is Professor Zenon J. Kokot, Ph.D. The total grant value is 798,590.00 Polish Zloty.

This project will provide new prospective characterization of reproductive system cancers including ovarian and prostate cancers. Due to low specificity and sensitivity of diagnostics methods, this malignancies often reach advanced stages, when the sufficient treatment become problematic. Today, diagnosis of prostate cancer is mainly based on per rectum examination and on measurement of Prostate Specific Antigen (PSA) in blood [1]. While ovarian cancer diagnosis is conducted with the use of transvaginal ultrasound examination and estimation of two markers: cancer antigen 125 (CA 125) and human epididymis protein 4 (HE 4)[2]. However, none of clinical tools provide early detection of these malignancies. If the proper

early diagnosis is made, cancers can be curable with standard therapies including surgery and chemotherapy. Bearing in mind, that malignancies, which affect reproductive system, are serious worldwide problem [3, 4], this project will focus on finding new potential indicators of these pathologies. Better characterization of diseases may contribute to understanding of tumorigenesis and to develop novel diagnostic and treatment approaches. Close cooperation between scientists and clinicians will allow for a thorough and complete insight into the subject.

Ethics

The project was approved by Bioethical Committee of Poznan University of Medical Sciences, Poland (Decision No. 165/16) on 4th February 2016.

Research Project Objectives

The goal of this project is to develop innovative model of discriminatory indicators of selected reproductive system cancers using modern methods including mass spectrometry techniques: nanoLC-MALDI-TOF MS (nano liquid chromatography – matrix assisted laser desorption ionization – time of flight mass spectrometry), LC-ESI-QqQ-MS/MS (liquid chromatography – electrospray ionization-triple quadrupole – tandem mass spectrometry) and advanced bioinformatics. Recently, proteomics and metabolomics studies are widely used to characterize many diseases and pathologies like cancers [5–7]. Mass spectrometry coupled with liquid chromatography enables detection and identification of thousands endogenous molecules (e.g. amino acids, proteins, peptides) in body fluids. Therefore, this project is focused on complementary metabolomics and proteomics characterization of serum and urine in order to identify compounds which may improve diagnostic and treatment of ovarian and prostate cancers. The term biomarker has been present in medical terminology for many years to define variable, which may be marked or measured in order to evaluate important physiological and pathophysiological processes. Biomarkers can play essential role in modern diagnostic methods [8] and also in undertaking therapeutic decisions [9]. Moreover, discovery of new potential disease indicators will provide knowledge about pathomechanisms. Currently, improvement of analytical methods led to increase of selectivity, resolution, precision, accuracy and specificity. It result in more detailed analysis and enable deeper description of tumorigenesis. Therefore, in this project, compilation of data derived from proteomics and metabolomics study based on advanced bioinformatics analysis will provide model of the most discriminative features describing reproductive system cancers. There are two main hypothesis in this project:

- Determination of the qualitative and quantitative correlation within a multiprotein/multipeptide panels of markers of ovarian and prostate malignancies will allow for development of rapid and non-invasive diagnostic methods.
- Analysis of amino acid and other low molecular components profiles can contribute to determination of the metabolomic differences between clinical and histological types of ovarian and prostate cancers.

Research Plan and Basic Concept

The main purpose of this project is to extend knowledge about indicators in selected reproductive system malignancies. This goal will be reached with the use of low molecular compounds (including amino acids), peptides and protein analysis. Comparison of healthy control group samples and group of patients with cancer will be possible. Proteomic and metabolomic studies will enable detection of novel peptides, proteins and low molecular compounds as potential biomarkers, which can differentiate healthy individuals and oncology patients. Deep analysis of those molecules is essential from the point of view of the project's goal.

In particular, the planned studies should allow for:

- Development and implementation of new, selective methods which will provide characterization of serum and urine biomarkers of ovarian and prostate cancer using mass spectrometry techniques
- Compilation data derived from proteomic and metabolomic analysis. Determination of correlation between them and health condition in patients, results of the routinely conducted diagnostic tests (CA125, PSA) and histopathological analysis.
- Creation of modern biomarker panel which may evaluate the risk of reproductive system cancer development and contribute to early diagnosis, which will minimize invasive methods currently used in clinical trials.
- Extension knowledge about tumorigenesis and neoplasia at metabolomic as well as proteomic levels. Deep analysis of the studied diseases will provide tools not only for diagnostics but also for monitoring results of undertaken treatment and to evaluate its efficacy.

Research Methodology

The project will be performed with the use of serum and urine samples collected from patients with ovarian and prostate malignancies. Moreover, samples from healthy individuals will be examined. The main analysis will be conducted with the use of 2 tandem mass spectrometers coupled with liquid chromatography systems: LC-ESI-QqQ-MS (4000 QTRAP, AB Sciex) and nanoLC-MALDI-TOF/TOF-MS (UltrafleXtreme, Bruker). The project is planned for 3 years and contains the following stages:

Stage I Optimization of the method for determination of levels of 42 amino acids in both serum and urine. The novel analytical method will be performed using a set of LC-ESI-QqQ-MS/MS.

- Selection and optimization of depletion and enrichment strategies for proteomics studies. Sample pretreatment which enables analysis of low abundance proteins and peptides is crucial step in analysis. Different methods including combinatorial ligand library, immunodepletion, magnetic beads and solid phase extraction will be tested.
- Optimization of approach for protein-peptide profiling of serum samples. Optimization of detection and identification of the most discriminative peaks using MALDI-TOF/TOF-MS.

Stage II

- Collection of serum and urine samples derived from patients with ovarian (around 100 samples) and prostate (around 100 samples) malignancies. Recruitment of healthy individuals to control group (around 200 samples). All participants of the study signed consent to publish their (anonymized) data for scientific purposes. Questionnaire was performed among all participants.
- Measurement of 42 amino acids levels in urine and serum samples using a LC-ESI-QqQ-MS system.
- Protein-peptide profiling of collected serum samples using MALDI-TOF-MS. Analysis will be performed in the mass range 1–10 kDa. Identification of the most discriminative peaks using nanoLC-MALDI-TOF/TOF-MS. Semi-quantitative analysis of identified proteins and peptides based on isobaric labeling iTRAQ.

Stage III

- Statistical and chemometric analysis of the obtained data using special software: Statistica, MetaboAnalyst, ClinPro Tools, MATLAB. Comparison and compilation of the results obtained from metabolomics, proteomic and clinical studies. Investigation of correlation between the results and patient's health condition.

Measurable Effects and Expected Results

The project combined proteomics and metabolomics studies to deep characterization of reproductive system cancers. According to close collaboration between scientists and clinicians, we expect that the use novel strategy will extend knowledge about these malignancies. Compilation of the obtained data will allow for understanding pathomechanisms accompanying development of the diseases. Moreover, this project will provide new data on potential biomarkers, which can be further used as diagnostic tools. Broadening the knowledge about tumorigenesis might also contribute to development of new targeted therapies. The improvement of unique advanced analytical strategies in the field of mass spectrometry will also allow for investigation other diseases in the future.

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Conflict of interest statement
The authors declare no conflict of interest.

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