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Original papers

Reviews

An experimental study on the use of lycopene to prevent infertility due to acute oxidative ovarian damage caused by a single high dose of methotrexate

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Abstract

Background. Methotrexate (MTX) is an antineoplastic agent, which increases the level of reactive oxygen species (ROS) and decreases the level of antioxidants. Lycopene, is a potent antioxidant, which is used because of its protective effect against tissue damage.

Objectives. The aim of this study was to determine the effect of lycopene on ovarian MTX-induced injury in rats.

Material and methods. Rats (n = 36) were randomly divided into 3 equal groups: a group with MTX only (MG, n = 12), a group with lycopene and MTX (LMG, n = 12), and a healthy control group (HCG, n = 12). Then, malondialdehyde (MDA), myeloperoxidase (MPO) and total glutathione (tGSH) levels and histopathological findings were examined in the ovaries of rats. Apart from the histopathological and biochemical evaluation, the reproductive performance of the experimental groups was also examined.

Results. Our study demonstrated that, in ovarian tissues of rats administered MTX, there was a decrease in the levels of tGSH, while MDA and MPO were increased, but it is observed that these ratios are reversed in the LMG ($p < 0.05$). It also has been proven that a single, high-dose use of MTX causes infertility in female rats, prolongs the gestation period and reduces the number of offspring.

Conclusions. Lycopene pretreatment ameliorates the MTX induced ovarian injury and infertility in rats through its antioxidative activities.

Key words: methotrexate, infertility, oxidative stress, rat, lycopene

Introduction

Methotrexate (MTX) is a chemotherapeutic agent that is a folic acid antagonist. This agent, which acts by inhibiting the activity of dihydrofolate reductase, is frequently used in the treatment of autoimmune and malignant diseases.[1] It has been found that this agent, which is also frequently used in ectopic and molar pregnancy treatments, increases the level of free oxygen radicals and pro-inflammatory molecules.[2] It is also gonadotoxic and decreases the number of ovarian follicles. In this way, it is thought to cause infertility, especially in young patients.[3] When published literature is examined, some researchers have stated that there is no negative effect on the ovarian reserve at the doses used for the treatment of ectopic pregnancy.[4] However, there is a controversy about the effect of MTX therapies on the ovarian reserve and infertility. Various methods have been used to evaluate ovarian reserve. Methods used to determine the ovarian reserve include a primordial follicle count, the amount of gonadotropins and the level of anti-Müllerian hormone.[5] However, the occurrence of pregnancy is the best clinical parameter illustrating the ovarian reserve and the fertility of a female.

Lycopene is a kind of carotenoid, which is a potent antioxidant. It has lipophilic properties and is found in large amounts in red fruits, for example in tomatoes. Lycopene supplementation is very important to stop the harmful effects of free oxygen radicals. Lycopene is one of the most studied antioxidant agents because of its protective effect against tissue damage. In addition, it shows anticancer activity.[6] It also has the effect of regulating detoxification systems, clearing reactive oxygen species (ROS) and increasing the transmission between gap-junctions.[7,8] An examination of the published literature reveals that lycopene has hepatoprotective and neuroprotective features.[9,10] A further effect of lycopene is that it decreases the complications associated with diabetes mellitus (DM).[11,12] However, there is no study indicating its protective effect against acute ovarian damage induced by a single, high dose of MTX.

In light of this information, the aim of this study was to determine the effect of lycopene on ovarian MTX-induced injury in rats. Apart from the histopathological and biochemical evaluation, the reproductive performance of the experimental groups was also examined.

Material and methods

Animals

The recommendations of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for animal care were taken into consideration. A total of 36 Wistar albino female rats weighing 260–272 g were randomly chosen for use in the study. The animals were housed and fed at room temperature (22–24°C) prior to the experiment. This study was carried out in accordance with international guidelines on the ethical use of animals (Ethics Committee Date and No.: 22.11.2018-12/210).

Experimental groups

Rats were randomly divided into 3 groups before the following experimental conditions were applied: a group with 20 mg/kg of MTX only (MG; n = 12), a group with 5 mg/kg of lycopene and 20 mg/kg of MTX (LMG; n = 12), and a healthy control group (HCG; n = 12).

Chemical substances

The MTX used in the study was provided by Med-Ilac (Istanbul, Turkey), thiopental sodium was provided by Ibrahim Etem Ulagay (Istanbul, Turkey) and lycopene was provided by Solgar (Leonia, USA).

Experimental procedure

Lycopene was given orally via gavage at a dose of 5 mg/kg to the LMG (n = 12) of rats.[13] In the other 2 groups, the same volume of normal sunflower oil (0.5 mL) was used as the solvent. One hour after the administration of the lycopene and the solvent, the LMG and MG were injected intraperitoneally (i.p.) with a single dose of MTX (20 mg/kg).[2] The lycopene and the solvent were administered once a day for 5 days. At the end of this application, 6 rats from each group (MG, LMG and HCG) were euthanized with a high dose of anesthesia (50 mg/kg of thiopental sodium i.p.) and the ovaries were removed. Malondialdehyde (MDA), myeloperoxidase (MPO) and total glutathione (tGSH) levels were measured in the ovarian tissues. The ovarian tissues were also examined histopathologically. All test results were evaluated by comparing the groups together.

Three mature male rats were added to each group of 6 female rats for reproduction. The groups with female and male rats together were kept in appropriate laboratory settings for 2 months. During this period, the pregnant rats were kept in a suitable environment in separate cages. In 2 months, the rats that did not get pregnant and did not give birth were considered infertile.

Biochemical analysis of ovarian tissues

Measurement of MDA

The levels of tissue lipid peroxidation were determined by predicting MDA levels (Cayman Chemical; Cat. No: 10009055; Michigan, USA) using the thiobarbituric acid test involving spectrophotometrical measurements at a wavelength of 532 nm used by Ohkawa et al.[14] The results were expressed as μmol/g of protein.

Measurement of MPO

A modified method by Wei and Frenkel was adopted for the determination of MPO activity (Cayman Chemical; Item No. 600620) in the homogenate.[15] The increases in the absorbance values were measured at a wavelength of 510 nm. Absorbance was measured spectrophotometrically at a wavelength of 412 nm. The results were expressed as U/g of protein.

Measurement of tGSH

The level of tGSH (Cayman Chemical; Cat. No. 703002) in the tissues was measured according to the process described by Sedlak and Lindsay.[16] The absorbance value was determined at a wavelength of 412 nm spectrophotometrically. The results were expressed as nmol/g of protein.

Histopathological analysis of ovarian tissues

After routine tissue follow-up, 5-micron sections were obtained for histopathological evaluation. These sections were stained with hematoxylin and eosin (H&E). The ovarian tissues were evaluated under an optical microscope (Olympus BX 51; Olympus Corp., Shinjuku, Tokyo, Japan) and the images were captured using a digital camera (Olympus DP 71). Histopathological examination was carried out using a blind histological evaluation method.

Statistical analysis

A statistical evaluation of the results was carried out using one-way analysis of variance (ANOVA) and SPSS v. 22.0 software (IBM Corp., Armonk, USA). The Tukey multiple comparison test was used to determine the differences between groups. The level of significance was set at $p < 0.05$.

Results

The biochemical results of this study are summarized in Fig. 1. The MDA level in the ovarian tissue was measured to be 1.2 ±0.2 µmol/g of protein in the HCG and 4.3 ±0.4 µmol/g of protein in the MG. When compared to the HCG, the increase in MDA level in MG was significant ($p < 0.05$). A 5 mg/kg dose of lycopene reduced the level of MDA (1.7 ±0.2 µmol/g of protein) significantly compared to the MG ($p < 0.05$).

When the other 2 groups were compared with the MG, MPO was significantly increased in the MG (7.1 ±0.7 U/g of protein), but the value in the LMG (2.8 ±0.6 U/g of protein) was close to the HCG (1.9 ±0.7 U/g of protein) ($p < 0.05$). Methotrexate application significantly decreased the tGSH level in the ovarian tissue of rats compared to the HCG ($p < 0.05$). The tGSH levels were measured to be 6.8 ±0.2 nmol/g of protein in the HCG and 2.6 ±0.4 nmol/g of protein in the MG. It was found that 5 mg/kg of lycopene, by comparison with the MG, significantly improved the tGSH level (5.9 ±0.5 nmol/g of protein) in the LMG ($p < 0.05$).

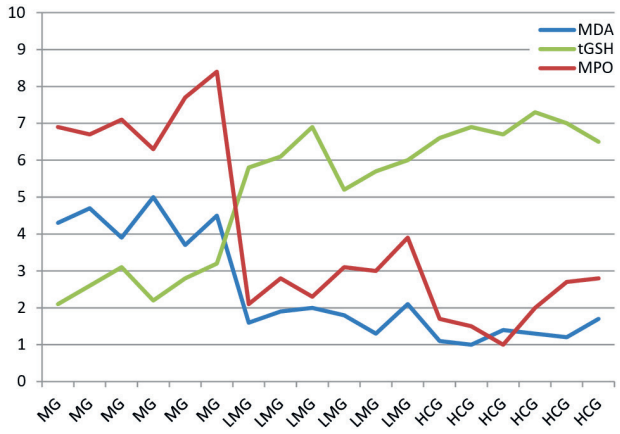

Fig. 1. Malondialdehyde (MDA), myeloperoxidase (MPO), and total glutathione (tGSH) levels of ovarian tissues for 3 groups.

HCG – healthy control group; MG – methotrexate group; LMG – lycopene + methotrexate group.

A histological examination of ovaries in the HCG revealed that the ovarian tissue structure had a normal cortex and medulla (Fig. 2). In the MG, a microscopic examination showed that there were apparent edema in the follicular cells and the interstitial area, vascular dilation and congestion, polymorphonuclear cell infiltration, and degeneration in developing follicles (Fig. 3,4). In the ovaries of rats treated with lycopene prior to MTX, we observed a marked decrease in edema in the follicular cells and interstitial area, mild

Table 1. The reproduction results of healthy control group

Rats	Gestation period [days]	Infertile rat	Number of offspring	Gender of offspring	
				male	female
1.	26	–	8	2	6
2.	29	–	6	3	3
3.	25	–	10	3	7
4.	31	–	8	4	4
5.	33	–	8	2	6
6.	30	–	9	3	6

Table 2. The reproduction results of Lycopene + MTX group

Rats	Gestation period [days]	Infertile rat	Number of offspring	Gender of offspring	
				male	female
1.	31	–	6	4	2
2.	28	–	8	5	3
3.	33	–	7	5	2
4.	–	+	–	–	–
5.	35	–	8	5	3
6.	30	–	6	3	3

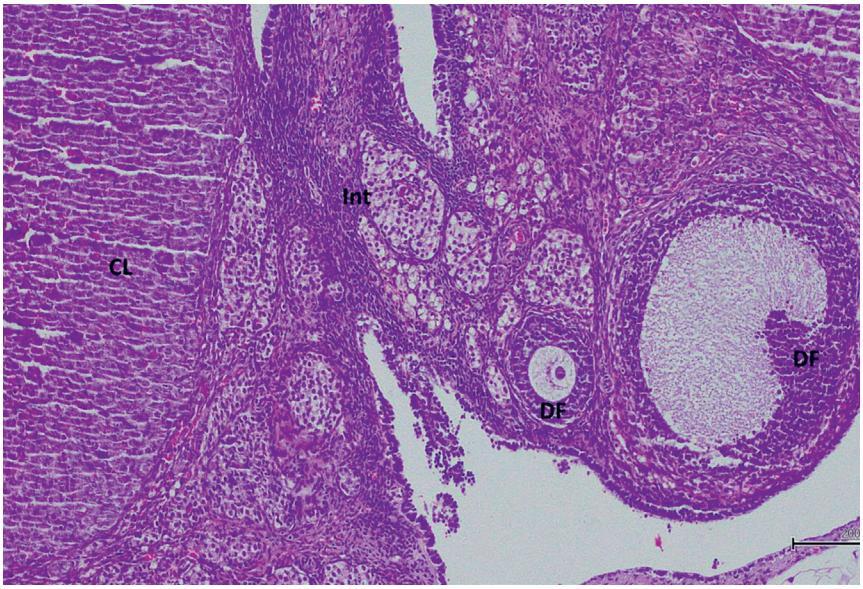

Fig. 2. Hematoxylin and eosin staining in ovarian tissue in the control group

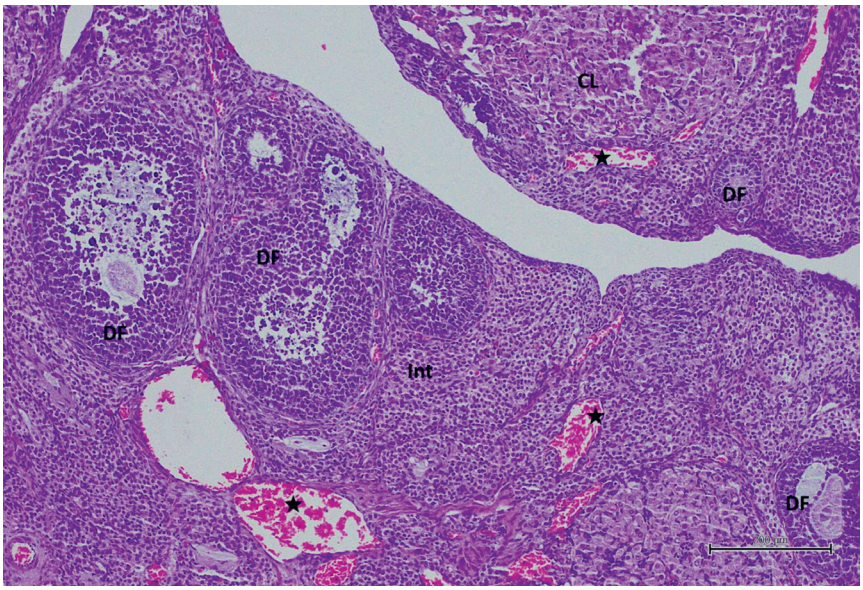

Fig. 3. Hematoxylin and eosin staining in ovarian tissue in the MTX group

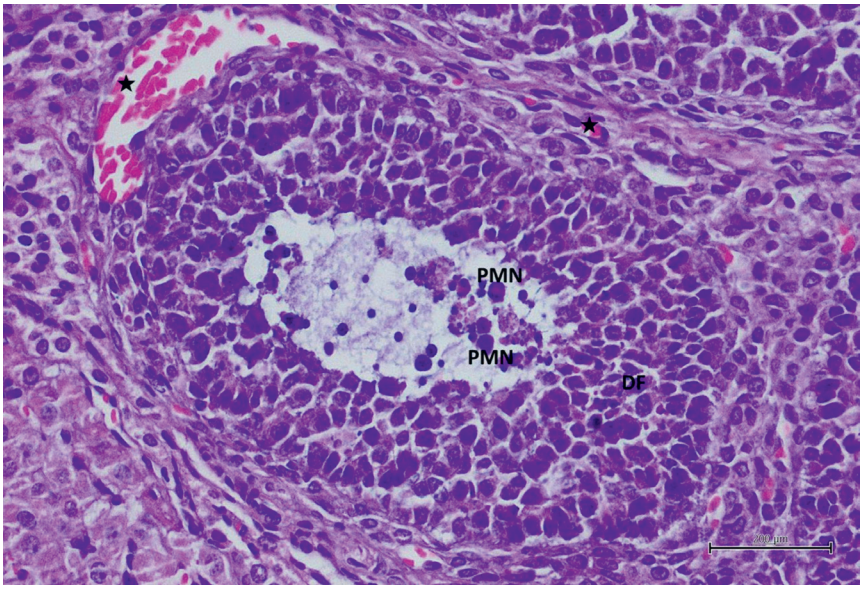

Fig. 4. Hematoxylin and eosin staining in ovarian tissue in the MTX group

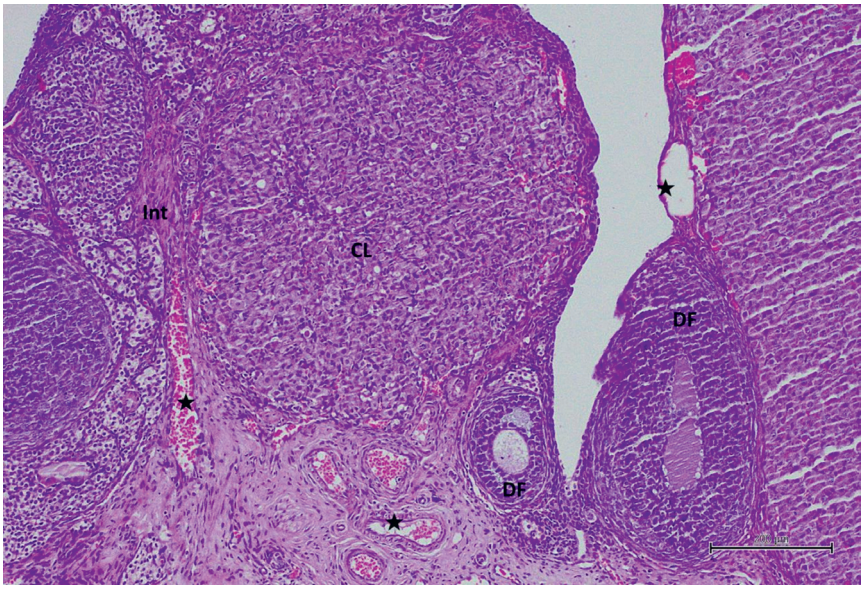

Fig. 5. Hematoxylin and eosin staining in ovarian tissue in the MTX + lycopene group

Table 3. The reproduction results of MTX group

Rats	Gestation period [days]	Infertile rat	Number of offspring	Gender of offspring	
				male	female
1.	–	+	–	–	–
2.	–	+	–	–	–
3.	47	–	4	3	1
4.	–	+	–	–	–
5.	–	+	–	–	–
6.	–	+	–	–	–

vascular dilatation and congestion, and a normal follicular structure (Fig. 5).

The reproduction results of the experimental groups are summarized in Table 1, 2 and 3. Table 1 shows that there were no infertile rats in the HCG, and the mean gestational period was 29 days. A total of 49 offspring, 17 male and 32 female, were produced by this group. Table 2 shows that there was 1 infertile rat in the LMG, and the mean gestational period was 31.4 days. A total of 35 offspring, 22 male and 13 female, were produced by this group. Table 3 shows that there were 5 infertile rats in the MG, and the mean gestational period was 47 days. A total of 4 offspring, 3 male and 1 female, were produced by this group. When the average number of offspring was examined, there were 8.1 in the HCG, 7 in the LMG and 4 in the MG. The fewness of the average number of offspring in the MG than the other 2 groups was statistically significant ($p < 0.05$).

Discussion

In recent years, the number of people diagnosed with cancer at an early age has increased due to the development of cancer awareness in the community, developments that allow early detection of cancer and perhaps environmental factors.[17] One of the most frequently asked questions among young patients diagnosed with cancer is whether they will have children after surgery, chemotherapy or radiotherapy. In particular, MTX is one of the most commonly used chemotherapeutics, both on its own and in combination therapy.

Published literature describes the use of MTX in various doses and at various times. Although the low-dose MTX used for ectopic pregnancy treatment does not affect the ovarian reserve, it is known that the use of high-dose MTX in chemotherapy does adversely affect it.[3,4,18] For this reason, ovarian tissue in this study was examined histopathologically in single and high-dose MTX-treated experimental groups, and the levels of oxidant/antioxidant molecules in the ovarian tissue were determined. It further investigated whether lycopene has a protective effect using this experimental model.

When the published literature is examined, there are many studies which prove that lycopene protects testicular tissue and improves the quality of sperm cells.[19,20] Therefore, lycopene and other antioxidant molecules have been used to treat male infertility.[21] However, there are no studies on the effects of lycopene on the reproductive capacity of women or female animals.

In this study, it was found that MDA and MPO levels increased in the ovarian tissues after MTX was applied and that the level of tGSH decreased. In the LMG, the antioxidant molecules (tGSH) increased and oxidant molecules (MDA and MPO) decreased. This investigation illustrated 2 results:

– using a single, high dose of MTX produces oxidative stress in ovarian tissues, and

– lycopene can combat this MTX-induced damage.

The histopathological examination of the ovarian tissues of the rats also supported these biochemical results.

Each group was subjected to reproductive testing on equal terms after biochemical and histopathological sampling. Reproduction occurred across the entire HCG over

the two-month reproductive period. However, in the MG, it was found that only 1 rat had given birth and that the duration of the pregnancy was quite long. In addition, only 1 rat was not pregnant in the LMG, and the duration of the pregnancy was similar to the HCG. The average number of offspring in the LMG and HCG was similar. Thus, it has been proven that a single, high dose use of MTX causes infertility in female rats, prolongs the gestation period and reduces the number of offspring. However, when we looked at the gender ratios of the offspring in the experimental groups, an unexpected result was seen. The female gender was dominant in the offspring obtained from the HCG, but this ratio was reversed in the LMG and MG. In a study by Lantinga et al., 124 female patients aged from 18 to 45 years who received chemotherapy and radiotherapy for childhood cancers were evaluated in terms of reproductive and menstrual cycles.[22] When they looked at the offspring of patients who had received chemotherapeutic treatment other than 6-mercaptopurine (6MP) and 6-thioguanine (6TG), the male gender was dominant, like in this study. This result is thought to be due to a defect of the X-chromosome caused by chemotherapeutic drugs. Testosterone, and its active form dihydrotestosterone, have a major role in the development of the male genital organs.[23] Also, we know that the production of adrenal gland hormones increase when the mother is stressed.[24] In our study, the second reason for the high number of male offspring in the experimental groups receiving MTX may be the oxidative stress caused by MTX in the mother and the resulting increase in the androgenic hormone level. Aksoy et al. found that the number of male offspring increased due to the oxidative stress on the remaining ovaries in unilateral ovariectomized rats.[25] This finding agrees with the published literature.

In a study on poultry, lycopene was found to inhibit both natural ovarian aging and d-galactose-induced ovarian aging.[26] In 1992, Stahl et al. reported that the measurements of beta-carotene and lycopene levels from the diet in human plasma and 7 different human tissues (liver, adrenal gland, testis, kidney, ovary, and fat and brain stem tissue).[27] In this study, it was reported that the lycopene level was found to be the lowest in the ovarian tissue. Considering the positive and curative effects of lycopene on ovarian tissue, lycopene supplementation is beneficial for clinical conditions caused by oxidative stress.

The experiment features some limitations. Firstly, there is no data about the ameliorative effect of lycopene on single, high-dose MTX-induced ovarian injuries in published literature. Secondly, the experiment used a single dose of lycopene (5 mg/kg). Future research should use different doses of lycopene to determine the mean effective dose for antioxidant activity. Thirdly, MTX-induced ovarian injury was demonstrated by the morphological modifications in the ovarian tissue. Any damage in the other organs and systems should be evaluated in future studies. Fourthly, in order to fully understand the reason for the high number of male offspring in the experimental groups receiving the MTX treatment, the offspring resulting from abortion should be examined.

Conclusions

When chemotherapeutic drugs such as MTX are used in high doses, they cause tissue damage through oxidative stress. If a woman undergoes this treatment at a young age, it is apparent that she has an infertility problem. This study showed that with lycopene, MTX-induced ovarian damage and infertility could be prevented. As a result, lycopene-like molecules with antioxidant properties may prevent infertility as an additional effect to chemotherapy.

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Roles of the mammalian target of rapamycin (mTOR) signaling pathway in the repair of hyperoxia-induced acute lung injury

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Abstract

Background. Rapamycin inhibits the mammalian target of rapamycin (mTOR) activity and has been proven effective for the treatment of lung injury.

Objectives. The objective of this study was to investigate the roles of the mTOR pathway and its inhibitor rapamycin in the repair of hyperoxia-induced acute lung injury (ALI).

Material and methods. Firstly, premature rat lung fibroblast L929 cells were cultured under different oxygen concentrations (40%, 60%, and 90%). At day 3, 7 and 14 after exposure, MTT assay and flow cytometry were used to evaluate the effect of oxygen stress on cell viability and apoptosis of L929 cells, respectively. Secondly, microscopy, MTT assay and flow cytometry was used to investigate the effect of 10 nM rapamycin on 90% O_2 exposed L929 cells. We also used small interfering RNAs (siRNAs) to abrogate the expression of mTOR in 90% O_2 exposed L929 cells, and then evaluated the apoptosis and cell viability using flow cytometry and the MTT assay, respectively. In addition, western blot was used to detect the protein expression of Bcl-2, p53, TGF-β and connective tissue growth factor (CTGF). A hyperoxia-induced lung injury model was established in Sprague Dawley (SD) rats in order to evaluate the histopathological changes in lung tissues and expression of the mTOR pathway and fibrosis related factors.

Results. Exposure to 40%, 60% or 90% oxygen all significantly inhibited the growth of L929 cells. Application of 10 nM rapamycin was found to effectively promote apoptosis of 90% O_2 exposed L929 cells. In addition, mTOR siRNA promoted the apoptosis and inhibited the growth of L929 cells. Rapamycin inhibited the activation of the mTOR signaling pathway, down-regulated the expression of downstream proteins p70S6K and 4EBP1, reduced the collagen deposition and the production of fibrosis-inducing factors, including TGF-β and CTGF in hyperoxia-induced lung injury rats.

Conclusions. Rapamycin may be useful for the treatment of hyperoxia-induced acute lung injury (ALI) by inhibiting the activation of mTOR signaling pathway.

Key words: mTOR, rapamycin, siRNA interference, hyperoxia-induced lung injury

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Introduction

Bronchopulmonary dysplasia (BPD), a form of chronic lung disease (CLD), is characterized by the abnormal formation of alveoli and chronic pulmonary vascular changes, especially in preterm infants.[1] BPD has a reported incidence of >60% in preterm infants, and is one of the leading causes of death and disability in preterm infants in China.[2] Oxygen therapy is a common method for the treatment of preterm infants, and appropriate oxygen therapy can effectively save the lives of preterm infants suffering from hypoxia.[3] However, sustained oxygen inhalation at high concentrations can lead to extensive and non-specific inflammatory responses in lung tissue, followed by pulmonary stromal hyperplasia, and pulmonary fibrosis, or even acute lung injury (ALI).[4] Lung fibroblasts (LFs) are the main cells involved in the repair of lung injury.[5] The accumulation of the abnormal extracellular matrix (ECM) that is produced by LFs plays an important role in the pathogenesis of BPD fibrosis.[6] Currently, one of the major problems in the prevention and early treatment of lung injury is the excessive proliferation of LFs in lung repair and reconstruction, which may end up replacing the normal terminal bronchioles and alveoli, resulting in irreversible damage of both the structure and function of the lung.[7,8]

As a serine/threonine protein kinase, mammalian target of rapamycin (mTOR) is a member of the phosphoinositide-3-kinase-related kinase family (PI3K). The mTOR signaling pathway can phosphorylate multiple target proteins for the regulation of transcription, translation initiation, protein synthesis, and degradation, mainly through the activation of p70S6K/S6 and the inhibition of 4EBPI/eIF4E.[9–11] Mammalian target of rapamycin signaling pathway has been shown to be able to regulate embryonic development,[12,13] and lung development[14], and is involved in a variety of lung diseases, such as chronic obstructive pulmonary disease (COPD),[15] cystic fibrosis,[16] and pulmonary fibrosis.[17]

Pulmonary fibrosis is characterized by the activation of the mTOR and its down-stream target, the ribosomal S6 kinase (p70S6K), pulmonary fibrosis generated fibrotic foci with abundant activated hepatic stellate cells and excessive collagen deposition, it has been reported that rapamycin, an inhibitor of p70S6K phosphorylation, could inhibit hepatic stellate cell proliferation and limits fibrogenesis.[18–20] Blocking the mTOR signaling pathway has been shown to suppress the proliferation of fibroblasts and the over-production of extracellular matrix (ECM) in liver tissue, which may reverse pulmonary fibrosis.[20] Rapamycin is a macrolide immunosuppressive agent, an inhibitor of mTOR, and it has been proven effective for the treatment of pulmonary fibrosis.[21] Nevertheless, the detailed molecular mechanisms underlying the role of the mTOR signaling pathway in the repair of hyperoxia-induced lung injury have not yet been fully elucidated and require further investigation.

In this study, we aim to investigate the roles of mTOR signaling pathway in the repair of hyperoxia-induced acute lung injury in vivo and in vitro.

Material and methods

Ethics statement

All animal handling procedures were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of University of South China (No. 2014).[11]

Animals and cell lines

The mouse lung fibroblast cell line L929 was purchased from the American Type Culture Collection (ATCC) (Manassas, USA). The L929 cells were thawed at 37°C, centrifuged at 1000 g for 5 min, and rinsed twice with RPMI-1640 medium containing 10% fetal bovine serum (Gibco, USA). Cells were harvested by centrifugation at 1000 g for 5 min, and cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, USA), which was replaced every day. Fifty-four specific-pathogen-free (SPF) SD rats (weighing 225–240 g and aged 8 weeks), including 36 females and 18 males, were obtained from the Experimental Animal Center of Nanhua University (Hunan, China). Female and male rats were co-housed at a ratio of 2:1 for mating. Females were checked for the identification of vaginal plugs each morning. The day a vaginal plug was identified was the 1st day of pregnancy. On the 21st day of pregnancy, 66 neonatal rats were obtained as the premature rats in this study. These 66 premature rats were used for 2 parts of the in vivo studies. In the first part of animal study, 42 premature rats were included and divided into 7 groups (n = 6 per group): 1) control, 2) 90% O_2 3d, 3) 90% O_2 7d, 4) 90% O_2 14d, 5) 90% O_2 + rapamycin 3d, 6) 90% O_2 + rapamycin 7d, 7) 90% O_2 + rapamycin 14d. In the second part of animal study, 24 premature rats were included and divided into 4 groups (n = 6 per group): 1) 90% O_2, 2) 90% O_2 + rapamycin 3d, 3) 90% O_2 + rapamycin 7d, 4) 90% O_2 + rapamycin 14d.

Oxygen exposure on L929 cells

L929 cells were cultured in 37°C with 5% of carbon dioxide and in saturated humidity until moisture began adhering to the wall. After aspiration of the culture medium, the cells were rinsed twice with 37°C pre-warmed PBS, and cultured in fresh complete medium. The L929 cells were cultured in distinct media and were randomly divided into 4 groups (control, 40% O_2, 60% O_2, and 90% O_2). The control group cells were cultured in fresh complete medium with regular air at 37°C. The other 3 groups were cultured in fresh complete medium under different oxygen concentrations (40%, 60%, and 90%) in independent

culture chambers. Air, including the specific concentrations of oxygen and nitrogen, as well as 5% CO_2, was injected into the culture chamber at a speed of 1 L/min for 30 min. The culture chamber was sealed once the oxygen monitor showed expected oxygen concentrations, and placed in an incubator for culture. The 40% oxygen group was cultured in 40% O_2, 55% N_2, and 5% CO_2. The 60% oxygen group was cultured in 60% O_2, 35% N_2, and 5% CO_2. The 90% oxygen group was cultured in 90% O_2, 5% N_2, and 5% CO_2. The corresponding concentration air was injected to the culture chambers every 12 h to confirm that the oxygen concentration was as expected. After being cultured for 3, 7, or 14 days, the cells of each group were harvested for follow-up experiments.

Rapamycin intervention

The 90% oxygen group of L929 cells was cultured in 37°C with 5% carbon dioxide and saturated humidity (until moisture adhered to the wall). After aspiration of the culture medium, the cells were rinsed twice with 37°C pre-warmed PBS, and were then cultured in fresh complete medium with 10 nM rapamycin in independent culture chambers. In order to keep the oxygen concentration in the culture consistent, mixed air of the specific oxygen and nitrogen concentrations, and 5% CO_2, was injected into the culture chambers every 12 h. After being cultured for 3 days under 90% concentration of oxygen concentration, the cells were harvested for follow-up experiments.

Flow cytometry for apoptosis

To evaluate the apoptosis of L929 cells, cells were double stained with annexin V-FITC and PI, and analyzed by flow cytometry (BD Biosciences, New York, USA). The L929 cells (10^5/mL) cultured under different conditions were incubated at 37°C and 5% CO_2 for 24 h before being digested by trypsin without EDTA. The cells were then rinsed with pre-cooled PBS, centrifuged at 1500 g for 5 min, and resuspended in 300 µL of ×1 binding buffer. After incubation with Annexin V-FITC (5 µL) for 15 min and PI (10 µL) for 5 min, the L929 cells were analyzed by FACSCalibur flow cytometry (BD Biosciences, USA).

MTT assay for cell viability

To evaluate the cell viability of L929 cells, the cell concentration of each group was adjusted to 3×10^4 cells/mL. The L929 cells were inoculated into a 96-well culture plate and 15 µL MTT was added to each well. Cells were incubated at 37°C in a humidified chamber and 5% CO_2 for 3–4 h. After aspiration, 200 µL DMSO was added to each well and incubated at room temperature in a shaker for 10 min; the absorbance of each well was measured at 492 nm. All samples were assayed in duplicate.

Inverted phase contrast microscopy

L929 cells exposed to 90% O_2 concentrations of oxygen, with rapamycin intervention, were harvested after 3 d. The morphology and number of L929 cells were evaluated under an inverted phase contrast microscope to investigate the effects of rapamycin on the morphology of oxygen exposed L929 cells.

Inhibition of mTOR expression using siRNA

The cells were diluted to a concentration of 1×10^5cells/mL and inoculated in a 6-well plate (3 mL/well). They were then cultured at 37°C and 5% CO_2 for 24 h. To prepare siRNA-Lipofectamine for (Invitrogen, Carlsbad, USA) L929 cell transfection, 75 pmol synthesized mTOR siRNA diluted in 100 µL serum-free RPMI1640 medium (Invitrogen Life Technologies, New York, USA), and 5 µL lipofectamine 2000 diluted in 100 µL serum-free RPMI1640 medium were mixed and incubated at room temperature for 20 min. After cells were washed with PBS, 200 µL siRNA-Lipofectamine 2000 was added to each well and cells were incubated at 37°C and 5% CO_2 for 4–6 h. The supernatant was removed and 3 mL RPMI1640 was added to each well. The siRNA-transfected L929 cells were divided into 4 groups: control, 90% O_2, 90% O_2 + rapamycin (10 nM), and the mTOR siRNA groups.

Quantitative real-time PCR

The mTOR siRNA transfected cells were harvested for RNA isolation using the Trizol reagent (Takara, Japan). The Takara kits were used for cDNA synthesis and qPCR, which was conducted in a 20 µL reaction system with 10 µL 2 × Mix buffer (Aidlab Biotechnologies, Beijing, China), forward and reverse primers (Table 1) (0.4 µL each), 1 µL DNA, and 15.4 µL double distilled water. The primers used in this study were shown in Table 1. The qPCR was conducted according to the following program: 94°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 40 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The relative mRNA level was

Table 1. The primers for real-time quantitative PCR

Gene names	Primers sequences (5′-3′)
GAPDH	F: CCGAGAATGGGAAGCTTGTC
	R: AAGCACCAACAGAGGAGAA
mTORC1	F: TCAACTGGGGAAGAAGTACC
	R:TCATGGGTCCTGTCTCAACT
p70S6K	F: AAATCTCCATGGCTTTGGGG
	R: AGGGGCCATGTATTCTATTG
4EBP1	F: CCAGGATTATCTATGACCGG
	R: AATTGTGACTCTTCACCGCC

calculated according to the $2^{-\Delta\Delta Ct}$ method with ABI Prism 7500 SDS software (Applied Biosystems, Foster City, USA).

Western blot

Harvested cells were mixed with 500 µL RIPA lysate with PMSF (Sigma-Aldrich, St. Louis, USA) and incubated on ice for 2 h, before being centrifuged at 12,000 g at 4°C for 10 min to obtain the supernatant. Protein quantification was performed using the BCA method. Western blotting was used to evaluate the protein expressions of p70S6K and 4EBP1 proteins in the control and mTOR siRNA groups. The expressions of Bcl-2, p53, TGF-β, and connective tissue growth factor (CTGF) in the control, 90% O_2, 90% O_2 + rapamycin, and mTOR siRNA groups were also evaluated using western blot. Relative density of western blot band was analyzed by using Image J software (National Institutes of Health, Bethesda, USA; http://imagej.nih.gov/ij/) and protein expression level of each group was normalized with β-actin.

Establishment of a model of hyperoxia-induced lung injury in SD rats and rapamycin intervention

On the 21st day of pregnancy, the rats were sacrificed in order to obtain premature neonatal rats. After 3, 7, and 14 days, the premature rats were sacrificed for follow-up experiments (n = 6 rats per group). The hyperoxia-induced lung injury rat model was established according to the study conducted by Zhang, et al.[22] Briefly, the premature rats were raised in 90% oxygen (5–6 L/min), at 22–25°C, and 65–75% humidity with unlimited food and water. The CO_2 level in the rat house was set to 5%, using the appropriate amount of barium hydroxide. Sun et al.[23] reported that mTOR signaling was blocked by intraperitoneal injection of rapamycin (1.5 mg/kg) in mice, thus 1.5 mg/kg dose of rapamycin was chosen in the animal study. Next, the 90% O_2 + rapamycin group received intraperitoneal injection of rapamycin (1.5 mg/kg) once a day. To avoid hyperoxicosis, the maternal rats of the control and hyperoxia groups were interchanged every 24 h.

Histopathological examination

Six rats were randomly selected from each group and sacrificed for lung dissection. The left lung was fixed in 4% paraformaldehyde to prepare paraffin sections for histopathological examination. The paraffin sections were stained with hematoxylin and eosin and examined under light microscopy (XDS-1A; Supore, Shanghai, China). Other lung tissue was washed with PBS at 4°C for 3 min and frozen in liquid nitrogen for follow-up experiments. In order to evaluate the effects of 90% oxygen and intraperitoneal injection of 1.5 mg/kg rapamycin on lung tissues of rats, histopathological scores were evaluated according to the protocol developed by Murakami et al.[24]

Enzyme-linked immunosorbent assay

Five lung tissue samples (10 mg per sample) randomly selected from each group were homogenized in cold PBS (pH = 7.4). The homogenate was centrifuged at 10,000 g for 20 min at 4°C. Total protein concentration of each sample was assayed using a BCA protein quantification kit (Sangon, Shanghai, China). The contents of collagen I, collagen III, and FN in ECM were measured using an ELISA kit (Cosmo Bio, Tokyo, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm on a microtiter reader (Thermo Scientific, USA). In addition, the levels (ng/mg) of TGF-β, CTGF, and collagen I in the lung tissue of premature rats were also determined with an ELISA kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Absorbance values were normalized to the standard curve.

Western blot analysis for the expression of mTORC1, p70S6K, and 4EBP1 in lung tissues

The lung tissue was washed with pre-cooled saline to remove residual blood and was then dried. One gram of lung tissue was used to prepare a 10% lung homogenate. After centrifugation at a low temperature, the supernatant from the lung homogenate was used for western blot to quantity the protein levels of mTORC1, p70S6K, and 4EBP1.

Statistical analyses

The data was expressed as mean ± standard deviation (x ±SD) and analyzed using SPSS V. 19.0 software (IMB Corp., Armonk, USA). One way analysis of variance (ANOVA) for repeated measures and post-hoc Tukey's test for pairwise comparison were performed. A p-value less than 0.05 was considered to be statistically significant.

Results

Oxygen exposure induced apoptosis and inhibited proliferation in L929 cells in a time and concentration-dependent manner.

The L929 cells exposed to 40%, 60%, and 90% oxygen exhibited significantly higher apoptosis rates than those cultured in regular air for the same time period (3, 7, or 14 days) in a time and concentration-dependent manner ($p < 0.05$) (Fig. 1A–B). The L929 cells exposed to 40%, 60%, and 90% oxygen also exhibited significantly lower cell viability than those cultured in regular air for the same time period (3, 7, or 14 days) in a time and concentration-dependent manner ($p < 0.05$) (Fig. 1C). These results suggest that exposure to 40%, 60% and 90% oxygen all could induced apoptosis and inhibited cell viability of L929 cells in a time and concentration-dependent manner.

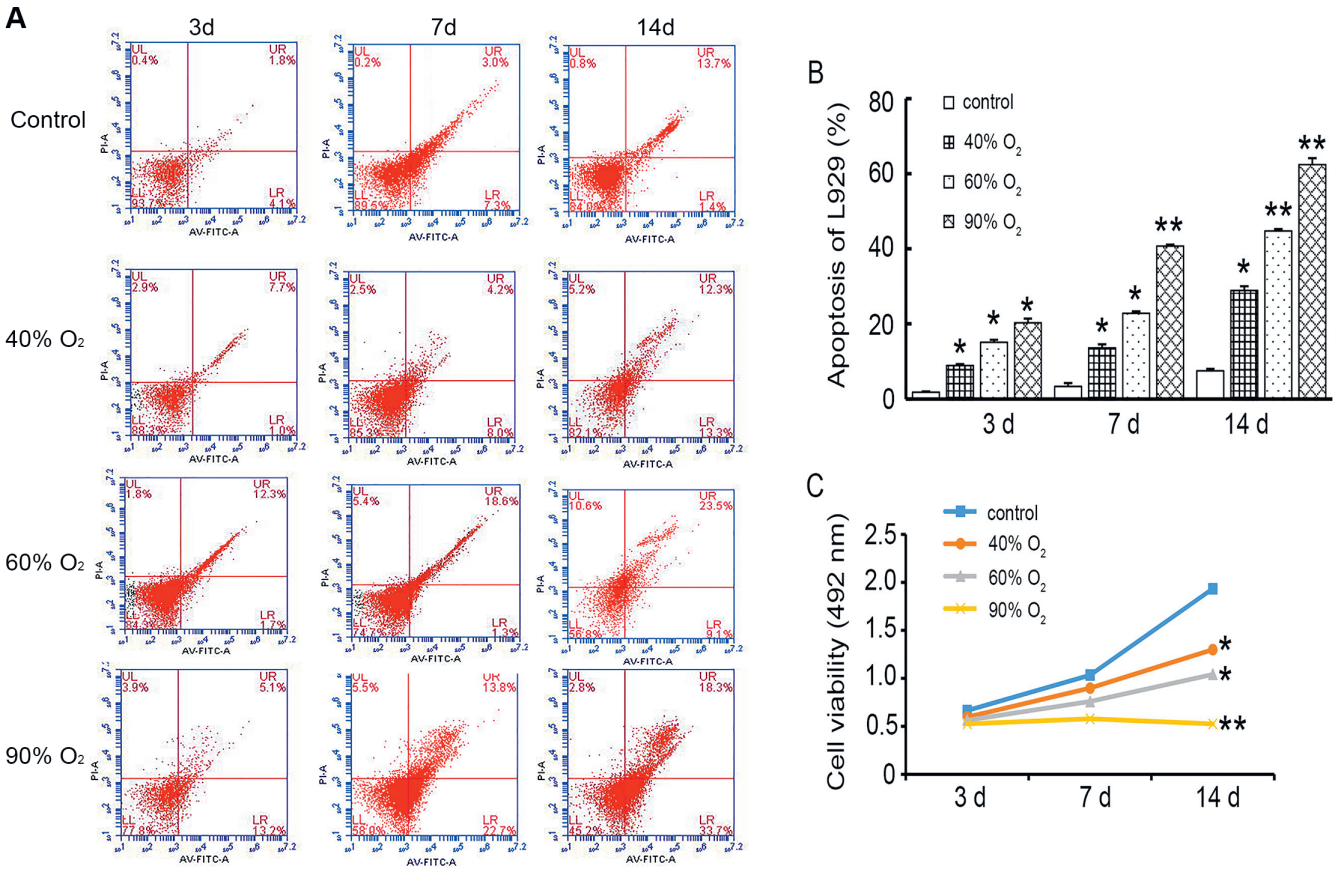


Fig. 1. A,B – flow cytometry analysis for apoptosis of L929 cells exposed to 40%, 60%, and 90% of oxygen; C – MTT assay for cell viability of L929 cells exposed to 40%, 60%, and 90% of oxygen

* $p < 0.05$; ** $p < 0.01$ vs control group.

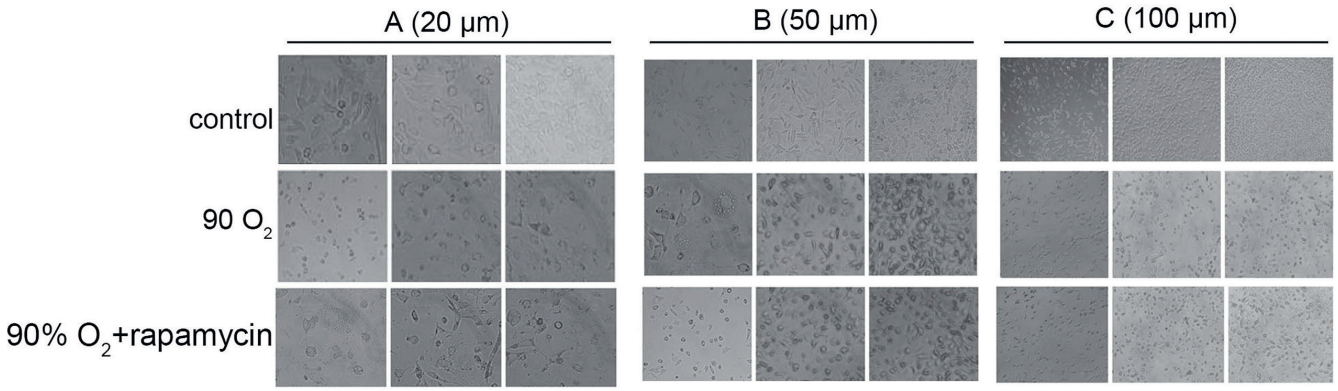


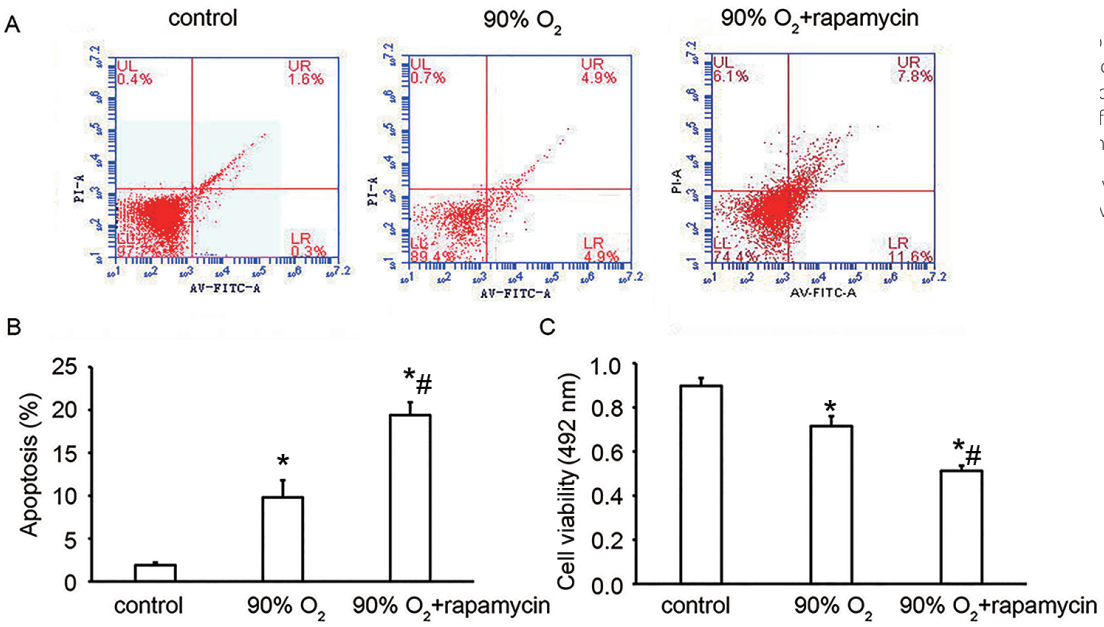
Fig. 2. Inverted phase contrast microscopy. L929 cells exposed to 90% O_2 with 10 nM rapamycin intervention were harvested after 3 days. The morphology and number of cells were evaluated under an inverted phase contrast microscope

Effects of 10 nM rapamycin on morphology of L929 cells exposed to 90% O_2

According to the results of above, 90% oxygen caused more severe damage to the L929 cells than 40% and 60% oxygen. Therefore, 90% oxygen was used in the follow-up experiments. We added 10 nM rapamycin to L929 cells exposed to 90% O_2 and cultured for 3 days, to investigate the effects of rapamycin on the morphology of L929 cells. As shown in Fig. 2, the majority of L929 cells cultured with regular air grew well and had bright, translucent, and compact cell bodies. With exposed to 90% O_2, cells cultured with rapamycin exhibited a reduced refractive index and had increased numbers of granules in the cytoplasm, while most cells were still viable and they exhibited a slightly different morphology (Fig. 2).

Rapamycin inhibited the growth and promoted apoptosis of L929 cells exposed to 90% O_2.

The L929 cells cultured with 10 nM rapamycin and exposed to 90% O_2 exhibited significantly higher apoptosis



– Flow cytometry
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vs control group;
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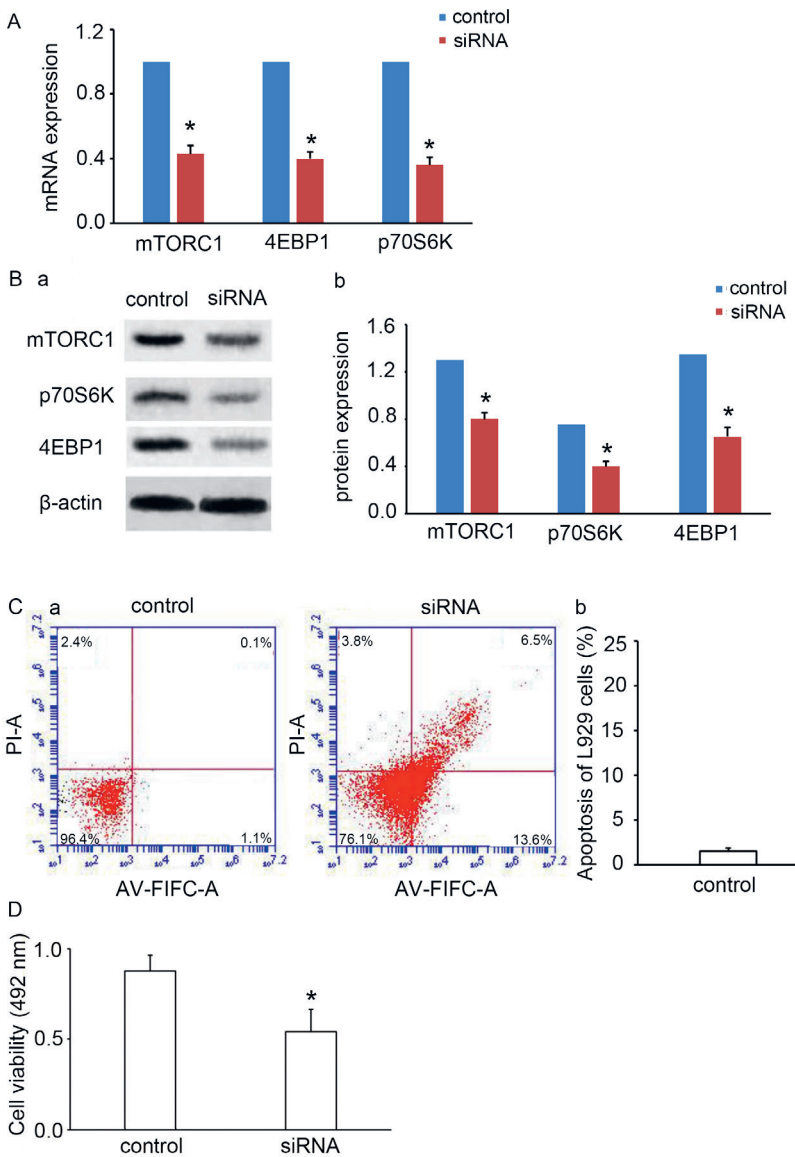

Fig. 4. A – qPCR analysis for the mRNA expression of mTORC1, 4EBP1, and p70S6K in L929 cells transfected with mTOR siRNA; B – western blot analysis for the protein expressions of mTORC1, 4EBP1, and p70S6K in L929 cells transfected with mTOR siRNA; C – flow cytometry analysis for the apoptosis of L929 cells transfected with mTOR siRNA; D – MTT assay for cell viability of L929 cells transfected with mTOR siRNA

* $p < 0.05$, ** $p < 0.01$ vs control group.

rates than the control group (*p < 0.05, Fig. 3A,B), and 90% O_2 exposure significantly inhibited the cell viability of L929 cells (*p < 0.05, Fig. 3C). In addition, the apoptosis ratio was increased and the cell viability was inhibited in the 90% O_2 + rapamycin group compared with 90% O_2 group (#p < 0.05) (Fig. 3A–C).

Validation of mTOR siRNA

After transfected with mTOR siRNA in L929 cells, we found that the mRNA and protein expression levels of mTORC1, 4EBP1 and p70S6K in L929 cells transfected with mTOR siRNA were significantly lower than in control cells (*p < 0.05, Fig. 4A,B). In addition, the rates of apoptotic cells transfected with mTOR siRNA (1.2%) were significantly increased than in the control (20.1%) (**p < 0.01, Fig. 4C), and the cell viability of cells transfected with mTOR siRNA was significantly decreased compared with control group (*p < 0.05, Fig. 4D). These results suggest that mTOR siRNA could down-regulate the expression of mTORC1, 4EBP1 and p70S6K, inhibit the cell viability and promote apoptosis of L929 cells.

Effects of rapamycin and mTOR siRNA on the protein expressions of Bcl-2, p53, TGF-β, and CTGF in L929 cells

We evaluated the protein expressions of apoptosis-related genes, including p53 and Bcl-2, as well as fibrosis-related genes, including TGF-β and CTGF, in L929 cells which were transfected with mTOR siRNA, treated with 10 nM rapamycin, and exposed to 90% O_2. As shown in Fig. 5, the expression of Bcl-2 in cells exposed to 90% oxygen and cultured with 10 nM rapamycin or transfected with mTOR siRNA was significantly lower than in cells exposed to regular air (*p < 0.05), and mTOR siRNA significantly increased the expression of Bcl-2 compared with 90% O_2 group (#p < 0.05). The expression level of p53 in L929 cells exposed to 90% oxygen and cultured with rapamycin or

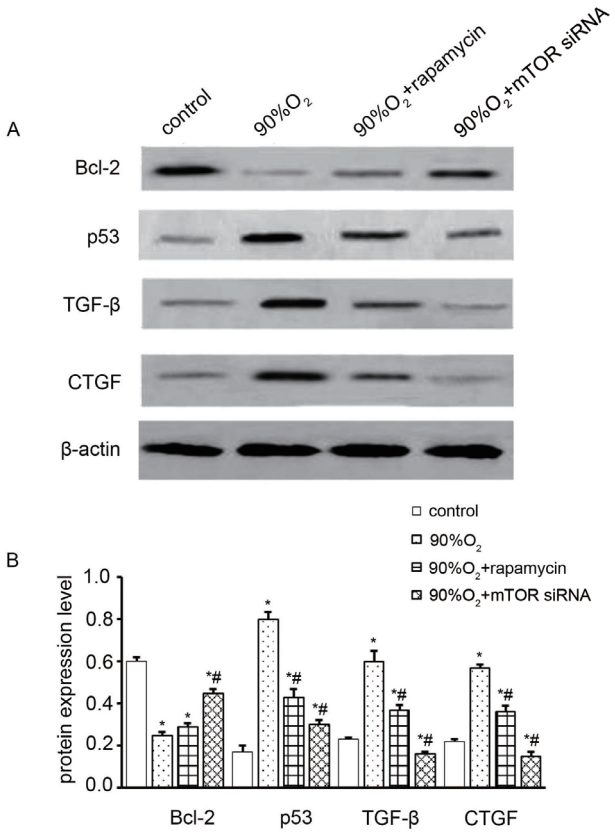

Fig. 5. A – western blot analysis of the protein expressions of Bcl-2, p53, TGF-β, and CTGF in the control, 90% O_2, 90% O_2 + rapamycin, and 90% O_2 + mTOR-siRNA L929 cell groups; B – relative density of western blot bands was analyzed using Image J software

* p < 0.05 vs control group; #p < 0.05 vs 90% O_2 group.

those transfected with mTOR siRNA was significantly higher than in cells exposed to regular air (*p < 0.05); however, the application of rapamycin or mTOR siRNA groups all exhibited lower expression level of p53 compared with 90% O_2 group (#p < 0.05). The expression levels of TGF-β and CTGF in L929 cells exposed to 90% oxygen was significantly higher than in the control group (*p < 0.05); however, the application of 10 nM rapamycin or mTOR siRNA groups

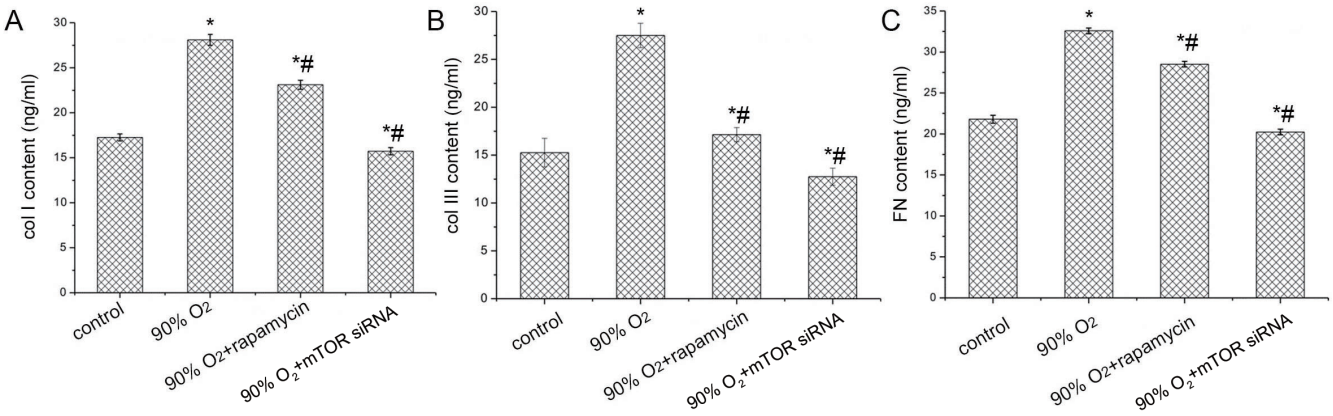

Fig. 6. The contents of col I (A), col III (B), and FN (C) in ECM of L929 cells from each group were determined by ELISA

col I – collagen I; col III – collagen III; FN – fibronectin; ECM – extracellular matrix; * p < 0.05 vs control group; #p < 0.05 vs 90% O_2 group.

all exhibited lower expression levels of TGF-β and CTGF compared with 90% O_2 groups. These results suggest that 90% O_2 exposure could promote L929 cell apoptosis and successfully inhibit the expression of CTGF and TGF-β.

The contents of collagen I, III and fibronectin in extracellular matrix

The contents of collagen I, collagen III (col I, col III), and fibronectin in extracellular matrix (ECM) of L929 cells exposed to 90% O_2 was significantly higher than those in cells exposed to regular air (*$p < 0.05$), suggesting that 90% O_2 could induce the production of FN and collagen. However, the contents of col I, col III, and FN in L929 cells treated with rapamycin or transfected with mTOR siRNA were significantly lower than that in 90% O_2 (#$p < 0.05$), suggesting that blocking the mTOR signaling pathway suppressed collagen deposition and decreased the production of FN (Fig. 6).

Histopathological changes in the lung tissues

The lung tissues of control group rats exhibited normal histopathology, without exudation of inflammatory cells (Fig. 7). Exudation of a few inflammatory cells and red cells was observed in the lungs exposed to 90% O_2 for 3, 7 and 14 days, and in a time-dependent manner. After 7 days of exposure to 90% oxygen, we observed alveolar rupture and fusion, and disordered distribution of cells along the tracheal arteries. After 14 days of exposure to 90% oxygen, extensive exudation of inflammatory cells and thickening blood vessels and airway walls were observed. The lungs of rats that had received intraperitoneal rapamycin injections and were exposed to 90% oxygen for 3 days exhibited normal histopathology. Alveolar rupture and fusion, as well as thickening blood vessels and airway walls, were observed in the lungs of rats that had received intraperitoneal injections of rapamycin and were exposed to 90% oxygen for 7 days. After 14 days of exposure to 90% oxygen, we observed more severe alveolar rupture and fusion in the lungs of rats that had received intraperitoneal injection of rapamycin.

The pathological scores of lung injury are shown in Table 2. The pathological scores of lung injury in the rats exposed to 90% oxygen for 3, 7, and 14 days were significantly higher than those of the rats from the control ($^a p < 0.05$). After 3 and 7 days, the pathological lung injury scores of the 90% O_2 + rapamycin group were (3.50 ±0.84) and (9.67 ±1.97), respectively, which were significantly lower than those of the 90% O_2 group for 3 (6.33 ±2.34) and 7 days (14.0 ±2.45), respectively ($^b p < 0.05$).

Table 2. The pathological scores of lung injury in rat model (n = 6)

Group	Pathological scores
Control	1.95 ±0.34
90% O_2 3d	6.33 ±2.34[a]
90% O_2 + rapamycin 3d	3.50 ±0.84[a,b]
90% O_2 7d	14.0 ±2.45[a]
90% O_2 + rapamycin 7d	9.67 ±1.97[a,b]
90% O_2 14d	23.83 ±3.71[a]
90% O_2 + rapamycin 14d	24.0 ±3.74[a]

[a] compared with control group, $p < 0.05$; [b] compared with 90% O_2 groups at the same time, $p < 0.05$.

Dynamic changes of col I, TGF-β, and CTGF in lung tissues

The col I concentration in the lung tissues of rats exposed to 90% oxygen for 3, 7, and 14 days were (471.87 ±5.72 ng/mg), (529.72 ±6.97 ng/mg), and (556.44 ±8.52 ng/mg), which were significantly higher than those of the air control group (414.43 ±8.97 ng/mg) ($^a p < 0.05$). The TGF-β concentration in the lung tissues of rats exposed to 90% oxygen for 3, 7, and 14 days were (33.74 ±2.84 ng/mg), (58.65 ±3.10

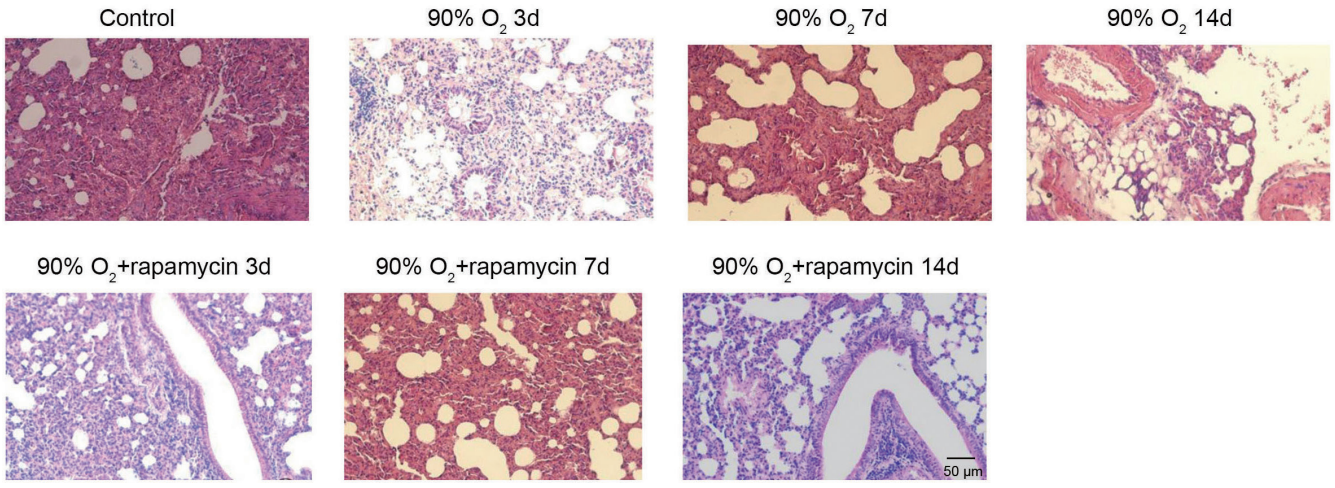

Fig. 7. Histopathological changes of the lung tissues from hyperoxia-induced lung injury rats

Hematoxylin and eosin stain; bar 50 μm; magnification ×200.

Table 3. The concentrations of TGF-β, CTGF and col I in the lung tissue determined by ELISA (n = 6)

Group	TGF-β [ng/mg]	CTGF [ng/mg]	Col I [ng/mg]
Control	25.50 ±1.86	41.23 ±1.08	414.43 ±8.97
90% O_2 3d	33.74 ±2.84[a]	50.72 ±1.80[a]	471.87 ±5.72[a]
90% O_2 + rapamycin 3d	25.72 ±1.44[b]	43.45 ±1.71[b]	425.11 ±3.50[b]
90% O_2 7d	58.65 ±3.10[a]	68.65 ±2.24[a]	529.72 ±6.97[a]
90% O_2 + rapamycin 7d	34.39 ±3.32[b]	50.92 ±2.42[b]	466.17 ±10.60[b]
90% O_2 14d	98.81 ±1.55[a]	94.39 ±2.48[a]	556.44 ±8.52[a]
90% O_2 + apamycin 14d	54.31 ±4.25[b]	63.64 ±2.78[b]	511.72 ±13.72[b]

[a] compared with control group, $p < 0.05$; [b] compared with 90% O_2 groups at the same time, $p < 0.05$; col I – collagen I.

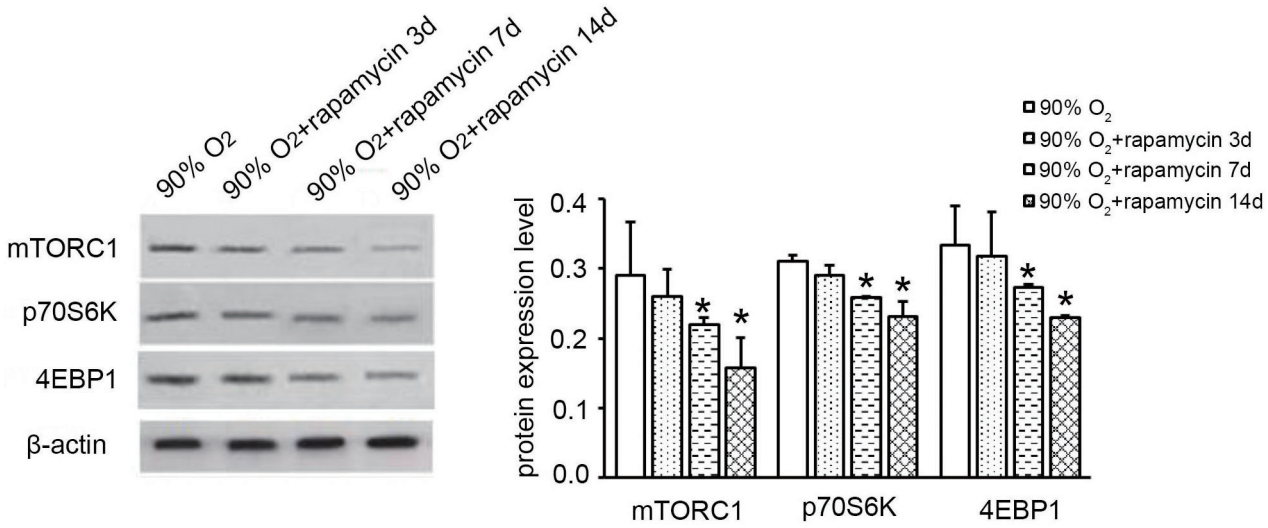

Fig. 8. The protein expression of mTORC1, p70S6K, and 4EBP1 in the lung tissues of rats of all experimental groups were detected by western blot

* $p < 0.05$ vs 90% O_2 group.

ng/mg), and (98.81 ±1.55 ng/mg), which were significantly higher than those of the control group (25.50 ±1.86 ng/mg) ([a] $p < 0.05$). The CTGF concentration in the lung tissues of rats exposed to 90% oxygen for 3, 7, and 14 days were (50.72 ±1.80 ng/mg), (68.65 ±2.24 ng/mg), and (94.39 ±2.48 ng/mg), which were significantly higher than those of the control group (41.23 ±1.08 ng/mg) ([a]$p < 0.05$). These results suggest that the production of pulmonary fibrosis factors was induced by 90% oxygen. Compared with the 90% oxygen group, rapamycin significantly reduced the concentrations of col I, TGF-β1, and CTGF in the lung tissues of rats exposed to 90% oxygen for 3, 7, and 14 days ([b]$p < 0.05$) (Table 3).

Rapamycin inhibits the mTOR pathway in the lung tissue of rats

The above results indicated that rapamycin attenuates acute lung injury induced by 90% oxygen. The objective of western blot was to determine whether the mTOR pathway was involved in the repair of lung injury. As shown in Fig. 8, the expression levels of mTORC1, p70S6K, and 4EBP1 in the lung tissue of rats in the 90% O_2 + rapamycin group were significantly decreased compared with control animal at day 7 and 14 (*$p < 0.05$), which suggested that the application of rapamycin could inhibit the activation of mTOR signaling pathway and inhibit the expression of downstream proteins p70S6K and 4EBP1.

Discussion

In the present study, we found that exposure to oxygen concentrations of 40%, 60%, and 90% all promoted the apoptosis of L929 cells in a time and concentration-dependent manner. Application of 10 nM rapamycin or transfected with mTOR siRNA could inhibit the activation of mTOR signaling pathway and promote the apoptosis of L929 cells exposed to 90% O_2, and down-regulate the expression of the downstream proteins 4EBP1, and p70S6K. Exposure to 90% oxygen increased the production of col I, col III, and FN in ECM of L929 cells; however, inhibition of the mTOR signaling pathway decreased the production of col I, III and FN, which could reduce hyperoxia-induced pulmonary fibrosis. In lung tissues of hyperoxia-induced lung injury rats, we found that rapamycin inhibited

the activation of the mTOR pathway, which protected the lung from the oxidative injury and fibrosis induced by high concentration of oxygen.

Lung fibroblasts play an important role in the repair of lung injury. Yang et al. found that high oxygen concentrations inhibited the proliferation of fibroblasts isolated from embryonic lungs.[25] The mTOR signaling pathway is essential for cell proliferation, survival, and migration by regulating the transcription and translation of proteins.[26] Rapamycin is a specific inhibitor of the mTOR pathway.[27] Sun et al. found that 0.01–10 nM rapamycin could inhibit the proliferation of lung cancer cells; however, only >1 nM rapamycin quickly inhibited the phosphorylation of downstream proteins and the growth of lung cancer cells.[28] In the present study, mTOR siRNA down-regulated the expression of mTORC1, 4EBP1, and p70S6K, suggesting that mTOR can specifically regulate the expression of 4EBP1 and p70S6K. The extracellular matrix is synthesized and secreted by animal cells to form a network structure between cells, which consists of protein and polysaccharide.[29] In the present study, we found that blocking the mTOR signaling pathway inhibited collagen deposition in L929 cells, which prevented the lung fibrosis caused by oxidative injury. Collagen deposition in the lung is one of the most important causes and major characteristics of pulmonary fibrosis.[30] In the pathology of pulmonary fibrosis, apoptosis-related proteins, such as Bcl-2, P53, and caspase-3, as well as other cytokines, interplay to promote the proliferation of lung fibroblasts, which replace alveolar type II epithelial cells (AT II), subsequently causing pulmonary fibrosis.[31–33] TGF-β and CTGF could activate the PI3K/Akt/mTOR signaling pathway, promoting the proliferation, collagen synthesis, and anti-apoptotic ability of lung fibroblasts. TGF-β, which is a key component in a number of cytokine networks, is currently recognized to be one of the most powerful factors causing fibrosis.[34] Lee and Kim reported that CTGF was closely associated with TGF-β in pulmonary fibrosis.[35] Connective tissue growth factor is a newly identified cytokine closely involved in pulmonary fibrosis and is widely distributed in human tissues and organs; it plays important roles in cell adhesion, fibroblast proliferation, and ECM synthesis.[36] In the present study, we observed increased expression of CTGF and TGF-β, which was induced by 90% oxygen. Additionally, we found that blocking the mTOR pathway could down-regulate the expression of CTGF and TGF-β, which suggests that rapamycin could decrease the expression CTGF and TGF-β through inhibiting the activation of mTOR signaling pathway. In other words, the mTOR pathway is involved in lung fibrosis by regulating the expression of CTGF and TGF-β.

The mammalian target of rapamycin is widely found in mammals in 2 complex forms: mTORC1 and mTORC2. As the target protein of rapamycin, mTORC1 is sensitive to rapamycin; however, mTORC2 is not.[27] The mammalian target of rapamycin complex 1 regulates cell growth, proliferation, and metabolism by phosphorylating 2 major downstream proteins, S6K1 and eukaryotic initiation factor 4E-BP1. In the present study, we found that 90% O_2 induced lung injury in premature rats in a time-dependent manner. We also observed increased expression of fibrosis-related proteins, including TGF-β, CTGF, col II and col III, in the lung tissue of rats, also in a time-dependent manner. These results suggest that lung injury induced by a high concentration of oxygen is consistent with lung fibrosis. High concentrations of oxygen induced changes in the expression of mTORC1, p70S6K, and 4EBP1, which are key components of the mTOR pathway, suggesting that the mTOR pathway is activated by high concentrations of inhaled oxygen. It has been reported that the mTOR pathway is activated in lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice and rapamycin reduced the expression of inflammatory factors in bronchoalveolar lavage fluid (BLF).[37] Lorne et al. reported that the phosphorylation of rpS6 and 4EBP1, 2 effector proteins of the mTOR signaling pathway, induced the over-production of inflammatory cytokines by neutrophils in ALI, which could be inhibited by rapamycin.[38] It has been reported that rapamycin administration causes a significant reduction of p70S6K phosphorylation, increased autophagy, decreases neuronal cells apoptosis and significantly reduces brain damage in neonatal rats, which testifies to the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia.[39] Furthermore, it was found that the inhibition of the mTOR signaling pathway by rapamycin prevented the development and progression of lung fibrosis in a rat pulmonary fibrosis model that was induced by TGF-α.[21] Tulek et al. also found that rapamycin could inhibit the progress of lung fibrosis in the early stage of bleomycin-induced pulmonary fibrosis in rats.[40] Therefore, we speculate that the mTOR signaling pathway plays an important role in the repair of hyperoxia-induced lung injury by regulating the expression of TGF-β and CTGF. Rapamycin can effectively inhibit the activation of the mTOR signaling pathway and downregulate downstream target proteins P70S6K and 4EBP1, and reduce the production of TGF-β and CTGF, inhibiting the development of pulmonary fibrosis induced by high concentration oxygen exposure.

Conclusions

In conclusion, our results suggest that rapamycin prevents the progression of lung fibrosis induced by high concentrations of oxygen by inhibiting the mTOR signaling pathway, including suppressing the expression of p70S6K, 4EBP1, TGF-β, and CTGF. Therefore, rapamycin may be useful for the treatment of hyperoxia-induced ALI. Our results help elucidate the molecular mechanism underlying hyperoxia-induced lung injury and may contribute to the identification of novel targets for its treatment.

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The association between *FTO* gene polymorphism rs9939609 and obesity is sex-specific in the population of PURE study in Poland

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Abstract

Background. Fat mass and obesity-associated gene (*FTO*) polymorphism remains the strongest known genetic determinant of common obesity. However, its influence depends on ethnicity, and the *FTO*-mediated predisposition to other metabolic disturbances is questionable.

Objectives. The aim of our study was to evaluate the association between *FTO* rs9939609 polymorphism and metabolic syndrome in a population of Prospective Urban Rural Epidemiology (PURE) study in Poland.

Material and methods. We enrolled 1,097 participants of the PURE study (683 women and 414 men) from the Lower Silesian voivodeship. Anthropometrical parameters and blood pressure were measured. Blood samples were taken for an examination of lipid profile and fasting glucose level. Genomic DNA was isolated and *FTO* polymorphism rs9939609 was genotyped.

Results. Male A-allele carriers had significantly higher mean body mass, body mass index (BMI), waist-to-hip ratio (WHR), and waist and hip circumferences than men without risk allele. They were also more often diagnosed with obesity on the basis of BMI and central obesity parameters. No such influence was observed in women. There were no significant associations between *FTO* polymorphism and metabolic syndrome or its components.

Conclusions. Our results suggest a sex-specific association between *FTO* polymorphism and obesity traits. The occurrence of metabolic syndrome or its components was not related with *FTO* gene variation in our cohort.

Key words: metabolic syndrome, obesity, *FTO* gene, PURE study

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Background

The rising prevalence of obesity and overweight has been described as a global pandemic, accounting for about 3.4 million deaths in 2010.[1] In order to decrease this tendency, we need to understand the pathogenesis of adiposity and its complications, such as diabetes, hypertension and dyslipidemia.

Since 2007, it has been shown that variations in the first intron of the fat mass and obesity-associated (*FTO*) gene contribute to excessive weight in many populations.[2–5] However, this relation is race-specific and not all studies have confirmed the influence of *FTO* gene variation on body mass index (BMI).[6,7] The association between *FTO* gene and cardiovascular complications is also controversial. Some authors have shown that risk allele carriers are predisposed to metabolic syndrome (higher fasting glucose, insulin, triglycerides, and lower high-density-lipoprotein (HDL) cholesterol serum levels),[8] or its components even after BMI adjustment.[9–11] Others studies found no link between *FTO* and diabetes[12] or other metabolic disturbances.[4,13]

The *FTO* gene is located in chromosome region 16q12.2 and encodes the nucleic acid demethylase. It is expressed in many tissues, most highly in hypothalamic regions responsible for energy homeostasis.[14] The major substrate for FTO protein is N^6-methyladenosine (m^6A) in nuclear RNA.[15] The m^6A is a prevalent internal modification of messenger RNA (mRNA), regulating gene expression[16]; thus, its demethylation plays an important role in mRNA processing.[17] People with *FTO* risk alleles are predisposed to obesity probably as a result of impaired central satiety processing and increased food intake[18,19] together with a preference for high-calorie foods.[20,21] *FTO* gene may also be involved in the regulation of adipogenesis,[17,22] lipolysis[23] and adipocyte thermogenesis.[24]

Although many previous studies concerned variations in the *FTO* gene with respect to obesity, there are a few important reasons to perform our analysis. Firstly, the influence of *FTO* gene on obesity and metabolic complications differs in various ethnic groups.[25] A recent association analysis in Poland confirmed the correlation between susceptibility loci in intron 1 of the *FTO* gene with obesity; however, it did not include the aspect of cardiovascular complications.[26] Other studies conducted in Poland were based on smaller sample sizes[13,27] or included only specific groups, like children with diabetes[28] or women with polycystic ovary syndrome (PCOS).[29] We examined the largest number of subjects from an unselected Polish population for the *FTO* variation and metabolic syndrome components.

Objectives

The aim of our study was to assess the association between *FTO* rs9939609 single nucleotide polymorphism (SNP) and obesity and metabolic syndrome components in the Polish group from the Prospective Urban Rural Epidemiology (PURE) study.

Material and methods

Study group and examination protocol

We enrolled 1,097 subjects (683 women and 414 men) from the Lower Silesian voivodeship in Poland (690 inhabitants of the city of Wrocław and 407 inhabitants of neighboring rural area), aged 30–80 years, who took part in the PURE study in years 2007–2010. The aims and design of the PURE study have been published elsewhere.[30] Each participant answered the PURE Questionnaire collected by a trained person, providing information about age, social background and medical history (including chronic medication and comorbidities). The physical examination included measurement of height with the subject's head in Frankfurt plane (accuracy of 0.5 cm) and weight on calibrated Tanita scales (accuracy of 0.1 kg). The BMI was calculated as follows:

$$\text{BMI} = \frac{\text{weight [kg]}}{[\text{height [m]}]^2}.$$

The waist circumference was measured with tape, halfway between the lowest rib and the top of the hipbone. The hip circumference was measured with tape, at the widest part of the buttocks. The waist-to-hip ratio (WHR) was calculated as follows:

$$\text{WHR} = \frac{\text{waist circumference [cm]}}{\text{hip circumference [cm]}}.$$

After 10 min of rest, the blood pressure was measured twice on the right arm with the use of Omron HEM-757 apparatus (Osaka, Japan). Blood samples from ulnar vein were taken in the morning after 8 h of fasting, and centrifuged at 1,000 × g for 20 min at 4°C. Each serum sample was stored at –80°C. Total serum cholesterol, triglycerides and HDL cholesterol were measured using standardized methods with the enzymatic assay SPINREACT (Sant Esteve De Bas, Girona, Spain). The level of low-density lipoprotein (LDL) cholesterol was estimated using Friedewald formula:

$$\text{LDL cholesterol} = \text{triglycerides} - \text{HDL cholesterol} - \frac{\text{triglycerides}}{5}$$

(in patients with triglycerides concentration lower than 400 mg/dL).

Plasma glucose was measured with standardized enzymatic methods using glucose oxidase (Dade Behring GmbH, Marburg, Germany). The metabolic syndrome was diagnosed if 3 or more of the following 5 criteria were met: waist circumference over 102 cm (men) or 88 cm (women), blood pressure over 130/85 mm Hg, triglyceride level over 1.7 mmol/L, HDL cholesterol level less than 1.03 mmol/L

(men) or 1.29 mmol/L (women), and fasting blood sugar over 100 mg/dL (National Cholesterol Education Program – Adult Treatment Panel III (NCEP-ATPIII) criteria).

Each participant submitted oral and written consent. The study was approved by Wroclaw Medical University Ethical Committee (approval No. KB 438/2014).

Genotyping

The *FTO* gene contains several polymorphic sites. In many populations, obesity susceptibility loci were found in intron 1 of the gene. The rs9939609 variant stays in strong linkage disequilibrium with other polymorphic sites in this intron, which gives the rationale to select this variant for genotyping in our study.

The genomic DNA was extracted from peripheral blood leukocytes in the blood samples according to the protocol of a commercial DNA isolation kit (NucleoMag® 96 Blood; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Polymerase chain reaction (PCR) conditions were optimized and a pair of specific primers was designed (synthesized by Generi Biotech s.r.o., Hradec Králové, Czech Republic) for the amplification and identification of the rs9939609 *FTO* gene polymorphism:

5'-CACTAACATCAGTTATGCAT-3' – forward primer

5'-CCATTTCTGACTGTTACCTA-3' – reverse primer.

Specific fragments of the *FTO* gene containing polymorphic sites were amplified with PCR using the TaKaRa Taq DNA Polymerase Amplification Kit (Takara Bio Inc., Shiga, Japan). The PCR mix (20 µL) contained forward and reverse primers (as above), 1 × PCR buffer containing 1.5 mM $MgCl^2$, 200 µM dNTPs, 2 units of Taq polymerase, and 200 ng of genomic DNA. Amplification was performed using a TPersonal Thermocycler (Biometra GmbH, Göttingen, Germany) with the following cycle conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of: denaturation at 95°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. The post-PCR products were purified of excess primers and nucleotides using a mixture of SAP and ExoI enzymes (Thermo Fisher Scientific, Waltham, USA).

The identification of gene polymorphism was performed using the minisequencing method according to the protocol of an ABI PRISM® SNaPshot™ Multiplex Kit (Thermo Fisher Scientific). The reaction was carried out in the presence of primers extended using single fluorescence-labeled dideoxynucleotide (ddNTP). This reaction consisted of 25 cycles: denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 30 s. The designed specific primer was:

5'-TGTCTGAATTATTATTCTAGGTTCCTTGC-GACTGCTGTGAATTT-3'.

Fluorescently labeled products of the reaction were separated with capillary electrophoresis in ABI PRISM® 3100 Genetic Analyzer (Thermo Fisher Scientific) and analyzed by GeneMapper® software v. 4.0 (Thermo Fisher Scientific).

Statistical analysis

Statistical analysis was performed using STATISTICA v. 12 for Windows (StatSoft Inc., Tulsa, USA). We described variables using elements of descriptive statistics that included the following: minimum and maximum value, mean and standard deviation (SD). Contingency tables were created for qualitative data. The Shapiro–Wilk test was used to verify how well the distribution of the studied characteristics fitted with normal distribution. If the hypothesis of normality was not rejected, then the Student's test or Welch's test was used to verify the equality of means, depending on the hypothesis of equality of variances could be accepted.

The distribution of most of the variables was significantly different from the normal distribution; thus, nonparametric methods were also used to conduct the analysis. The hypotheses of homogeneity (equality) of distributions were verified using the Kolmogorov–Smirnov test and the Mann–Whitney test, depending on the assumption of the Mann–Whitney test could be accepted. We used the χ^2 test to verify the hypothesis of independence between qualitative data. Frequencies of the observed alleles were tested against the Hardy–Weinberg equilibrium. Differences were considered statistically significant at $p < 0.05$.

Results

We genotyped SNP rs9939609 in 1,097 individuals (683 women and 414 men). The frequency of A-allele (obesity risk allele) among participants was 0.44 and of T-allele – 0.56. Variant frequencies followed the Hardy–Weinberg equilibrium and the genotyping success rates exceeded 98%. Briefly, 20.2% of the study group were homozygous for A-allele (AA-group), 32.4% had 2 T-alleles (TT-group) and 47.3% were heterozygotes (AT-group) – see Table 1. The occurrence of alleles was sex-independent ($p = 0.6659$).

The characteristics of the study group are shown in Table 2. Mean BMI among participants was 28.2 kg/m^2. About 31% of the group had obesity defined with a BMI over 30 kg/m^2 – similarly in both sex groups, while overweight (BMI 25–29.9 kg/m^2) affected 48.6% of men and 36.6% of women ($p = 4 \times 10^{-5}$). Central obesity according to waist circumference criterion (over 88 cm in women

Table 1. The frequencies of *FTO* polymorphism rs9939609 alleles in the study group

Allele	Whole group	Women	Men
AA	222 (20.24%)	144 (21.08%)	78 (18.84%)
AT	519 (47.31%)	319 (46.71%)	200 (48.31%)
AA + AT	741 (67.55%)	463 (67.79%)	278 (67.15%)
TT	356 (32.45%)	220 (32.21%)	136 (32.85%)

Table 2. Characteristics of the study group

Characteristics	Study group	Women	Men	p-value
Number of subjects	1,097	683	414	–
Age [years], mean ±SD	54.20 ±9.26	54.38 ±8.86	53.98 ±9.89	p < 0.05*
BMI [kg/m^2], mean ±SD	28.2 ±5.0	28.0 ±5.4	28.5 ±4.4	p < 0.001*
Waist circumference [cm], mean ±SD	92.1 ±13.9	88.1 ±13.4	98.8 ±12	p < 0.001*
WHR, mean ±SD	0.88 ±0.09	0.84 ±0.07	0.96 ±0.07	p < 0.001*
SBP [mm Hg], mean ±SD	146.6 ±21.6	143.0 ±21.7	152.4 ±20.3	p < 0.001*
DBP [mm Hg], mean ±SD	86.2 ±11.3	84.3 ±10.7	89.2 ±11.7	p < 0.001*
Blood glucose [mg/dL], mean ±SD	99.4 ±21.6	99.3 ±21.5	99.7 ±21.7	p > 0.1*
Total cholesterol [mmol/L], mean ±SD	5.05 ±1.02	5.1 ±1.0	4.96 ±1.05	p = 0.01**
LDL cholesterol [mmol/L], mean ±SD	2.90 ±0.92	2.89 ±0.93	2.92 ±0.92	p = 0.448 **
HDL cholesterol [mmol/L], mean ±SD	1.50 ±0.43	1.60 ±0.41	1.34 ±0.40	p < 0.001*
Triglycerides [mmol/L], mean ±SD	1.44 ±0.98	1.36 ±0.84	1.58 ±1.16	p < 0.005*

* Kolmogorov–Smirnov test; ** Mann–Whitney U test; SD – standard deviation; BMI – body mass index; DBP – diastolic blood pressure, SBP – systolic blood pressure, BDP – diastolic blood pressure; WHR – waist-to-hip ratio; LDL – low-density-lipoprotein; HDL – high-density-lipoprotein.

Table 3. *FTO* polymorphism rs9939609 and anthropometric parameters in the whole study group

Characteristic	*FTO* rs9939609 genotype			p-value*	
	AA	AT	TT	AA vs TT	AA + AT vs TT
Body mass [kg], mean ±SD	77.65 ±16.26	78.15 ±17.00	75.7 ±14.5	0.1	0.1
BMI [kg/m^2], mean ±SD	28.45 ±5.35	28.41 ±5.12	27.80 ±4.84	0.1	0.1
Waist circumference [cm], mean ±SD	92.84 ±14.60	92.59 ±14.10	91.1 ±12.9	0.1	0.1
Hip circumference [cm], mean ±SD	104.55 ±9.99	104.66 ±10.29	103.35 ±9.36	0.1	0.1
WHR, mean ±SD	0.89 ±0.098	0.88 ±0.09	0.88 ±0.09	0.1	0.1

* Kolmogorov–Smirnov test; BMI – body mass index; WHR – waist-to-hip ratio; SD – standard deviation.

Table 4. *FTO* polymorphism rs9939609 and anthropometric parameters in male group

Characteristics	*FTO* rs9939609 genotype			p-value	
	AA	AT	TT	AA vs TT	AA+AT vs TT
Body mass [kg], mean ±SD	86.76 ±14.75	87.82 ±16.52	82.38 ±12.98	p = 0.025**	p = 0.0005***
BMI [kg/m^2], mean ±SD	28.99 ±4.63	28.99 ±4.52	27.60 ±3.96	0.05 < p < 0.1*	p < 0.01*
Waist circumference [cm], mean ±SD	100.80 ±12.91	99.86 ±12.20	95.99 ±10.70	p = 0.004**	p = 0.0005***
Hip circumference [cm], mean ±SD	103.33 ±7.70	103.73 ±8.50	101.26 ±7.60	p > 0.1*	p < 0.005*
WHR, mean ±SD	0.97 ±0.08	0.96 ±0.07	0.94 ±0.07	p > 0.1*	0.05 < p < 0.1*

* Kolmogorov–Smirnov test; ** Student's t-test; ***Welch's test; *FTO* – obesity-associated gene; SD – standard deviation; BMI – body mass index; WHR – waist-to-hip ratio.

and 102 cm in men) was diagnosed in 45.3% of women and 35.6% of men (p = 0.0017). According to WHR criterion, central obesity was present in 39% of women (WHR > 0.85) and 81.8% of men (WHR > 0.9). In our examination, 42.9% of men and 25.6% of women had blood pressure over 140/90 mm Hg, indicating hypertension ($p < 10^{-5}$). Impaired fasting glucose (100–125 mg/dL) occurred in 31.9% of the participants, while hyperglycemia suggestive for diabetes (>126 mg/dL) was present in 6.6% of the study group (no difference between men and women, p = 0.948). Mean total and HDL cholesterol levels were higher in women than in men, while men had higher levels of triglycerides in the blood. There was no significant difference in LDL cholesterol levels between sex groups. Three hundred eighty-two participants (37% of the group) fulfilled the NCEP-ATPIII criteria for the presence of metabolic syndrome, and there was no difference between men and women (p = 0.68).

There were no significant associations between obesity parameters and *FTO* rs9939609 polymorphism in the whole study group (Table 3). However, when we analyzed men and women separately, obesity traits were significantly associated (p < 0.01 after adjustment for multiple testing) with the occurrence of A-allele in men (Table 4). The differences were present both in the recessive and co-dominant model. A-allele carriers had higher mean

Table 5. *FTO* polymorphism rs9939609 and anthropometric parameters in female group

Characteristics	*FTO* rs9939609 genotype			p-value*	
	AA	AT	TT	AA vs TT	AA+AT vs TT
Body mass [kg], mean ±SD	72.71 ±14.88	72.07 ±14.30	71.57 ±13.90	p > 0.1	p > 0.1
BMI [kg/m^2], mean ±SD	28.17 ±5.69	28.05 ±5.44	27.93 ±5.30	p > 0.1	p > 0.1
Waist circumference [cm], mean ±SD	88.53 ±13.60	88.02 ±13.30	88.04 ±13.30	p > 0.1	p > 0.1
Hip circumference [cm], mean ±SD	105.20 ±11.02	105.25 ±11.25	104.64 ±10.10	p > 0.1	p > 0.1
WHR, mean ±SD	0.84 ±0.07	0.83 ±0.07	0.84 ±0.07	p > 0.1	p > 0.1

* Kolmogorov–Smirnov test; *FTO* – obesity-associated gene; SD – standard deviation; BMI – body mass index; WHR – waist-to-hip ratio.

Table 6. *FTO* polymorphism rs9939609 and blood pressure measurements

Parameter	*FTO* rs9939609 genotype			p-value	
	AA	AT	TT	AA vs TT	AA + AT vs TT
SBP [mm Hg] – whole group, mean ±SD	145.4 ±20.5	145.7 ±21.4	148.5 ±22.3	0.1*	0.1*
DBP [mm Hg] – whole group, mean ±SD	85.3 ±10.4	85.9 ±11.5	87.2 ±11.6	0.1*	0.1*
SBP [mm Hg] – men, mean ±SD	152.6 ±17.1	151.2 ±21.0	153.8 ±20.7	0.66**	0.3**
DBP [mm Hg] – men, mean ±SD	87.0 ±10.1	89.3 ±11.6	90.4 ±12.5	0.05**	0.16**
SBP [mm Hg] – women, mean ±SD	141.5 ±21.2	142.3 ±20.8	145.2 ±22.6	0.1*	0.1*
DBP [mm Hg] – women, mean ±SD	84.2 ±10.5	83.7 ±10.9	85.2 ±10.5	0.1*	0.1*

* Kolmogorov–Smirnov test; ** Shapiro–Wilk test; SBP – systolic blood pressure; DBP – diastolic blood pressure.

Table 7. *FTO* polymorphism rs9939609 and lipid levels

Parameter	*FTO* rs9939609 genotype			p-value*	
	AA	AT	TT	AA vs TT	AA + AT vs TT
Total cholesterol in whole group [mmol/L], mean ±SD	4.95 ±1.02	5.06 ±1.00	5.09 ±1.05	0.1	0.1
LDL cholesterol in whole group [mmol/L], mean ±SD	2.82 ±0.92	2.91 ±0.91	2.93 ±0.95	0.05 < p < 0.1	0.1
HDL cholesterol in whole group [mmol/L], mean ±SD	1.47 ±0.42	1.51 ±0.43	1.53 ±0.43	0.1	0.1
Triglycerydes in whole group [mmol/L], mean ±SD	1.48 ±0.89	1.45 ±1.04	1.41 ±0.94	0.1	0.1
Total cholesterol in women [mmol/L], mean ±SD	5.07 ±1.05	5.12 ±0.97	5.10 ±1.02	0.1	0.1
LDL cholesterol in women [mmol/L], mean ±SD	2.84 ±0.94	2.89 ±0.89	2.90 ±0.98	0.1	0.1
HDL cholesterol in women [mmol/L], mean ±SD	1.58 ±0.41	1.61 ±0.39	1.62 ±0.44	0.1	0.1
Triglycerydes in women [mmol/L], mean ±SD	1.44 ±0.89	1.37 ±0.86	1.30 ±0.76	0.1	0.1
Total cholesterol in men [mmol/L], mean ±SD	4.70 ±0.91	4.96 ±1.05	5.09 ±1.11	0.05 < p < 0.1	0.1
LDL cholesterol in men [mmol/L], mean ±SD	2.77 ±0.87	2.93 ±0.95	2.96 ±0.89	0.05 < p < 0.1	0.1
HDL cholesterol in men [mmol/L], mean ±SD	1.26 ±0.34	1.35 ±0.43	1.37 ±0.38	0.05 < p < 0.1	0.1
Triglycerydes in men [mmol/L], mean ±SD	1.56 ±0.86	1.57 ±1.27	1.59 ±1.15	0.1	0.1

* Kolmogorov–Smirnov test; LDL – low-density lipoprotein; HDL – high-density lipoprotein; SD – standard deviation.

BMI (AA-group and AT-group – 28.99 kg/m^2, TT-group – 27.6 kg/m^2, p < 0.01) and were more often diagnosed with obesity defined with BMI > 30 kg/m^2 (37% of AA-group and 24% of TT-group, p = 0.04). Male A-allele carriers had higher mean waist circumference and WHR and they were more often diagnosed with central obesity than men without risk allele (44% of AA-group and 28% of TT-group had waist circumference over 102 cm, p = 0.02; 88% of AA group and 76% of TT group had WHR > 0.9, p = 0.023). We did not observe any significant associations between *FTO* polymorphism and obesity traits in the female group (Table 5). In order to find a possible reason why *FTO* polymorphism had a weaker effect on obesity in women, we divided the female group on the basis of menopausal status and age, but this did not significantly influence the results (data not shown).

We also analyzed the association between *FTO* rs9939609 polymorphism and blood pressure measurements, lipid and glucose levels in the blood, but there were no significant associations (Tables 6–8). The associations remained insignificant also in separate analyses in men and women. The metabolic syndrome occurred more frequently in risk-allele groups, but the associations were statistically insignificant (Table 9).

Table 8. *FTO* polymorphism rs9939609 and glucose level

Parameter	*FTO* rs9939609 genotype			p-value*	
	AA	AT	TT	AA vs TT	AA + AT vs TT
Glucose level in whole study group [mg/dL], mean ±SD	101.7 ±31.4	99.4 ±19.5	98.3 ±16.8	0.1	0.1
Glucose level in women [mg/dL], mean ±SD	100.5 ±27.5	99.5 ±21.0	98.2 ±17.6	0.1	0.1
Glucose level in men [mg/dL], mean ±SD	104.0 ±37.5	99.2 ±16.0	98.5 ±15.5	0.1	0.1

* Kolmogorov–Smirnov test; SD – standard deviation.

Table 9. The occurrence of metabolic syndrome in study group according to *FTO* polymorphism rs9939609

Parameter	*FTO* rs9939609 genotype			p-value*	
	AA	AT	TT	AA vs TT	AA + AT vs TT
Frequency of metabolic syndrome in whole study group	40.1%	37.4%	35.4%	0.27	0.39
Frequency of metabolic syndrome in female group	38%	38%	37%	0.96	0.78
Frequency of metabolic syndrome in male group	43%	36%	33%	0.33	0.29

* χ^2 test.

Discussion

The frequency of obesity-risk A-allele in our group was 0.44, which remained in accordance to that of other European populations.[3,25] The analyzed *FTO* SNP rs9939609 had, together with rs1558902, rs1421085, rs9930506, and rs12149832, the strongest influence on obesity phenotype in the recent association study in Polish population. These SNPs are located in intron 1 and stay in almost complete linkage disequilibrium (LD) ($|D'| > 0.98$, $r^2 > 0.80$).[26]

Many studies confirmed that the *FTO* gene variation predisposes to excessive weight. This association varies significantly in different ethnic groups.[2–5] Nonetheless, it remains the strongest genetic determinant of common obesity known so far, with an estimated population risk of 22%.[2] In this context, we have confirmed such an association in the male population of Lower Silesia in Poland. However, our most interesting result is the significant difference in the influence of *FTO* polymorphism on obesity traits between men and women in our cohort. Male A-allele carriers had higher mean BMI, waist circumference and WHR, and were more often diagnosed with obesity on the basis of all those criteria than men without genetic risk factor. We did not observe such associations in the female group. Sex-dependent contribution of *FTO* gene on obesity is a novel finding. To our knowledge, there are only 2 previous studies, 1 in the Polish and 1 in the Mexican population, where sex-specific differences have been shown. Sobalska-Kwapis et al. demonstrated a strong association of *FTO* intronic variants in block 8 with overweight in a group of men only; however, this sex difference was no longer valid in the case of obesity. When authors considered obesity and overweight groups together, there were only 8 statistically significant SNPs in women comparing with 27 SNPs in men. It has been also revealed in this study that obesity-associated SNPs had a different inheritance model in men (recessive) than in women (dominant).[26] In the Mexican population, Saldaña-Alvarez et al. have shown, in turn, that 2 *FTO* SNPs were specifically associated in men under a dominant model, while 3 were associated with women under additive and recessive models.[31] Together with our results, this highlights the need to consider sex as an important factor modulating *FTO* genetic association with overweight and obesity. The outcomes of our study and the one of and Sobalska-Kwapis et al. suggest that in Polish women, obesity is less influenced by *FTO* polymorphism than in men.[26]

To further explore this problem, we analyzed the *FTO* influence on obesity in female subgroups according to menopausal status and age, but it did not change our results. There are suggestions that the association between *FTO* gene and obesity is age-dependent.[32] We also know that many other factors (socioeconomic, cultural, environmental, psychological) play an important role in such a complex trait as body mass. In our other publication, we presented a list of important environmental determinants of obesity in the population of PURE study in Poland.[33] Some of those factors (like rural inhabitancy, unemployment and stress) had a greater impact on body mass than *FTO* polymorphism, particularly in women. We can presume that environmental factors play a more important role in the pathogenesis of female obesity in our study, which blurs the role of *FTO* gene. Moreover, other regions in genome may regulate overweight/obesity in a sex-dependent manner. It has been shown that *FTO* sequences are functionally connected with other genes, for example homeobox gene *IRX*.[34] Landgraf et al. reported that *FTO* variants affected adipocyte-specific expression of *IRX5* and *IRX3*.[35] There are more studies needed in this area with a distinction between men and women.

To our knowledge, this is the most representative Polish study, in which participants were examined for *FTO* polymorphism and metabolic syndrome components. Previous studies in Poland suggested that the variation in the *FTO* gene was related to some metabolic disturbances indicative

of insulin resistance. For example, Łuczyński et al. have shown that children with AA genotype rs9939609 had higher values of blood pressure (systolic blood pressure (SBP) and diastolic blood pressure (DBP)), triglycerides, fasting insulin, and homeostatic model assessment – insulin resistance (HOMA-IR) index, although only the latter was not attenuated after adjusting for BMI.[36] In another Polish study consisting of women with PCOS, there was an association between the occurrence of risk allele and lower insulin sensitivity, but it was mediated by adiposity.[29] The recent analysis of 425 women from West Pomeranian voivodeship revealed that AA homozygotes of *FTO* rs9939609 presented a higher risk of fasting hyperglycemia than those with at least 1 T allele, but no associations were found in the case of lipids, blood pressure or waist size.[27] In a study concerning other *FTO* polymorphism – rs9930506 in 442 Polish adults, there was no link between this genetic variation and lipid disturbances or higher fasting glucose level.[13] Recently, Ślęzak et al. presented a study where *FTO* gene variation was related to single metabolic disturbances in a homogenous male group, but the risk of metabolic syndrome was not increased in risk allele carriers.[37] In our analysis, we have found no association between carrying *FTO* rs9939609 risk allele and the onset of metabolic syndrome or its components in men and women from Lower Silesia.

In the context of mentioned studies, we can presume that the *FTO* influence on metabolic disturbances in Polish population is secondary to its influence on adiposity. Because the link with obesity was not very strong in our group, the association with metabolic syndrome components could not be revealed. Many studies in other countries also suggest that *FTO* gene variation modifies metabolic disturbances only indirectly through adiposity. For example, the analyses of very big European populations by Frayling et al. and Freathy et al. supported this thesis.[3,8] However, there are also studies showing that the influence of *FTO* variation on diabetes onset, age at diagnosis or even diabetes-related complications did not disappear after BMI adjustment.[38–40] The latter work was performed in the Czech population, which is genetically similar to Poles.

The association between *FTO* gene and metabolic traits may be explained in different ways. First, it can be only an effect of adiposity. Our results support this thesis – if the influence on obesity is weak, there are consequently no associations with metabolic traits. However, the mentioned works showing that diabetes-related complications are associated with FTO independently of BMI may suggest other mechanism of FTO gene action on metabolism. The expression in different tissues and regulatory role of *FTO* gene (FTO protein is a nucleic acid demethylase) allows it to be involved in pathogenesis of different disturbances. More studies are needed to explain the exact role of *FTO* gene in metabolism.

There were a few limitations of our study. Firstly, our results come from cross-sectional analysis, while longitudinal study is more suitable to draw conclusions about obesity and its complications. However, there is an ongoing observation of our cohort and we plan to analyze results of the follow-up after a few years. Secondly, our cohort is not strictly population-based, with some possible snowball sampling biases (according to the PURE study design, people were allowed to register themselves voluntarily in selected urban and rural districts). In other Polish studied, minor allele frequency (MAF) of *FTO* rs9939609 were higher (Łuczyński et al.[36] – 0.49, Sobalska-Kwapis et al.[26] – 0.69). This may be the reason why the associations between *FTO* risk allele and obesity traits were weaker in our cohort than in other studies. Another important factor is that our cohort was older than in most Polish studies and we know that in the older age genetic influence on obesity is blurred by environmental factors. Nevertheless, our results stay in accordance with other studies from Poland and Europe and we were able to detect sex-specific difference of *FTO* influence on obesity, which is a novel and interesting finding. More studies in this area are needed.

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Diagnostic equivalency of mobile CTG devices and remote analysis to conventional on-site nonstress test

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

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Abstract

Background. Remote pregnancy monitoring is one of the most promising applications of telemedicine; however, the diagnostic value of self-examination using mobile cardiotocography (CTG) devices and remote analysis of the subsequent results has never been properly studied.

Objectives. The study aimed to compare the diagnostic usefulness of CTG self-examination using a mobile device to examination performed by a medical professional using a stationary device; and to evaluate the quality of CTG analysis performed remotely.

Material and methods. Eighty-two pairs of CTG recordings were collected; each pair consisted of a single recording from an examination performed by a midwife using a stationary device, and another recording from an unassisted patient self-examination using a mobile device. Recordings were performed with a maximum time interval of 30 min. Each recording was analyzed twice. Primary analysis included a comparison of the assisted examination evaluated on-site vs the self-examination evaluated remotely in pairs. Secondary analysis was conducted by an independent expert who evaluated the unpaired recordings. Baseline fetal heart rate (BFHR) values were compared independently.

Results. We found that patients were more likely to perform inconclusive recordings than experienced midwives; however, the self-examination feasibility was satisfactory. The primary analysis showed 88.4% agreement of the recorded pairs; 11.6% of inconsistent pairs were due to inter-observer variability or medical reasons. The independent expert's analysis showed 97.1% agreement between the assisted and unassisted examinations. Paired t-test for BFHR values showed a statistically significant but clinically negligible mean difference between the 2 devices at 1.75 bpm.

Conclusions. The CTG examinations performed using mobile devices present satisfactory feasibility and equivalent diagnostic value compared to conventional devices, while the remote evaluation of recordings is as reliable as on-site analysis. Remote pregnancy surveillance is safe, effective and may be implemented into everyday obstetric care.

Key words: teleCTG, mobile CTG, telemedicine, eHealth, remote pregnancy monitoring

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Background

Healthcare is facing new challenges with regard to the growing number of chronic diseases, resulting in increasing direct and indirect costs, an insufficient number of medical professionals in relation to patient needs, and the growing expectations to provide quality care. One of the single most effective approaches to dealing with such challenges is to improve patient engagement by enabling individuals to manage their own health. Concomitantly, we are witnessing the emergence of new systems and services using electronic communication, referred to as eHealth or telemedicine, including web-based or mobile informative and decision-making applications, teleconsultations and remote monitoring.[1] These technologies enable a shift from hospital-centered to patient-centered care. Telemedicine can also be used to improve access to medical services, which makes it applicable for rural areas and underdeveloped countries,[2,3] where the concentration of health centers is low.

Telemedicine is increasingly used in obstetrics, as young women in their reproductive years are frequent users of the Internet, social media and smartphone applications.[4] At the same time, the demand for fetal monitoring and constant reassurance is high amongst pregnant women.[5] Telemedicine applications in obstetrics can be categorized depending on the form of service provided (educational, teleconsultations, remote monitoring) or depending on the problem addressed (lifestyle issues, gestational diabetes, mental health, etc.). Many of the applications have been tested in the past few years and positive effects have been shown (reviewed by van den Heuvel et al.[6]); however, most of the studies lack an endpoint related to its clinical impact, which still remains to be determined.

Remote pregnancy monitoring is perceived to be one of the most promising telemedical applications. Antepartum fetal assessment is used in pregnancies with a high risk of perinatal morbidity and mortality owing to pre-existing maternal conditions, pregnancy-related conditions,[7] intrauterine fetal death in the past, or in the case of overdue pregnancy. Testing options include the nonstress test (NST) based on a fetal heart rate (FHR) recording, which has been in clinical use for over 4 decades. The NST is based on the premise that the FHR, which is not acidotic or neurologically depressed, will temporarily accelerate with fetal movement. Heart rate reactivity is thought to be a good indicator of normal fetal autonomic function[8] and a normal NST result is, in most cases, highly reassuring, as indicated by the high negative predictive value.[9,10] Loss of reactivity is most commonly associated with fetal sleep cycles but may result from any cause of central nervous system depression, including fetal hypoxia and academia.[8] The primary objective of perinatal patient management is to discover signs and symptoms of early stage hypoxia. This has implications not only in acute care, but may also affect the quality of an entire future life burdened with the consequences of hypoxia.[11]

The method routinely used for antenatal monitoring is cardiotocography (CTG), which allows for simultaneous detection of FHR (C – cardio) with an ultrasound transducer, and uterine contractions with a tocodynamometer (T – toco), and a graphic (G) presentation of their values as a function of time.[12] Contemporary CTG devices are also able to detect maternal heart rate (MHR) and fetal movements, or allow the mother to manually mark fetal movements. The CTG examination is easy to perform, safe and completely non-invasive, which makes it a viable tool for patient self-monitoring. Even more importantly, CTG-based NST are considered safe for remote monitoring due to its low false-negative rate.[7]

Hod and Kerner[7] identified the usefulness criteria for remote monitoring: 1) feasibility in patient's self-use and diagnostic similarity to traditional methods; 2) improvement of access to healthcare; 3) similarity in maternal and neonatal outcomes, compared to traditional methods; 4) patient and clinician satisfaction; and 5) cost-effectiveness. Even though it has been 16 years since the publication, none of these areas have been sufficiently examined and all still need more research, especially clinical outcomes, feasibility and diagnostic equivalence, as technology is constantly changing.

Maternal and neonatal outcomes

Kitagawa et al.[13] found that home monitoring was equally as effective as ambulatory hospital management in preventing adverse events in high-risk women. Another study compared modes of delivery and complications postpartum between home-monitored high-risk patients and patients admitted to the hospital.[14] There were no statistically significant differences between the 2 groups. Moore and Sill[15] showed that the number and duration of hospital admissions for fetal surveillance were reduced in a home-monitored, high-risk patient group, without an increase in C-section and forceps delivery rates. Di Lieto et al.[16,17] described the clinical results of the first 5 working years of a telemedicine project based on computerized telecardiotocography. The project embraced 1,873 low- and high-risk patients who delivered 5,830 CTG tracings. The authors found good neonatal outcomes, no false-negative results and no increase in C-section rate.

Due to the function of uterine contraction activity monitoring, CTG is also suitable for early detection of threatened premature delivery symptoms in order to initiate treatment earlier.[13] Studies have demonstrated that transmitting uterine activity by telecommunication resulted in significantly prolonged pregnancy survivals,[18,19] while newborns were less likely to be of low birth weight or to be admitted to the neonatal intensive care unit, when a telemonitoring group was compared with a standard care group.[19,20]

Feasibility and diagnostic equivalence

A prerequisite for positive clinical outcomes from remote monitoring is the ability for a pregnant patient to properly perform an examination herself. It was shown that patients were equally as able as midwives to perform accurate CTG fetal monitoring[15,21]; however, the quality of the recorded data can be correlated with maternal body mass index (BMI).[21] Reece et al.[22] performed a controlled clinical trial on 60 patients who performed home based non-stress tests, followed by NCT performed by a midwife at the hospital within 60 min. The pairs of tests were independently reviewed by 2 investigators and all were judged satisfactory for interpretation. Remote maternal-fetal monitoring is also feasible in resource-constrained environments, as was shown on a group of 153 high-risk pregnant indigenous Mayan women receiving either remote monitoring (n = 74) or standard of care (n = 79). Health outcomes were not statistically different between the 2 groups, while remote monitoring resulted in a markedly increased adherence (94.3% vs 45.1%).[23]

Altogether, the data so far shows that home FHR monitoring is safe and feasible even in high-risk patients, as it can be easily and reliably performed without worsening medical outcomes, while patient satisfaction and overall acceptance remains promising.[21]

As more than 10 years have passed since the publication of most of the cited papers, during which the technology has evolved, and since diagnostic equivalence of remote versus conventional CTG monitoring was never properly studied, we aimed 1) to compare the diagnostic usefulness between an examination performed by a patient with a mobile CTG device and an examination performed by a medical professional with a stationary CTG device; and 2) to evaluate the quality of CTG analysis performed remotely.

Material and methods

The study was conducted at the 1st Department and Clinic of Gynecology and Obstetrics at Wroclaw Medical University, Poland. The protocol was approved by the Bioethics Committee at the university (approval No. KB-370/2018). Participants were recruited from patients admitted to the Gynecology and Obstetrics unit based on the following inclusion criteria: gestational age between 27 and 42 weeks, singleton pregnancy, and BMI before pregnancy 18.50–29.99 kg/m^2. Patients were excluded from the study in the case of: multiple pregnancy, active medical implants, lethal fetal defects, chronic or acute skin lesions, or allergy to any of the substances found in the device probes, belts or ultrasound gel. Twenty-seven patients were enrolled after providing informed consent, of which 18 provided CTG recordings for analysis. The remaining 9 patients were excluded from the study as they were unable to deliver results that met the protocol criteria (i.e., the interval between examinations was exceeded or no recording from the mobile device was delivered). A total of 164 recordings were collected, including 82 from a stationary device (Corometrics model 172; GE Healthcare, Chicago, USA) and 82 from a mobile device (Pregnabit; Nestmedic, Wrocław, Poland).

Study group

The CTG examinations included in the analysis were performed on 18 women, 27–37 years old (mean age: 32.2 years). Body mass index before pregnancy ranged from 17.8 kg/m^2 to 29.0 kg/m^2 (mean: 21.5 kg/m^2), while gestational age at the moment of inclusion was between 32 weeks and 40 weeks (mean: 35.4 weeks). All participants had an indication for increased CTG supervision, with 8 patients having a history of miscarriage or fetal death.

Study design

The patients included in the study were trained in operating the mobile device. The CTG examinations with a stationary device were carried out by midwives in accordance with standard of care, twice daily for at least 30 min per examination. Within 30 min of completing the conventional recording, the patient carried out the examination using the mobile device on her own, without any assistance from a medical professional. The 2nd examination took another 30 min. Afterwards, the patients were asked to complete the questionnaire.

A single result meant a pair of CTG recordings, one using the stationary device and the second recorded from the mobile device, obtained from the same patient, at an interval of no more than 30 min. The recording was considered technically incorrect when there was less than 20 min of FHR signal recorded or if there was no TOCO signal. Results where one of the recordings was technically incorrect were excluded from further analysis.

Primary analysis (on-site vs remote)

Tracings from the stationary device were analyzed visually on-site by the investigators (physicians); reactivity, presence of accelerations and decelerations, variability, number of fetal movements, and uterine contraction activity were assessed. Baseline fetal heart rate (BFHR) was calculated using Monako software (Institute of Medical Technology and Equipment, Zabrze, Poland). Based on the above factors, the following status' were assigned to the recordings: normal, suspicious, pathological, or technically incorrect.

The tracings obtained from the mobile device were sent directly from the device to the telemedical center (Medical Telemonitoring Center (MTC), Wrocław, Poland) via GSM communication and were assessed remotely

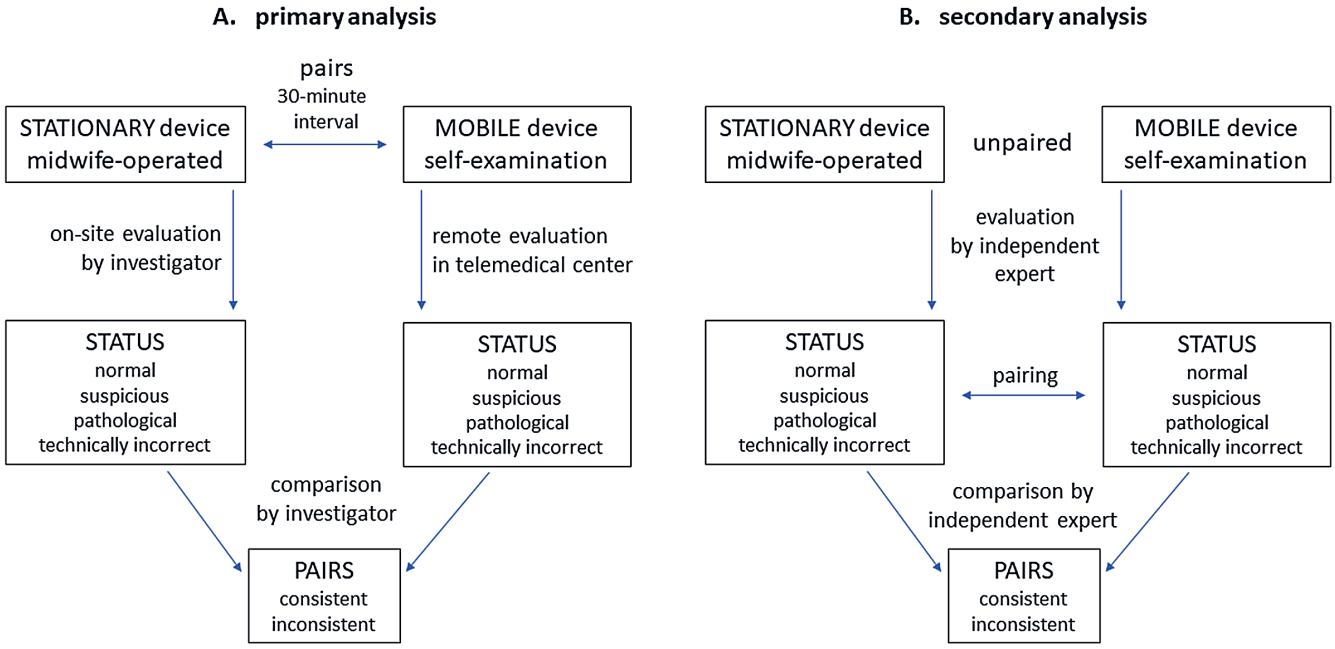

Fig. 1. Primary (A) and secondary (B) analysis schemes

by the MTC staff according to UK National Institute for Health and Care Excellence (NICE) 2001 guidelines. Reactivity, the presence of accelerations and decelerations, variability, number of fetal movements, and uterine contraction activity were assessed. Baseline fetal heart rate was calculated using the Pregnabit telemedical platform. Based on the above factors, the recordings were assigned the status normal, suspicious, pathological, or technically incorrect. Descriptions were available via the Pregnabit platform to the investigators, who further compared the recording statuses and marked the results as consistent or inconsistent. The primary analysis scheme is presented in Fig. 1A.

Secondary analysis (independent expert)

After all 82 results (pairs of tracings) were collected, and the results were coded, unpaired and delivered to an independent expert (obstetrician) who assessed them individually. All recordings were assigned a status as above (normal, suspicious, pathological, or technically incorrect) and then were paired again. The degree of agreement between the recordings in a pair was measured as consistent or inconsistent. The secondary analysis scheme is presented in Fig. 1B.

Statistical analysis

The frequency of a particular status in groups was assessed using the McNemar's χ^2 test with continuity correction. The inter-rater agreement was tested using the prevalence-adjusted bias-adjusted kappa (PABAK) coefficient for qualitative variables. Normality of BFHR measurements was assessed using the Shapiro–Wilk test. The BFHR measurements were compared with a paired t-test and Cohen's d coefficient. The agreement between BFHR measurements was analyzed using Bland–Altman methodology. The relationship between the patient's status and BMI or gestational age was examined using analysis of variance (ANOVA). Analyses were performed in R for Windows statistical software v. 3.5.2 (Microsoft Corp., Redmond, USA).[24]

Results

Feasibility

Eighty-two pairs of CTG tracings were collected (164 in total). Each pair consisted of a single tracing from the stationary device registered by a midwife and a single tracing from the mobile device registered by the patient herself, taken with an interval time no longer than 30 min.

Among the 82 recordings from the stationary device, we found 2 results (2.4%) that were inappropriate for analysis (technically incorrect) because of a recording time that was too short (less than 20 min). Among the recordings from the mobile device, 13 recordings (15.9%) were technically incorrect, 8 due to interruptions in the FHR signal longer than 10 min and 5 due to a lack of TOCO signal. Two of the recordings with no TOCO signal were marked by the telemedical center as normal due to the correct recording of FHR; however, there were annotations in the descriptions with recommendations for the following examination, to correct the positioning of the TOCO probe and to restart it after positioning.

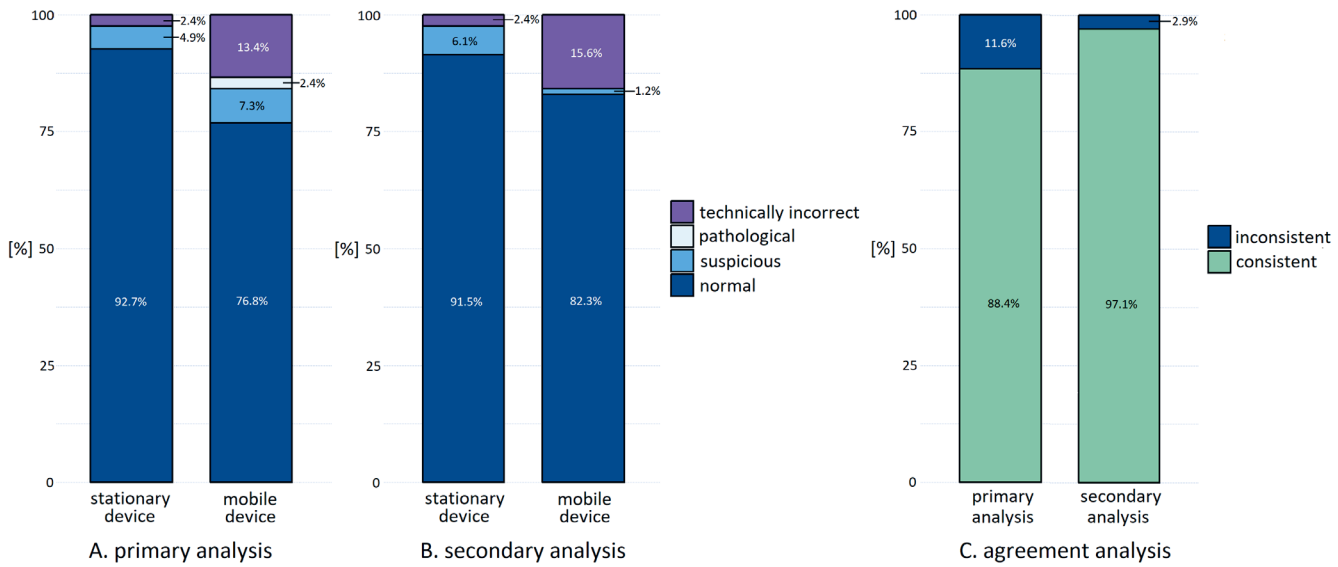

Fig. 2. A. Results of primary analysis (on-site vs remote). B. Results of secondary analysis (independent expert); C. Agreement analysis

McNemar's χ^2 test showed that the difference in frequency of the technically incorrect recordings was statistically significant (p = 0.027). Six out of 13 technically incorrect recordings using the mobile device came from only 2 patients, who we consider uncooperative (case 1: 3/6 technically incorrect recordings; case 2: 3/4 technically incorrect recordings). The poor cooperation was likely due to the fact that the patients were monitored either way and felt safe, so they did not feel the need to control the placement of the probes. Differences between the frequency of the other statuses were not statistically significant. We were unable to show that technically incorrect results occur significantly more often among patients with a higher BMI or lower gestational age.

In summary, patients were more likely to perform inconclusive recordings than experienced midwives; however, the feasibility of self-examination was satisfactory (84.1% of conclusive recordings). After removal of the 2 uncooperative patients, the percentage of conclusive examinations rose to 90.1%. Each time, in the case of a technically incorrect recording, the telemedical center gave the recommendation to 'repeat the examination' along with directions on how to place the probes correctly over the abdomen.

Diagnostic comparability

Pairs in which one of the recordings was technically incorrect were removed from further analysis; in pairs categorized as technically incorrect only due to a lack of TOCO signal, BFHR value was included into BFHR agreement analysis.

The primary analysis was performed by comparing the recording status in pairs – the status for the stationary recording was given by the investigator on-site, while the status for the mobile recording was provided remotely by the telemedical center. Both statuses were compared by the investigator. Sixty-nine pairs were included; agreement was found in 61 pairs (88.4%), while inconsistency was in 8 pairs (11.6%). The PABAK coefficient for the conformity of the quality variables was 0.77 (95% CI = 0.57–0.90) and indicated substantial agreement in the primary analysis. The results of the primary analysis are summarized in Fig. 2A. Inconsistent pairs were analyzed once again by direct comparison of the recordings to identify the causes of the inconsistency; the findings are listed in Table 1A.

There was no disagreement identified that could be attributed to the differences between the 2 devices. Two cases of disagreement occurred because of physiological or medical reasons due to the time shift between the 2 consecutive recordings. This is a consequence of the study design, as it is practically impossible to place 4 probes on the patient's abdomen simultaneously. Additionally, there is only 1 optimal location for FHR detection, thus, in the case of simultaneous recordings, one of the devices would necessarily be condemned to a worse position, which could lead to even more technically incorrect results. Five cases of disagreement resulted from inter-observer variability; this was an effect of the cautious approach of specialists in the telemonitoring center, who assessed the recordings remotely with no contact with the patients and no prior knowledge about their gestational age, which normally affects the diagnosis. One mistake was identified in the status given by the telemedical center; however, it did not change the final recommendation, which would be to 'repeat the examination' either way, for both 'suspicious' or 'technically incorrect' results.

Secondary analysis was carried out by an independent expert in order to eliminate inter-observer variability. The analysis was partially blinded as the expert was provided unpaired recordings and assessed them independently.

Table 1. Results of direct comparison of tracings from pairs assigned as inconsistent during primary (A) and secondary (B) analysis

No.	Stationary device		Mobile device		Cause of inconsistency
	status	justification of the status	status	justification of the status	
A. Primary analysis					
1	normal	normal FHR tracing despite uterine contraction activity	pathological	regular uterine contractions, normal FHR tracing	inter-observer variability – pathological status in the remote evaluation is equivalent to recommendation 'contact the doctor' according to the telemedical center standards, which is justified in case of regular uterine contractions, without the possibility of contacting the patient by phone; the specialist in the telemedical center had no prior knowledge regarding gestational age of the study participants which affected the interpretation; the signal of uterine contraction activity was identical in both recordings
2	suspicious	short-term FHR decreases <100 bpm	normal	normal FHR tracing, no uterine activity	medical cause – FHR decreased below 100 bpm, which requires further observation; however, the most probable cause in this case was accidental detection of MHR by the FHR probe (recordings shown in the Fig. 3B)
3	normal	normal FHR tracing	suspicious	no fetal movements marked by the patient	inter-observer variability – while there was no possibility to contact the patient, telemedical center indicated the need for further observation by giving the status 'suspicious'
4	normal	normal FHR tracing	suspicious	transient tachycardia	physiological cause – during the recording with mobile device, twice as many fetal movements were marked by the patient compared to the recording using the stationary device; this was probably reflected by acceleration of the heart rate (BFHR value from mobile device was 156 bpm vs 140 bpm from stationary device)
5	normal	normal FHR tracing	suspicious	FHR variability temporarily reduced; only 1 fetal movement	inter-observer variability – assessment of the telemedical center was conservative due to the lack of telephone contact with the patient
6	normal	normal FHR tracing	suspicious	lack of uterine activity tracing	wrong status given by the telemedical center – the right status was 'technically incorrect'; it was advised to repeat the examination
7	normal	normal FHR tracing	pathological	regular uterine contractions, normal FHR tracing	as in No. 1
8	normal	normal FHR tracing	suspicious	regular uterine contractions, normal FHR tracing	inter-observer variability – pathological status in the remote evaluation is equivalent to recommendation 'contact the doctor' according to the telemedical center standards, which is justified in case of regular uterine contractions, without the possibility of contacting the patient by phone; the specialist in the telemedical center had no prior knowledge regarding gestational age of the study participants which affected the interpretation; the signal of uterine contraction activity was identical in both recordings
B. Secondary analysis					
1	suspicious	FHR variability temporarily reduced	normal	normal FHR tracing, not regular uterine contractions	physiological cause – reduced variability occurring during the last 30 min of recording with the stationary device (total recording length – 1 h) may be, but not necessarily, an indication for further monitoring; result at the borderline of the standard
2	suspicious	FHR variability temporarily reduced	normal	normal FHR tracing, not regular uterine contractions	physiological cause – reduced variability in the recording with the stationary device may be, but not necessarily, an indication for further monitoring; result at the borderline of the standard

FHR – fetal heart rate; MHR – maternal heart rate; BFHR – baseline fetal heart rate.

Fifteen recordings were assigned as 'technically incorrect' – 13 from the mobile device and 2 from the stationary device. After assessment, the recordings were paired again; pairs with technically incorrect recordings (14; 17.1%) were excluded from further analysis. The remaining 68 pairs were analyzed for agreement based on the status given. Sixty-six pairs (97.1%) were found consistent, while 2 pairs (2.9%) were found to be inconsistent. The PABAK coefficient for the secondary analysis was 0.94 (95% CI = 0.80–0.99), indicating an excellent agreement. The results from the secondary analysis are summarized in Fig. 2B and 2C. Inconsistent pairs were analyzed as previously by direct comparison of the tracings to identify the causes of inconsistency; the findings are listed in Table 1B. An example of consistent tracings is shown in Fig. 3A, while an example of inconsistent recordings is shown in Fig. 3B (case 2 from Table 1A – inconsistency due to medical reason).

As before, there was no inconsistency that resulted from the differences between devices. Both pairs assigned as 'inconsistent' were due to physiological variability of FHR and referred to results at the borderline of the standard. It must be noted that neither of the 2 inconsistent pairs from the secondary analysis overlaps with any inconsistent pair from the primary analysis. This confirms that CTG is largely affected by inter-observer variability on one hand, and on the other, that the pairs identified as inconsistent were in fact technically consistent.

In order to assess the quality of remote analysis, we compared statuses given to the same recordings from the mobile device by the telemedical center and the independent expert. We found 8 disagreements amongst the assessed recordings, of which 7 coincided with inconsistencies from the primary analysis. Identified differences were attributed to the excessively conservative assessment by the telemedical center (6 cases) and to erroneous evaluation of the recordings that should not be analyzed (technically incorrect – 2 cases).

Next, the calculated BFHR values were compared. Paired t-test showed that the mean difference between 2 devices was 1.75 bpm (p = 0.017; 95% CI = 0.33–3.17), which we consider very low and clinically irrelevant. Cohen's d coefficient, which evaluates the strength of the relationship between the device used and the BFHR measurement, was 0.29 (95% CI = 0.04–0.62) and showed little effect of the device type on the BFHR value. The Bland–Altman plot suggests an acceptable range of difference between BFHR measurements of –10.1–13.6. According to the Gaussian distribution in the population, approx. 95% of observations should be contained in less than 2 standard deviations (SD). In our study, 3 observations (4.2%) go beyond the range, which is less than 5% of the permissible error. The results of the BFHR analysis are shown in Fig. 4.

Altogether, the data presented indicates that there is an excellent technical diagnostic comparability between conventional and mobile CTG devices. Remote evaluation by the telemedical center is reliable – however, sometimes too conservative and cautious, but in a way that does not adversely affect the final recommendations.

Adverse events

During the course of the study, 1 adverse event was noted. A single patient reported mild and transient skin irritation, with redness in the area where the ultrasound gel was applied while using the mobile device. The redness resolved itself within ½ h.

Patient survey

Eighteen completed questionnaires were collected. Most patients reported that CTG self-examination using the mobile device was easy (28%) or rather easy (61%), even if localization of the FHR signal was sometimes difficult (difficult – 28%; rather difficult – 50%). Eighty-nine percent of the patients declared that the possibility to perform the examination at home would increase their sense of security. The most frequently listed situations in which patients would likely use remote fetal monitoring included: 1) days off when access to the physician is limited (72%); 2) when there are identified factors that threaten the child or there is an increased risk of premature birth, but to an extent that does not require hospitalization (72%); 3) overdue pregnancy (56%).

Discussion

In a situation in which an indication for intensified CTG surveillance occurs, a physician faces the decision whether to admit the patient to the ward or recommend that she visit the clinic several times a week. Both options are inconvenient for the patient since, instead of being at home, she must stay at the hospital or in the best-case scenario travel there a few times a week. This situation also results in an overcapacity in hospital wards, extended queues at the clinic and medical personnel overwork, as well as an increase in the cost of prenatal care.

Another option that has emerged in recent years is remote fetal surveillance with the use of mobile CTG devices; however, this is still rarely used due to limited trust in telemedical solutions. The lack of trust results partly from poor experiences and lack of knowledge of these solutions, and partly from limited scientific data that confirms its clinical effectiveness and safety.

In this study, we aimed to compare the diagnostic usefulness of CTG self-examination using a mobile device to examination performed by a medical professional using a stationary device, and to evaluate the quality of CTG analysis performed remotely. We found that technically, the mobile and the stationary devices are indistinguishable, as shown by the mean difference in the BFHR

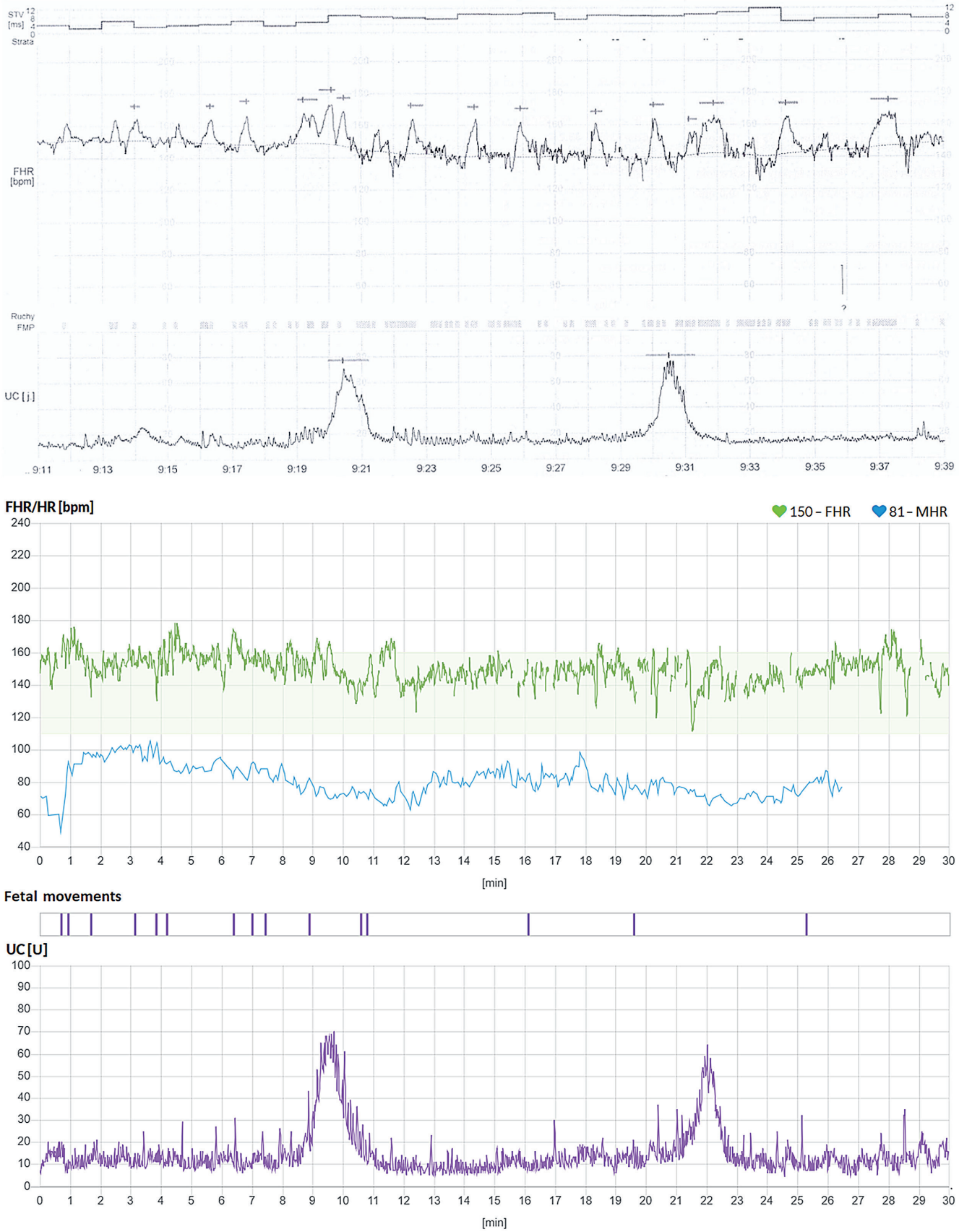

Fig. 3. A – pair of consistent recordings: upper – stationary device, lower – mobile device: upper – stationary device (suspicious), lower – mobile device (normal). Identified reasons of evaluation disagreement were FHR decreases <100 bpm during the 1st recording, which most likely resulted from accidental detection of maternal heart rate by the FHR probe

Fig. 3. B – pair of inconsistent recordings: upper – stationary device (suspicious), lower – mobile device (normal). Identified reasons of evaluation disagreement were FHR decreases <100 bpm during the 1st recording, which most likely resulted from accidental detection of maternal heart rate by the FHR probe

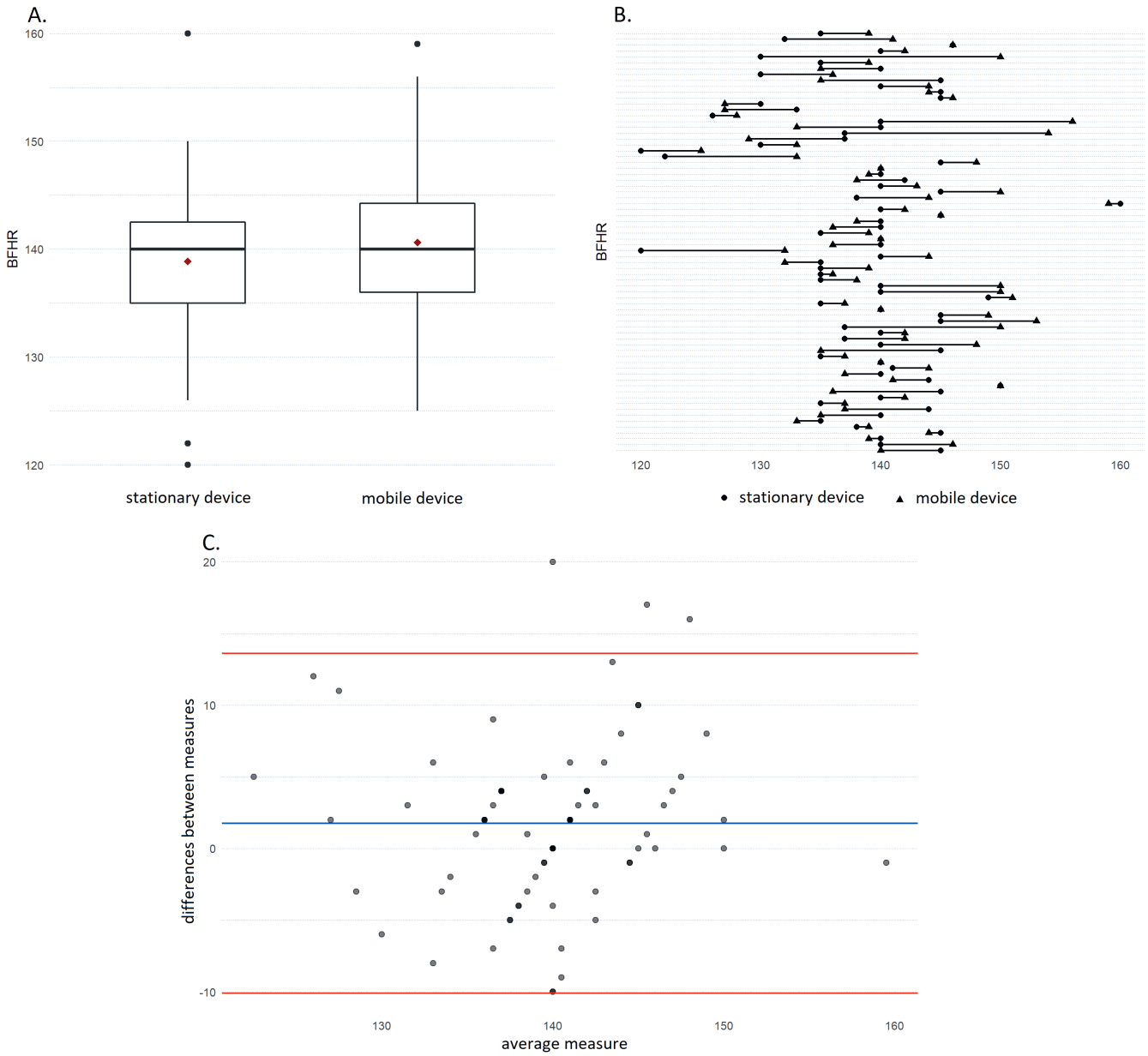

Fig. 4. Results of BFHR analysis: A. Box chart of BFHR measurements – the horizontal line represents the median, the dot represents the mean; B. Differences in BFHR values in pairs; C. Bland–Altman plot

measurement at 1.75 bpm (less than the permitted measurement error of FHR probes, which is 2 bpm), comparison of CTG tracing assessments (97.1% of consistent diagnoses in the independent expert analysis) and direct comparison of the recording graphs – even if the assessment of the recorded pairs was inconsistent, we were not able to identify any technical differences. Discrepancies resulted either from inter-observer variability or the study design, in which the time shift between the 2 examinations led to a physiological change of the fetus's state, which was reflected in the FHR recording.

Inter-observer variability is a known characteristic of CTG,[25,26] as the diagnosis is mostly based on the subjective assessment of the graphical record and can be related to the professional experience of the interpreter. Variability is the most prominent in borderline situations when the decision made can be either more or less cautious. A conservative approach was seen in telemedical center interpretations; however, under the study conditions, we found them justifiable. There were 2 main limitations for the center staff to make more accurate assessments: 1) no access to additional information about the patient and her condition, as it was impossible to contact the patient by phone (patients were hospitalized), which is standard in the center's normal operation; and 2) no information about the patient's gestational age (due to data anonymization), which strongly influences the diagnosis. In the situations described in Table 1 (No. 1, 7 and 8), the recordings were remotely described as pathological or suspicious only because of regular contraction activity

of the uterus, while the FHR signal was physiological. It may seem exaggerated, however. If it concerned a patient under 36 weeks of pregnancy, regular contractions could be a sign of preterm delivery, which would definitely be a reason to contact the doctor ('pathological' status in the center's operation is equal to the recommendation to contact a doctor) or at least for further intensified surveillance. Such doubts do not apply to investigators on-site who had constant supervision over the patient.

One can conclude that excessively cautious assessments can lead to unnecessary visits to the clinic or unnecessary medical interventions; however, the literature does not confirm that remote fetal monitoring leads to either of them. On the contrary, the number of unnecessary visits decreases, while the number of C-sections remains at a comparable level. The experience of an American obstetrics team running a remote prenatal care program, called ANGEL shows that based on a single telephone contact with the midwife, 676 emergency visits were avoided in 2016.[27] This number could possibly be even greater with access to remote NST. This is why we consider direct contact with the patient crucial in remote monitoring. In a study where women undergoing labor induction with slow-releasing dinoprostone at home with constant remote monitoring of the fetus, it was shown on the basis of women's declarations, that contact with a medical professional is a prerequisite for feeling safe and secure. Women who had problems with reaching the hospital by phone experienced increased anxiety.[28] The C-section rate for patients with diabetes managed by the ANGEL program was 26%, compared to the 34% US national rate.[27]

Based on our study results, we assess the feasibility of CTG self-examination as satisfactory. After exclusion of the results of 2 poorly cooperating patients, the percentage of recordings appropriate for analysis was 90.1%. Eighty-nine percent of patients who performed unassisted CTG examinations judged it as easy or rather easy to perform. Also 89% of the study participants declared that the possibility of performing NST at home would increase their sense of security. This often-neglected aspect is, however, worth considering in obstetric practice, especially when taking care of high-risk patients or patients with a history of intrauterine fetal death. Pregnancy with such a history is an emotionally challenging life event for women and adequate support during pregnancy should be considered as an essential component of quality maternal care.[29]

The academic community needs to develop evidence that will constitute the basis for the development of new care delivery models, including remote monitoring, as well as methods for providing the best patient-centered care. It is important that the future of medicine be determined by solid research and education.[30] Our study provided the evidence that mobile CTG devices present an equivalent diagnostic value to conventional devices, and that remote recording evaluation is as reliable as on-site analysis and thus can be implemented in perinatal care.

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Psychometric validation of Corah's Dental Anxiety Scale in the Polish population

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Abstract

Background. Corah's Dental Anxiety Scale (DAS) is one of the most popular psychological scales used for diagnosing odontophobia worldwide. Despite being used by Polish researchers, it has never been validated in the Polish population. Also, there are no similar tools that could be used by dentists for screening.

Objectives. The aim of this study was to validate and present the psychometric properties of the Dental Anxiety Scale adapted to Polish. The scale is a self-assessment tool designed to measure odontophobia and dental anxiety.

Material and methods. The sample consisted of 162 adults. The adaptation to Polish of the DAS scale included a back-translation and a think-aloud study. The validation procedure incorporated 3 basic methods to be applied in the reliability analysis – the test-retest method, the statistical properties analysis of test items as well as a factor analysis. The general result of the responders was also compared to the anxiety-trait STAI scale and the neuroticism scale form IPIP-BMF-20.

Results. The Cronbach's α was 0.9. The solution obtained in the exploratory factor analysis was a one-factor model, explaining 76.24% of the variance of responses. The test-retest gave a strong correlation (rho = 0.69, $p < 0.001$). The correlations between the DAS score, the anxiety-trait STAI score and the neuroticism scale form IPIP-BMF-20 were moderate, as expected. The percent of responders with odontophobia and intense dental anxiety was almost similar to the results of other studies.

Conclusions. The psychometric characteristics of the Polish adaptation of the DAS scale are similar to those reported in the original version. The results allow us to recommend the method for scientific research and patient screening. However, further analyses are necessary to assess if the scores indicating odontophobia and dental anxiety are similar in Poland and in the USA.

Key words: dental anxiety, odontophobia, psychology

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Introduction

The ability to both psychologically and physically adapt to the surrounding, ever-changing world is a prerequisite for the survival of every species. One of the main defense mechanisms against threats is escape, which in the psychological sphere is reflected by the feeling of anxiety and fear. Properly functioning neurological centers of anxiety fulfill the evolutionary protective function and enable the individual to react adequately in order to avoid danger.[1]

Anxiety is defined as an unpleasant emotional state and is a physiological reaction to a stimulus being a potential threat. It accompanies every human being almost from the moment of birth. Fear, on the other hand, is a fully conscious reaction to danger, promoting the cautious behavior of the individual.[2] Excessive fear may paralyze and interfere with the rational thought processes, and in the long-term it may intensify or become an independent etiological factor of many different somatic diseases, such as cardiovascular disorders or diabetes.[3,4]

A particular type of anxiety disorder is phobia. Its main symptom is the persistent fear of specific situations, phenomena or objects, leading to their avoidance at all costs. The stimuli that induce pathological anxiety reactions are in this case situations or objects that even in the patient's own opinion do not pose a threat, and people affected by the phobia are aware of the irrationality of their feelings.[5] These anxiety disorders are a significant social problem and have even been included as a disease entity in the International Statistical Classification of Diseases and Related Health Problems ICD-10.[6]

Odontophobia is one of the most common phobic disorders and may affect up to 20% of the population, but the results depend to a large extent on the questionnaire used.[7,8] Corah's Dental Anxiety Scale (DAS) usually shows slightly lower results than other psychological tools. Odontophobia is defined as an irrational and difficult to control fear of visiting a dentist, which is often the effect of past experiences, especially from childhood, a time when the physician had no possibility of providing total pain control. This fear is sometimes so strong that despite the existence of disturbing oral complaints associated with negligence in the dentition, patients postpone going to the dentist.[9]

Dental fear consists of 3 components: fear of pain, of injury to the body and of the unknown. Physiological, behavioral and psychological methods are used to assess the anxiety of dental patients. Physiological methods are based on the assessment of measurable and observable reactions of the body to stress stimuli. They include the following: measurement of the blood pressure, heart rate assessment, changes in muscle tone, perspiration, and reduction of salivation. Behavioral methods refer to the use of behavioral scales that use subjective doctor's opinion about changes in the patient's behavior and are mainly used in children. Psychological methods are based on the analysis of special self-assessment questionnaires, elaborated by the psychologists.[10]

One of the most popular psychological tools used to assess the level of dental anxiety is DAS, created by Corah in 1969. It is a simple questionnaire consisting of 4 items, each containing 5 possible answers. Each answer has an assigned numeric value (1–5). The sum of the values assigned for each response is the result of the test and can vary from 4 to 20 points. A score of 17–20 points indicates odontophobia (Table 1).

Table 1. The relationship between the DAS test result and the patient's level of anxiety according to Corah[29]

DAS result	Dental anxiety level
<9	low
9–12	moderate
13–14	high
15–20	very high

Despite its creation in 1969 and the existence of slight modifications, usually enriching the original test with 1 additional question, it is still one of the most frequent tools used to analyze the level of dental anxiety and is applicable worldwide.[11–14] In the studies of the Polish population, DAS was used numerous times at the turn of the 20th and 21st centuries, yet it has never been validated, only translated.[15–21]

The aim of this study is to present the psychometric properties of the Polish adaptation of the Corah's DAS. By validation we mean measuring the accuracy and reliability of the method.[22] In the case of DAS, it was impossible to determine its validity by comparing it with other methods examining the severity of dental anxiety, as it is the first tool of this type in Poland. In order to estimate the accuracy of the scale, it was decided that it should be combined with the anxiety-trait subscale of the State-Trait Anxiety Inventory (STAI) questionnaire, where anxiety is understood as a permanent personality attribute, as well as with the neuroticism subscale from the International Personality Item Pool-Big Five Markers-20 (IPIP-BFM-20) questionnaire based on Big Five personality theory.[23–25] Both tools have excellent psychometric properties. Neuroticism, as well as its cortical aspect, the tendency to react with fear,[26] are predictors of anxiety in the situation of a visit to the dentist, although the correlation is weak.[27] It was assumed that the percentage of people with odontophobia and severe anxiety according to the original scale would help us gauge the accuracy of the Polish adaptation. The reliability of the tool was examined by calculating Cronbach's α, as well as using the test–retest method.

Description and psychometric characteristics of the original scale

The DAS by Norman L. Corah is a simple tool, perfectly suited to screening dental anxiety and odontophobia in dental patients who require special treatment and special attention from their dentist.[28,29] The questionnaire consists of 4 items describing successively situations related to dental

treatment: appointment at the dentist the next day, waiting in the dentist's waiting room, waiting on a dental chair for drilling, and finally a rich description of waiting for tooth scraping near the gums during the removal of tartar. For each item, the subject has to respond using a 5-point scale.

The original validation studies performed on the student population showed that the scale is characterized by a high reliability ratio – Kuder–Richardson coefficient was 0.86, although due to the fact that the scale is not dichotomous, it would be more appropriate to calculate Cronbach's α.[30] The correlation between the first result and the retest after about 3 months was 0.82, which indicates a high stability of results over time. In addition, because fear of the dentist is met with considerable understanding and social acceptance, the respondents did not show a tendency to provide insincere answers.

Material and methods

The language validation procedure was carried out in accordance with the guidelines described in the literature.[31] Because the author of the DAS scale, N.L. Corah, died in 2001, we first obtained the official written permission of the publisher. Then, the instructions and test items were translated into English, leading to the development of the initial language version of the DAS scale, which was then subjected to the retranslation procedure. The language version created this way has been checked and corrected by experts fluent in English and with expert knowledge – psychologists and linguists. The scale obtained satisfactory results of Gunning's fog index. The 4 items were translated into Polish as follows:

1. If you had to go to the dentist tomorrow, how would you feel about it?
2. When you are waiting in the dentist's office for your turn in the chair, how do you feel?
3. When you are in the dentist's chair waiting while she/he gets her/his drill ready to begin working on your teeth, how do you feel?
4. You are in the dentist's chair to have your teeth cleaned. While you are waiting and the dentist is getting out the instruments which she/he will use to scrape your teeth around the gums, how do you feel?

For the 1st question, the Polish adaptation of the scale of answers looks as follows:

a) I would look forward to it as a reasonably enjoyable experience.
b) I wouldn't care one way or the other.
c) I would be a little uneasy about it.
d) I would be afraid that it would be unpleasant and painful.
e) I would be very frightened of what the dentist might do.

For the remaining 3 test items, the response scale, also 5-level, describes the feelings of a person using dental services:

a) relaxed,
b) a little uneasy,
c) tense,
d) anxious,
e) so anxious that I sometimes break out in a sweat or almost feel physically sick.

After considering experts' comments, an experimental version of the scale was created, which was used in a think-aloud pilot study conducted among 23 psychology students.[32] These persons informed the investigators how they understand individual test items, and the researchers checked the compatibility of the interpretation with the original meaning of the items. This allowed for the official approval of the Polish language version of the questionnaire and for it to be subjected to a psychometric assessment process.

Results

One hundred sixty-two students participated in the study of the validation procedure. The specificity and number of participants is similar to the one in the original scale. Both the Bartlett sphericity test (χ^2 = 387,738, degrees of freedom (df) = 6, $p < 0.001$) and the Kaiser–Meyer–Olkin test (0.828) indicate the adequacy of analyzing the matrix for the existence of common factors, and thus mean that the sample number and diversity are adequate. Then, an analysis of the discriminative power of the questionnaire items was carried out. The results are presented in Table 2.

The discriminatory power of the analyzed items is satisfactory – the discrimination factor of all 4 significantly exceeds 0.2. In order to verify the internal structure, an exploratory factor analysis was carried out, which showed that all the items are loading 1 strong factor, explaining 76.24% of the responses variance.

In the Polish adaptation of the DAS, the Cronbach's α reliability coefficient was 0.898, which is a very good result for a scale of only 4 items and shows that it is a reliable tool that can successfully be used for screening the level of dental anxiety. To determine the absolute stability coefficient of the tool, 47 students were examined twice at an interval of 2 months. The correlation of the obtained results was 0.69 ($p < 0.001$), which indicates satisfactory stability of the scale over time. To determine the validity of the tool, the results of 32 subjects on the DAS scale were compared with the scale of anxiety as a trait of the STAI questionnaire and with the neuroticism scale of the IPIP-BFM-20 questionnaire. As expected, the result was moderate in both cases – rho = 0.314 and rho = 0.38, respectively ($p < 0.05$).

Discussion

The aim of the above studies was to create the Polish adaptation of the DAS scale, allowing screening to identify patients with odontophobia and increased anxiety, and

Table 2. Discrimination rate of test items (N = 162)

Item	Item-total correlation	M	SD
1. If you had to go to the dentist tomorrow, how would you feel about it?	0.795	2.97	1.043
2. When you are waiting in the dentist's office for your turn in the chair, how do you feel?	0.845	2.41	1.092
3. When you are in the dentist's chair waiting while she/he gets her/his drill ready to begin working on your teeth, how do you feel?	0.718	2.82	1.005
4. You are in the dentist's chair to have your teeth cleaned. While you are waiting and the dentist is getting out the instruments which she/he will use to scrape your teeth around the gums, how do you feel?	0.691	2.48	1.018
Total DAS score		10.68	3.632

M – mean; SD – standard deviation.

to determine its psychometric characteristics. In the sample collected during the validation of the DAS-PL scale, 5.9% of the responders reached a score of 17 points and higher, allowing for odontophobia diagnosis, and another 18.7% obtained the result between 15 and 17 points, allowing us to suspect quite intense anxiety. This result is almost similar to the one obtained in the study of the Łódz region population, in which, however, another non-validated method was used.[33] It is also similar to the results achieved in other studies with the original version of the DAS scale and its adaptations, which is another premise of its validity.

Numerous studies of the level of dental anxiety and odontophobia with the use of tools that have only been translated without a full cultural adaptation and validation procedure indicate the urgent need for such a tool. It will also be useful in the dental practice as a quick and easy screening tool. The use of such scales is recommended as the golden standard for treatment,[34] and for example in the UK, they are used by about 20% of dentists, surprisingly men more often than women.[35]

Conclusions

On the basis of all the analyzed psychometric characteristics, it can be concluded that the Polish adaptation of the DAS scale does not deviate significantly from the original one and that it has good psychometric properties. However, it has not yet been normalized. The adopted values of 15–17 points for high anxiety and 17–20 points for odontophobia have been established on the basis of American population studies; however, a similar percentage of people with odontophobia and intense anxiety in the Polish sample indicate that they are probably adequate. We can, therefore, recommend using the DAS scale with the awareness of its limitations for screening in dental offices and for scientific research.

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Micromorphological assessment of bone tissue remodeling in various hip degeneration conditions

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Abstract

Background. The reorganization of bone tissue is closely associated with its metabolism and changes in its internal structure. Metabolism of the bone, which results from the simultaneous processes of resorption and formation of new bone tissue, may depend on the presence and type of arthritis.

Objectives. The objective of the study was to assess, based on the morphological features and mineral composition of bone tissue, changes in the femoral head in various types of hip joint degeneration.

Material and methods. The study group consisted of 21 patients surgically treated for hip joint degeneration. They included 17 women, aged 30–70 years (mean age 52.5 years), and 4 men, aged 38–51 (mean age 48.5 years). The assessment of the morphological condition of the bone and the mineral composition of bone tissue took into account quantitative and qualitative relationships among the mineral components and bone matrix. The structure of spongious bone tissue was analyzed in histological studies, with special attention paid to osteogenesis and osteoclastic processes and the advancement of degeneration.

Results. Three main types of degenerative changes in bone tissue of the examined femoral head were recognized: osteoporosis with a prevalence of coarse-fiber bone tissue and decreased osteogenic activity; osteolysis with few osteogenesis centers; and intensified reorganization of bone tissue. In more than half of the examined samples, coarse-fiber bone tissue was replaced by newly formed bone tissue. We observed bone resorption and osteogenesis, which indicate normal homeostasis of the bone tissue. Uneven saturation of spongious bone with mineral components was found. The content of organic and inorganic bone components measured with Ca : P and C : Ca + P ratios had similar values in all types of changes. Only the bone with intense osteolysis contained a smaller quantity of carbon (4.96–8.13%).

Conclusions. Our observations indicate an intense adaptive reorganization of bone tissue depending on external and internal factors, including biomechanical condition.

Key words: bone, remodeling, micromorphometry, hip joint degeneration

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Introduction

Bone tissue is a specialized connective tissue which plays a mechanical and metabolic role; its characteristic feature is constant reorganization and adaptation of its structure to external factors.[1] Changes in internal structure and metabolism of bone which result from the simultaneous processes of resorption and formation of new bone tissue have been described by Frost[2] and termed the adaptive restructuration of bone. Contrary to intra-osseous changes, Frost[3] called shaping of external structure of the bone modelling. According to Frost's theory, tensions arising in bone tissue under the effect of load cause microcracks of the trabeculae, which in turn stimulates the processes of bone reorganization.[4]

There are many theories on the effect of electric potential and inter-cellular liquid on bone tissue reorganization,[5,6] though most authors believe that the main role in the process is played by mechanical factors, such as form distortion, the energy of form distortion and the density or index of form distortion of the bone.[3,7–13] The reorganization of bone tissue is closely associated with bone metabolism.[14] The mechanical properties of bone are determined by the quantity of organic and inorganic components and the structure of bone trabeculae, which are composed of collagen fibers and the hydroxyapatite crystals deposited on them.[15,16] Experimental studies on the mechanical resistance of bone tissue indicate that the Young elasticity module for a hydroxyapatite crystal is 114 GPa and is nearly 10 times higher than the Young axial module of a collagen molecule, which is 1.41 GPa,[17–19] thus the quantity and proper structure of hydroxyapatite has a decisive effect on the mechanical resistance of the bone. The organic components (organic matrix) of bone tissue constitute 34.9–41% of the bone mass, mineral components approx. 50%, water 15.5–27%, and volatile inorganic components 4.2–4.6%. The bone matrix consists of approx. 90% collagen; the remaining 10% is non-collagen proteins. About 50% of the mineral substances form a part of hydroxyapatite Ca10(PO4)6(OH)2. In its combusted form, the mineral substances constitute 33.9–39.9% of the bone mass.[20]

The proportions and quantitative changes of organic (collagen) and mineral (hydroxyapatite) components are described using the ratio of carbon (C) to the combined quantity of calcium and phosphorus (Ca + P). Normal proportions of calcium and phosphorus in an ideally built hydroxyapatite crystal in vitro are expressed using a molar calcium-to-phosphorus ratio (Ca/P) of 1.67:1.[21] Experimental studies on hydroxyapatite structure revealed that in vivo other elements – especially heavy metal ions – could replace calcium ions in hydroxyapatite crystals, change their chemical composition and structure, and lead to a deterioration of their mechanical properties. Considering the effect of habitat factors and diet, the molar calcium-to-phosphorus ratio (Ca : P) in the hydroxyapatite crystals of human bones under normal conditions was determined to be 2:1.[22,23] There are no corresponding estimates for a normal carbon-to-combined calcium and phosphorus (C : Ca + P) ratio. It is believed that disturbances in the quantity of mineral components, especially hydroxyapatite, affect the quality of bone tissue.

This study was aimed at determining, on the basis of morphological state and the mineral composition of bone tissue, the changes taking place in the femoral head in various types of hip joint degeneration.

Material and methods

Samples from 21 patients surgically treated for hip joint degeneration at the Department and Clinic of Orthopedic and Traumatological Surgery in the Faculty of Postgraduate Medical Training of Wroclaw Medical University, Poland, were used in the study. The study group consisted of 17 women aged 30–70 years (mean 52.5 years) and 4 men aged 38–51 (mean 48.5) (Table 1).

Secondary reasons for hip joint degeneration were diagnosed in 13 patients (61.9%) and primary reasons in 8 patients (38.1%). Degeneration following inborn hip dysplasia developed in 5 patients (23.8%), stemming from rheumatoid arthritis in 7 patients (33.3%) and as a result of injury in 1 patient (4.8%).

Table 1. Number, sex and age (range and mean) of patients in the study population

Variable	Number of patients	Age range	Mean age
Whole population	21	30–70	51
Women	17	30–70	52.5
Men	4	38–51	48.5

The assessment of morphological condition and the mineral composition of bone tissue, considering the quantitative and qualitative relationships between mineral components and bone matrix for various types of hip degeneration, was based on the femoral heads extracted from 21 patients during hip replacement operations. The histological examination and mineral composition analysis were carried out at the Department of Histology and Embryology, Chair of Anatomy and Histology of the Faculty of Veterinary Medicine at the Natural History University in Wrocław.

Depending on the type of morphological changes observed in histological examination, the samples were divided into 3 groups and a mineralographic analysis was carried out. Statistical analysis of the results of the mineralographic studies was then performed using the data on the morphological changes observed in the tissue during histological examination.

Assessment of histological structure and mineral composition of the bone tissue

The surgically removed femoral heads were cut along the diameter and samples of spongious bone tissue were taken from the central and peripheral parts (Fig. 1). The material for histological examination was fixed in 4% buffered formalin. After decalcification, the bone block was dehydrated and embedded in paraffin. The material for electron microscope studies was fixed in glutaraldehyde on phosphate buffer 0.1N, dried in critical point and gold-coated. Sections with 10 μm in thickness were stained with hematoxylin and eosin (H&E), according to van Gieson and Schmorl.

The structure of spongious bone tissue was examined histologically, with special attention being paid to osteogenic and osteoclastic processes and the advancement of degenerative changes. Electron microscope analysis was conducted with a LEO 435 Scanning Electron Microscope (SEM) with a ROENTEC Roentgen microprobe (Leo/Zeiss, Jena, Germany). Surface analysis of the mineral composition was carried out at constant magnification of ×500 and an accelerating voltage of 20 keV.

Fig. 1. Cross-section of a femoral head from which samples for histological and mineralogical analysis were taken

Results

Histological examination

Three types of changes were observed during histological examination of the femoral heads which were surgically removed from the patients:

1. osteoporosis with immature coarse-fiber bone tissue and low osteogenic activity;
2. osteolysis with osteogenesis centers; and
3. intense bone reorganization.

Osteoporosis with immature coarse-fiber bone tissue and low osteogenic activity, of varying intensity, were observed in the femoral heads of 6 patients.

The histological slides of this group of patients showed numerous areas occupied by fibrous bone tissue with blood vessels and fat cells within it. The trabeculae were thinner than in other groups of patients; on their surfaces, osteoclasts were observed sporadically and, more often, osteoblasts (Fig. 2A). In extensive areas, the trabeculae were covered by bone surface cells, which indicates low metabolic activity of bone tissue in such areas (Fig. 2B). Some slides showed degenerative changes within the cartilage, with numerous lesions of joint cartilage (Fig. 3).

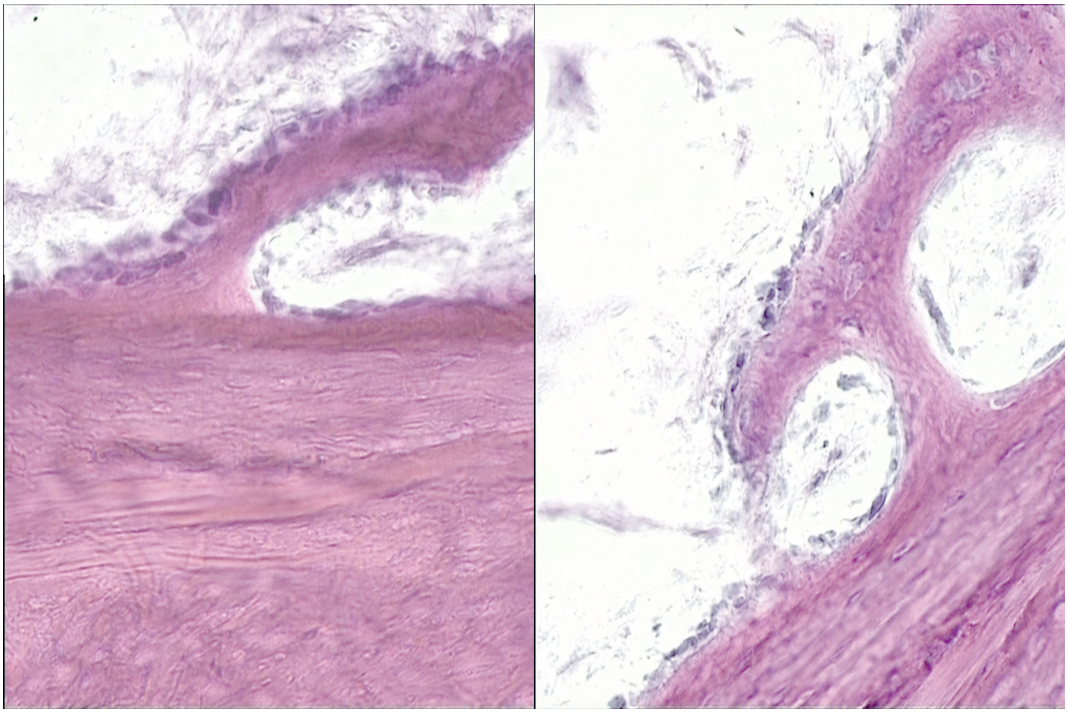

Fig. 2. A. Osteoblasts on the surface of bone trabeculae. H&E staining, ×400 magnification. B. Trabeculae without cells on the surface or between them; visible fat cells and reticulate tissue; center of trabeculae built of coarse-fiber bone tissue covered by 1 lamella of secondary bone with few osteocytes. H&E staining, ×100 magnification

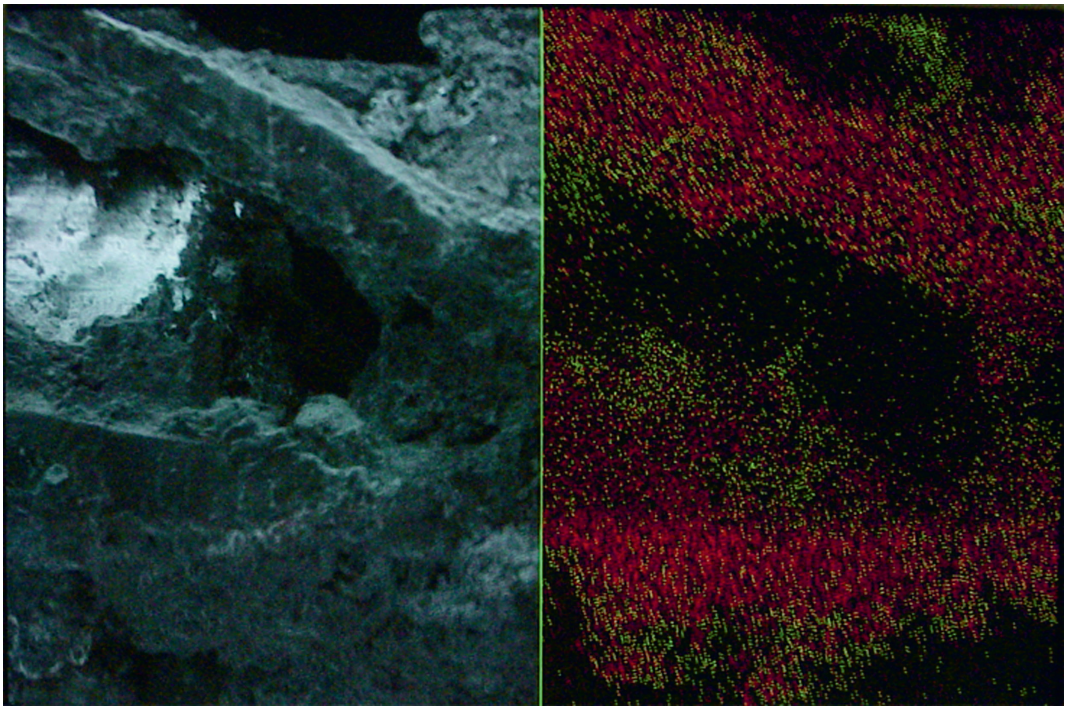

Fig. 3. Elements distribution in the bone beneath the articular surface of a femoral head surface

Red – carbon;
green – calcium;
yellow – phosphorus SEM, ×1000.

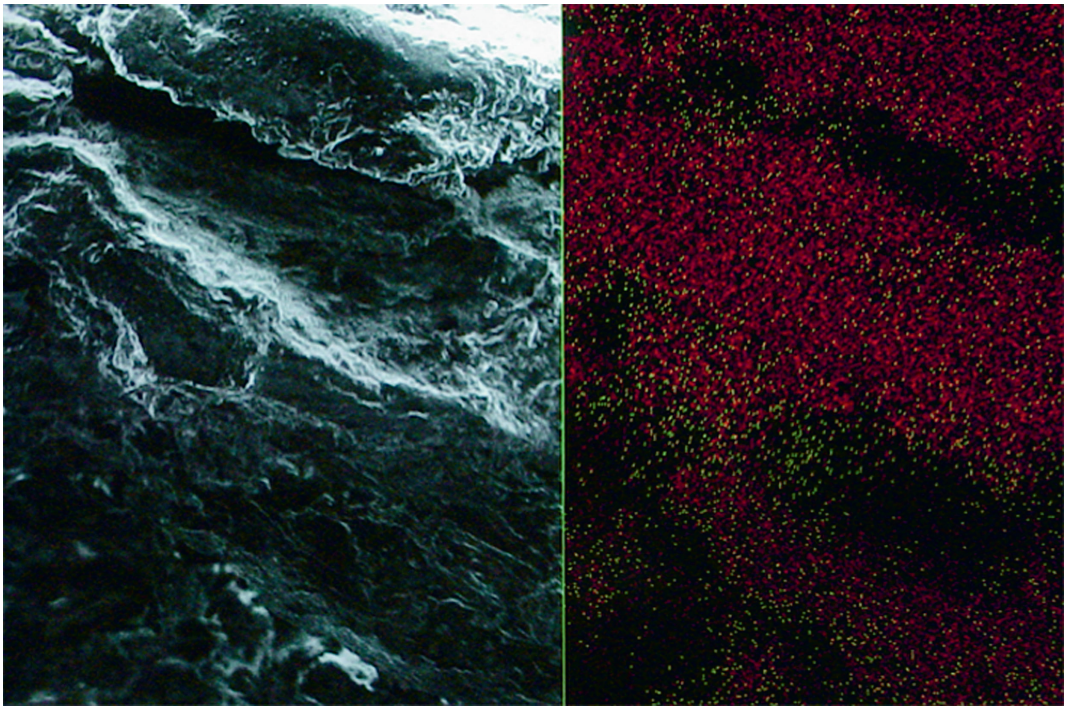

Fig. 4. Elements distribution in area near "cyst-like changes". On the surface of the trabecula there are no active cells of bone tissue. In the center, there are fibers of connective tissue with blood vessels.

Red – carbon;
green – calcium;
yellow – phosphorus SEM, ×1000.

No activity of osteogenic cells or osteoclasts was observed in the subcartilaginous part and within the spongious bone. Fibroblasts synthesizing collagen fibers were visible in areas below the joint cartilage. In 1 case, among irregularly arranged trabeculae, numerous fibrous structures were present; they were built of collagen fibers with fat cells, with numerous fibrocytes and fibroblasts in between (Fig. 4).

Osteolysis with few osteogenesis centers, with varying intensity, was observed in 4 patients. In areas located close to the articulation surface, extensive osteolysis was observed, aided by neutrophil granulocytes; in its center, numerous fibroblasts, fat cells and developing blood vessels were found. Outside the osteolysis area, the multiplication of connective tissue cells and intensified angiogenesis were observed. In places remote from the osteolysis areas, few centers of repair osteogenesis were noted (Fig. 5A).

Extensive "cyst-like changes" were observed within the trabeculae. Such areas inside were filled by numerous cells of fibroblastic lineage synthesizing connective tissue

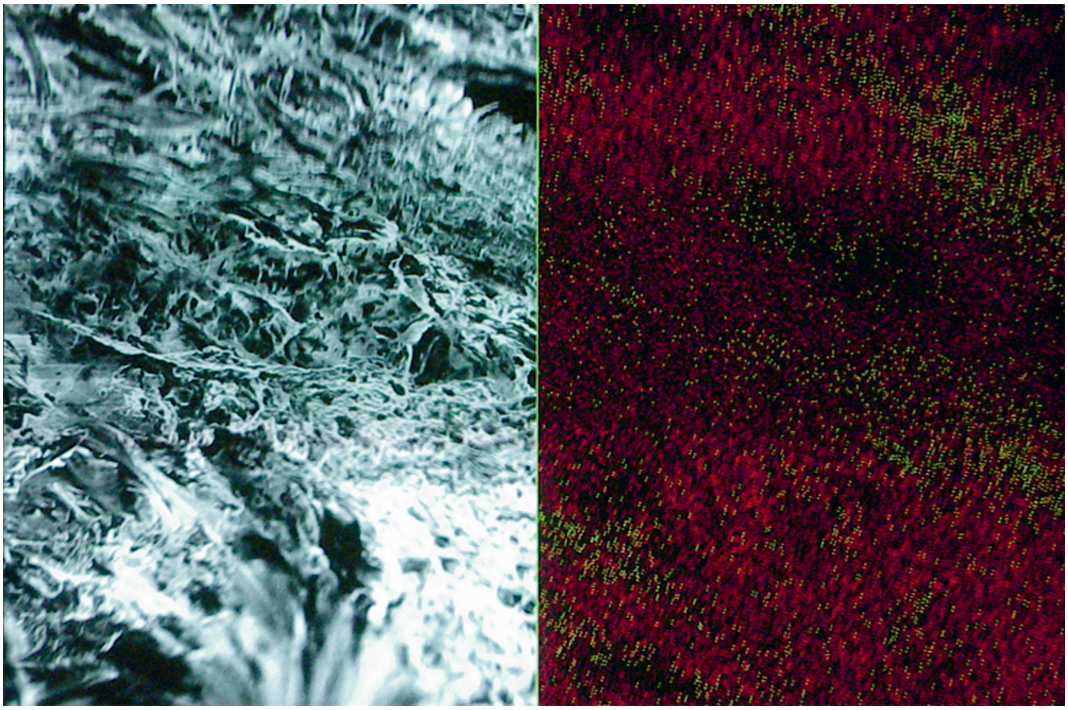

Fig. 5. Elements distribution in area near "cyst-like changes". In the center, there are numerous collagen fibers of connective tissue.

Red – carbon; green – calcium; yellow – phosphorus SEM, ×1000.

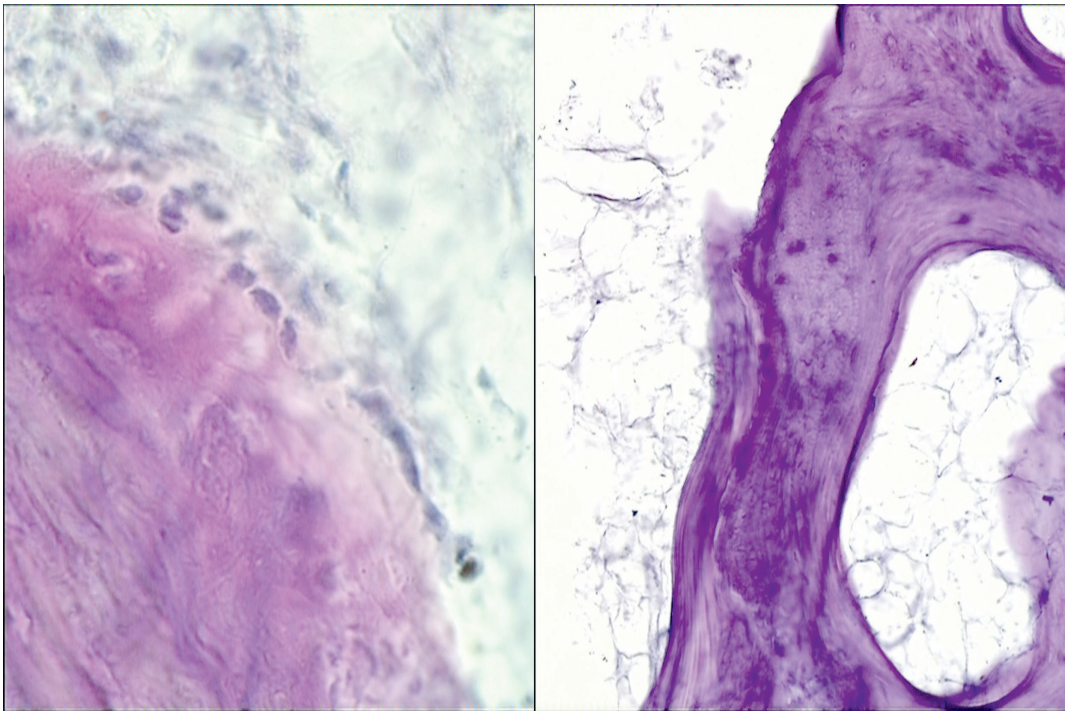

Fig. 6. A. Coarse-fiber bone tissue resorbed by osteoclasts and mononucleate cells (left). H&E staining, ×200 magnification. B. Bone trabecula; no cells are on the surface or between the trabeculae. Fat cells and reticulate tissue are visible. H&E staining, ×100 magnification

fibers which were randomly arranged (Fig. 5B). Outside these areas, osteoclasts, mononuclear macrophages and cells of the lymphatic–granulocytic system were visible (Fig. 6A). In the remaining areas, the trabeculae showed an irregular course and abundant fibrous connective tissue between them. Histological slides from 2 patients showed trabeculae which were much thinner than those of the remaining patients from this group, and small quantities of connective tissue and fat cells were observed between them (Fig. 6B).

Degeneration of joint cartilage was also observed, with numerous lesions and degenerating chondrocytes (Fig. 7). Intense bone reorganization, with varied intensity, was observed in 14 patients. The joint cartilage showed large surface lesions and a considerable degeneration of the chondrocytes. On some slides near joint cartilage, the proliferation of fibroblastic lineage cells was also observed, appearing as fibrous areas below the joint cartilage (Fig. 8).

Differentiated areas of coarse-fiber bone tissue predominated in the subcartilage tissue (Fig. 9). Areas of drastic

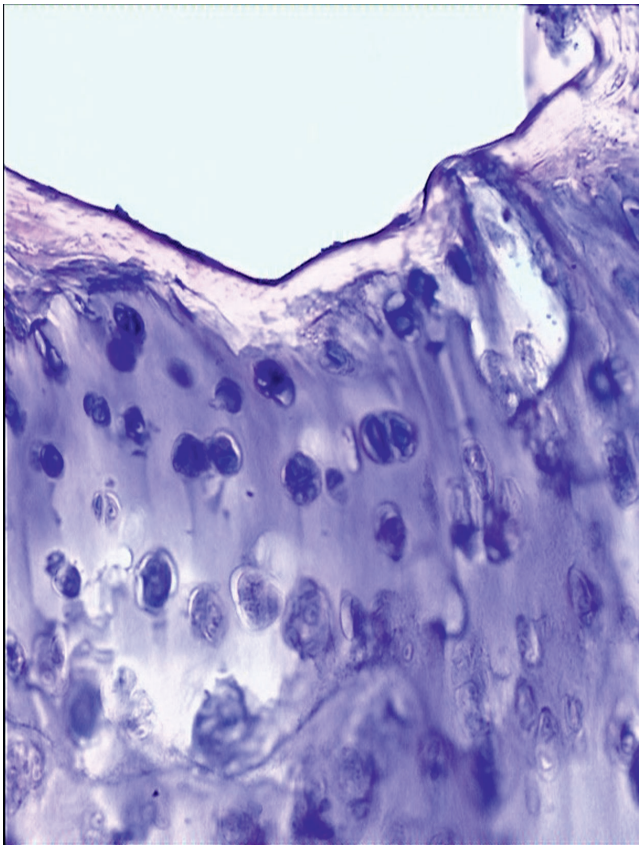

Fig. 7. A. Abnormal joint cartilage; visible stratification (right) of cartilage located directly above a biologically inactive area of bone tissue. H&E staining, ×100 magnification. B. Degenerating joint cartilage. The visible changes within the chondrocytes indicate progressing degenerative changes. H&E staining, ×200 magnification

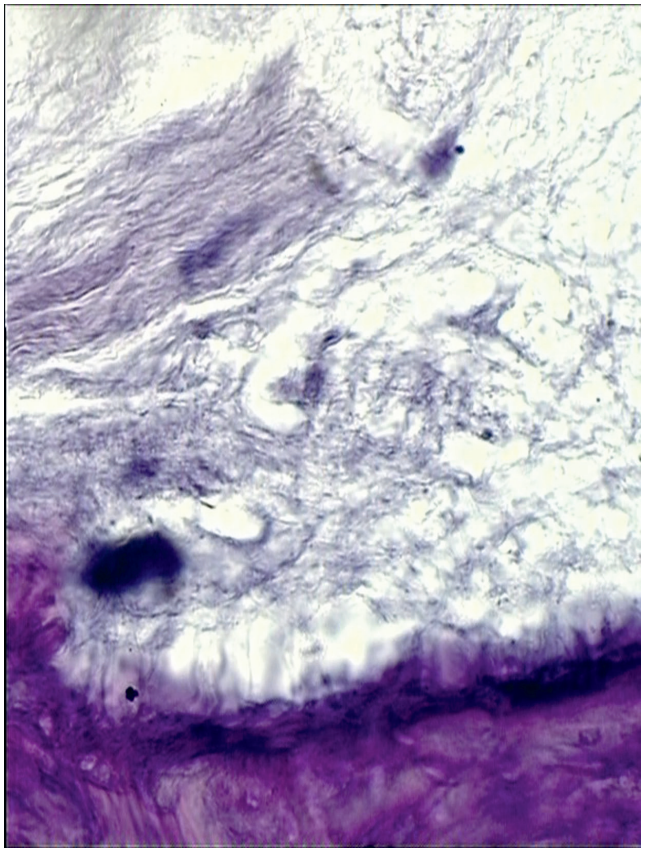

Fig. 8. Greatly reduced articular surface of the femoral head. Few isogenic groups containing degenerating chondrocytes. Cartilage on the side opposite of the articular surface shows structural disturbances manifested as irregular invaginations between the trabeculae. Articular surface is irregular, distinctly mechanically damaged. H&E staining, ×200 magnification

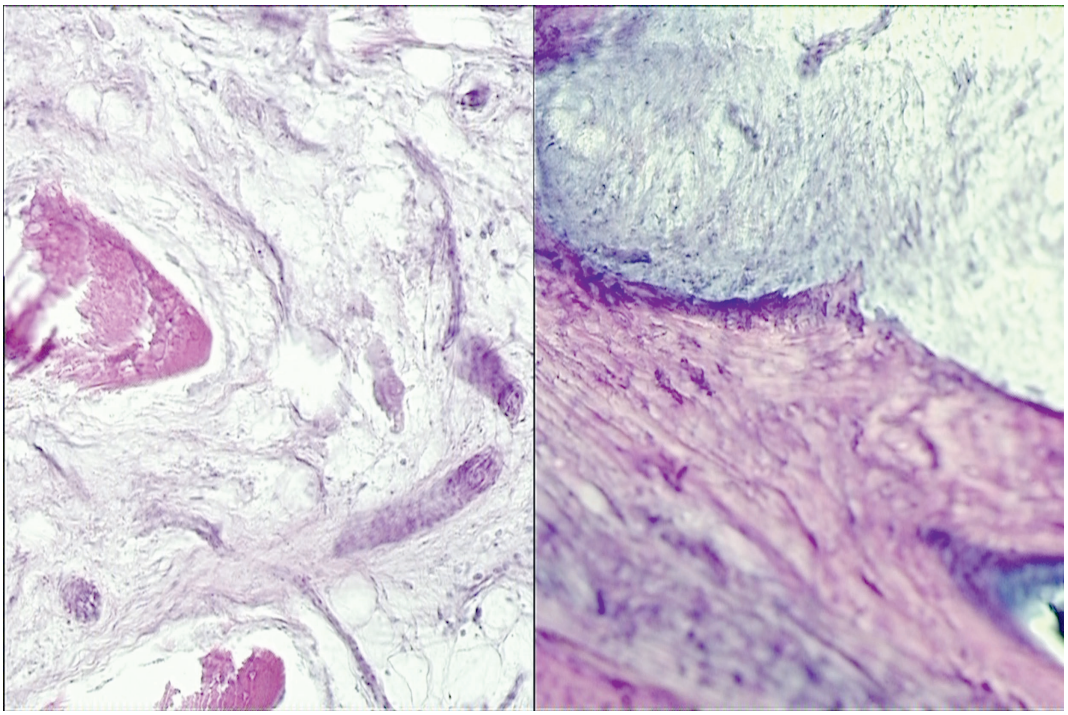

Fig. 9. A. Metabolically active bone; in the center, areas of osteolysis with osteoclasts; above, bone tissue with osteoblasts. H&E staining, ×100 magnification. B. Synthesis of bone tissue matrix (center); osteoblasts surrounding an island of coarse-fiber bone tissue are visible. H&E staining, ×200 magnification

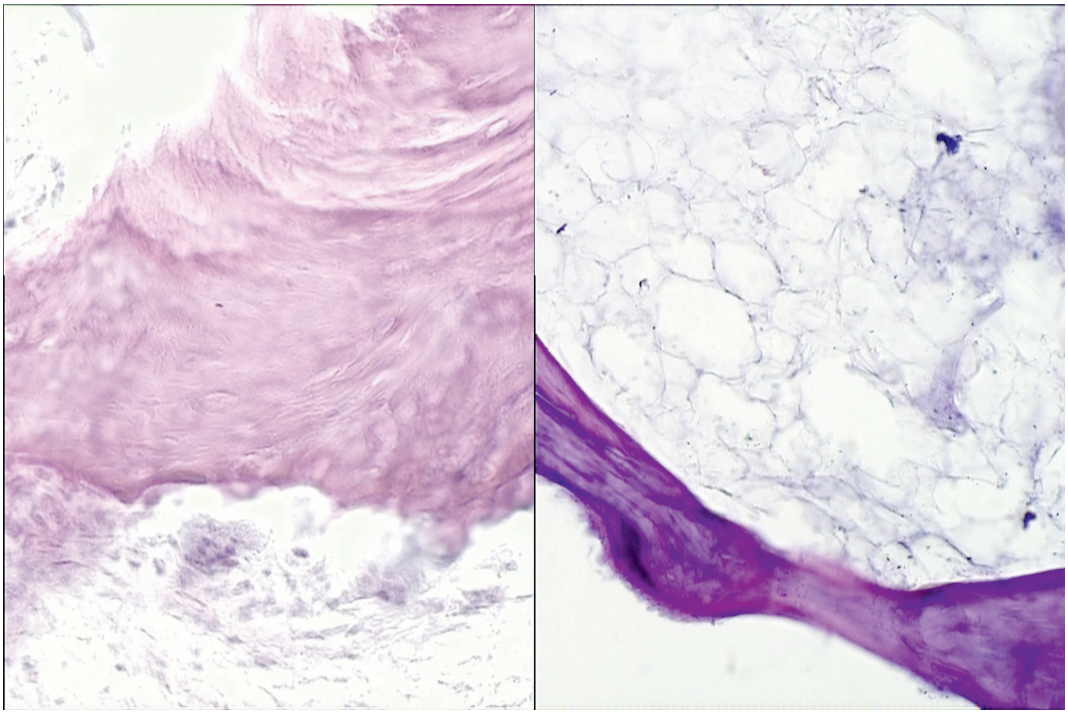

Fig. 10. A. Numerous osteoblasts on the surface of the trabeculae. H&E staining, ×400 magnification. B. Numerous osteoblasts on the surface of the trabeculae. H&E staining, ×200 magnification

reorganization of bone tissue were visible in the central part of the femoral head. Areas of coarse-fiber bone tissue were replaced by new bone tissue, hence the presence of osteoclasts and osteoblasts on their surface (Fig. 10).

Results of mineral composition analysis

The analysis of mineral composition of bone tissue included all samples from the patients. The data was then analyzed within each of the 3 groups distinguished based on histological changes.

Among the whole study group, the carbon content was 52.04 ±9.78%, calcium 10.21 ±2.69% and phosphorus 5.18 ±1.98%. The calcium-to-phosphorus ratio was 2.05 ±0.89 and the ratio of carbon to calcium plus phosphorus was 4.56 ±1.23 (Table 2).

For the osteoporosis group, the electron microscope examination revealed an uneven distribution of elements on the sample surface (Fig. 11). In all samples, the calcium-to-phosphorus ratio (Ca : P) was normal and amounted to 2.16 ±0.78. The ratio of carbon to calcium plus phosphorus (C : Ca + P) was 6.01 ±0.12, but in 6 cases it was low and in 1 case very high (21.33). The very low calcium and phosphorus content was likely the effect of a very low quantity of hydroxyapatite.

The distribution of the mean content of elements in the examined samples, determined using mineralographic analysis, was typical for the whole set and amounted to 49.79 ±5.23% for carbon, 10.42 ±1.34% for calcium and 5.02 ±0.59% for phosphorus (Table 2). The 2nd group of patients – with intensified osteolysis with few osteogenesis centers – showed an uneven distribution of elements in the bone with a mean carbon content of 44.83 ±8.45%, calcium 9.99 ±1.11% and phosphorus 5.34 ±0.78% (Fig. 12). The Ca : P ratio was 2.20 ±0.78 and C : Ca + P ratio was 4.28 ±1.13. The occurrence of small quantities of cadmium (Cd), copper (Cu), aluminum (Al), and silicon (Si) is noteworthy (Table 2). In the 3rd group – intense bone reorganization – electron microscope examination showed an uneven saturation of the surface with calcium and phosphorus

Table 2. Mean content of elements (%), calcium-to-phosphorus ratio and carbon-to-calcium plus phosphorus ratio in bone tissue in the whole study population and by group according to histology

Element	Mean content of element per sample [%]			
	Whole population	Group 1	Group 2	Group 3
C	52.04	49.79	44.83	45.92
O	30.89	33.28	36.89	30.19
Na	0.44	0.36	1.18	0.51
Mg	0.23	0.19	0.29	0.20
P	5.18	5.02	5.34	4.42
Cl	0.43	0.38	0.34	0.37
Ca	10.21	10.42	9.99	8.87
Fe	0.08	0.07	0.08	0.07
K	0.34	0.19	0.47	0.27
S	0.44	0.42	0.57	0.14
Cu	0.24	0.03	0.44	0.22
Al	0.16	0.13	0.31	0.10
Si	0.50	0.50	0.73	0.30
Ag	0.62	0.24	0.00	0.87
Ca : P	2.05	2.1589	2.2042	2.0429
C : CaP	4.56	6.0145	4.2858	4.2646

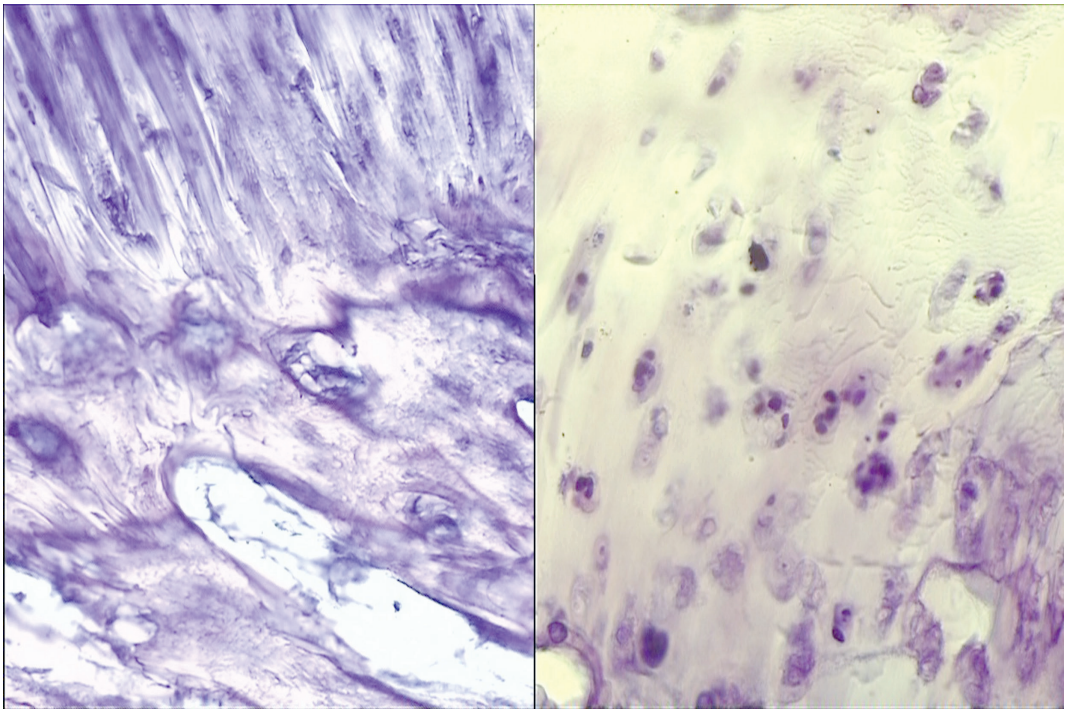

Fig. 11. AK slide (Hist. Ch. 2456/99). Example of spongious bone tissue under electron microscope and distribution of elements on the surface of the slide in group 1 (osteoporosis with a prevalence of coarse-fiber bone tissue and decreased osteogenic activity). Red points correspond to carbon atoms; green-yellow points correspond to calcium and phosphorus ions. Uneven distribution of elements on the surface

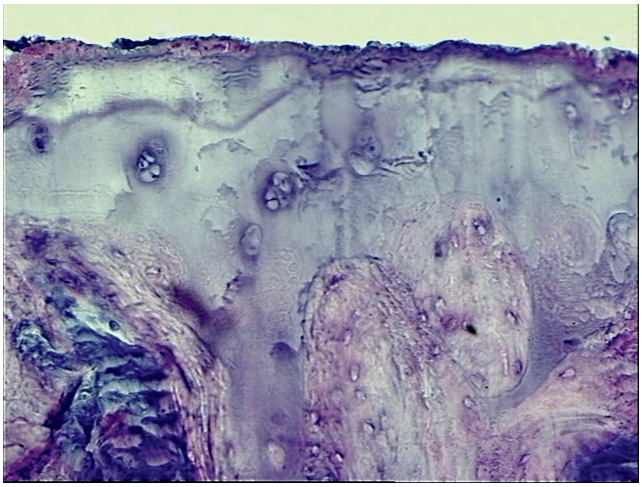

Fig. 12. RM slide (Hist. Ch. 2367/99). Example of spongious bone tissue under electron microscope and distribution of elements on the surface of the slide in group 2 (osteolysis with few centers of osteogenesis). Red points correspond to carbon atoms; green-yellow points correspond to calcium and phosphorus ions. Uneven distribution of elements on the surface

(Fig. 13). The mean carbon content was 52.96 ±11.34%, calcium 9.40 ±2.34% and phosphorus 4.82 ±1.9%. Ca : P was 2.04 ±0.59 and C : Ca + P was 4.26 ±1.15 (Table 2).

Statistical analysis of the data on the mineral composition of the examined bone samples revealed a statistically significant dependence between calcium (Ca) and phosphorus (P), weak dependence between phosphorus (P) and carbon (C) and very weak negative dependence between calcium (Ca) and carbon (C). Oxygen (O) showed a statistically significant negative dependence with carbon (C) and a slightly weaker one with phosphorus (P). The statistically significant negative dependence between Ca : P ratio and carbon (C) indicates that this index may potentially be very useful in estimating quantitative and qualitative correlations among the main organic and inorganic components of bone tissue. The C : Ca + P index, reflecting relations between the organic and inorganic components of hydroxyapatite, showed a statistically significant negative dependence with calcium (Ca) and phosphorus (P). It showed no statistically significant dependence with carbon (C), the correlation being 0.0066 (Table 3).

Discussion

The histological and electron microscope analyses showed degeneration and joint cartilage disappearance in all of the samples examined. It revealed 3 main types of bone tissue changes in the femoral heads: osteoporosis with a prevalence of coarse-fiber bone tissue and decreased osteogenic activity, osteolysis with few centers of osteogenesis, and intensified reorganization of bone tissue. In more than half of the samples, the coarse-fiber bone tissue was replaced by newly formed bone tissue. The observed morphological changes point to ongoing processes of bone resorption and osteogenesis, which indicate a normal homeostasis of bone tissue.

The mineralogical analysis of the bone samples showed an uneven saturation of mineral components within the spongious bone tissue. The indices of the content of organic and inorganic bone components – Ca : P and C : Ca + P – were similar across all 3 groups; only bones with intense osteolysis contained less carbon: 4.96–8.13% less than the osteoporotic and intensely reorganized bone samples.

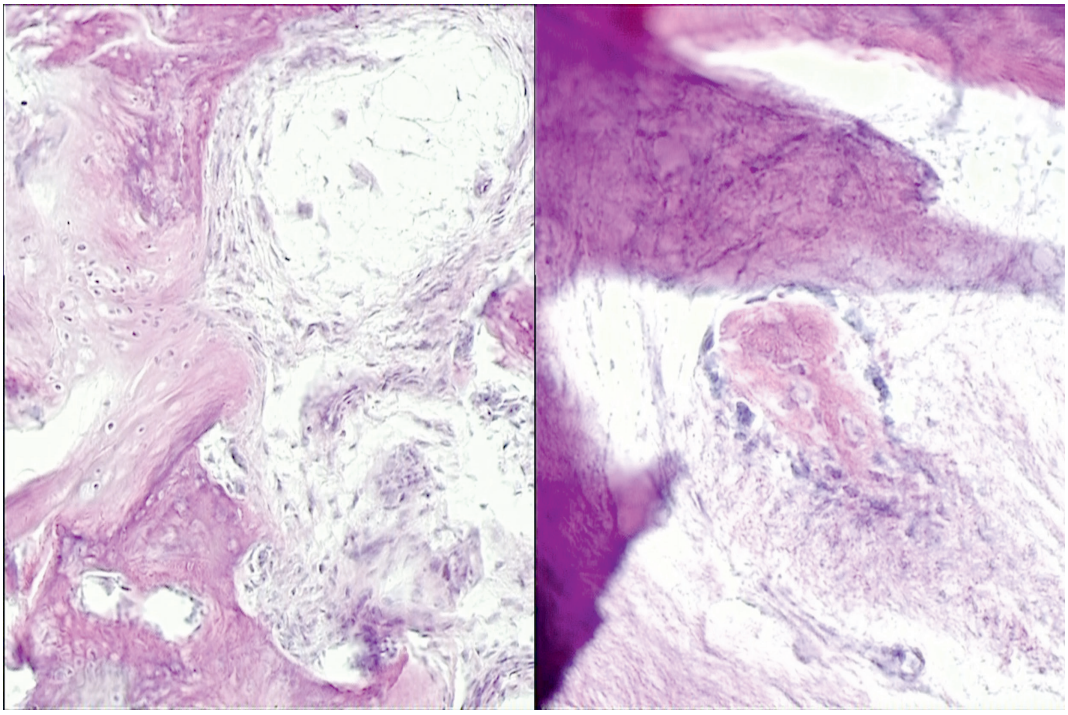

Fig. 13. JZ slide (Hist. Ch. 1387/99). Example of spongious bone tissue under electron microscope and distribution of elements on the surface of the slide in group 3 (intensified reorganization of bone tissue). Red points correspond to carbon atoms; green-yellow points correspond to calcium and phosphorus ions. Uneven distribution of elements on the surface

Table 3. Correlation between inorganic and organic components and Ca : P and C : Ca + P ratios in the bone tissue of the studied femoral heads

Correlation coefficient	Carbon (C)	Calcium (Ca)	Phosphorus (P)	Oxygen (O)	Ca : P ratio	C : Ca + P ratio	BMD [g/cm^2]	BMC [g]
Carbon (C)	1							
Calcium (Ca)	−0.1547	1						
Phosphorus (P)	0.0326	0.9526	1					
Oxygen (O)	−0.7800	−0.4877	−0.6409	1				
Ca : P ratio	−0.7739	−0.2855	−0.5194	0.8816	1			
C : Ca + P ratio	−0.0066	−0.7661	−0.7315	0.4977	0.2304	1		
BMD [g/cm^2]	0.3755	−0.0452	0.0934	−0.3468	−0.3787	−0.0527	1	
BMC [g]	0.0511	−0.0348	0.0287	−0.0744	−0.0767	0.3369	0.0143	1

BMD – bone mineral density; BMC – bone mineral content.

The histological and electron microscope analyses demonstrated that, irrespective of the etiology or advancement of degenerative changes in the hip joint, the processes of bone reorganization take place in the spongious bone tissue of the femoral head. In these processes, apart from the disappearance of bone tissue with a prevalence of fibrous bone tissue formation and osteolysis with centers of osteogenesis, in more than half of the samples examined, processes of transforming coarse-fiber bone tissue into mature lamellate bone tissue were observed. These changes were observed within the femoral head originating from patients at a mean age of 51, even in those with long-term hip joint arthrosis. For this reason, the centers of disappearance and the formation of bone tissue in such patients observed histologically – and sites of denser and sparser arrangement of bone tissue seen on X-ray scans – indicate that bone reorganization of the femoral head takes place not only in the initial period of arthrosis, but also during late-advanced degenerative changes of the hip. Bone reorganization, characterized by the prevalence of osteogenesis processes even in advanced hip arthrosis, suggests that bone tissue has a considerable osteoconductive potential, which creates the proper biological conditions for applying cementless replacements.

The degree of bone tissue mineralization is also important for the mechanical properties of bone. Mineralogical analyses of femoral heads in hip joints with arthrosis were carried out by Bohatyrewicz[24] and Niedźwiedzki et al.[25] Niedźwiedzki et al.[25] limited his studies to the mineral composition of femoral head cartilage in patients with degenerative changes. He found an elevated degree of saturation with mineral components (calcium, phosphorus, magnesium, and potassium) in cartilage with degenerative changes, especially the cartilage covering bone outgrowths. Calcium and phosphorus occurred in the form of "mineral granules". In the cartilage covering bone outgrowths, he also observed changes in ossification.

Bohatyrewicz,[24] studying the mechanical properties of bone tissue in the vicinity of the hip joint, found similar values and a positive correlation of calcium content in compact bone tissue with calcium content in spongious bone tissue from the head and neck of the femur. According to the author, the mean calcium content in spongious bone tissue in elderly people was 6.09 ±1.22 mol/kg, while in the compact tissue it was 5.39 ±0.99 mol/kg.

Our mineralogical analysis showed that irrespective of the type of morphological changes, the bone tissue was unevenly saturated with mineral components. In the bone tissue, besides elements typical of bone, small quantities of cadmium, copper, aluminum, and silicon were detected, which might be explained by the effects of diet and environment.[9,18,22,26–28]

According to McLean and Budy,[21] Kleerekoper et al.,[22] Parfitt et al.,[23] Glimcher,[26] and Bohatyrewicz,[24] quantitative analysis of the mineral composition of bone tissue based on calcium-to-phosphorus and carbon-to-calcium plus phosphorus ratios makes it possible to determine the quantity of organic components and hydroxyapatite and enables characteristics of phosphate-calcium metabolism of the bone. In all of the samples of spongious bone tissue examined in this study, irrespective of the ongoing processes of bone tissue reorganization, the calcium-to-phosphorus ratio (Ca : P) was normal, ranging from 2.04 to 2.20, which indicated a normal structure of hydroxyapatite crystals in the bone. The carbon-to-calcium plus phosphorus ratio (C : Ca + P) seemed more useful when assessing reorganization of bone tissue. In bone tissue undergoing an intense reorganization, the value of the C : Ca + P ratio ranged from 4.26 to 4.28, while in the osteoporotic tissue it was 6.01 – indicating disturbances of mineralization, low hydroxyapatite content and thus decreased mechanical resistance of the bone. The usefulness of these indices in assessing the structural and mechanical properties of bone tissue is also supported by the statistically significant negative correlation between carbon and the Ca : P ratio (–0.7739) and the C : Ca + P ratio (–0.7661) found in our studies (Table 2). The micromorphometric results indicate that chondrolytic changes take place and that bone resorption and osteogenesis progress simultaneously in both joint cartilage and bone tissue. The processes testify to an intense adaptive reorganization of bone tissue depending on the internal and external factors, including biomechanical condition. Analysis of the histophysiological condition of the femoral heads removed during hip surgery, along with biomechanical studies, may help to determine the osteointegrative capacity of a patient's bones. Progress in this field will not completely eliminate the risk of unfavorable adaptive reorganization of bone tissue, especially tissue surrounding a hip replacement, hence there is a need for further studies on the adaptive reorganization of bone tissue and a search for methods boosting the osteogenic potential of bones and improving the saturation of bone with mineral components. These future studies should make use of biological materials containing pharmacologically-aided osteoprogenitors, e.g., through-cutaneous administration of bone marrow, which is used in clinical practice in cases of healing disturbances after bone fracturing.

Conclusions

1. Morphological features and mineral composition of spongious bone tissue of the femoral head are disordered in degenerative changes of the hip joint.
2. Continuous bone reorganization of the femoral head takes place not only in the initial period of arthrosis, but also during late-advanced degenerative changes of the hip.
3. We revealed 3 main types of morphological changes to the bone tissue in femoral heads: osteoporosis with a prevalence of coarse-fiber bone tissue and decreased osteogenic activity; osteolysis with few centers of osteogenesis; and intensified reorganization of bone tissue.
4. Irrespective of the etiology or advancement of degenerative changes to the hip joint, saturation of spongious bone tissue with mineral components is uneven.
5. Hydroxyapatite crystals in the bone are normal, but low hydroxyapatite content in the bone increases osteoporotic disorders and decreases the mechanical resistance of the bone.

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Cardiovascular risk factors and the concentration of asymmetric dimethylarginine

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Abstract

Background. The most commonly recognized cardiovascular risk factors (CVRF) include smoking cigarettes, manifestation of arterial hypertension (AH), hypercholesterolemia, hypertriglyceridemia, manifestation of type 2 diabetes mellitus (DM), and the presence of overweight or obesity. In recent years, investigations have documented the significance of asymmetric dimethylarginine concentration (ADMA) in the pathogenesis of diseases affecting the cardiovascular system.

Objectives. To evaluate the relationship between the number of CVRF and blood ADMA concentration.

Material and methods. The study was conducted on a sample of 138 individuals (mean age 54.90 ±10.38 years). Among the participants, we distniguished subgroups with no CVRF (group A, n = 21), with 1–2 CVRF (group B, n = 53), with 3–4 CVRF (group C, n = 55), and with 5–6 CVRF (group D, n = 9). Plasma concentrations of arginine and of endogenous methylarginines were estimated.

Results. Plasma ADMA concentrations proved to be significantly higher in groups B, C and D than those in group A. Regression analysis allowed us to demonstrate that in the studied population of patients, manifestation of type 2 DM, followed by AH and hypercholesterolemia, were linked to the highest probability of elevated plasma ADMA concentration.

Conclusions. Higher concentration of ADMA in the blood may be a marker for higher cardiovascular risk, especially associated with hypertension, type 2 DM and hypercholesterolemia.

Key words: type 2 diabetes mellitus, arterial hypertension, cardiovascular risk factors, endogenous methylarginines, asymmetric dimethylarginine

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Introduction

The most commonly recognized cardiovascular risk factors (CVRF) include smoking cigarettes, arterial hypertension (AH), hypercholesterolemia, hypertriglyceridemia, type 2 diabetes mellitus (DM), and the presence of overweight or obesity.[1] According to the World Health Organization (WHO), smoking cigarettes affected 21% of the world population ≥15 years of life in 2012 (36% men and 7% women, in Poland around 26% individuals). In 2014, AH was diagnosed in 22% of persons over 18 years of age worldwide (in Poland around 33.5%, including 30.4% of women and 36.8% of men). In 2008, hypercholesterolemia was present in about 39% of the world population and around 54% of the European population.[2] According to the results of the WOBASZ study in Poland, elevated total cholesterol concentration was found in 67% of men and 64% of women.[3] Patients with diabetes accounted for around 9% of the world population; in Poland, 1,134,000 people had diabetes in 2000. On the other hand, obesity affected 13% of people worldwide in 2014; simultaneously, overweight was detected in 39% of people. In the European population, overweight was present in around 50% of women and men and obesity in 23% of women and 20% of men.[2]

Endogenous methylarginines represent products of arginine metabolism. Arginine residues in protein molecules may undergo post-translational modification, including methylation, involving the addition of 1 or 2 methyl groups to guanidine nitrogens of arginine, with the mediation of protein arginine methyl transferases (PRMTs). Two types of PRMTs are distinguished: type 1 catalyzes the addition of 2 methyl groups to a single guanidine residue, which results in the formation of the asymmetric dimethylarginine (ADMA). On the other hand, type 2 of PRMTs induces the addition of a single methyl group to each of the 2 guanidine residues, thus yielding a synthesis of symmetric dimethylarginine (SDMA). The 2 types of PRMT may also add a single methyl group, yielding N-monomethyl-arginine (L-NMMA). In those reactions, S-adenosyl-methionine (SAM) serves as a donor of methyl groups, and the reaction product is S-adenosyl-homocysteine (SAH). Following proteolysis, free L-NMMA, ADMA and SDMA are released to the cytosol. L-NMMA and ADMA act as competitive inhibitors for all 3 isoforms of nitrogen oxide synthase (NOS), while SDMA does not manifest such an activity.[4,5] Nitrogen oxide synthase catalyzes conversion of L-arginine to citrulline and NO. Asymmetric dimethylarginine acts as the competitive inhibitor of NOS due to its competition with L-arginine. Asymmetric dimethylarginine and L-NMMA are eliminated from the body in part through the kidneys and also by enzymatic degradation with the mediation of enzymes, namely, the dimethylarginine dimethyl-aminohydrolases (DDAH). Symmetric dimethylarginine is almost completely eliminated through the kidneys. However, ADMA becomes eliminated mostly through enzymatic decomposition. Citrulline and dimethylamine are the products of enzymatic decomposition of ADMA. Nitrogen oxide (NO) is a direct inhibitor of DDAH, which probably provides a homeostatic mechanism, through which an increased concentration of NO may inhibit its further synthesis.[4,5] Nitrogen oxide constitutes one of the main, endothelium-dependent vasoactive mediators, playing a principal role in the maintenance of tonus and the structure of vascular walls. In the cardiovascular system, NO is responsible for numerous activities such as endothelium-dependent vasodilation, inhibition of platelet aggregation, and inhibition of leukocyte adhesion and of smooth muscle cell proliferation. Moreover, NO manifests an anti-oxidative effect, which reduces the synthesis of superoxide radicals and oxidation of low-density lipoprotein (LDL) molecules. Since all the mechanisms are known as factors promoting the development of atherosclerosis, NO has been termed the anti-sclerotic molecule.[6] Asymmetric dimethylarginine, due to its ability to inhibit synthesis of NO, is regarded to represent a marker of endothelial dysfunction.[6]

In recent years, investigations have documented the significance of endogenous methylarginines in the pathogenesis of cardiovascular system disease. Endogenous methylarginines concentration in blood has been evaluated in various diseases of the cardiovascular system, including in individuals with various CVRF.[5–12] However, the available literature contains no unequivocal data related to the relationship between the coexistence of CVRF and the concentration of ADMA in the blood.

This study evaluated the relationship between the number of CVRF and blood ADMA concentration. Moreover, we have attempted to determine CVRF linked to the highest probability of elevated blood ADMA concentrations.

Material and methods

The studies were conducted on 138 individuals, 39–79 years of age. Among the participants, men accounted for 33.33% (46 persons), and women for 66.67% (92 persons). The mean age in the study group was 54.90 ±10.38 years. The general characteristics of the entire studied group are presented in Table 1.

The participants were subjected to questionnaire studies, after which basic anthropometric measurements were performed; arterial blood pressure was measured and routine blood tests were carried out, such as total cholesterol, triglycerides and glucose concentrations (Boehringer Mannheim GmbH, Mannheim, Germany). Also, concentrations of L-arginine and of endogenous methylarginines in plasma were estimated using high-performance liquid chromatography (HPLC). Arginine and its methyl derivatives were isolated from serum using a powerful cation exchanger: the mixture of concentrated ammonia, water and methanol in the volume ratios of 10:40:50 and, then,

using o-phthalic aldehyde (OPA), in an alkaline medium, they were transformed to derivatives of fluorescent properties and separated using HPLC column (Symmetry C18 HPLC; Waters Co., Milford, USA). The separation and detection was conducted at room temperature using a fluorescence detector (Varian Pro Star 363, Varian Analytical Instruments, Walnut Creek, USA) (excitation: 340 nm, emission: 455 nm).

An anamnesis, physical examination and laboratory tests allowed us to characterize every participant in respect of CVRF, i.e., smoking habit, AH, hypercholesterolemia, hypertriglyceridemia, type 2 DM, and overweight/obesity. Cardiovascular risk factors have been evaluated, which, according to the INTERHEART study, account for 80% of the population risk.[1]

The active cigarette smoking habit was defined as a single cigarette smoked daily at least for the last year. Arterial hypertension was diagnosed in line with the Joint National Committee (JNC 8) recommendations, when a mean of 2 measurements amounted to ≥140 mm Hg in the case of systolic blood pressure and/or 90 mm Hg in the case of diastolic blood pressure. Hypercholesterolemia was diagnosed in cases when total cholesterol concentration was ≥200 mg%. Hypertriglyceridemia was diagnosed when triglycerides concentration was ≥150 mg%. Diabetes was diagnosed when incidental glycemia amounted to ≥200 mg/dL and it was accompanied by typical diabetic signs or when 2 separate measurements of fasting glycemia amounted to ≥126 mg/dL, or when glycemia in the 2-hour glucose tolerance test with the use of 75 g glucose amounted to ≥200 mg/dL. In a situation when a participant declared taking any hypotensive drugs, AH was diagnosed independently of the measured values of arterial blood pressure. Analogously, individuals taking hypolipidemic agents were qualified as patients with dyslipidemia and the patients taking hypoglycemic drugs as patients with DM. Overweight was diagnosed upon body mass index (BMI) values in the range of 25–30 kg/m^2 and obesity upon BMI values exceeding 30 kg/m^2.

On the basis of the number of detected CVRF, subgroups of participant were distinguished: 1) with no CVRF (group A, n = 21), 2) with 1–2 CVRF (group B, n = 53), 3) with 3–4 CVRF (group C, n = 55), or 4) with 5–6 CVRF (group D, n = 9).

Written informed consent was obtained from all persons taking part in the study. The study was approved by the Wroclaw Medical University Ethics Committee.

The statistical analysis was conducted using the STATISTICA v. 12.0 (StatSoft Polska, Kraków, Poland) software. For quantitative variables, arithmetic means and standard deviations (SD) of the estimated parameters were calculated in the examined groups. Distribution of the variables was tested using the Lilliefors and W-Shapiro–Wilk tests. In cases involving quantitative variables manifesting the normal distribution, the subsequent statistical analysis involved a t-test for unlinked variables or analysis of variance (ANOVA) (unifactorial, parametric). The Mann–Whitney U test was applied in cases involving variables manifesting a distribution divergent from the normal one, whereas for quantitative variables the non-parametric equivalent of ANOVA or Kruskal–Wallis test was applied. Statistically significant differences between arithmetic means were detected using the Newman–Keuls post-hoc test. For the quantitative variables, results were presented in the form of lists of percentages. For qualitative variables, in further statistical analysis, the Chi-square test of maximum likelihood was used. In order to define the relationship between studied variables, correlation and regression were analyzed. In the case involving quantitative variables manifesting the normal distribution, Pearson's r correlation coefficients were estimated, while in cases involving quantitative variables manifesting distribution divergent from the normal one, Spearman's r coefficients were calculated. Parameters of the model obtained in logistic analysis were estimated using the quasi-Newton method. Moreover, evaluations of the test accuracy were performed based on the receiver operating characteristic (ROC) curve analysis. Results at the level of $p < 0.05$ were accepted as statistically significant.

Results

Mean concentrations of L-arginine, ADMA and SDMA in plasma of participants are presented in Table 1.

Concentrations of L-arginine and endogenous methylarginines in groups distinguished on the basis of single CVRF are presented in Table 2. When groups of patients differing in single CVRF were compared, ADMA concentration in plasma was demonstrated to be significantly higher in the following participants: those with AH as compared to participants with no AH, among participants with hypercholesterolemia as compared to participants with no hypercholesterolemia, among participants with hypertriglyceridemia as compared to participants with no hypertriglyceridemia, and among participants with type 2 DM as compared to participants with no type 2 DM. Moreover, the mean values of arginine/ADMA ratio were significantly lower among participants with AH, as compared to participants with no AH, in participants with hypercholesterolemia, as compared to participants with no hypercholesterolemia, and among participants with hypertriglyceridemia as compared to participants with no hypertriglyceridemia.

Concentrations of L-arginine and endogenous methylarginines in the groups distinguished on the basis of the number of detected CVRF are presented in Table 3. The comparative analysis of participant groups distinguished on the basis of the number of detected CVRF demonstrated that concentrations of ADMA in plasma were significantly higher in groups B, C and D than in group A. No significant differences were detected

Table 1. Clinical and laboratory parameters in the study group

Variable	Whole study group	Men	Women
Number[a]	138/100.0	46/33.3	92/66.7
Age[b] [years]	54.90 ±10.38	56.09 ±11.57	55.30 ±9.40
BMI[b] [kg/m^2]	26.23 ±2.70	27.20 ±3.32	25.75 ±2.19
Overweight/obesity[a]	63/45.6	23/50.0	40/43.5
Systolic blood pressure[b] [mm Hg]	125.03 ±26.28	124.67 ±28.62	125.16 ±25.19
Diastolic blood pressure[b] [mm Hg]	74.82 ±13.46	74.02 ±13.61	75.22 ±13.44
AH[a]	56/40.5	17/37.0	39/42.4
Total cholesterol[b] [mg/dL]	164.68 ±39.07	154.64 ±38.58	169.69 ±38.54
Triglycerides[b] [mg/dL]	122.53 ±66.86	155.25 ±69.91	106.67 ±59.38
Hypercholesterolemia[a]	73/52.9	27/58.7	46/50.0
Hypertriglyceridemia[a]	58/42.0	17/37.0	41/44.6
Glucose[b] [mg/dL]	103.28 ±15.14	103.87 ±16.59	102.98 ±14.45
Diabetes mellitus[a]	16/11.6	5/10.9	11/12.0
Smoking[a]	54/39.1	19/41.3	35/38.0
Smoking years[b]	276.98 ±148.40	309.71 ±163.93	259.59 ±139.04
ADMA[b] [µmol/L]	1.15 ±0.70	1.15 ±0.71	1.16 ±0.69
SDMA[b] [µmol/L]	0.97 ±0.58	0.88 ±0.50	1.01 ±0.62
Arginine[b] [µmol/L]	22.66 ±6.94	23.79 ±6.76	22.10 ±7.00
Arginine/ADMA[b]	24.59 ±12.63	26.05 ±12.70	23.85 ±12.60
Arginine/SDMA[b]	28.99 ±14.65	31.41 ±13.77	27.78 ±14.99

[a] numbers/percentages; [b] mean values ± standard deviation (SD); ADMA – plasma asymmetric dimethylarginine concentration; BMI – body mass index; AH – arterial hypertension; SDMA – plasma symmetric dimethylarginine concentration; smoking years – number of cigarettes/24 h × years of smoking

Table 2. L-arginine and endogenous methylarginines in groups distinguished on the basis of single risk factors of cardiovascular diseases

Variable	Presence of the risk factor	ADMA[a] [µmol/L]	SDMA[a] [µmol/L]	Arginine[a] [µmol/L]	Arginine/ADMA[a]	Arginine/SDMA[a]
Overweight/obesity	yes	1.17 ±0.66	1.00 ±0.57	22.82 ±7.31	23.46 ±11.55	28.66 ±15.33
	no	1.14 ±0.74	0.94 ±0.59	22.53 ±6.66	25.53 ±13.47	29.27 ±14.14
	p-value	NS	NS	NS	NS	NS
AH	yes	1.52 ±0.65	0.88 ±0.51	22.79 ±6.55	22.32 ±11.31	30.59 ±14.08
	no	0.90 ±0.73	1.02 ±0.62	22.58 ±7.24	26.13 ±13.30	27.90 ±15.01
	p-value	0.001	NS	NS	0.041	NS
Hypercholesterolemia	yes	1.28 ±0.72	0.94 ±0.60	22.99 ±7.20	21.78 ±11.11	30.06 ±14.80
	no	1.01 ±0.65	1.00 ±0.56	22.30 ±6.68	27.73 ±13.55	27.79 ±14.49
	p-value	0.020	NS	NS	0.005	NS
Hypertriglyceridemia	yes	1.35 ±0.76	0.92 ±0.64	22.97 ±7.90	20.58 ±10.80	31.58 ±16.03
	no	1.01 ±0.62	1.00 ±0.53	22.44 ±6.20	27.49 ±13.12	27.11 ±13.35
	p-value	0.005	NS	NS	0.001	NS
DM	yes	1.49 ±0.58	0.86 ±0.56	24.07 ±3.20	23.21 ±12.04	33.67 ±11.43
	no	1.11 ±0.61	0.98 ±0.58	22.48 ±7.28	25.21 ±12.75	28.37 ±14.95
	p-value	0.020	NS	NS	NS	NS
Smoking	yes	1.07 ±0.54	1.00 ±0.55	21.40 ±7.37	23.14 ±11.33	26.48 ±15.06
	no	1.21 ±0.78	0.94 ±0.60	23.48 ±6.57	25.52 ±13.38	30.60 ±14.24
	p-value	NS	NS	NS	NS	NS

[a] mean values ± standard deviation (SD); AH – arterial hypertension; DM – diabetes mellitus; ADMA – plasma asymmetric dimethylarginine concentration; NS – non significant; SDMA – plasma symmetric dimethylarginine concentration.

Table 3. L-arginine and endogenous methylarginines in groups distinguished on the basis of the number of cardiovascular risk factors

Group	Number of CVRF	ADMA[a] [µmol/L]	SDMA[a] [µmol/L]	Arginine[a] [µmol/L]	Arginine/ADMA[a]	Arginine/SDMA[a]
A (n = 21)	0	0.58 ±0.11	0.98 ±0.51	22.40 ±3.06	39.98 ±9.60	26.66 ±10.12
B (n = 53)	1–2	1.32 ±0.78	1.03 ±0.61	22.56 ±8.11	20.56 ±10.37	27.65 ±16.01
C (n = 55)	3–4	1.22 ±0.67	0.93 ±0.62	22.68 ±7.17	22.55 ±11.70	30.24 ±14.95
D (n = 9)	5–6	1.12 ±0.52	0.76 ±0.22	23.83 ±5.09	24.86 ±10.99	34.66 ±13.10
p-value		A vs B: 0.002 A vs C: 0.006 A vs D: 0.009	NS	NS	A vs B: 0.001 A vs C: 0.001 A vs D: 0.001	NS

[a] mean values ± standard deviation (SD); ADMA – plasma asymmetric dimethylarginine concentration; CVRF – cardiovascular risk factor; NS – non significant; SDMA – plasma symmetric dimethylarginine concentration.

Table 4. Results of estimation for model obtained in progressive step-wise multivariable analysis of regression. Model for ADMA [µmol/L]

Variable	Regression coefficient	SEM of Rc	p-value	p-value of the model	SEM of the model
Intercept	0.953	0.114	0.001	0.028	±0.680
DM	0.380	0.200	0.040		
AH	0.355	0.193	0.037		
Hypercholesterolemia	0.284	0.148	0.047		

ADMA – plasma asymmetric dimethylarginine concentration; AH – arterial hypertension; DM – diabetes mellitus; SEM of Rc – standard error of the mean of regression coefficient.

Table 5. The sensitivity and specificity of ADMA concentration as a predictor of CVRF number

Variable	CVRF ≥ 1	CVRF ≥ 2	CVRF ≥ 3	CVRF ≥ 4	CVRF ≥ 5
ADMA concentration as predictor of CVRF number [µmol/L]	≥0.76	≥0.78	≥0.85	≥0.95	≥0.97
Sensitivity	0.952	0.719	0.554	0.530	0.519
Specificity	0.786	0.792	0.672	0.652	0.778
Accuracy	0.812	0.775	0.609	0.551	0.536
Positive predictive values	0.444	0.511	0.661	0.884	0.971
Negative predictive values	0.989	0.903	0.566	0.217	0.101
Likelihood ratios positive	4.457	3.463	1.689	1.525	2.337
Likelihood ratios negative	0.061	0.355	0.664	0.720	0.618

ADMA – plasma asymmetric dimethylarginine concentration; CVRF – cardiovascular risk factor.

in ADMA concentrations in plasma between groups B, C and D. Moreover, in groups B, C and D mean values of arginine/ADMA quotient were significantly lower than in group A.

An analysis of correlation revealed no linear relationship between the number of CVRF on the one hand, and concentrations of L-arginine and of endogenous methylarginines on the other. Nevertheless, significant linear relationships were detected: the positive one between ADMA concentration in plasma and cholesterol concentration in the blood ($r = 0.19$, $p = 0.023$) and negative ones between the value of arginine/ADMA ratio and cholesterol concentration in the blood ($r = -0.22$, $p = 0.007$), and also between the value of arginine/ADMA ratio and triglyceride concentration in the blood ($r = -0.18$, $p = 0.030$).

Results of estimations using the model reflecting the regression analysis are presented in Table 4. In an analysis of multifactorial progressive stepwise regression, taking into account dichotomic, potentially independent variables, i.e., smoking of cigarettes, AH, hypercholesterolemia, hypertriglyceridemia, type 2 DM, and overweight/obesity, the following model was obtained: ADMA = 0.953 + 0.380 type 2 DM + 0.355 AH + 0.284 hypercholesterolemia ±0.680. The model allowed us to conclude that, out of the evaluated CVRF, type 2 DM, followed by AH and hypercholesterolemia, were linked to the highest probability of elevated ADMA concentrations in plasma.

The sensitivity and specificity of ADMA concentration as a predictor of the CVRF number were presented in Table 5. The ROC analysis indicated ADMA concentration values constituting predictor conditions for the CVRF number, being for CVRF ≥ 1 ≥0.76 µmol/L, for CVRF ≥ 2 ≥0.78 µmol/L, for CVRF ≥ 3 ≥0.85 µmol/L, for CVRF ≥ 4 ≥0.95 µmol/L, and for CVRF ≥ 5 ≥0.97 µmol/L. The highest prediction sensitivity of 95.2% was demonstrated for the ADMA criterion ≥0.76 µmol/L as a predictor

of the CVRF ≥ 1 number, and the highest specificity equal to 79.2% for the ADMA criterion ≥0.78 μmol/L as a predictor of the CVRF ≥ 2 number. In general, the highest prediction accuracy of 81.2% was obtained for the ADMA criterion ≥0.76 μmol/L as a predictor of CVRF ≥ 1.

Discussion

We showed that higher blood ADMA levels may be the marker for higher cardiovascular risk. The comparative analyzes and the one-way analysis of dependencies (correlation analysis) demonstrated a relationship between selected CVRF and higher levels of ADMA in the blood. Higher levels of ADMA in the blood were observed in patients with hypertension, hypercholesterolemia, hypertriglyceridemia, and type 2 DM. A positive correlation between total cholesterol and ADMA concentration in the blood as well as negative correlations between total cholesterol and triglyceride concentrations and ADMA concentration in the blood were demonstrated. In the multivariate analysis of dependencies (regression analysis), it was confirmed that type 2 DM, hypertension and hypercholesterolemia are independently associated with higher levels of ADMA in the blood. Analysis of the dependency between CVRF and ADMA concentration in the blood indicated that in the context of ADMA concentration, the occurrence of the first CVRF is more important. Asymmetric dimethylarginine concentration in the blood was significantly higher in each group of patients with CVRF than in the group without CVRF. At the same time, there were no differences between the groups of patients with 1–2 CVRF, 3–4 CVRF and 5–6 CVRF. In addition, there was no linear dependency between CVRF and ADMA concentration in the blood, and the analysis of ROC curves and associations indicated the highest accuracy of the concentration criterion of ADMA > 0.76 μmol/L as a predictor of CVRF ≥ 1.

The results of the current study are consistent with the previous reports regarding CVRF and ADMA blood concentration. At the same time, they fill the gap in literature in the context of the relationships between the number of CVRF and ADMA.

In patients with AH, representing the other important CVRF, the elevated ADMA concentrations were described. In one of the studies, significantly higher ADMA levels in patients with AH and cardiovascular complications were noted, and especially in individuals with uncontrolled or not appropriately controlled AH as compared to the control group. Additionally, concentrations of ADMA correlated positively with the traditional CVRF.[13] Moreover, results obtained by Vallance et al. showed that in patients with renal failure, the AH developed significantly more frequently in patients with elevated blood ADMA level,[4] while Fliser et al.[14] and Schulze et al.[15] could not detect any relationship between ADMA concentration and values of arterial blood pressure. The other investigations[16,17] allowed us to clarify the distinct results obtained in various studies: it has been found that the presence of additional risk factors, such as age, insulin resistance, dyslipidemia, or administered drugs, may modify ADMA levels and in this way they may disturb the relationship between ADMA and arterial blood pressure. Results of studies conducted by Poręba et al. showed that mean concentrations of ADMA were significantly higher and the arginine/ADMA ratio was significantly lower in people with AH and co-existing left-ventricular diastolic dysfunction (LVDD) as compared to the group without LVDD. Also, in the group of patients with AH diagnosed within the recent 5 years, similar relations were found. The independent factors of LVDD risk included higher concentrations of ADMA and LDL, higher values of BMI, of left ventricular myocardium (LVMI) and of higher mean arterial blood pressure. Thus, the authors proved that higher values of ADMA may carry a prognostic value in the context of the development of LVDD in individuals with AH.[18]

An interesting publication by Achan et al. described the effects of intravenous administration of low ADMA dose on heart and systemic vascular resistance. In the study group consisting of 12 men without any serious disease, the application of ADMA caused a reduction in the heart rate and an decrease in cardiac output, and additionally an increase in mean arterial blood pressure and systemic vascular resistance. The effects resembled those detected in pathologies associated with accumulation of ADMA.[19] Our study clearly confirmed the relationship between hypertension and higher levels of ADMA in blood.

In a couple of studies, the relationships between elevated ADMA levels and a range of pathologies were investigated and among them some seem to be important in the aspect of cardiovascular risk. In a group of patients with hypercholesterolemia, elevated concentrations of ADMA and decreased values of L-arginine/ADMA ratio were present. The increase in ADMA concentration was linked to the inhibited endothelium-dependent vasodilatation and reduced excretion of nitrates.[9] In other reports, hypertriglyceridemia was accompanied by elevated levels of ADMA in a group of persons with insulin resistance,[20] while in the group of patients with dyslipidemia, higher values of endothelial dysfunction markers such as ADMA as well as plasma viscosity and the level of oxidized LDL molecules were documented, together with lower NO levels.[21]

Results of several investigations indicate that ADMA concentrations are higher in diabetics as compared to patients without DM.[22,23] Higher levels of ADMA were described, as previously mentioned, also in persons with insulin resistance.[20] Moreover, the data was then supported by the different studies in the group of diabetics, where higher concentrations of ADMA were linked to the progression of atherosclerosis and an elevated risk

of cardiovascular events. Therefore, the conclusion was drawn that ADMA represents the independent marker of progression involving cardiovascular complications in diabetic patients.[24–26] Nevertheless, the results of certain studies failed to confirm this relationship; for example, Anderssohn et al. and Böger et al. could not find the relationship between ADMA and cardiovascular events in diabetic patients.[27,28] Such discrepancies may reflect the distinct activity of enzymes linked to the degradation of ADMA (DDAH), influenced by numerous variables: genetic lesions, including polymorphism of genes responsible for synthesis of DDAH 1 and DDAH2, presence of DM itself, and the applied hypoglycemic drugs.[27] The other authors detected lower concentrations of ADMA and uric acid and, at the same time, higher levels of HbA1c, LDL-cholesterol and NO in 99 women with type 1 DM, as compared to the control group. The results, which seem to be completely distinct from the previously discussed, may reflect the selection of the investigated group, preferring women with type 1 DM with no additional risk factors, probably affected by less pronounced oxidative stress, which could inhibit the activity of enzymes engaged in ADMA degradation, than in the groups subjected to other studies.[29] Current studies confirm the relation between the occurrence of type 2 DM and higher levels of ADMA in the blood. Simultaneously, it should be noted that type 2 DM, when compared with various examined CVRF, proved to be the strongest predictor of higher ADMA levels in the blood in a regression analysis.

The results of the present study allow us to recognize higher levels of ADMA as an indicator of higher cardiovascular risk associated with the occurrence of recognized risk factors. However, the higher concentration of ADMA still cannot be considered as an independent CVRF. In the methodology of the current study, the data was collected at the same time in a single determination. To consider higher levels of ADMA in the blood as an independent CVRF, a long-term analysis with an assessment of long-term adverse cardiovascular events would be most appropriate.

In the light of previous publications, higher cardiovascular risk results mainly from endothelial dysfunction. Asymmetric dimethylarginine represents a competitive inhibitor of NOS. Nitrogen oxide performs a controlling function in several mechanisms of the cardiovascular system and, among others, it controls the tension of vascular walls; simultaneously, it is considered to be the principal factor linked to endothelium vasodilatation. Moreover, it controls the structure of vascular walls (inhibits smooth muscle proliferation) as well as interactions between cells of blood vessels (inhibiting adhesion and aggregation of platelets and adhesion of monocytes). Increased levels of ADMA lead to decreased concentrations of NO, abrogation of its biological functions and, in this way, to endothelial dysfunction. Endothelial dysfunction is linked to an elevated cardiovascular risk.[10]

In addition to the formerly discussed relationship between the CVRF number and ADMA blood concentration, the currently discussed study complements the existing scientific literature of the topic regarding the CVRF prediction attempts using a single determination of ADMA concentration. The results of the performed analyses indicated the highest accuracy of prediction (amounting to 81.2%) for the ADMA criterion ≥0.76 μmol/L as a predictor of the CVRF ≥ 1 number, although it should be noted that the accuracy levels in the range of 53.6–77.5% for other CVRF numbers also appear to be acceptable.

The current study is not devoid of several limitations. In the field of study material, a significant limitation is the number of patients examined. Despite the inclusion of 138 people in the study, in a comparative analysis, a subgroup of people with 5–6 CVRF is represented by only 9 people, which significantly limits the validity of the obtained results. The overrepresentation in the studied group of women, who accounted for 2/3 of the respondents, is also worth noting. In the field of study methodology, the selection of analyzed CVRF may be considered controversial. When selecting them, the INTERHEART study criteria were used and those CVRF can attribute to 80% of the population risk. In addition, the study is limited by the single stage of measurements made and the data collected. Consequently, the results of the study only allow for the recognition of higher levels of ADMA as an indicator of higher cardiovascular risk.

Conclusions

A higher concentration of ADMA in the blood may be a marker for higher cardiovascular risk, especially associated with hypertension, type 2 DM and hypercholesterolemia.

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Paraoxonase 1 decline and lipid peroxidation rise reflect a degree of brain atrophy and vascular impairment in dementia

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Abstract

Background. Paraoxonase 1 (PON1) is an enzyme with the capability to protect against lipid oxidation and atherosclerotic lesions formation. Impaired antioxidative capacity and enhanced lipid peroxidation (reflected by malondialdehyde rise) accompany dementias.

Objectives. The aim of this study was to discern the possible differences in the activity and phenotype distribution of PON1, and lipid peroxidation level in dementias of neurodegenerative and vascular pathology, to assess whether they reflect structural changes in the brain, and to evaluate their potential as dementia markers.

Material and methods. Paraoxonase 1 arylesterase activity and polymorphisms (dual-substrate method), and malondialdehyde/thiobarbituric acid reactive substances (MDA/TBARS) levels were determined spectrophotometrically in 257 serum samples derived from 136 dementive patients (with Alzheimer's disease (AD; n = 63), vascular dementia (VaD; n = 40) and mixed-type dementia (MD; n = 33), as well as from 121 non-dementive individuals. The results were analyzed with reference to dementia type and severity (assessed with Mini Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) scales), structural brain changes (estimated with magnetic resonance imaging (MRI) – Global Cortical Atrophy (GCA), Medial Temporal lobe Atrophy (MTA) and Fazekas scales)) and brain ischemia (Hachinski Ischemic Scale (HIS) index), and evaluated using receiver operating characteristic (ROC) analysis.

Results. Malondialdehyde/thiobarbituric acid reactive substances were increased in dementia (more in VaD than AD). In patients with vascular involvement, MDA/TBARS elevation reflected a degree of global cortical atrophy. Paraoxonase 1 activity was decreased in patients with dementia, especially in patients with severe cognitive deficits. In VaD, a drop in PON1 reflected a degree of MTA and brain ischemia. MDA/TBARS displayed 75% accuracy as a general dementia marker, but, similarly to PON1, were a poor differential marker.

Conclusions. Both indices were more associated with vascular involvement and the severity of brain atrophy or ischemia rather than with degree of cognitive decline.

Key words: Alzheimer's disease, vascular dementia, paraoxonase 1, mixed-type dementia, MDA/TBARS

Introduction

Progressive degeneration of neurons, a hallmark of neurodegenerative disorders, leads to serious deficits in cognitive performance and general functioning.[1] Due to the progressing aging of societies, the number of people suffering from various forms of dementia is projected to double every 20 years and will become a serious burden for public healthcare in the near future. Alzheimer's disease (AD) is the main cause of dementia in the elderly population, followed by vascular dementia (VaD). Alzheimer's disease is estimated to account for 60–80% of cases and is characterized by the accumulation of extracellular β-amyloid plaques and intracellular neurofibrillary tangles.[1] Vascular dementia is the result of the blockage or damage of the cerebral blood vessels, leading to infarcts, bleeding and/or ischemia and ultimately to brain injury. It was initially considered to be the sole form of dementia attributable to vascular lesions; however, several studies have demonstrated that the development of cardiovascular risk factors also play a substantial role in AD. Indeed, neuropathological indices of brain infarcts can be found in up to half of AD patients. The co-existence of AD and VaD pathologies is observed in the vast majority of mixed dementia (MD) cases.[1]

Epidemiological studies have revealed that lipid peroxidation caused by oxidative imbalance is a common etiology of both cardiovascular[2] and neurocognitive diseases.[3] Among others, oxidative stress (OS) alters the permeability of vascular and cerebral endothelium and promotes inflammatory responses.[3] Brain tissue is particularly vulnerable to OS due to high content of oxidative damage-prone polyunsaturated fatty acids and intense oxidative metabolism combined with low antioxidant capacity.[3] Accordingly, the decline in antioxidants as well as the accumulation of oxidative damage markers, e.g., malondialdehyde (MDA), considered as the most abundant aldehyde derived from lipid peroxidation, have been reported in both AD and VaD.[3,4]

Paraoxonase 1 (PON1) is a liver-synthesized enzyme of multiple biological functions, which has gained particular attention as an anti-atherogenic agent. It displays antioxidative properties and protects low-density lipoprotein (LDL) and high-density lipoprotein (HDL) lipids from oxidation, decreases oxidative status of macrophages and increases cholesterol efflux, thus contributing to the prevention and attenuation of the development of atherosclerotic lesions.[5] Moreover, PON1 participates in the detoxification of homocysteine thiolactone, another pro-atherogenic compound, and plays an anti-inflammatory role by inhibiting the expression of monocyte chemoattractant protein (MCP)-1 and activity of myeloperoxidase, which serve as key players in vascular inflammation and OS. It has also been reported to stabilize lipid membranes and enhance their integrity under the conditions of oxidative imbalance. Accordingly, decreased enzyme activity has been repeatedly linked with an increased atherogenic risk.[5]

The study evaluated the potential relationship between oxidative imbalance and the degree of structural and functional impairment of the brain in patients with dementia of neurodegenerative and vascular pathology.

Material and methods

Study population

The study population consisted of 257 individuals: 136 with dementia and 121 without dementia who served as a control group. Among patients with dementia, 63 were diagnosed with AD, 33 with MD and 40 with VaD. The following diagnostic criteria were used: 1) DSM-IV[6] and NINCDS-ADRDA[7] for AD; 2) ICD-10[8] and NINDS-AIREN[9] for VaD, and 3) ICD-10 with the Hachinski Ischemic Scale (HIS)[10] for MD. Patients with dementia were recruited from the Alzheimer Center, Wroclaw Medical University, Ścinawa, Poland (48 AD patients and all patients with MD and VaD) and from the Department of Psychiatry, Wroclaw Medical University (15 AD patients). All of the patients underwent a routine medical examination. Global cognitive function was assessed using the Mini Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) scales. The presence of vascular involvement and global or focal atrophy were evaluated using magnetic resonance imaging (MRI) and estimated with the application of the following scales: the Global Cortical Atrophy (GCA) scale, the Medial Temporal lobe Atrophy (MTA) scale, and the Fazekas scale for white matter lesions.[11] Computed tomography (CT) was used in patients in whom contraindications for MRI were observed (e.g., pacemaker or other metal elements in the body or fear of staying in confined spaces). The MRI scans were assessed by 2 trained persons (one of the authors (MZ) and an independent blinded radiologist), whose scores were averaged. Nutritional status of patients with dementia was evaluated using the body mass index (BMI) and the Mini Nutritional Assessment (MNA).[12] Clinical characteristics of patients with dementia are presented in Table 1.

Control group consisted of the following:

(1) BD group – 68 apparently healthy blood donors (age >45 years, no significant health history, no active inflammation, no pregnancy, no complaints concerning memory and cognitive function), recruited from the Regional Center for Blood Donation and Therapeutics in Wrocław, Poland;

(2) HA group – 38 otherwise healthy individuals suffering from headaches, dizziness and/or complaining about weak memory in a degree justifying neuroimaging, but in whom neither loss of cognitive function nor any significant somatic or mental illnesses were diagnosed. They were recruited at the Alzheimer Center;

(3) MCI group – 15 otherwise healthy individuals diagnosed with mild cognitive impairment (MCI) according

Table 1. Characteristics of the study population

Variables	Patients with dementia (D)				Not demented controls			P_{all}
	AD	MD	VaD	$P_{dementia}$	BD	HA	MCI	
Number of cases	63	33	40		68	38	15	
Demographics								
Age [years]	75.5 ±8.0	75.6 ±7.0	72.8 ±8.3	0.185[1]	55.1 ±7.0	61.6 ±8.8	66.5 ±10	<0.001[1]
Sex, F/M	40/23	22/11	22/18	0.551[2]	42/26	28/10	9/6	0.559[2]
Nutritional status								
BMI [kg/m^2]	27.1 ±4.1	27.7 ±4.2	28.7 ±5.4	0.300[1]		27.7 ±5.4		0.523[1]
MNA	12.8 ±0.6	12.9 ±0.4	12.9 ±0.3	0.811[1]		13.8 ±0.7[4]		<0.001[1]
Mental deficits								
CDR	1.48 ±0.58	1.42 ±0.50	1.48 ±0.55	0.895[1]		0[4]		<0.0001[3]
MMSE	17.5 ±4.2	19.4 ±4.3	18.7 ±4.0	0.137[1]		29.1 ±0.9[4]		<0.001[1]
Neuroimaging and Hachinski Ischemic Scale								
MTA	2.5 (2.4–3.0)	2.5 (2.0–3.5)	2.63 (2.3–3.0)	0.725[3]		0.5 (0.0–0.5)[4]		<0.0001[3]
GCA	2.3 ±0.5	2.3 ±0.5	2.5 ±0.5	0.133[1]		0.9 ±0.5[4]		<0.001[1]
Fazekas	1.3 (0.5–1.5)[5,6]	2 (1.5–2.5)[6,7]	2.5 (2–3)[5,7]	<0.00001[3]		0.5 (0.5–1.0)[4]		<0.0001[3]
HIS	3 (2–3)[5,6]	5 (5–6)[6,7]	7 (7–8)[5,7]	<0.00001[3]		2 (1–3)[4]		<0.0001[3]

Data presented as means ± standard deviation (SD) or medians with interquartile range. AD – Alzheimer's disease; MD – mixed-type dementia; VaD – vascular dementia; BD – healthy blood donors; HA – patients with headaches, dizziness and/or complaining about weak memory but without dementia or MCI; MCI – mild cognitive impairment; F/M – female-to-male ratio; BMI – body mass index; MNA – Mini Nutritional Assessment; CDR – Clinical Dementia Rating; MMSE – Mini Mental State Examination; MTA – Medial Temporal lobe Atrophy; GCA – Global Cortical Atrophy; HIS – Hachinski Ischemic Scale; [1] – one-way analysis of variance (ANOVA); [2] – χ^2 test; [3] – Kruskal–Wallis H test; [4] – significantly different from all remaining groups (AD, MD and VaD); [5] – significantly different from MD; [6] – significantly different from VaD; [7] – significantly different from AD.

to Petersen's criteria[13] were recruited from the Department of Psychiatry.

Basic demographic data of the control groups, including clinical characteristics of HA patients, are summarized in Table 1.

Ethical considerations

The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University (approval No. KB-679/2011 and KB-367/2017). The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013, and informed consent was obtained from all study participants. In the case of patients with severe dementia, legal guardians were consented.

Analytical methods

Blood samples were obtained following overnight fasting through venipuncture, clotted for 30 min, and centrifuged (15 min, 720 × g). Serum was collected, aliquoted and kept frozen at –80°C until examination.

Malondialdehyde and malondialdehyde-like substances

Serum concentrations of MDA/TBARS were determined spectrophotometrically with thiobarbituric acid (TBA) assay.[14] To increase the specificity of the reaction, MDA/TBARS were assessed in the presence of butylated hydroxytoluene (BHT) (Fluka Chemie, Buchs, Switzerland).[15]

Measurement of PON1 activities and PON1 phenotyping

To express PON1 activity, we determined its arylesterase activity against phenyl acetate as a substrate. Since phenyl acetate is equally metabolized by PON1 alloforms, which result from the most frequent Q192R polymorphism, the obtained enzymatic activity is considered as a surrogate for the enzyme concentration.[16]

To evaluate PON1 phenotype distribution, we used a dual substrate method, employing a determination of arylesterase and paraoxonase activities of the enzyme.[17] Subsequent plotting of arylesterase against paraoxonase activity results in the separation of 3 forms of PON1, representing individuals homozygous for the Q alloenzyme (A phenotype of PON1 with Q at 192), individuals homozygous for the R alloenzyme (B phenotype of PON1 with R at 192), and heterozygous individuals (AB phenotype of PON1). Due to the very low occurrence of B phenotype, for the purpose of the present study, phenotypes AB and B were combined and are further described as phenotype B.

Paraoxonase 1 arylesterase and paraoxonase activities were determined spectrophotometrically by measuring the rates of hydrolysis of respective substrates: phenyl acetate (Sigma-Aldrich, St. Louis, USA) according to the Arylesterase/Paraoxonase assay kit protocol (ZeptoMetrix

Co., Buffalo, USA) (CV = 3.0%) and paraoxon (ChemService Inc., West Chester, USA) with the method designed by Charlton-Menys et al.[18] One unit (U) of enzyme activity was defined as 1 mmol of released phenol (arylesterase activity) or 1 µmol of released p-nitrophenol (paraoxonase activity) per 1 L of serum per 1 min. Intra-assay coefficients of variation (CV) for these methods were 1.1% (paraoxonase activity) and 3% (arylesterase activity). All measurements were conducted in duplicates and technical replicates were averaged.

Statistical analysis

Normality of data distribution was tested using the χ^2 test and homogeneity of variances using Levene's test. Log-transformation was used, if appropriate. If not otherwise stated, data is presented as medians or means with 95% confidence interval (95% CI). Continuous variables were analyzed using the Kruskal–Wallis H test or one-way analysis of variance (ANOVA) with Bonferroni correction for multiple testing and the t-test for independent samples with the Welch correction, if required. Correlation analysis was conducted using either the Spearman test (ρ) or the Pearson's test (r). Frequency analysis and comparison of 2 proportions were conducted using the χ^2 test. Age- and sex-adjusted analyses were conducted using the analysis of covariance (ANCOVA). The discriminative power of PON1 and MDA/TBARS was evaluated using the ROC analysis. Overall accuracy was expressed as an area under the ROC curve (AUC). Additionally, an optimal cut-off was determined and corresponding sensitivities and specificities were calculated. Backward method of multiple regression was used to discern independent predictors of PON1 activity and MDA/TBARS concentrations with $p < 0.05$ as inclusion and $p > 0.1$ as exclusion criteria. A two-tailed probability <0.05 was considered significant. The analyses were performed using MedCalc Statistical Software v. 17.4.4 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2017).

Results

PON1 and MDA/TBARS in dementia

Patients with dementia had significantly higher concentrations of MDA/TBARS and lower levels of arylesterase activity of PON1 (Fig. 1).

A detailed analysis revealed differences in MDA/TBARS in both control and dementia groups (Fig. 2). Among individuals recruited as controls, the BD group had significantly lower concentrations of MDA/TBARS than the HA or MCI group. Among patients with dementia, the presence of vascular impairment was responsible for significant upregulation of MDA/TBARS concentrations. Arylesterase activity of PON1 was significantly higher

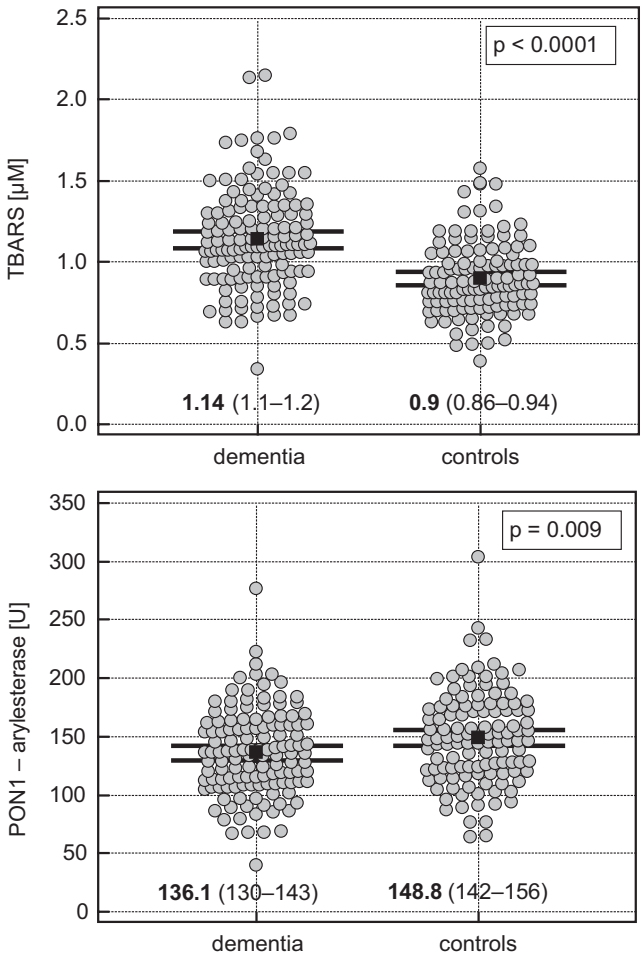

Fig. 1. Comparison of MDA/TBARS concentrations and PON1 activity between non-demented and demented individuals. Circles represent individual MDA/TBARS or PON1 values. Solid squares with whiskers represent means with 95% confidence intervals (95% CI)

in BD as compared to other groups, with an exception of MCI, but did not show significant differences with respect to the type of dementia.

There was a significant age discrepancy between the control and dementia groups (Table 1). MDA/TBARS concentrations and PON1 arylesterase activities did not correlate with age in any of the studied groups (MDA/TBARS: $p = 0.790$ for AD group, $p = 0.405$ for MD group, $p = 0.526$ for VaD group, $p = 0.811$ for BD group, $p = 0.418$ for HA group, and $p = 0.720$ for MCI group; PON1: $p = 0.515$ for AD group, $p = 0.435$ for MD group, $p = 0.543$ for VaD group, $p = 0.935$ for BD group, $p = 0.498$ for HA group, and $p = 0.562$ for MCI group). However, there were significant correlations with age when the study population was analyzed as a whole ($r = 0.43$, $p < 0.001$ for MDA/TBARS and $r = -0.24$, $p < 0.001$ for PON1). Therefore, for the whole cohort evaluation, age- and sex-adjusted analysis was employed. Analysis of covariance revealed a significant effect of health status ($p < 0.001$) and insignificant effects of age ($p = 0.931$) and sex ($p = 0.339$) on MDA/TBARS. Similarly, health status ($p = 0.041$), but not age ($p = 0.163$) or sex ($p = 0.071$), was a significant predictor of PON1 arylesterase activity.

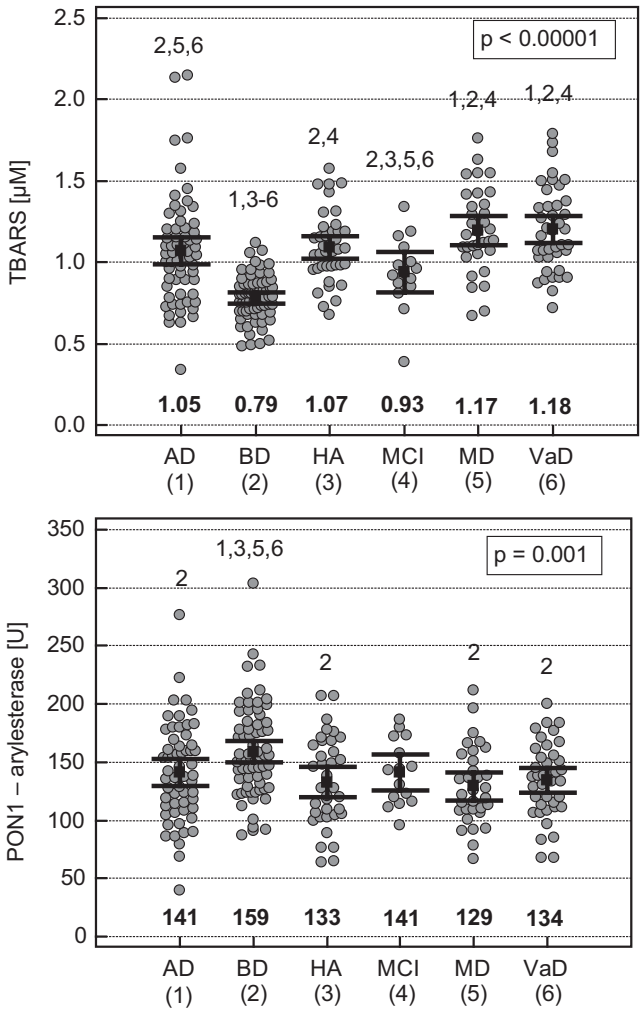

Fig. 2. Comparison of MDA/TBARS concentrations and PON1 activity between healthy individuals, individuals with benign headaches and memory deficits, patients with mild cognitive impairment, and patients with dementia of different pathology. Circles represent individual MDA/TBARS or PON1 values. Solid squares with whiskers represent means with 95% confidence intervals (95% CI). The numbers above the data indicate significance of differences between groups in: Alzheimer's disease (1); blood donors (2); individuals with headaches, dizziness and/or complaining about weak memory but without dementia or MCI (3); mild cognitive impairment (4); mixed-type dementia (5); and vascular dementia (6)

MDA/TBARS and PON1 as dementia and dementia differential markers

The ROC analysis was employed to evaluate MDA/TBARS and PON1 power in discriminating demented from non-demented individuals (dementia markers) as well as in differentiating dementia with and without vascular component (differential markers).

The AUCs for MDA/TBARS and PON1 as dementia markers were 0.75 ($p < 0.0001$) and 0.59 ($p = 0.011$), respectively. At the cut-off >1.0 μM and ≤142.2 U for MDA/TBARS and PON1, the sensitivities and specificities were as follows: 71% and 75% for MDA/TBARS and 60% and 59% for PON1.

The AUCs for MDA/TBARS and PON1 as differential markers were 0.58 ($p = 0.150$) and 0.57 ($p = 0.269$), respectively. At the cut-off >1.061 μM and ≤153.5 U for MDA/TBARS and PON1, the sensitivities and specificities were as follows: 73% and 46% for MDA/TBARS and 75% and 48% for PON1.

PON1 phenotype distribution

The prevalence of the A phenotype of PON1 was 63% in AD group, 59% in VaD group, 50% in MD group, 47% in MCI group, 61% in HA group, and 48% in BD group. There was a 15% difference in the prevalence between AD and BD groups at the trend level significance ($p = 0.095$).

In patients with vascular component (MD and VaD) stratified by the presence of strategic infarcts, the prevalence of the A phenotype was 61.5% in patients without and 55.5% in patients with strategic infarcts ($p = 0.671$).

Interrelationship between PON1 alloforms and MDA/TBARS

Controls with the phenotype A of the enzyme had significantly lower concentrations of MDA/TBARS than those with the phenotype B (0.85 μM (0.8–0.9) vs 0.94 μM (0.87–1.0), $p = 0.044$). Similarly, MD patients with the phenotype A had significantly lower concentrations of MDA/TBARS than those with the phenotype B of the enzyme (1.1 μM (0.97–1.23) vs 1.29 μM (1.15–1.42), $p = 0.045$). In the other studied groups, the phenotype-related difference in MDA/TBARS was not significant.

The correlation of PON1 and MDA/TBARS with the degree of cognitive deficits

The MDA/TBARS were positively correlated with MMSE in demented patients with vascular involvement (MD and VaD groups: $\rho = 0.28$, $p = 0.017$). Paraoxonase 1 did not exhibit any association with MMSE, but it correlated with CDR scale in the VaD group; the patients with CDR-1 grade had significantly higher PON1 activity than those with CDR-2 and CDR-3 grades (140.6 U (132–149) vs 127.4 U (118–137), $p = 0.040$).

PON1 and MDA/TBARS and the level of brain atrophy and vascular changes

MDA/TBARS positively correlated with GCA scores in all patients who underwent neuroimaging ($\rho = 0.21$, $p = 0.020$, $n = 127$). The association was stronger in patients with dementia and vascular involvement ($\rho = 0.30$, $p = 0.022$, $n = 57$), particularly those with strategic infarcts ($\rho = 0.58$, $p = 0.015$, $n = 17$). Arylesterase activity of PON1 negatively correlated with the MTA score in patients with dementia and with vascular involvement ($\rho = -0.27$,

p = 0.049, n = 57), particularly those with strategic infarcts (ρ = −0.55, p = 0.024, n = 17). None of the parameters correlated with the Fazekas score.

PON1 and MDA/TBARS and ischemia

Arylesterase activity of PON1 negatively correlated with the HIS in VaD patients (ρ = −0.46, p = 0.004) and in patients with strategic infarcts (ρ = −0.56, p = 0.015). A similar tendency was observed in MD patients (ρ = −0.34, p = 0.061). MDA/TBARS displayed only a tendency towards a weak correlation with the HIS score in a whole studied population (ρ = 0.14, p = 0.073).

Multivariate analysis

The effect of mental deficits on MDA/TBARS and PON-1 was co-examined with the effect of brain abnormalities detected with neuroimaging and with the HIS. The GCA score was the only variable retained in the multiple regression model explaining the MDA/TBARS variability with partial correlation coefficient r = 0.23. The HIS was the only variable retained in the multiple regression model explaining PON1 variability with partial correlation coefficient r = −0.44.

Discussion

In this study, we demonstrated an increase in MDA/TBARS accompanied by a drop in PON1 activity in patients with dementia, particularly these with vascular involvement, which was related to the severity of brain atrophy or ischemia rather than to the degree of cognitive decline.

Oxidative stress has been shown to contribute to the pathogenesis of AD with the markers of oxidative imbalance evident both locally and systemically.[19] Accordingly, the accumulation of MDA has been consistently reported in AD patients.[4] There is a scarcity of studies comparing MDA levels between AD and VaD patients. In addition, these studies have yielded conflicting results, showing either equally high MDA levels in both types of dementia[20] or significantly higher MDA accumulation in AD[21] or, contrarily, in VaD.[22] Taking into account difficulties in strict AD and VaD differentiation, a need for easily accessible biomarkers of vascular involvement has been stressed, and possible application of MDA has been suggested but not tested.[22] In this study, we corroborated previous reports on elevated levels of MDA/TBARS in dementia. By showing MDA/TBARS to be significantly higher in VaD/MD than AD, we confirmed its particular association with vascular involvement. Our finding is in line with close cause-and-effect relationship between OS and vascular diseases supporting its involvement in the pathogenesis of VaD.[3] Adding to existing knowledge, we assessed MDA suitability as a differential dementia marker. However, despite its higher levels in dementia with vascular involvement, MDA/TBARS failed to display significant diagnostic power to be applied as a differential biomarker. Yet, MDA/TBARS showed a moderate accuracy as a general dementia marker in our mixed cohort with AUC identical to previously reported ones for AD.[23]

Similarly to many other authors,[24–28] we demonstrated a significant decrease in PON1 arylesterase activity in AD. However, studies comparing PON1 activity between distinct subtypes of dementia have yielded conflicting results. Bednarska-Makaruk et al.[24] observed that a drop in PON1 activity was particularly expressed in the case of AD-related pathology, whereas Paragh et al.[28] showed no differences. Others, like us, have found it to be lowered in dementias with vascular involvement.[26,27,29] Moreover, Cervellati et al.[30] reported low PON1 activity to be associated with an increased risk of MCI progression to VaD rather than AD. Even though the difference in PON1 activity between patients with dementia with and without vascular involvement in our cohort did not reach statistical significance, the enzyme association with vascular involvement seems to be confirmed by the negative correlation between PON1 activity and HIS index, reflecting the degree of ischemia. This finding is in agreement with the biological activity of PON1[5] and the results of epidemiological studies showing that the PON1 activity is inversely correlated with carotid and cerebral atherosclerosis.[31]

A direct correlation between MDA accumulation and cognitive decline is not unanimously observed. While some studies[32] have reported erythrocyte and serum MDA to be inversely related to MMSE scores in AD patients, this relationship has not been mentioned by others.[22,23,33] Liu et al.[34] found this correlation in the general stroke patients but not in the subgroup suffering from post-stroke cognitive impairment. Similarly, the correlation between PON1 and MMSE has been reported only by 1 group of researchers.[25] We, in turn, found MDA/TBARS to correlate with MMSE, but this association was counterintuitive, implying an improvement of cognitive function with the exacerbation of oxidative status. As expected, the association of PON1 with CDR suggested impaired protection against OS with the deterioration of cognitive functions. However, both correlations were weak and might have resulted from an interference with confounding factors. Indeed, when co-examined with the degree of brain alterations and HIS, the correlations between MDA/TBARS levels with MMSE, as well as between the PON1 activity and the CDR, lost their significance.

Quantitative structural MRI has evidenced structural changes in the brain already in the early phases of cognitive deterioration and the degree of regional atrophy has been associated with the severity of cognitive decline. Therefore, a more widespread application of neuroimaging in clinical routine practice, allowing for early and more accurate detection of dementia as well as its reliable

differential diagnosis, has been postulated.[11] The issue of possible correlation between biochemical markers of OS and neuroimaging has been addressed only recently. Bulboacă et al.[35] demonstrated a positive correlation between markers of nitro-OS and posterior cortical atrophy. To our knowledge, neither MDA nor PON1 have been previously analyzed in the context of presence and the degree of brain alterations accompanying dementia. We found that in patients with dementia, particularly those with strategic infarcts (within VaD group), systemic MDA/TBARS accumulation was proportional to the degree of structural brain changes expressed in terms of GCA rating scale. Also, PON1 activity negatively correlated with the degree of medial temporal lobe atrophy (MTA rating scale). However, structural changes in the brain were independently associated only with MDA/TBARS, whereas they lost significance when their effect was co-evaluated with ischemia in the case of PON1.

Results from animal studies have shown OS to precede plaque formation characteristic for AD.[36] The MCI is viewed as a transitional condition between physiological aging and AD and, as such, has gained increased attention. However, reports on OS in MCI are scarce and inconclusive.[4] Although some authors have reported the lack of a significant difference in MDA between controls and MCI patients[37] or its gradual increase along the sequence from healthy individuals to MCI and AD,[32] we and others found serum MDA/TBARS to accumulate in MCI and AD patients to a similar extent,[33] substantiating the notion that lipid peroxidation is a phenomenon that occurs early in the progression of dementia.[34] Likewise, in the case of MDA/TBARS, there is no consensus in the literature concerning PON1 in MCI, the activity of which has been reported to resemble that in healthy controls[29] or, contrarily, to be decreased similarly to patients with dementia.[26,27,38] Also, our results are inconclusive – the PON1 activity in MCI was similar to that observed in AD; yet, MCI was the only group for which the difference, as compared to the healthy controls, did not reach statistical significance.

An interesting observation of our study was an evident oxidative imbalance in HA subgroup of controls comprising individuals admitted to the Alzheimer Center due to persistent headaches, dizziness and/or memory loss complaints. The etiology of their problems was not elucidated; neither the loss of cognitive function nor any significant somatic or mental illnesses were diagnosed. Nevertheless, these patients had significantly lower PON1 activity than BD controls recruited through the Blood Donation Center. Moreover, their concentration of lipid peroxidation markers (MDA/TBARS) exceeded that observed in both BD group and MCI patients. Therefore, our results implicate OS also in the pathogenesis of seemingly benign conditions.

Genetic studies addressing variations in the *PON1* gene have revealed a lower frequency of the 192R allele among AD patients than in the general population, suggesting its role as a protective factor against disease development.[38] Although not consistently observed, this finding seems to corroborate our observations on the PON1 phenotype B that tended to be less prevalent in AD patients compared to healthy blood donors. The protecting effect attributed to the 192R allele might be associated with the PON1 activity as a natural inhibitor of cholinesterase. Cholinesterase inhibitors are employed as the first-line treatment for AD to prevent further loss of cognitive function via an increase in the availability of acetylcholine.[39] On the other hand, the alloenzyme Q of PON1 is believed to be more efficient in counteracting lipid peroxidation.[40] Accordingly, the concentrations of MDA/TBARS were lower in the control group and MD patients with phenotype A of the enzyme at the trend level significance.

Our study has certain limitations that need to be discussed. Due to a natural history of neurocognitive disorders and the fact that the age over 65 years is considered a contraindication for blood donation, there was a significant difference in age distribution between controls and patients. However, the PON1 activity and MDA/TBARS levels correlated with age exclusively in the whole cohort and the issue was addressed in age- and sex-adjusted analysis. The ANCOVA confirmed that observed differences in the PON1 activity and MDA/TBARS levels could be attributed solely to a diagnosis of dementia and not to age or sex. The low number of patients with MCI as well as the lack of neuroimaging data or the assessment of degree of cognitive impairment is yet another limitation, negatively affecting the relevance of our findings concerning this particular group.

Conclusions

In this study, we confirmed an impaired oxidative status in patients with dementia and, for the first time, showed that an increase in MDA/TBARS level and a decrease in the PON1 activity reflects the severity of brain atrophy established with neuroimaging. We found ischemia, assessed with the application of HIS index, to be an independent predictor of the PON1 activity. Additionally, our findings suggest that OS also accompanies pathological changes in benign conditions.

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Assessment of visfatin concentrations in the serum of male psoriatic patients in relation to metabolic abnormalities

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Abstract

Background. Visfatin is one of the pro-inflammatory adipokines secreted by adipose tissue cells. Recent scientific research has drawn attention to the role of adipokines in the pathophysiology of metabolic disorders and their association with inflammatory diseases, including psoriasis. Visfatin may be one of the important links explaining the connection between psoriasis and diseases which are components of metabolic syndrome.

Objectives. The aim of this study was to assess the serum visfatin concentration in patients with psoriasis and to evaluate its possible correlations with parameters of metabolic syndrome and the clinical severity of psoriasis.

Material and methods. A group of 102 patients with psoriasis and a control group of 40 healthy subjects were examined. The clinical severity of psoriasis was assessed according to Psoriasis Area and Severity Index), BSA (Body Surface Area) and DLQI (Dermatology Life Quality Index) indicators, the presence and type of obesity, and hypertension. In both the study and control groups, laboratory tests (C-reactive protein (CRP), glucose concentration, total cholesterol, low-density-lipoprotein (LDL0 cholesterol, high-density-lipoprotein (HDL) cholesterol, and triglycerides (TG)) were performed and serum visfatin concentrations were determined. The clinical data, results of laboratory tests and visfatin concentrations were then subjected to statistical analysis.

Results. There was a significantly higher concentration of visfatin in the psoriatic patients ($p < 0.001$) than in the control group. Significant positive correlations between visfatin concentration and PASI ($p = 0.008$) and BSA ($p = 0.007$) were observed. In the psoriatic group, there were positive correlations between the concentrations of visfatin and the concentrations of CRP ($p = 0.008$) and total cholesterol ($p = 0.002$). Visfatin concentration was elevated in the psoriatic patients who had elevated total cholesterol ($p = 0.001$), LDL cholesterol ($p = 0.012$) and TG levels ($p = 0.001$) compared to the psoriatic patients with normal levels of these lipid profile components.

Conclusions. The results indicate the possible participation of visfatin in pathophysiological and inflammatory processes in the course of psoriasis. Adipokines may be an important link connecting psoriasis with coexisting metabolic disorders.

Key words: psoriasis, visfatin, metabolic syndrome

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Introduction

Psoriasis is a chronic, genetically determined inflammatory skin disease with autoimmune involvement and a pathogenesis which still has not been completely revealed. Recent literature data indicates that psoriasis can be included in the group of chronic inflammatory systemic diseases (CIDs). The presence of various mediators – including cytokines and adipokines – responsible for systemic inflammation and its metabolic consequences has been demonstrated not only in the affected skin but also in peripheral circulation. Inflammatory mediators, also secreted by adipose tissue cells, participate in inducing vascular endothelial dysfunction and insulin resistance, increasing the concentration of thrombogenic factors and promoting oxidative stress and lipid oxidation disorders. Adipokines may be an important link connecting psoriasis with coexisting metabolic disorders.

Visfatin is a 52 kDa protein composed of 473 amino acids secreted mainly by hepatocytes, macrophages and adipocytes of visceral adipose tissue.[1–8] The intracellular form of the protein is crucial in cellular metabolism regulation, adaptation to extracellular stressors and cell survival. The extracellular form detected in the circulation and extracellular environment has an influence on the inflammatory and metabolic processes.[3,5,9,10] Some studies have indicated the involvement of visfatin in the pathogenesis of abdominal obesity, atherosclerosis, type 2 diabetes mellitus, and vascular and inflammatory diseases,[1,4–6,9,11] because the protein has enzymatic, metabolic, inflammatory, and immunomodulatory properties.[5] Moreover, the protein affects vascular endothelial cells and vascular smooth muscle cells (VSMC) and it stimulates neoangiogenesis.[5,12] Visfatin contributes to increased expression of adhesion molecules, i.e., intercellular adhesion molecule *1* (ICAM-1), vascular cell adhesion molecule *1 (*VCAM-1), selectin, and vascular endothelial growth factor (VEGF), which results in increased recruitment and adherence of the immune cells to the vascular endothelium, increased proliferation and migration of the endothelium, and the formation of new blood vessels. Visfatin affects the inflammatory reaction in the vessel, inducing increased secretion of the molecules interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor α (TNF-α), matrix metalloproteinase 2 (MMP-2), and MMP-9 by endothelial cells and monocytes,[5,13–17] and activating nicotinamide adenine dinucleotide oxidase.[3] Exogenous protein administration has been shown to increase the synthesis of the inducible nitric oxide synthase (iNOS) enzyme, impairing the production of nitric oxide (NO) and enhancing oxidative stress in atherosclerotic plaque.[4,5,9,12,13,18,19] On the other hand, recently published scientific reports point to a beneficial effect of visfatin on the course of ischemic processes. It seems that due to its antiapoptotic properties, it promotes the prolonged survival of myocardial and neuronal cells; therefore, the protein may prove to be a useful therapeutic tool in reducing the area of myocardial or cerebral ischemia in the course of acute necrosis of these organs.[3,5,20–22] The proangiogenic, antiapoptotic and pro-inflammatory properties of visfatin may indicate possible involvement in the development of cancer.[5,9]

Objectives

Due to the relationship between psoriasis and metabolic syndrome, as well as the important role of visfatin in the induction of vascular endothelial dysfunction, our research was undertaken to investigate the relationship between the serum visfatin concentrations, psoriasis and individual components of metabolic syndrome.

Material and methods

The study included 102 adult, male psoriatic patients and 40 healthy, age-matched men. The patients with psoriasis were interviewed about the duration of their disease, the coexistence of psoriatic arthritis, any medications taken, and the presence of systemic comorbidities – with special attention to disorders that are components of metabolic syndrome, such as hypertension, diabetes, lipid disorders, and coexisting ischemic heart disease. The clinical condition of the patients was assessed by measuring blood pressure; determining the extent and severity of psoriatic skin lesions with the use of the indicators PASI (Psoriasis Area and Severity Index), BSA (Body Surface Area) and DLQI (Dermatology Life Quality Index); and assessing the presence and extent of obesity with body mass index (BMI) and waist–hip ratio (WHR). Patients with BMI values above 30 were considered obese. The WHR values higher than 1 confirmed the presence of abdominal obesity. The PASI values exceeding 10 indicated a moderate course of the disease, whereas patients with values over 18 were classified as having severe psoriasis. Patients in both the psoriasis group and the control group were assessed for serum concentrations of visfatin and laboratory parameters: C-reactive protein (CPR) concentration and components of the lipid profile (total cholesterol, high-density-lipoprotein (HDL) cholesterol, low-density-lipoprotein (LDL) cholesterol, triglycerides (TG), and glucose). Serum visfatin concentrations were determined with the immunoenzymatic enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's recommended procedure using an AdipoGen Nampt (Visfatin/PBEF) human ELISA Kit (test sensitivity: 3.0 pg/mL, absorbance reading: 450 nm) (AdipoGen Corp., San Diego, USA). For the analysis of the results obtained, using IBM SPSS software v. 19 (IBM Corp., Armonk, USA), statistical significance was assumed at a p-value <0.05.

Results

There was a significantly higher concentration of visfatin in psoriatic patients than in the control group ($p < 0.001$) (Fig. 1, Table 1). In the study population, a significant correlation between serum visfatin concentration and CRP value ($p = 0.008$) was found; a positive correlation was also observed between the serum concentration of visfatin and PASI ($p = 0.008$) (Fig. 2) and BSA ($p = 0.007$) (Fig. 3). The protein concentrations were also significantly higher in the patients with severe psoriasis, as measured with PASI, compared to patients with mild disease severity ($p = 0.013$). However, no significant correlations were observed between the serum concentration of visfatin and DLQI ($p = 0.544$) or duration of the disease ($p = 0.576$). There were no correlations between the serum concentration of visfatin and BMI or fat distribution (WHR) ($p = 0.380$ and $p = 0.095$, respectively). In the psoriatic group, there was a significant positive correlation between serum visfatin concentration and total cholesterol level ($p = 0.002$). Serum visfatin concentration was significantly higher in the psoriatic patients who also had elevated total cholesterol, LDL cholesterol and TG levels compared to the patients with normal values of these parameters ($p = 0.001$, $p = 0.012$ and $p = 0.001$, respectively). The concentration of visfatin was not different in the psoriatic patients with and without concomitant arterial hypertension ($p = 0.749$).

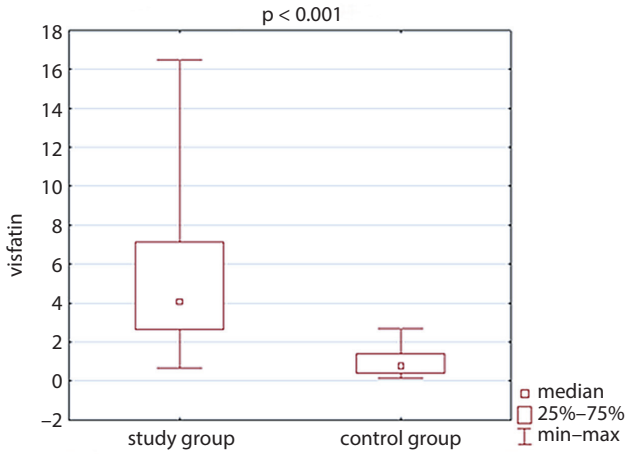

Fig. 1. Assessment of visfatin concentration in the experimental and control groups

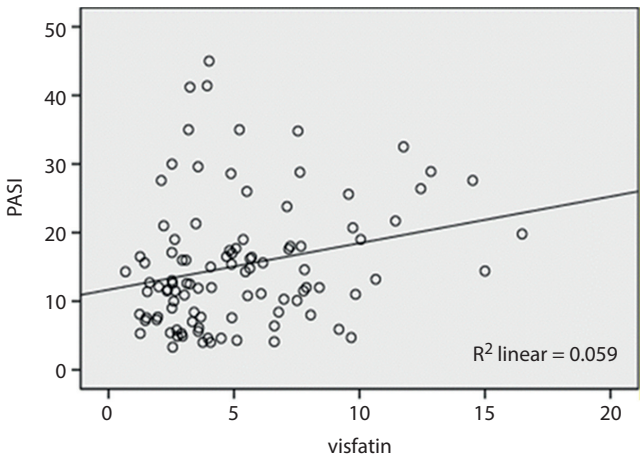

Fig. 2. Correlation between visfatin and PASI values

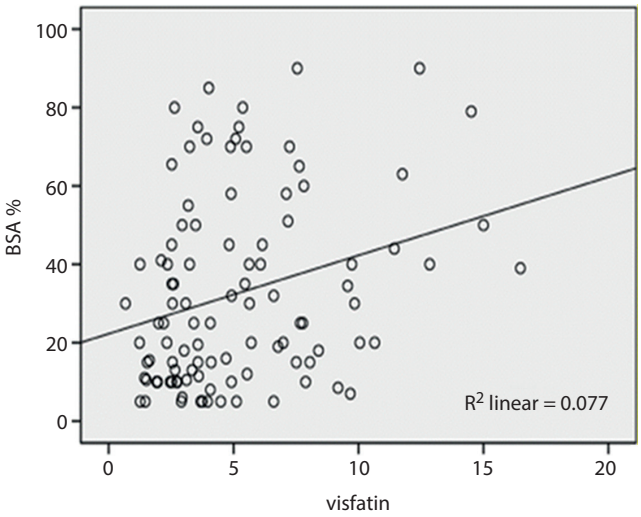

Fig. 3. Correlation between visfatin and BSA values

Discussion

Visfatin belongs to the group of adipokines produced by adipose tissue cells. The literature indicates visfatin involvement in the pathogenesis of many inflammatory diseases, e.g., ulcerative colitis, Crohn's disease or hepatitis C.[9,17] It is possible that the protein also participates in pathophysiological processes in psoriasis. In our own study, significantly higher concentrations of visfatin were observed in the group of psoriatic patients than in the control group. Moreover, in the experimental group, positive correlations between serum visfatin concentration and PASI and BSA values were also found.

Similar results have been presented by Ismail et al.[23] in investigating the serum concentrations of visfatin and

Table 1. The concentration of visfatin in patients with psoriasis and in the control group

Visfatin	Number of patients	Average	Median	Minimum	Maximum	Lower quartile	Upper quartile	SD
Experimental group	102	5.19	4.07	0.67	16.48	2.66	7.11	3.31
Control group	40	0.93	0.78	0.15	2.67	0.40	1.38	0.62

$Z = 8.694$; $p < 0.001$; SD – standard deviation.

omentine in a group of 46 patients with plaque psoriasis. The authors found a significantly elevated concentration of visfatin in the psoriatic patients compared to a group of 42 healthy people. In addition, they observed positive correlations between visfatin concentration and PASI value and disease duration. Moreover, visfatin concentration was significantly higher in the patients with severe psoriasis. The results of our own study are also in agreement with the ones presented by Okan et al.[24] who observed a positive correlation between visfatin concentration and disease severity in a group of 45 patients with plaque psoriasis, as assessed with PASI.

Campanati et al.[25] evaluated the serum adipokines in patients with psoriasis before and after the 24-week period of treatment with TNF-α inhibitors. As in our own study, elevated concentrations of visfatin were found in the patients with psoriasis compared to the control group. A significant difference in the serum visfatin concentration between the experimental and control groups was maintained even after 24 weeks of biological treatment. The authors, however, did not find any relationship between the concentration of visfatin and the severity of the disease.

On the contrary, Sereflikan et al.[26] assessed the concentrations of visfatin, adiponectin and TNF-α in the group of 42 psoriatic patients and noticed that although the levels of visfatin in the experimental group were higher than in the controls, the difference was not statistically significant. The authors did not observe a significant correlation between serum visfatin concentration and PASI value.

In our study, higher concentrations of CRP were recorded in the study group than in the control group ($p = 0.008$), and a positive correlation between serum CRP and visfatin concentration ($p = 0.008$) was found. Similarly, Catalán et al.,[27] studying a group of 30 obese men and a 14 controls, found a positive correlation between visfatin concentration and not only CRP concentration but other inflammatory parameters as well, i.e., the levels of fibrinogen and von Willebrand factor. Lu et al.[28] investigated a group of 173 patients with chronic kidney disease and also showed positive correlations between serum visfatin concentration and CRP value and the total number of leukocytes and neutrocytes.

Visfatin is known to be a protein produced by macrophages of the visceral adipose tissue, among other tissues. However, previous studies have yielded conflicting data on concentrations of the protein in correlation with BMI and adipose tissue distribution. In our own study, there was no statistically significant correlation between serum visfatin concentration and BMI ($p = 0.380$) or WHR ($p = 0.095$) in the group of psoriatic patients. This relationship was still not observed after dividing the study population into subgroups of normal body mass, overweight and obesity, as well as subgroups with gluteal-femoral and abdominal fat distribution.

Other authors[23,25,29] investigating the concentration of visfatin in patients with psoriasis and lipid disorders, insulin resistance and hypertension obtained similar results. In the psoriatic patients, the authors did not find any correlation between serum visfatin concentration and obesity rate. Similarly, Ozal et al.[30] did not observe a significant correlation with BMI values in patients with hypertension with and without coexisting obesity.

Interestingly, the results obtained by Sereflican et al.[26] were different from the results of the present study and the abovementioned ones. The authors observed a correlation between serum visfatin concentration and BMI value in a group of 42 Turkish psoriatic patients. Similarly, elevated concentrations of visfatin in groups of obese patients and a positive correlation of visfatin with BMI and abdominal obesity have also been observed by other authors.[27,31–35]

Dyslipidemia is an essential element of metabolic syndrome, occurring more often in people suffering from psoriasis. Data on the relationship between serum visfatin level and lipid profile components is divergent and does not give a definite answer as to whether the protein can serve as a predictor of these disorders.

While studying lipid disorders, we found significantly higher total cholesterol levels in our group of psoriatic patients in comparison to the control group ($p < 0.001$). There was also a significant positive correlation between serum visfatin and total cholesterol concentrations ($p = 0.002$). However, no significant correlation was observed between serum visfatin concentration and the level of LDL cholesterol ($p = 0.059$), HDL cholesterol ($p = 0.249$) and TG ($p = 0.224$). However, after dividing the subjects into groups with normal and abnormal lipid profiles, a significantly higher concentration of visfatin in patients with both psoriasis and elevated total cholesterol ($p = 0.001$), LDL cholesterol ($p = 0.012$) and TG ($p = 0.001$) was found in comparison with the patients without dyslipidemia.

While Catalán et al.[27] showed a positive correlation between the concentration of visfatin and lipid profile components – i.e., total cholesterol, HDL cholesterol and TG – in their study of 30 individuals with morbid obesity, independent of other factors, Coban et al. observed a negative correlation between visfatin concentration and total cholesterol and LDL cholesterol levels in a group of psoriatic patients.[36]

Some data indicate a significant effect of visfatin on the cardiovascular system.[5] In addition to involvement in the induction of vascular endothelial dysfunction, vascular damage and atherosclerosis, there is more and more evidence on the association of this adipokine with the regulation of arterial blood pressure, though it remains the subject of numerous controversies.

In our own analysis, after dividing the patients into those suffering from arterial hypertension and those without the disease, there was no statistically significant difference in visfatin concentration between these subgroups ($p = 0.749$). Similar results were presented by Kocelak et al.[37] from a study on a large group of 2,789 older Polish

patients. The authors divided the patients into groups composed of people without hypertension, patients with pharmacologically regulated hypertension and those untreated for the disease. The authors did not observe significantly higher visfatin values in patients with hypertension compared to patients not suffering from the disease, nor did they find any influence of treatment or disease severity on the hormone levels. In contrast, significantly higher visfatin values were observed in patients with elevated CRP levels (hs-CRP) and homeostatic model assessment of insulin resistance (HOMA-IR).

Likewise, other authors have reported that there was no significant correlation between serum visfatin concentrations and systolic and diastolic blood pressure values in groups of patients with ischemic heart disease and metabolic disorders.[28,38,39]

Different results were presented by Ozal et al.[30] when they compared the serum visfatin concentrations of a group of 71 patients with refractory hypertension with a group of 94 pharmacologically controlled patients. The results showed significantly higher adipokine concentrations in the first group and a positive correlation with systolic and diastolic blood pressure. Moreover, in this study, visfatin turned out to be an independent predictor of the severity of hypertension.

Liakos et al.,[40] whose results were consistent with the previous authors' reports, compared serum visfatin and apelin concentrations in patients with upper-limit values with the concentrations in healthy subjects. Significantly higher concentrations of visfatin and lower apelin concentrations were found in the study group, suggesting a possible contribution of both adipokines in the prognosis of hypertension.

Conclusions

Visfatin is a pro-inflammatory adipokine and some literature data indicate its involvement in metabolic disorders, autoimmune diseases and cancer. In our study, the significantly higher concentrations of visfatin found in the patients with psoriasis compared to the control group indicate a possible involvement of this adipokine in the pathophysiological processes present in the course of psoriasis. Since positive correlations between serum visfatin concentration and CRP level and PASI and BSA values have been demonstrated, visfatin may prove to be a useful biomarker of systemic inflammation and psoriasis severity.

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Moderate-to-severe ovarian hyperstimulation syndrome: A retrospective multivariate logistic regression analysis in Chinese patients

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Conflict of interest
None declared

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Abstract

Background. Ovarian hyperstimulation syndrome (OHSS), a life-threatening complication occurring in stimulated ovarian cycles, arises from treatment with gonadotropin for inducing follicular maturation.

Objectives. The aim of this study was to compare the risk factors between patients with severe OHSS and those without OHSS after in vitro fertilization by intracytoplasmatic sperm injection/embryo transfer (IVF-ICSI/ET). Identifying the associated risk factors may provide guidance for clinicians on how to prevent OHSS.

Material and methods. The retrospective study involved patients who had completed IVF-ICSI/ET cycles. The difference in markers for predicting the occurrence of OHSS between groups was compared. The potential protective and risk factors, as well as the predictive markers, were identified.

Results. Patients with OHSS were younger ($p = 0.015$), had higher basal antral follicle counts (AFC) ($p < 0.001$) and lower total dosages of gonadotropin (Gn) ($p = 0.011$). On the day of human chorionic gonadotropin (hCG) administration, significantly higher total numbers of follicles ($p < 0.001$), serum estradiol (E2) ($p < 0.001$) and progestrone (Pg) ($p = 0.001$) levels, numbers of oocytes ($p < 0.001$) and metaphase II (MII) oocytes ($p < 0.001$) were also observed in the OHSS group when compared to the non-OHSS group. A univariate regression analysis revealed that age (OR = 0.898, 95% CI = 0.822–0.981) and total dosage of Gn (OR = 0.999, 95% CI = 0.999–1.000) were protective factors, whereas AFC (OR = 1.090, 95% CI = 1.051–1.131) and, on the day of hCG injection, the number of follicles (OR = 1.185, 95% CI = 1.027–1.230), serum E2 (OR = 1.000, 95% CI = 1.000–1.000) and Pg (OR = 2.773, 95% CI = 0.510–3.370) levels, the number of oocytes (OR = 1.254, 95% CI = 0.894–1.472) and MII oocytes (OR = 1.238, 95% CI = 0.747–1.217) were risk factors for OHSS. However, a multivariate regression analysis showed that the total number of follicles (OR = 1.124, 95% CI = 1.027–1.230) was the only predictive factor for the occurrence of OHSS.

Conclusions. The study demonstrated that the follicle count measured on the day of hCG administration was the only predictive factor for the occurrence of OHSS. This provides basic guidance to clinicians on the prevention of the complication when using assisted reproductive technologies (ART).

Key words: in vitro fertilization, ovarian hyperstimulation syndrome, human chorionic gonadotropin

Ovarian hyperstimulation syndrome (OHSS), a life-threatening complication occurring in stimulated ovarian cycles, arises from treatment with gonadotropin for inducing follicular maturation. The syndrome is commonly observed in women undergoing treatment with controlled ovarian hyperstimulation (COH) employed during assisted reproductive technology (ART). It is characterized by a significant increase in vascular permeability and ovarian enlargement,[1,2] as well as hemoconcentration with increased blood viscosity and leakage of fluid into the extravascular space. Several possible mediators, such as vascular endothelial growth factor (VEGF), renin-angiotensin system and other cytokines, may be involved the development of this complication.[3] Patients with severe OHSS may also develop ascites, pleural and pericardial effusion, thromboembolism and multiple organ failure.[2,4]

The incidence of moderate-to-severe OHSS is approx. 3.1–8% of in vitro fertilization (IVF) cycles, but could be as high as 20% in high-risk women.[2,5,6] Monitoring serum estradiol (E2) levels was deemed effective in reducing the incidence of OHSS[7,8]; however, its relevance to predicting the occurrence of OHSS, especially during COH, remains controversial.[9,10] Several factors associated with OHSS have been identified, including young age, low body mass index (BMI), polycystic ovary syndrome (PCOS) or polycystic ovarian morphology, high anti-Mullerian hormone levels, a high antral follicle count (AFC), high gonadotropin (Gn) dosage, previous OHSS, high serum E2 levels, a high number of follicles or collected eggs, luteal support with human chorionic gonadotropin (hCG), and pregnancy.[11–13] Since there is no specific effective treatment for OHSS, there has been considerable research aimed at identifying predisposing factors in order to predict early onset of the complication.

In this study, patients undergoing in vitro fertilization procedures by intracytoplasmic sperm injection/embryo transfer (IVF-ICSI/ET) at our reproductive center were retrospectively analyzed to explore the risk factors associated with the occurrence of severe OHSS.

Materials and methods

Study population

Between January 2013 and December 2014, a total of 350 IVF-ICSI/ET cycles were performed at our hospital. Excluding 50 cycles cancelled for cycle stimulation or with incomplete clinical data, 300 complete ovarian stimulation cycles were retrospectively analyzed. Among the 300 cycles included in this study, clinical data obtained from the 33 cycles with moderate-to-severe OHSS were grouped into the OHSS group and the other 267 cycles were grouped into the non-OHSS group. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki; written informed consent was obtained from all the participants enrolled; and this study was approved by the local Ethics Committee.

IVF-ICSI/ET treatment

Gonadotropin-releasing hormone agonist (GnRH-a)/Gn/hCG was used as treatment to induce ovulation. In this study, induction therapy included short-acting (n = 183), long-acting (n = 65) and ultra-long-acting (n = 23) long-term treatment, micro-stimulation treatment (n = 15), short-term treatment (n = 9), antagonist treatment (n = 3) and improved ultra-long term treatment (n = 2).

Short- and long-acting long-term treatment

A one-third-dose or half-dose depot of long-acting GnRH-a, or a dosage of 0.05–0.1 mg per day of short-acting GnRH-a until the day of hCG injection was administered 5–7 days after ovulation or after 15 days of Marvelon. Following 10–14 days of GnRH-a, hCG was injected when the following criteria were met: a) at least 1 follicle with a diameter of 19 mm, 2 follicles with a diameter of 18 mm or 3 follicles with a diameter of 17 mm; b) an average blood E2 level of 250–300 ng/L when each dominant follicle had a diameter of 16 mm or greater or at least 60% of follicles had a diameter greater than 16 mm. The dosage of the hCG injection was 5,000–10,000 IU, and oocytes were retrieved 34–36 h after the injection.

Short-term treatment

Starting on the 2nd day of the menstrual cycle, short-acting GnRH-a was given at a dosage of 0.1 mg per day, and on the first 2–3 days of the menstrual cycle, Gn was given until the day of hCG administration. The condition for hCG injection was the same as in the long-term treatment, and oocytes were retrieved 34–36 h after the injection.

Ultra-long-term treatment

On the 2nd day of the menstrual cycle, the 1st dose of long-acting GnRH-a was given at a dosage of 3.75 mg, followed by a 2nd dose after 28 days. A total of 2 or 3 subsequent doses was given at the same interval; 14–16 days after the last dose of GnRH-a, Gn was initiated at a dosage of 1.875 mg until the day of hCG administration. The conditions for hCG injection were the same as in the long-term treatment, and oocytes were retrieved 34–36 h after the injection.

Antagonist treatment

On the 2nd–3rd day of the menstrual cycle, Gn treatment was initiated to induce ovulation. When a follicle with a diameter of nearly 14 mm or greater was observed,

GnRH-a was given at a dosage of 0.25 mg per day until the day of hCG administration. Again, the conditions for hCG injection were the same as in the long-term treatment, and oocytes were retrieved 34–36 h after the injection.

Micro-stimulation treatment

On the 3rd day of the menstrual cycle, oral clomiphene citrate (50–100 mg per day) or letrozole (2.5–5 mg per day) in combination with Gn (150 IU per day) was started for a total of 5 days. On the 8th day of the menstrual cycle, the size of the follicles was measured using vaginal ultrasonography. If the follicle diameter was less than 10 mm, the dosage of Gn was increased by 75 IU per day. Otherwise, the dosage was maintained. Again, the condition for hCG injection was the same as in the long-term treatment, and oocytes were retrieved 34–36 h after the injection.

Collection of the clinical data

Prior to IVF-ICSI/ET therapy, the patients' demographics, including age, BMI, infertility duration and AFC, were collected. During the therapy, the Gn dosage, number of days of Gn treatment, the FSH, LH and E2 levels on the day of Gn treatment initiation, and the LH level, E2 level, Pg level, the total number of follicles, the number of oocytes and the number of metaphase II (MII) oocytes on the day of hCG injection were obtained. The basal antral follicular count was measured using vaginal ultrasonography between the 2nd and 3rd day of the menstrual cycle, and antral follicles with a diameter of 2–10 mm were counted. The number of follicles with a diameter greater than 12 mm was counted on the day of hCG administration using vaginal ultrasonography. MII oocytes, defined as the presence of the nuclear materials or polar body after removing the cumulus granulosa cells, were collected and counted 16–18 h after conventional IVF fertilization. The MII oocytes fertilized using intra-cytoplasmic sperm injection during the microinjection of spermatozoa were counted when the first polar body was observed.

Diagnosis and classification of OHSS

According to expert consensus on the diagnosis and treatment of polycystic ovarian syndrome by the Endocrinology Group of Chinese Society of Obstetrics and Gynecology, OHSS can be classified as mild, moderate, severe, and life-threatening on the basis of clinical signs and symptoms, and ultrasound and laboratory findings. In this study, only those with moderate-to-severe OHSS patients were included in the OHSS group.

Statistical methods

All data was analyzed using SPSS v. 17.0 software (SPSS Inc., Chicago, USA). Quantitative data were expressed as means ± standard deviation (SD) and differences in parameters between the groups were compared using independent sample t-tests or χ^2 tests as appropriate. Logistic regression was used to identify risk factors.

Results

Comparison of general conditions between the OHSS and non-OHSS groups

The differences in general conditions between the OHSS and non-OHSS patients are displayed in Table 1. The mean age of the patients in the OHSS groups was 2 years younger than in the non-OHSS group; this difference was statistically significant ($p = 0.015$). A significant statistical difference in AFC was observed between the patients in the OHSS and non-OHSS groups (24.82 ±8.11 vs 16.30 ±8.86, $p < 0.001$). The results demonstrated that patients with bilateral multiple follicular development and age less than 30 years were a high-risk population for OHSS. No statistical difference in BMI or the duration of infertility was observed between the 2 groups.

Table 1. Comparison of general conditions in the OHSS and non-OHSS groups

Variables	OHSS group (n = 33)	Non-OHSS group (n = 267)	p-value
Age [years]	29.79 ±3.45	31.77 ±4.52	0.015*
BMI	21.66 ±2.94	21.46 ±2.81	0.706
Duration of infertility [years]	3.76 ±2.21	4.41 ±3.28	0.267
AFC	24.82 ±8.11	16.30 ±8.86	<0.001*

*statistically significant; OHSS – ovarian hyperstimulation syndrome; BMI – body mass index; AFC – antral follicle count.

Comparison of markers before and after treatment between the OHSS and non-OHSS groups

The differences in marker levels between OHSS and non-OHSS groups are displayed in Table 2. The total Gn dosage used in the OHSS group was 353 IU lower than in non-OHSS group; this difference was statistically significant ($p = 0.011$). On the day of hCG injection, serum E2 and Pg levels were significantly higher in the OHSS group than in the non-OHSS group ($p < 0.001$) and the total number of follicles was nearly double in the OHSS group compared with the non-OHSS group ($p < 0.001$). After egg retrieval, a significantly higher numbers of oocytes and MII oocytes was observed in the OHSS group than in the non-OHSS group ($p < 0.001$). On the day of initiation of Gn treatment, no significant difference was observed in the levels of FSH, LH and E2 between the groups; on the day of hCG injection, no significant difference was observed in the level of LH.

Table 2. Comparison of pre- and post-treatment marker levels in the OHSS and non-OHSS groups

Variable	OHSS group (n = 33)	Non-OHSS group (n = 267)	p-value
Total dosage of Gn [IU]	1823.48 ±640.58	2,176.08 ±2,176.08	0.011*
Number of days of Gn	10.39 ±1.77	10.40 ±2.27	0.987
On the day of Gn treatment			
FSH level [IU/L]	3.40 ±1.56	4.08 ±2.86	0.18
LH level [IU/L]	1.84 ±1.04	2.08 ±1.55	0.408
E2 level [ng/L]	10.50 ±9.61	14.90 ±15.95	0.123
On the day of hCG injection			
E2 level [ng/L]	8,476.18 ±5,007.85	4,479.02 ±4,154.47	<0.001*
LH level [IU/L]	1.51 ±0.77	2.25 ±2.15	0.051
Pg level [ng/L]	1.10 ±0.56	0.82 ±0.46	0.001*
Total number of follicles	22.42 ±6.83	12.31 ±7.30	<0.001*
Number of oocytes	14.52 ±5.29	8.90 ±4.47	<0.001*
Number of MII oocytes	12.73 ±5.27	7.68 ±4.26	<0.001*

*statistically significant; OHSS – ovarian hyperstimulation syndrome; E2 – estradiol; FSH – follicle stimulating hormone; Gn – gonadotropin; hCG – human chorionic gonadotropin; LH – luteinizing hormone; Pg – progesterone.

Table 3. Multivariate regression analysis results for predicting OHSS syndrome

Variable	Regression coefficient	Standard error	p-value	OR (95% CI)
Number of follicles on the day of hCG administration	0.11	0.05	0.011*	1.124 (1.027–1.230)

*statistically significant; OHSS – ovarian hyperstimulation; hCG – human chorionic gonadotropin; OR – odds ratio; 95% CI – 95% confidence interval.

Table 4. Univariate regression analysis results for risk of ovarian hyperstimulation syndrome

Variable	Regression coefficient	Standard error	p-value	OR (95% CI)
Age [years]	–0.11	0.05	0.017*	0.898 (0.822–0.981)
BMI [kg/m^2]	0.02	0.06	0.705	1.025 (0.903–1.163)
Duration of infertility [years]	–0.08	0.07	0.267	0.926 (0.808–1.061)
Basal AFC	0.09	0.02	<0.001*	1.090 (1.051–1.131)
Total Gn dosage [IU]	0.00	0.00	0.011*	0.999 (0.999–1.000)
On the day of Gn treatment				
Number of treatment days	–0.00	0.08	0.987	0.999 (0.848–1.176)
FSH level [IU/L]	–0.14	0.10	0.178	0.872 (0.867–1.577)
LH level [IU/L]	–0.12	0.15	0.408	0.886 (0.636–1.669)
E2 level [ng/L]	–0.03	0.02	0.122	0.969 (0.909–1.010)
On the day of hCG injection				
E2 level [ng/L]	0.00	0.00	<0.001*	1.000 (1.000–1.000)
LH level [IU/L]	–0.39	0.19	0.051	0.685 (0.551–1.467)
Pg level [ng/L]	1.02	0.34	0.002*	2.773 (0.510–3.370)
Total number of follicles	0.17	0.03	<0.001*	1.185 (1.027–1.230)
Number of oocytes	0.23	0.04	<0.001*	1.254 (0.894–1.472)
Number of MII oocytes	0.21	0.04	<0.001*	1.238 (0.747–1.217)

*statistically significant; OR – odds ratio; 95% CI – 95% confidence interval; OHSS – ovarian hyperstimulation syndrome; BMI – body mass index; AFC – antral follicle count; E2 – estradiol; FSH – follicle stimulating hormone; Gn – gonadotropin; hCG – human chorionic gonadotropin; LH – luteinizing hormone; Pg – progesterone.

Logistic regression analysis of risk factors for OHSS

The univariate logistic regression analysis demonstrated that age and the total dose of gonadotropin were protective factors, whereas baseline AFC, E2 and Pg levels, the total number of follicles, and the numbers of oocytes and MII oocytes on the day of hCG injection were risk factors for OHSS. Other parameters were not associated with the occurrence of OHSS. In a multivariate model using logistic stepwise regression analysis, the total number of follicles was selected as an independent risk factor for OHSS (Table 3). When the total follicle count was more than 17 on the day of hCG injection, the sensitivity and specificity of predicting the occurrence of OHSS were 84.85% and 77.15%, respectively, according to the model. Details of these results are presented in Table 4.

ROC curve of predicting the occurrence of OHSS by the total number of follicles on the day of hCG injection

The area under the ROC curve was 0.847 (95% CI = 0.775–0.919) for predicting the occurrence of severe OHSS with measuring the total number of follicles on the day of hCG administration (Fig. 1). An optimal cut-off value of 17 was determined with the ROC curve analysis.

Discussion

Ovarian hyperstimulation syndrome is a complication caused by the stimulation of ovarian cycles arising from medical treatment with gonadotropin for inducing follicular maturation. With the increasing use of assisted reproductive techniques and ovulation-induction drugs, there is a growing trend for the occurrence of OHSS.[14] During IVF treatment, the incidence of moderate-to-severe OHSS was approx. 3.1–8%, but could reach 20%

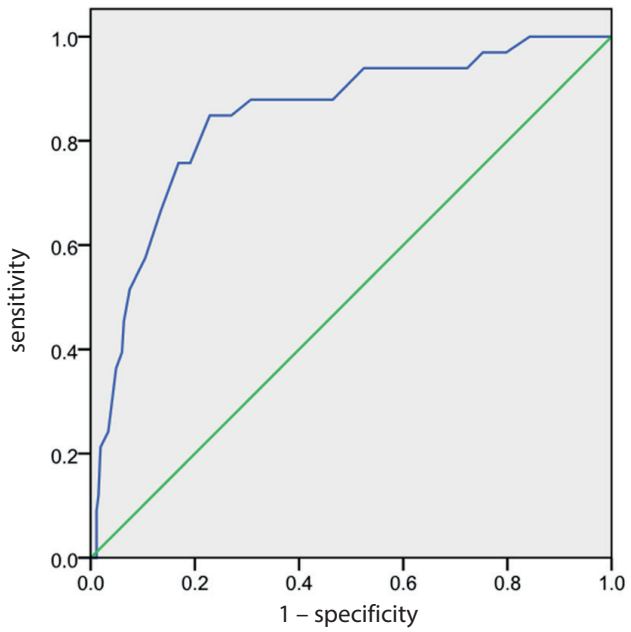

Fig. 1. Receiver operating characteristic (ROC) curve for predicting the occurrence of OHSS by the total number of follicles on the day of hCG injection

in high-risk women.[2,5,6] Severe OHSS is life-threatening and could lead to hospitalization in 0.1–2% of IVF cases.[15,16] Although the pathogenesis of OHSS is still unclear, VEGF, renin-angiotensin system and other cytokines may be involved. It is now recognized that hCG exposure is a risk factor for OHSS. During controlled ovarian stimulation (COS), injection of hCG induces follicular maturation and stimulates LH to the peak level. However, a high concentration of exogenous hCG exposure is considered to be a key factor in the onset of early OHSS, whereas late OHSS is mainly associated with endogenous hCG produced by corpus luteum.[17,18] Currently, there is lack of effective clinical treatment for OHSS, but mainly preventive measures.

Relationship between ovarian reserve and moderate and severe OHSS

Age and AFC are reliable indicators of ovarian reserve function. Our study demonstrated that patients at a younger age (less than 30 years) and with higher AFC (>24), which indicated that they had better ovarian reserve function, had a higher chance of experiencing moderate or severe OHSS in COS. These findings were consistent with a report presented at the 2013 European Society of Human Reproduction and Embryology (ESHRE) annual conference that patients with PCOS are at high risk for OHSS.[19] These patients had multiple antral follicles, increased numbers of follicles and high gonadotropin receptor densities, and therefore became more responsive to gonadotropin and subsequently more prone to the occurrence of OHSS.

Relationship between the use of Gn for COS in IVF cycles and the occurrence of OHSS

During COS in women undergoing the IVF-ICSI/ET cycle, in order to select more dominant follicles, the use of exogenous Gn, which increases the number of follicles, overcomes the inherent selectivity of a single dominant follicle so that multiple follicles can grow and become mature or near mature at the same time. In this study, the average total dosage of Gn in the OHSS group (1,823.48 ±640.58 IU) was 353 IU lower than that in the non-OHSS group (2,176.08 ±2,176.08 IU), but the duration of the use of Gn did not reveal any significant difference. This demonstrated that patients in the OHSS group have better ovarian reserves: Although they required fewer dosage of Gn, more follicles were obtained within the same duration of Gn usage as in the non-OHSS group. This suggested that pre-COS assessment of the ovarian reserve function is important and when appropriate follicles were obtained from young PCOS patients, the initial dosage and the duration of Gn usage should be reduced in order to prevent the occurrence of OHSS.

Relationship between the total number of follicles, E2 and Pg levels, the number of oocytes and MII oocytes, and the occurrence of OHSS

During an IVF cycle, GnRH-a is used for downregulation to increase the number of follicles obtained. Following the use of Gn for a period of time to obtain the dominant follicle, exogenous hCG is injected to promote final follicular maturation. Therefore, the total number of follicles and E2 levels on the day of hCG injection is critical. In our study, it was found that the total number of follicles with diameters greater than 12 mm was 22.42 ±6.83 in the OHSS group, which was significantly more than the number in the non-OHSS group (12.31 ±7.30). At our center, hCG injection was applied by the number of dominant follicles obtained. As a result, there were not many dominant follicles although the total number of follicles obtained was higher in the OHSS group and the number of days of Gn use were basically the same as in the non-OHSS group. This increased the probability of OHSS occurrence. On the other hand, in this study, the E2 and Pg levels and the number of oocytes and MII oocytes on the day of hCG injection were significantly different between the OHSS and non-OHSS groups. According to the multivariate logistic regression analysis, the total number of follicles on the day of hCG administration was the only predictive factor for the occurrence of OHSS. An optimal cut-off value of 17 was determined by the ROC curve analysis. In other words, when the total number of follicles was greater than 17, the sensitivity and specificity of the model in predicting the occurrence of moderate

and severe OHSS were 84.85% and 77.15%, respectively. Kwan et al.[20] and Aramwit et al.[21] reported that the E2 level and the number of oocytes retrieved can effectively predict the occurrence of OHSS. In our study, although both E2 levels and the number of oocytes were statistically different between the OHSS and non-OHSS groups, the multivariate logistic regression analysis did not reveal them as independent predictive factors. This may possibly be because the highest measurable value for the E2 marker was set at 4,300 ng/L, requiring dilution for measurement, which may have introduced a measurement error. However, in clinical settings, it is very frequent that the levels of E2 and the number of follicles do not match. As for the number of oocytes, since it depends on the surgical skills of doctors and the condition of the patients, it may not fully represent the total number of follicles, especially in patients who have difficulty in egg retrieval.

In summary, the multivariate logistic regression analysis revealed that the total number of follicles on the day of hCG administration is an independent predictive factor for OHSS. These findings could guide clinicians regarding the timing and dosage for the use of Gn during COS. When the total number of follicles exceeds 17, appropriate measures including Gn dose reduction, hCG administration or GnRH-a injection should be taken, and patients should be recommended for embryo cryopreservation to prevent the occurrence of OHSS.

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Non-standard AGE4 epitopes that predict polyneuropathy independently of obesity can be detected by slot dot-blot immunoassay

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

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Abstract

Background. Advanced glycation end-products (AGEs) are formed during cascade reactions between reducing sugars or reactive aldehydes and proteins, lipids or DNA molecules. They constitute a group of various stable compounds. Advanced glycation end-products are considered potential biomarkers of metabolic disorders. However, so far only a few methods to determine the level of individual AGEs have been developed.

Objectives. The aim of the study was to compare the efficiency of the slot-dot blot method and direct enzyme-linked immunosorbent assay (ELISA) in detecting non-standard epitopes of methylglyoxal (MGO)-modified proteins (AGE4) found in diabetes serum in trace amounts, and to assess AGE4 in diabetes and associated metabolic abnormalities.

Material and methods. The presence of AGE4 was detected using 2 methods: direct ELISA and the slot-dot blot method – a newly developed immunoassay based on monoclonal, commercially available antibody detection of non-standard AGE epitopes. AGE4 quantification, expressed as median AGE4 in arbitrary units (AU) and AGE4 positivity (the percent of samples with detectable AGE4) was related to diabetes-associated metabolic abnormalities, complications and treatment.

Results. Slot-dot blot was significantly more efficient than ELISA in detecting non-standard AGE4 epitopes. AGE4 positivity was less frequent in patients with microangiopathy and in those with polyneuropathy. In patients with abnormal glucose metabolism, metformin treatment was associated with higher AGE4. AGE4 positivity was significantly lower in gliptin-treated patients. Multivariate analysis showed that polyneuropathy and obesity were independently associated with AGE4 positivity, with odds ratios (ORs) of 0.21 and 3.02, respectively. Moreover, logistic regression showed that AGE4 positivity and HbA1c are independent predictors of polyneuropathy. Considering both indicators allows correct classification of 70.4% of cases with a general accuracy of 76%.

Conclusions. The slot dot-blot method detects compounds found in serum in trace amounts. Accumulation of AGE4 was associated with glucose metabolism abnormalities. A tendency toward AGE4 positivity was less frequent in patients with microangiopathy and in non-treated and gliptin-treated diabetes patients.

Key words: ELISA, diabetes, methylglyoxal, advanced glycation end-products (AGEs), slot dot-blot

Cite as
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Introduction

Advanced glycation end-products (AGEs) play a pivotal role in the development of complications in individuals with diabetes.[1] They have recently gained attention as prospective biomarkers and targets for pharmacotherapy for diabetes, atherosclerosis and related metabolic abnormalities. They are also being investigated as probable contributors to the pathogenesis of neurodegenerative diseases and cancer.[2] Advanced glycation end-products are formed as a result of non-enzymatic reaction between reducing carbohydrates or oxo-aldehydes and amino groups of proteins, nucleic acids or lipids. Glycation negatively affects the functionality of modified macromolecules. It is a multi-step cascade prompted by hyperglycemia and oxidative stress, yielding compounds of varying structures and stability.[3] Glycation usually affects proteins with a long half-life, such as extracellular matrix proteins, myelin sheath proteins, crystallins in the eye, or serum albumins.[4] Proteins with a short half-life also undergo glycation. However, the resulting products are not AGEs, but Amadori compounds, such as glycated hemoglobin (HbA_1c), a routinely assessed marker of glycemic control.[5]

Studies on the importance of AGEs in human pathology and potential applications of AGEs in diagnostics and therapy are hindered by their heterogeneity and difficulties in quantification. Only a limited number of AGE structures have been identified, characterized and evaluated in a clinical context. At present, N^{ε}-carboxymethyl-lysine (CML) is the most extensively studied AGE, followed by N^{ε}-(1-carboxyethyl)-lysine (CEL), pentosidine, argpyrimidine (AP), methylglyoxal (MGO)-derived hydroimidazolone, glyoxal-derived hydroimidasolone, and MOLD (MGO-lysine dimer).[6] The relevance of other AGEs remains largely unknown.[7] The detection and quantification of AGEs in easily available biological material, such as blood, is difficult. They are characterized by low solubility combined with high viscosity, and unlike in tissues, AGEs in blood are present in trace amounts. Therefore, sophisticated instrumental techniques, such as mass spectrometry, are employed to detect specific AGE epitopes.[8] Moreover, extraction of AGEs from sera using affinity or liquid chromatography is frequently required prior to their identification and quantification.[9] The necessity of advanced equipment and specialists limits the possibilities of applying these techniques in clinical practice on a large scale. More accessible techniques such as immunoassays are used mostly to quantify so-called "total AGEs", a pool of various AGEs with fluorescent properties. Although useful for getting a general overview of the degree of glycation, "total AGEs" do not encompass all AGEs (non-fluorescent species are not accounted for), nor discern the concentrations of any specific AGE. This makes the assessment of their individual contributions to a pathology impossible. Data on specific AGEs determined using immunoassays is scanty, and limited mostly to CML and pentosidine.[10]

Methylglyoxal is a low-molecular-weight reactive aldehyde formed endogenously as a by-product of glycolysis and lipid peroxidation.[11] It evokes inflammatory[12] and immune responses,[13] and contributes to mitochondrial dysfunction and the formation of both AGEs and advanced lipoxidation end-products (ALEs). Methylglyoxal has been implicated in the development of complications in diabetes patients[13] as well as those with cancer[14] and multiple sclerosis.[15] The presence of MGO-modified proteins is usually detected indirectly, by evaluating the titer of the respective autoantibodies. Hydroimidazolone-1 (MG-H1) is the only known specific epitope formed on MGO-modified proteins. Others, namely CML and CEL, can be formed not only by MGO but also by glucose modification. Hydroimidazolone-1 has recently been proposed as a biomarker of reduced diastolic heart function in type 1 diabetes mellitus (T1DM).[16]

To our knowledge, no immunoassay allowing quantification of MGO-derived epitopes (AGE4) other than CML, CEL or MG-H1 has been published. In this paper, we therefore present a slot dot-blot method for determining MGO-derived non-standard epitopes using commercially available monoclonal antibodies. The suitability of the assay for patient evaluation was tested on individuals with diabetes and related metabolic abnormalities and complications.

Material and methods

Serum samples

Serum samples were obtained from clotted (15 min, room temperature) and centrifuged (15 min, 400 × g) blood drawn by venipuncture following a 12-hour overnight fast and collected into serum-separator tubes.

For the purpose of validation of the method performance in a clinical setting, serum samples were obtained from 46 individuals, of whom 26 were diagnosed with diabetes (10 men and 16 women), and the remaining 20 were apparently healthy blood donors (10 men and 10 women). Similarly, AGE4 was determined in 131 individuals: 91 with abnormalities in glucose metabolism (85 diagnosed with diabetes) and 30 apparently healthy blood donors.

Patients with deregulated glucose metabolism were recruited from the Department of Angiology, Diabetes and Hypertension of Wroclaw Medical University (Poland). Apparently healthy blood donors, without known glucose metabolism abnormalities, were recruited from the Regional Center of Blood Donation and Therapy (Wrocław, Poland) using the following inclusion criteria: age >50 years; no known systemic disease, dementia or depression; no ongoing inflammation; and fasting glucose <100 mg/dL.

Diabetes was diagnosed using the World Health Organization (WHO) criteria. The disease was considered uncontrolled if HbA_1c exceeded 6.4%.[17] Overweight was defined according to WHO classifications as body mass index (BMI)

25–30 kg/m^2, and obesity as BMI ≥ 30 kg/m.[2,18] Metabolic syndrome (MetS) was diagnosed using the International Diabetes Federation (IDF) criteria[19] as the coexistence of central obesity plus any 2 of the following: hypertriglyceridemia (≥150 mg/dL) or receiving treatment, low high-density lipoprotein (HDL)-cholesterol (<40 mg/dL in men and <50 mg/dL in women), hypertension (≥130 mm Hg for systolic or ≥85 mm Hg for diastolic) or receiving treatment, glucose ≥100 mg/dL or diagnosed with diabetes and receiving treatment. Hyperlipidemia was diagnosed using the guidelines of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)[20] as elevated triglycerides (≥150 mg/dL) and/or low-density lipoprotein (LDL)-cholesterol (≥115 mg/dL) and/or total cholesterol (≥190 mg/dL), or receiving treatment. Hyperuricemia was diagnosed using the National Health and Nutrition Examination Survey III (NHANES-III) criteria[21] as uric acid ≥7.0 mg/dL in men and ≥5.7 mg/dL in women.

Macroangiopathy (atherosclerosis of the arteries) was diagnosed if any of the following was present: myocardial infarct, stroke, percutaneous coronary intervention, coronary artery bypass graft, acute coronary syndrome, objective evidence of coronary artery disease (defined as a positive exercise test and angiography with at least 1 stenosis >50), symptomatic peripheral arterial obstructive disease (confirmed by an ankle/brachial pressure index <0.90 or an amputation), stenosis of the carotid artery, or cardiovascular death. Microangiopathy was diagnosed in patients with diabetic nephropathy (albuminuria >30 mg/g, creatinine and glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration Equation (CKD-EPI)), diabetic retinopathy (microaneurysms, dot-blot hemorrhages, flame-shaped hemorrhages, retinal edema and hard exudates, soft exudates, venous loops and venous beading, intraretinal microvascular abnormalities, hemorrhage into the vitreous, traction retinal detachments, macular edema etc., detected using ophthalmoscopy, fundus fluorescein angiography or optical coherence tomography) or polyneuropathy (diagnosed after excluding other possible causes with the pinprick test to establish absent or reduced pain sensations, electromyography and clinical evaluation using a quantitative diagnostic tool such as the Neuropathy Impairment Score).

The patients' demographic, laboratory and clinical data was collected prospectively and is presented in Table 1. Data on the implemented treatment as well as the co-existence of other abnormalities is given in Table 2.

Ethical considerations

The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University (approvals No. KB-303/2010 and No. KB-384/2012) and was in accordance with the ethical standards formulated in the Helsinki Declaration of 1975. Informed consent was obtained from all participating individuals.

Table 1. Characteristics of patients according to metabolic abnormalities

Metabolic abnormalities	n (%)
Diabetes	85 (93.4)
T1DM	3 (3.5)
T2DM	76 (89.4)
T3DM	6 (7.1)
Overweight/obesity	
overweight	28 (30.8)
obesity I/II/III	37 (40.7)
Macroangiopathy	69 (75.8)
IHD	32 (35.2)
ACS	18 (19.8)
IS	11 (12.1)
AO	28 (30.8)
CAP	62 (68.1)
Microangiopathy	46 (50.5)
retinopathy	11 (12.1)
nephropathy	20 (22.0)
polineuropathy	35 (38.5)
Hypertension	83 (91.2)
Hyperlipidemia	78 (85.7)
Hyperurykemia	26 (33.8)
FLD	12 (13.2)
CKD	10 (11.0)

IHD – ischemic heart disease; ACS – acute coronary syndromes; IS – ischemic stroke; AO – arteriosclerosis obliterans; CAP – carotid artery plaques; FLD – fatty liver disease; CKD – chronic kidney disease.

Table 2. Characteristics of patients according to treatment

Treatment	n (%)
Antihypertensive drugs	77 (84.6)
Dyslipidemia medications	55 (60.4)
Aspirin	60 (65.9)
Anticoagulants	13 (14.3)
Clopidogrel	11 (12.1)
Metformin	65 (71.4)
Acarbose	10 (11.0)
Sulphonylureas	31 (34.1)
Gliptins	12 (13.2)
Insulin	38 (41.8)

Antibodies

Specific anti-AGE4 monoclonal antibodies (clone No. 14B5; Cosmo Bio Co. Ltd., Tokyo, Japan) – i.e., MGO-modified bovine serum albumin (BSA) – were used as the primary antibodies. These antibodies have been shown not to cross-react with glucose-modified BSA (AGE-1), glyceraldehyde-modified BSA (AGE-2), glycolaldehyde-modified BSA (AGE-3), glyoxal-modified BSA (AGE-5), anti-3-deoxyglucose-modified BSA (AGE-6), CML-modified BSA, CEL-modified BSA, or with native BSA. The method for obtaining and purifying anti-AGE4 monoclonal antibodies was described by Takeuchi et al.[22]

Polyclonal goat anti-mouse IgG (H+L) antibodies conjugated with horseradish peroxidase (HRP) (Jackson ImmunoResearch Europe Ltd., Ely, UK; cat. No. 115-035-166) were used as secondary antibodies at the dilution recommended by the manufacturer (1:2,000).

Direct ELISA assay

The wells of flat-bottom Maxisorp microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 50 µL of serum samples diluted in 50 mM carbonate buffer (pH 9.6) and incubated for 4 h at 37°C, followed by overnight incubation at 4°C. The following serum dilutions were tested: 200×, 100×, 50×, 20×, 10×, 5×, 4×, 2×, and undiluted. After intensive washing with 3 changes of 300 µL of tris buffered saline with tween 20 (TBS-T), pH 7.4, the plates were blocked with 300 µL of 5% skimmed milk for 20 h at 4°C. Unbound milk was removed by washing with TBS-T, pH 7.4 (3 × 300 µL), and the plates were incubated for 2 h at 37°C with 50 µL of the primary antibodies per well. The following dilutions of the primary antibodies were tested: 1:2,000, 1:1,000, 1:500, and 1:250. Subsequently, following a wash step (3 × 300 µL of TBS-T, pH 7.4), the plates were treated with the secondary antibodies conjugated with HRP (50 µL per well; 1:2,000) for 1.5 h at 37°C. After washing (3 × 300 µL of TBS-T, pH 7.4), the emptied wells were filled with 50 µL of o-phenylenediamine dihydrochloride (OPD) (Thermo Fisher Scientific, Rockford, USA) and incubated for 10 min at room temperature. The appearance of an orange-brown product, proportional to the AGE4 concentration in serum samples, was measured at λ = 450 nm using an EnSpire® microplate reader (Perkin Elmer Inc., Waltham, USA).

Additionally, the following negative controls were run simultaneously with the samples: antigen control (no antigen), blockage control (without the primary or secondary antibodies), primary antibody control (no secondary antibody), and secondary antibody control (without the primary antibody). All the samples and controls were run in triplicate.

Slot dot-blot assay

Fifty microliters of twice-diluted sera in phosphate-buffered saline (PBS) pH 7.2 were applied in duplicate to methanol-activated polyvinylidene difluoride membrane (Immobilon®-P Transfer Membrane; Bio-Rad Laboratories, Hercules, USA) using slot apparatus (Bio-Rad Laboratories). The prepared membranes were soaked in 5% skimmed milk in TBS-T for 2 h at room temperature, and then for 18 h at 4°C. After this and every subsequent step, the membranes were rinsed 3 times with TBS-T, pH 7.4. Subsequently, the membranes were incubated with the primary antibodies for 2.5 h at 37°C, using the following dilutions: 1:2,000, 1:1,000, 1:500, and 1:250 in TBS. After washing, the membranes were incubated for 1.5 h at 37°C with the secondary antibodies conjugated with HRP, using the 1:2,000 dilution recommended by the manufacturer. 3-amino-9-ethylcarbazole (Sigma Aldrich, St. Louis, USA) was used as a substrate for peroxidase. The density of the developed bands, proportional to AGE4 concentration, was determined using densitometry and was expressed in arbitrary units (AU). The readings were conducted using the Gel Doc XR+ (Bio-Rad Laboratories), and the band density was analyzed using Image Lab software (Bio-Rad Laboratories). All detected signals were normalized against the background density of the membrane.

Except for determination of the coefficients of variation (CV%), all samples were run in duplicate. Parallel control assays were performed, in which the incubation step with the primary antibodies was omitted to control for the specificity of the secondary antibodies. Any signals observed in the control assays were subtracted from the corresponding samples in the test assay.

Statistical analysis

Prior to the analysis, the results of the technical replicates were averaged. Data distribution was tested using the Kolmogorov–Smirnov test. Data on AGE4 is presented as medians with 95% confidence intervals (95% CI) and was analyzed using the Mann–Whitney U test. Normally distributed data was analyzed using the t-test for independent samples with Welch's correction if appropriate. The frequency analysis was conducted using Fisher's exact test. Comparisons of 2 proportions were conducted using χ^2 statistics. Correlations were analyzed using Spearman's rank correlation coefficient. Stepwise logistic regression with $p < 0.05$ as inclusion and $p > 0.1$ as exclusion criteria was applied to identify independent predictors of AGE4 positivity or polyneuropathy. Odds ratios (ORs) with 95% CI were calculated for significant variables. The accuracy of the model based on explanatory variables was determined using receiver operating characteristics (ROC) curve analysis and expressed as the area under the ROC curve (AUC). Additionally, the sensitivity and specificity of the model corresponding with the optimal cut-off were calculated.

All calculated p-values were two-sided and $p \leq 0.05$ was considered statistically significant. The statistical analysis was conducted using MedCalc Statistical Software v. 17.9.6 (MedCalc Software bvba, Ostend, Belgium).

Results

Assay development

Competitive ELISA

Our first attempt to develop an immunoassay for the detection and quantification of AGE4 was a competitive enzyme-linked immunosorbent assay (ELISA). In order

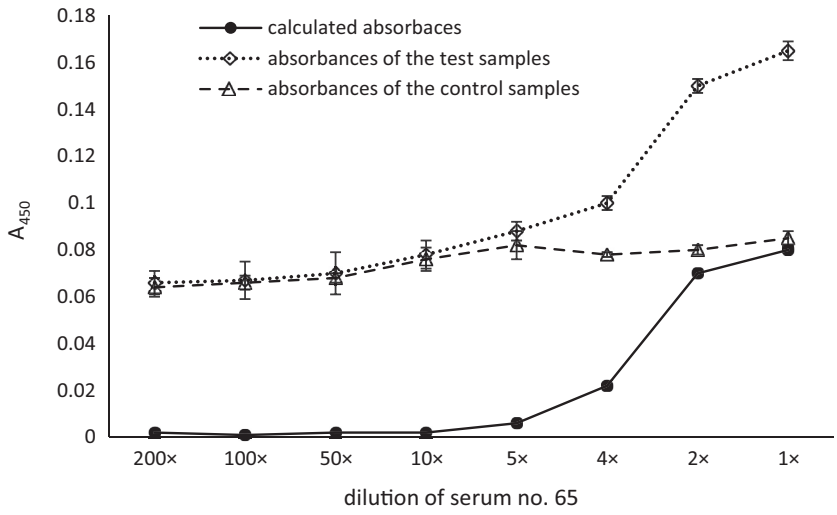

Fig. 1. The absorbance values of the test samples (serum No. 65), control samples and the calculated absorbance

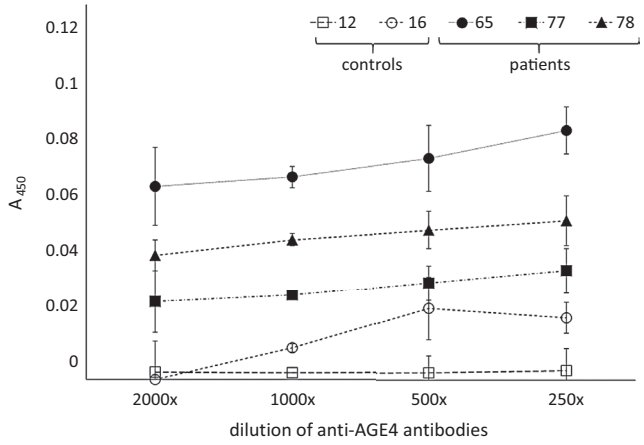

Fig. 2. Absorbance values (results after taking into account the absorbance values of the control samples, i.e., corresponding anti-AGE4 untreated sera) obtained using direct ELISA. Serum dilutions in carbonate buffer were 1×. The anti-AGE4 dilution in TBS was 250×

to establish the optimal assay conditions, microtiter plate wells were coated with different dilutions of serum samples, from undiluted sera up to 200-fold dilutions. Except for the least diluted sera, the readings recorded were almost identical with their respective controls (without anti-AGE4 antibodies). Therefore, when the control readings were subtracted from the samples, only the undiluted sera and 200-fold diluted sera had absorbance readings exceeding 0.05. An example of the effect of serum dilution on AGE4 detection is presented in Fig. 1.

The assay was further optimized by testing various dilutions of anti-AGE4 antibodies using plates coated with either undiluted or 200-fold diluted sera. Of the tested antibody dilutions, the highest sample-to-control absorbance ratios were obtained for the lowest dilution (1:250). There was no significant difference between assays conducted using undiluted or 200-fold diluted sera for the coating. The effect of antibody dilution factors on AGE4 detection is presented in Fig. 2 for 6 different sera (2 derived from healthy individuals and 4 from patients with diabetes).

Using the conditions yielding the highest sample-to-control readings, 23 randomly selected serum samples were used to detect and quantify AGE4. In 7 samples out of 23, reactions were clearly observed in anti-AGE4-treated wells, yielding 30% AGE4 positivity. Absorbance of the control wells (sera untreated by anti-AGE4) for these 7 samples was significantly lower. The median AGE4 absorbance was 0.118 (95% CI = 0.13–0.09). The absorbance values for the samples were in the 0.06–0.17 range for 250× anti-AGE4 dilution and in the 0.06–0.09 range for corresponding control samples. Based on the results including the control test, it was found that trace amounts of AGE4 were detected in some sera, but the direct ELISA method was insufficiently sensitive.

Slot dot-blot

Since the control-adjusted readings in ELISA were generally low, the slot dot-blot method, which allows more antigens to be loaded on a membrane, was tried. The serum dilution was fixed at 1:200 and only the anti-AGE4 antibody step was adjusted. As in the ELISA test, the starting antibody dilution was 1:2,000. Since there was no signal on the membranes, lower dilutions were tested; a 1:250 dilution was found to yield signals in a number of sera (Fig. 3).

Using a 1:250 antibody dilution, 24 randomly selected sera were tested (22 from individuals with abnormalities in glucose metabolism and 2 from healthy blood donors). Simultaneously, a control assay, without anti-AGE4 antibodies, was run. A number of bands could be clearly observed in an anti-AGE4-treated blot: 17 samples out of 24, yielding 71% AGE4 positivity. However, there were only 2 responses in the control assay (Fig. 4, samples #66 and #68). The density of these control bands was relatively small, constituting 3.6% and 1.4% of the density recorded for corresponding samples treated with anti-AGE4. Nonspecific signals from the controls were subtracted from the test samples to calculate AGE4 levels. The median AGE4 level was 29,494 AU (95% CI = 7,950–41,369). Data

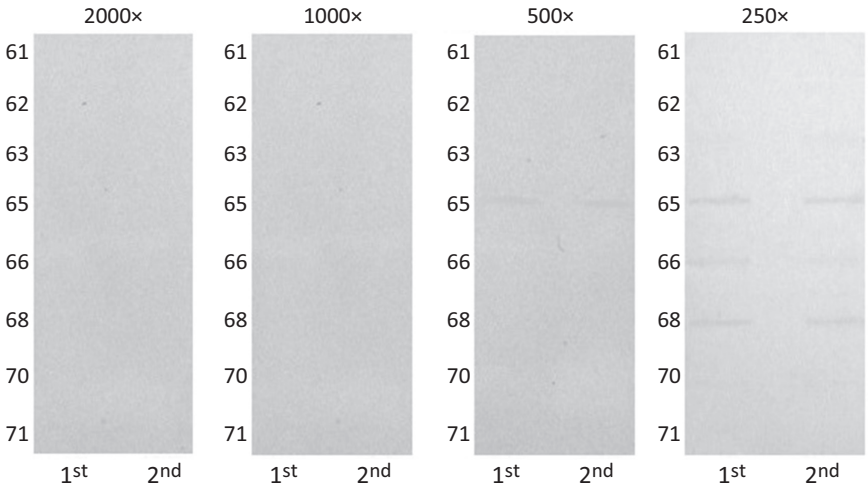

Fig. 3. Results of the slot dot-blot test carried out at the following anti-AGE4 dilutions: ×2,000, ×1,000, ×500, and ×250. The number of the sample tested is given on the left side of the blot and the number of the technical replicate is given at the bottom

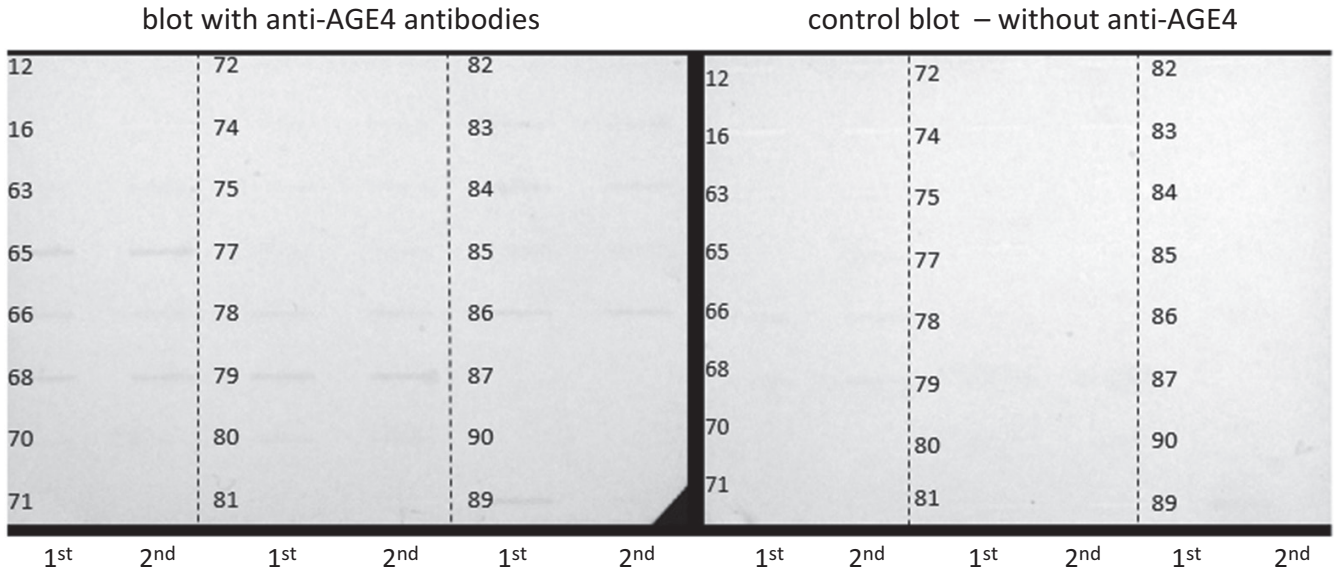

Fig. 4. Slot dot-blot results of diabetes sera treated with A) anti-AGE4 and secondary anti- mice IgG antibodies, and B) only secondary anti-mice IgG antibodies (control slot dot-blot). The number of sample tested is given on the left side of the blot and the number of the technical replicate is given at the bottom

on individual AGE4 levels for both assays (test and control) is presented in the Table 3. The mean CV% calculated for technical replicates was 9.9%.

In order to determine the intra- and inter-assay CV%, 12 replicates of selected serum samples were run within the same assay on 9 separate runs. The calculated intra-assay CV% was 10.8% and the inter-assay CV% was 10.6%, both within the range acceptable for immunoassays.

The slot dot-blot method was used to quantify AGE4 levels in the healthy individuals and in the patients with diabetes with respect to disease complications, other metabolic abnormalities and treatment.

AGE4 and diabetes

As shown in Table 1, the healthy controls had lower AGE4 and AGE4 positivity than the patients. When analyzed in terms of the relationship with diabetes, the non-diabetic study participants had lower median AGE4 than the diabetics: 0 AU (0–0) vs 11,501 AU (3,061–26,905) ($p < 0.0001$) as well as lower AGE4 positivity (19.4% vs 64.7%, $p < 0.0001$).

There was no significant difference between the diabetics and the controls in terms of median AGE4 ($p = 0.485$) or AGE4 positivity ($p = 0.992$). However, the patients with uncontrolled disease had significantly lower median AGE4 than the patients with controlled disease (3,906 AU (0–21,426) vs 26,979 AU (6,605–45,322), $p = 0.033$) and tended to have lower AGE4 positivity (58.2% vs 76.6%, $p = 0.102$). There was also a negative correlation between AGE4 and HbA_1C: $\rho = -0.29$, $p = 0.008$, particularly in males: $\rho = -0.42$, $p = 0.018$.

AGE4 and other metabolic abnormalities

To evaluate correlations between AGE4 and various metabolic abnormalities, we compared the median AGE4

Table 3. Band density of studied samples and controls. Errors of measurement

Serum sample#	Band density in test assay (with anti-AGE-4 antibodies) Mean [AU]	CV [%]	Band density in control assay (without anti-AGE-4 antibodies) Mean [AU]	Calculated AGE-4 (test assay – control assay) Mean [AU]
12	0	-	0	0
16	0	-	0	0
63	33,573	1.8	0	33,573
65	82,128	3.6	0	82,128
66	55,975	13.7	2,000	55,975
68	81,450	1.4	1,100	81,450
70	24,570	7.5	0	24,570
71	53,975	-	0	53,975
72	80,340	-	0	80,340
74	26,650	16.4	0	26,650
75	20,215	8.2	0	20,215
77	34,060	13.8	0	34,060
78	49,693	7.3	0	49,693
79	84,858	7.8	0	84,858
80	10,646	16.8	0	10,646
81	0	-	0	0
82	0	-	0	0
83	38,545	11.7	0	38,545
84	52,358	13.9	0	52,358
85	38,220	10.8	0	38,220
86	66,593	6.0	0	66,593
87	16,315	17.3	0	16,315
89	32,338	-	0	32,338
90	0	-	0	0

Measurement error was calculated as the ratio of absolute difference between 2 replicates to higher measurement and expressed as percent.

and the distribution of AGE4-positive samples among patients with and without obesity, hypertension, hyperlipidemia, hyperuricemia, fatty liver disease, and chronic kidney disease. Of these, obese patients had a significantly higher median AGE4 than non-obese ones: 33,573 AU (15,786–46,033) vs 3,242 AU (0–11,391) ($p = 0.004$) and a higher percentage of AGE4 positivity: 75.7% vs 55.6% ($p = 0.051$). Moreover, there was a positive correlation between AGE4 and BMI: $\rho = 0.27$, $p = 0.018$.

AGE4 and diabetic complications

We also compared the median AGE4 and the distribution of AGE4-positive samples between patients with and without diabetic complications: macroangiopathy in general and as ischemic heart disease, acute coronary syndromes, ischemic stroke, arteriosclerosis obliterans, and carotid artery plaques; as well as microangiopathy in general and as retinopathy, nephropathy and neuropathy. AGE4 positivity tended to be less frequent in patients with microangiopathy in general as compared to patients without microangiopathy (54.3% vs 73.3%; $p = 0.061$). Detailed analysis showed a significant correlation between AGE4 positivity and polyneuropathy: Patients with this complication had significantly lower AGE4 positivity than those without it (45.7% vs 75%, $p = 0.005$).

AGE4 and treatment

We compared the median AGE4 and the distribution of AGE4-positive samples between patients treated and untreated with insulin, sulfonylurea, metformin, gliptins, acarbose, antihypertensive drugs, dyslipidemia medications, anticoagulants, aspirin, and clopidogrel. The median AGE4 was higher in metformin-treated than in non-treated patients: 20,215 AU (6,533–35,430) vs 384 AU (0–11,318) ($p = 0.011$). AGE4 positivity tended to be higher in metformin-treated patients as well (69.2% vs 50% for non-treated; $p = 0.086$) but was significantly lower in gliptin-treated than non-treated patients (33.3% vs 68.4%; $p = 0.019$).

Independent predictors of AGE4 positivity: multivariate analysis

To identify independent factors associated with AGE4 positivity, stepwise logistic regression was employed. Obesity, HbA_1C, polyneuropathy, and treatment with gliptins were entered as explanatory variables. Polyneuropathy (coefficient 1.55, $p = 0.003$) and obesity (coefficient 1.1, $p = 0.039$) were found independently associated with AGE4 positivity with the following odds ratios (ORs): 0.21 (95% CI = 0.08–0.58) for polyneuropathy and 3.02 (1.1–8.6) for obesity. The model built on both variables correctly classified 71.1% of the cases and its overall accuracy was 73%.

When treatment with metformin was entered as an additional explanatory variable, it replaced obesity in the model. Polyneuropathy had a coefficient of –1.67 ($p = 0.002$), while the coefficient for metformin was 1.33 ($p = 0.027$). The ORs for polyneuropathy and metformin treatment were 0.19 (0.07–0.53) and 3.76 (1.16–12.18), respectively. The model correctly classified 70% of the cases and its accuracy was 72%.

AGE4 positivity as an independent predictor of polyneuropathy

To test the strength of the association of AGE4 positivity with polyneuropathy, stepwise logistic regression was employed. To select variables associated with polyneuropathy in a univariate analysis, categorical data was tested using Fisher's exact test, while continuous data was submitted to a t-test for independent samples. Fasting glucose, HbA_1C, and triglycerides were significantly increased in patients with polyneuropathy. They were therefore entered into

the model of logistic regression as explanatory variables in addition to AGE4 positivity. Of these, AGE4 positivity (coefficient –1.42, p = 0.015) and HbA_1C (coefficient 0.37, p = 0.010) were found to be independent predictors of polyneuropathy with ORs of 0.24 (95% CI = 0.08–0.75) for AGE4 positivity and 1.6 (1.1–2.3) for HbA_1C. The model built on both variables correctly classified 70.4% of the cases and its overall accuracy was 76%. The combined markers were characterized by perfect specificity but low sensitivity in detecting polyneuropathy (Fig. 5).

Discussion

There is growing interest in detecting and quantifying individual AGE epitopes, as various AGEs may evoke different, even contradictory, responses in the body.[23] However, the available tools remain limited. Of these, immunoassays are likely to have a potential for a widespread application due to their relative simplicity and low cost, but the available ELISAs for AGEs are usually universal assays not dedicated to quantifying individual AGEs epitopes.[22,24]

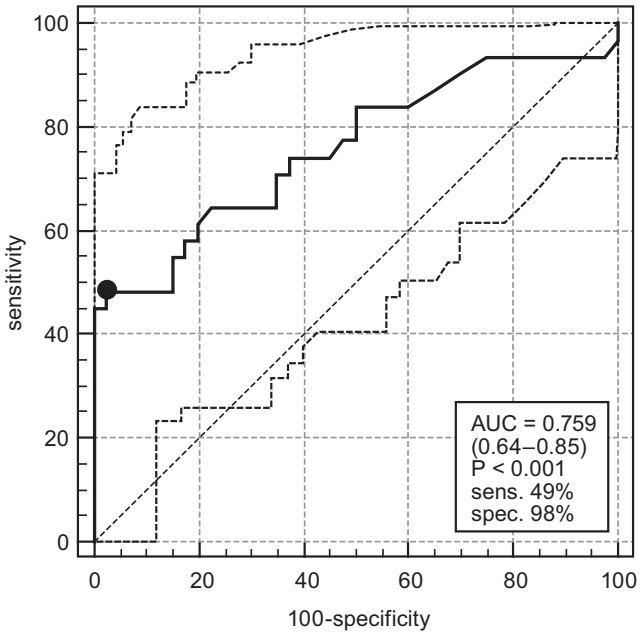

Fig. 5. Receiver operating characteristic (ROC) curve for combined AGE4 positivity and HbA1c as markers of polyneuropathy. Data is presented as area under ROC curve (AUC) with 95% CI and sensitivity (sens.) and specificity (spec.) corresponding with the optimal cut-off (represented as solid circle on the graph)

In this paper, we present a slot dot-blot method for the semi-quantitative determination of non-standard epitopes of AGE4 present in trace amounts in sera. In a classic ELISA using a microtiter plate format, the sensitivity of the assay is limited by the volume of the serum sample loaded into the well. The amount of antigen successfully bound to the microtiter plate well is directly correlated with its concentration in a medium. Therefore, the lower the concentration of an antigen of interest in the medium, the lower the probability of its being bound and not removed in subsequent washing steps. Moreover, the more complex the medium, the greater the presence of competing antigens, and the binding of a particular antigen of interest is even less probable. Although the problem can be solved by prior extraction of the antigen of interest, allowing for its relative concentration, such an approach is expensive and time- and labor-consuming. It may also require sophisticated procedures and equipment, thus hindering the widespread application of such a method in clinical and diagnostic laboratories. In this respect, the slot dot-blot method is superior, as it allows for more of the antigen of interest to be loaded on a carrier membrane. There is no volume limit of the serum sample to be loaded, and the use of a vacuum ensures that all the antigens are successfully bound and retained on the membrane. Therefore, as our study demonstrated, the slot dot-blot method allowed us, with satisfying reproducibility, to detect and semi-quantify AGE4 antigens undetectable with a competitive ELISA. The superior sensitivity of the slot dot-blot method as compared to a classic ELISA has also been demonstrated by others, as it was previously used to detect the presence of malaria-causing *Plasmodium falciparum* in mosquitos.[25,26]

Using monoclonal antibodies to target non-standard epitopes of AGE4 (MGO-modified albumin) that do not cross-react with other AGEs or albumin modified by standard AGE epitopes such as CML and CEL, we showed that their increased serum levels correlate with diabetes and obesity. This observation is in line with AGE formation being accelerated not only by hyperglycemia but by hyperlipidemia, as well as by the oxidative stress and low-grade inflammation accompanying these conditions.[27] Methylglyoxal represents low-molecular aldehydes derived from normal metabolism as well as pathological glycation and lipoxidation. The reactivity of MGO is about 20,000 times higher than the reactivity of glucose. As such, MGO has been called (by van der Heijden et al.) "a key compound involved in the very fast generation of glycation adducts", also produced in reactions with short-lived proteins.[28] Elevated concentrations of MGO in serum have previously been shown in diabetes[20] and held responsible for inducing maladaptive responses in the vasculature.[29] Similarly, MGO has been linked with diabetic complications, increasing risk of cardiovascular events and mortality,[31] as well as impairing bone regeneration in patients with diabetes.[32] Mechanistically, MGO has been shown to interfere with the platelet-derived growth factor B/platelet-derived growth factor receptor β (PDGFB/PDGFRβ) axis in smooth muscle cells and fibroblasts through formation of CML and CEL adducts on the PDGFB receptor. PDGFRβ inhibition, in turn, destabilizes the fibrous cap on atherosclerotic plaque, contributing to its rapture by inducing growth arrest and apoptosis of smooth muscle cells and fibroblasts.[33] However, animal studies have shown that MGO-derived

AGEs also accumulate in the pancreatic β cells of healthy rats, and the levels of these AGEs were not affected by streptozotocin-induced diabetes. Therefore, it has been suggested that MGO-derived AGEs may play a positive role in the post-translational maturation of insulin.[34]

To our knowledge, there are no studies in the literature that analyze the amount of a specific epitope of an MGO-modified protein in the context of metformin; only the influence of metformin on MGO content in human serum has been checked.[35] Beisswenger et al. used high-performance liquid chromatography to examine MGO in plasma from 57 subjects with T2DM, half of whom were being treated with metformin. Those authors found that metformin reduces MGO in a dose-dependent fashion and minimizes the effect of worsening glycemic control on MGO levels.[36] A similar conclusion was reached by Kiho et al., who incubated BSA or RNase with MGO, and metformin turned out to be an inhibitor of AGE formation.[37] In our study we found that the level of AGE4 is higher in patients treated with metformin. This is somewhat contradictory to the finding that the drug reduces systemic MGO levels in T2DM, because it is logical that the more MGO is present, the higher AGE4 levels are expected. Additional studies of the potential effects of metformin on MGO production and modifying proteins by MGO are required to further elucidate the role of metformin in diabetic complications.

The effect of gliptins (dipeptidyl peptidase-4 inhibitors, an established anti-inflammatory drug) on MGO-induced glycation has not been thoroughly researched. It has been reported that linagliptin improves MGO-induced peritoneal fibrosis. In a study carried out on mice, it was found that linagliptin suppressed the expression of fibrotic markers, such as alpha-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP-1) and type I and III collagen, and inhibited macrophage infiltration in MGO-induced peritoneal fibrosis.[38] It has also been shown that gemigliptin inhibits MGO-modified AGE-BSA formation and traps MGO in a concentration-dependent manner.[39] Regarding drugs of this group, our study found the expected results: AGE4 positivity was significantly lower in gliptin-treated patients than in non-treated patients, which confirmed its anti-glycation properties.

Conclusions

The extent to which high glucose levels in diabetic patients, as estimated by measuring HbA_{1c} levels, contributes directly to vascular disease remains controversial. Recent studies have suggested that postprandial spikes of high glucose may be a more robust determinant of vascular risk than average glucose levels as determined by HbA_1c. However, we should recognize that HbA_1c levels are unlikely to provide a fully accurate picture of temporal glycemic fluctuations, and thus there is a difference between diabetic individuals with and without regular occurrences of hyperglycemic spikes. This may be one of the underlying reasons why glycemic control, as measured by decreasing HbA_1c levels, has not been consistent in successfully reducing vascular disease.[40] The formation of MGO might be a mechanism that causes repeated glucose spikes to have a more damaging effect than high fasting or mean glucose levels on endothelial cells and the development of vascular complications. Methylglyoxal is a strong candidate for use as a more robust clinical marker of glycemic fluctuation than HbA_{1c}. A major challenge for the future would be to systematically analyze MGO-modified proteins in order to identify a better risk predictor than HbA_1c. Methylglyoxal triggers maladaptive responses in vascular tissue.[41] AGE4 positivity and HbA_1C were found to be independent predictors of polyneuropathy.

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Multiplex ligation-dependent probe amplification as a screening test in children with autism spectrum disorders

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Abstract

Background. Autism spectrum disorders (ASDs) are a heterogeneous group of neurodevelopmental disorders, characterized by the presence of various symptoms related to deficits in communication and social interactions as well as stereotyped and repetitive behavior. Increasing evidence indicates the contribution of genetic factors in the etiology of ASDs. Genetic diagnosis in ASDs is based on identifying chromosome aberrations, microaberrations and point mutations in specific genes. One of the diagnostic tools is multiplex ligase-dependent probe amplification (MLPA) with a set of probes dedicated to ASDs (SALSA MLPA P343 Autism-1; MRC-Holland BV, Amsterdam, the Netherlands) targeting the genes located in the regions 15q11-q13, 16p11 and the *SHANK3* gene in the 22q13 region.

Objectives. Our study included 240 patients referred to the clinical genetics unit because of ASDs and/or developmental delay and/or an intellectual disability. Before genetic testing, the patients underwent a comprehensive medical work-up.

Material and methods. Multiplex ligase-dependent probe amplification was performed in 256 DNA samples from 240 probands and 16 family members using the SALSA MLPA P343 Autism-1 probe mix (MRC-Holland BV) according to the manufacturer's protocol.

Results. We obtained 234 normal results and 22 abnormal results (15 probands and 7 abnormal results for probands' parents or siblings). We diagnosed 1 16p11 microdeletion syndrome and 1 16p11 microduplication syndrome. We also found 3 deletions and 1 duplication in 15q13 region including 2 or 3 genes and 9 single probe alterations in the regions examined (1 duplication and 7 deletions).

Conclusions. Due to the low costs, MLPA test may be a good tool for the genetic screening of ASD patients.

Key words: diagnostics, autism, MLPA

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The prevalence of autism spectrum disorders (ASDs) has been estimated at 20–60/10,000 children, corresponding to 1–2% of the general population.[1–3] Importantly, ASDs are highly heterogeneous neurodevelopmental disorders, characterized by the presence of various symptoms, including deficits in communication and social interactions as well as stereotyped and repetitive behaviors. Moreover, patients with ASDs present high rates of comorbid intellectual disability, sensory abnormalities or sleep disturbances.[2] The role of genetic factors and metabolic conditions in the development of ASDs is strongly suggested on the basis of family studies, especially twin studies.[4] Although several genes have been identified and their association with ASD susceptibility is well-documented, a large group of genes is still suspected to be involved in this disease.[2] Additionally, diagnoses of several genetic syndromes need to be excluded before a final diagnosis of ASD is established.[5] Diagnostic considerations are even more complex due to multifactorial inheritance and a number of environmental risk factors potentially involved in the etiology of ASDs.[6]

Over the last few years, the laboratory methods used for ASD diagnosis have moved from classical cytogenetics (banding analysis) or fluorescent in situ hybridization (FISH) to array comparative genomic hybridization (aCGH) and next-generation sequencing (NGS). Several single nucleotide variants (SNV) have been identified in many genes and confirmed by family-sequencing using whole-exome sequencing (WES) and whole-genome sequencing (WGS). These studies have revealed that ASDs may be caused by numerous alterations in genes acting in different molecular pathways with incomplete penetrance and variable expressivity.[2,7]

The WES and WGS methods make it possible to screen large parts or the entire genome and to identify all potential genomic alterations, but their costs and laboratory equipment requirements are high. Taking into account these considerations, multiplex ligation-dependent probe amplification (MLPA) is an appealing alternative for initial screening in patients with ASD.[5,8] The dedicated MLPA probe mix for ASD contains probes for 3 chromosomal regions: 15q11-q13, 16p11 and 22q13. Therefore, the aim of our study was to evaluate the rate of positive results in a large sample of ASD patients, using the MLPA mix dedicated to this disease.

Material and methods

Materials

The analyses were performed in 240 individuals with ASDs and 16 healthy family members. The patients' group included 54 women and 202 men, with a mean age of 8.7 ±8.06 years. The patients had been referred to the clinical genetics unit with ASDs and/or developmental delay and/or intellectual disability, with or without dysmorphic features or additional congenital abnormalities. The diagnosis of ASD was established according to ICD-10 criteria.[9] All the probands and/or their legal guardians signed a consent form. The MLPA test was performed after excluding genetic disorders that had been diagnosed based on clinical examinations or specific genetic tests (cytogenetics and fragile-X testing). The study was accepted by the Ethics Committee of Wroclaw Medical University (approval No. 320/2018).

DNA isolation

Genomic DNA was isolated from 200 µL of peripheral blood lymphocytes, using the Prepito DNA Blood kit and the chemagic Prepito-D isolator (both from PerkinElmer Inc., Waltham, USA) according to the manufacturer's protocol. DNA was diluted to 20 ng/µL and 5 µL (100 ng) was used in the MLPA reaction.

The MLPA analysis

The SALSA MLPA P343 Autism-1 probe mix (MRC-Holland BV, Amsterdam, the Netherlands) containing MLPA probes for 3 chromosomal regions 15q11-q13 (including, among others, *UBE3A*, *GABRB3* and in 15q13 *CHRNA7*), 16p11 (including, among others, *LAT, MAZ, MAPK3*, and *HIRIP3*) and 22q13 (including *SHANK3*) was used to assess the deletions and duplications in these regions. The MLPA reaction was prepared using a TC512 thermocycler (Techne Inc., Burlington, USA). The producer's protocol for MLPA was followed precisely.

The MLPA products were separated using the ABI 310 Genetic Analyzer with GeneScan Analysis v. 3.1.2 software, POP-4 Polymer and GeneScan™ 500 LIZ™ dye Size Standard (all from Thermo Fisher Scientific, Waltham, USA). The analysis of the results was prepared using GeneMarker v. 1.85 software (SoftGenetics LLC, State College, USA).

In the analysis of copy number variants (CNVs), a change in the peak values of over +0.3 was considered a duplication and –0.3 a deletion.

Microarray comparative genomic hybridization analysis

In aCGH analysis, 300 ng of each DNA sample was labeled with Cy-5 fluorescent dye using the CGH Labelling Kit for Oligo Arrays (Enzo Life Sciences Inc., Farmingdale, USA) according to the manufacturer's protocol. As a reference genotype for hybridization, 300 ng of gender-matched human reference DNA (Agilent Technologies, Santa Clara, USA) was simultaneously labeled with the Cy-3 dye. The hybridization of the samples and the processing of the microarrays were performed according to a standard oligonucleotide array-based CGH protocol

(Agilent Technologies). The microarray design used for the analysis was SurePrint G3 CGH ISCA v. 2, 8 × 60K (Agilent Technologies). The design focuses on 498 regions in the human genome of high clinical significance, defined on the basis of the International Standards Cytogenomic Arrays (ISCA) Consortium.[10] The approximate coverage of the genome is about 60 kb, with median practical resolution for accurate detection of imbalances estimated at 300 kb (lower in ISCA regions). The data was analyzed using CytoGenomics software (Agilent Technologies) with the default CGH analysis method, and was interpreted in reference to available databases (DGV [last update: 2016-05-15], ClinVar [2018-06-01 03:26], DECIPHER v. 9.23 [2018-05-23] May, 2018]).

Results

The MLPA analysis was performed on 256 DNA samples from patients and their families. We obtained 234 normal results and 22 abnormal results (in 15 patients and 7 parents or siblings of patients) (Table 1).

In the patients' group, we diagnosed 1 16p11 microdeletion syndrome and 1 16p11 duplication syndrome. We also found 1 duplication in the 15q13 region, 3 deletions in the 15q13 region, including *KLF1* and *CHRNA7* or *ABPA2*, *NDNL1* and *TJP1* genes, as well as 9 single-probe alterations in all the regions examined (2 duplication and 7 deletions) (Table 1).

15q11-13

In our study, alterations of region 15q11-13 were found in 11 out of 15 patients with a positive result: 4 out of 15 patients had abnormal results for more than 1 probe in this region (3 deletions and 1 duplication) while 7/15 had an alteration of 1 exon in this region (5 deletions and 2 duplications). In 1 case, the deletion of 2 probes in the 15q13 region (*KLF13*ex2 and *CHRNA7*ex4) was confirmed using aCGH; it occurred as part of a 1.1 Mb deletion (arr 15q33.3[31516639_32635959]x1). In the group of individuals with alterations in the 15q11-13 region, we observed ASDs with other clinical symptoms (6 cases) or without (4 cases); only 1 patient had developmental delay with epilepsy (Table 1).

In 5 patients we observed a deletion of 1 exon in the *GABRB3* gene (15q12), 4 in exon 9 (exon 6 according to new numbering) and 1 in exon 4. Three of these deletions were also present in 1 healthy parent (2 families were not examined). In this group of patients, we observed the following clinical picture: 1) ASDs without other clinical problems in 3 cases (1 with ASD family history); 2) ASD with developmental delay and epilepsy in 1 case; 3) other problems in 1 case; and 4) developmental delay with epilepsy in 1 case (Table 1).

We found 2 duplications and 2 deletions in the *CHRNA7* gene (the probe for exon 4), 1 duplication and 3 alterations being parts of a more complex rearrangement. In this group of patients, we observed ASDs in all cases. The patients with deletions presented with other comorbidities, such as intellectual disability in 1 case as well as developmental delay, speech delay and short stature in another 1 (Table 1). A single duplication of *CHRNA7* exon 4 was also present in a healthy sister of the proband.

16p11.2

Out of 15 patients, 3 were carriers of 16p11.2 alterations. We found 2 deletions: a small deletion (represented by 1 probe for *LAT4*), confirmed with aCGH as a 187 kb deletion (arr 16p11.2[28843773_29031059]x1]; 1 deletion exposed by 9 out of 11 probes for this region in the MLPA analysis; and 1 duplication, also for 9 out of 11 probes. In this group of patients, we observed that patients with deletions presented with more clinical symptoms than patients with duplications (Table 1).

22q13

In 1 patient, 1 probe found a deletion of exon 15 of the *SHANK3* gene.

Discussion

Genetic testing is an important part of the diagnostic process in children with ASDs and/or developmental delay and/or intellectual disability. Identification of a genetic cause of a disorder may elucidate the issue of etiology, enable assessment of the prognosis, facilitate care and management planning, and permit an estimation of the risk of recurrence. Using currently available standard laboratory methods (other than WES or WGS), it is possible to find a genetic cause in about 10% of ASD cases.[11] According to the literature, the aCGH analysis is recommended as a first-tier diagnostic method for ASD patients.[11] Additionally, single-gene tests, such as fragile-X syndrome testing, should be applied.[11] Given that aCGH and WES are expensive procedures, it is reasonable to use more cost-effective methods for screening and selecting patients for further analysis. One of these methods is MLPA – a method mainly used for the detection of small deletions and duplications. The SALSA MLPA P343 Autism-1 probe mix (MRC-Holland BV) is dedicated for ASD patients and allows deletions and duplications to be found in the 15q11-13, 16p11.2 and 22q13 chromosomal regions. Alterations identified in our study include deletions and duplications in regions 15q11-13, 16p11.2, and 22q13, as well as deletions and duplications in the *GABRB3* and *CHRNA7* exons.

Table 1. Results of the MLPA analysis

Patient number	Sex	Age [years]	Clinical diagnosis	MLPA result	Chromosomal region	MLPA family results	aCGH result
1	M	10	Fragile X syndrome suspected (excluded)	del *SHANK3ex15*	22q13	nd	nd
2	M	5	ID, ASD	del *KLF13ex2*, *CHRNA7ex4*, *TRPM1ex27*	15q13	nd	nd
3	M	9	DD, epilepsy, epilepsy in family	del *GABR3ex9 (6)*	15q12	nd	nd
4	F	15	DD, ASD	dup *CHRNA7ex4*	15q13	mother – normal result, healthy one sister – duplication, healthy	nd
5	M	7	ASD	del *GABR3ex9(6)*	15q12	mother – deletion, healthy father – normal results, healthy	nd
6	F	5	ASD	del *GABR3ex4*	15q12	mother – deletion, healthy	nd
7	M	8	ASD	dup *HIRIP3ex3*, *SEZ6L2ex1*, *DOC2Aex4*, *MAZex5*, *MVPex5*, *SPNex3*, *MAZex6*, *HIRIP3ex4*, *MAPK3ex5*	16p11	mother – deletion, healthy father – normal results, healthy	nd
8	F	7	ASD, DD, ID	del *HIRIP3ex3*, *SEZ6L2ex1*, *DOC2Aex4*, *MAZex5*, *MVPex5*, *SPNex3*, *MAZex6*, *HIRIP3ex4*, *MAPK3ex5*	16p11	nd	nd
9	M	2	DD, dysmorphy	del *LATex4*	16p11	mother – deletion, healthy father – normal results, healthy	arr 16p11.2(28843773_29031059)x1
10	M	11	DD, ADS, ID, speech delay, short stature, short stature in family	del *KLF13ex2*, *CHRNA7ex4*	15q13	3 siblings – healthy, normal result	arr 15q33.3(31516639_32635959)x1
11	M	13	ASD, epilepsy, gynecomastia	del *ABPA2ex14*, *NDNL2ex1*, *TJP1in1*	15q13	mother – normal result, healthy father – deletion, healthy	nd
12	F	11	DD, speech delay, epilepsy, ASD, short stature, autoagression	del *GABR3ex9 (6)*	15q12	mother – deletion, healthy father – normal results, healthy	nd
13	M	4	ASD, ID, ASD in family	del *GABR3ex9 (6)*	15q12	nd	nd
14	F	4	ASD	dup *HIRIPex3*	15q13	nd	nd
15	F	6	ASD	dup *HIRIPex3*, *CHRNA7ex4*, *TRPM1ex27*	15q13	nd	nd

MLPA – multiplex ligase-dependent probe amplification; aCGH – array comparative genomic hybridization; ID – intellectual disability; ASD – autism spectrum disorder; DD – development delay; nd – no data.

Deletions and duplications in region 15q11-13

Copy number variants in the 15q11-13 region are among the most common autosomal alterations in ASD patients.[5] A substantially higher rate of ASDs has been found in individuals with Prader–Willi syndrome, especially in those with the uniparental disomy (UPD) subtype.[12]

The proximal part of chromosome 15 is one of the most unstable regions in the human genome, because it contains 6 low copy repeat (LCR) elements that are grouped in 6 breakpoints involved in non-homologous recombination. Microdeletions within this region are observed in patients with Prader–Willi and Angelman syndromes, while microduplications are associated with ASDs, learning disabilities and seizures.[13] Similarly, CNVs in the 15q13.3 region, located downstream to the 15q11-13 locus, are reported in patients with neuropsychiatric phenotypes, attention deficit hyperactivity disorder and ASDs.[13]

In 1 of our patients (No. 10), we found a 15q13 deletion not observed in 3 healthy siblings; using the aCGH method, it was characterized as a 1.1 Mb deletion. Moreover, a smaller deletion in this region, covering genes *ABPA2* (exon 14), *NDNL2* (exon 1) and *TJP1* (intron 1) was diagnosed in patient No. 11. Although this alteration was also observed in the presumably healthy father, it may still be the cause of ASD in the proband if the penetrance of the alteration is incomplete. To verify this hypothesis, further genetic testing in numerous family members is necessary.

Deletions and duplications of *GABRB3* exons

Alterations in the *GABRB3* gene reported in ASD patients mainly involve variations of single or several nucleotides, and most of them are familial.[14] Due to high incidence of this deletion (5 patients and 3 familial cases, with no data for 2 families) it is likely that this alteration is not causative of clinical features in these patients.

Deletions and duplications of *CHRNA7* exons

The α7 subunit of the nicotinic acetylcholine receptor is encoded by *CHRNA7* (15q13). This ion channel is reported to be expressed in the brain. A homopentameric form of the α7 subunit is involved in mediating signal transduction at synapses, the regulation of neurotransmitter release, synaptic plasticity, learning, and memory. The *CHRNA7* gene is located in the 15q13.3 region and has been associated with neurodevelopmental and neuropsychiatric disorders.[13] Deletion of the whole *CHRNA7* gene is observed in 1% of patients with idiopathic generalized epilepsies, while duplication of the gene occurs in patients with ASDs and cognitive impairment.[13,15–17] For patients with CNVs in the 15q13.3 regions, incomplete penetrance (about 80%) and variable expressivity have been reported.[13] It is difficult to conduct a sequence analysis of the *CHRNA7* gene because of the presence of a large identical sequence in the *CHRFAM7A* gene, which is therefore sequenced together with *CHRNA7*, but no rare damaging variants were reported in the *CHRNA7* gene in a group of 135 patients with ASD.[17] It should be noted that because of the limitations of MLPA analysis, this alteration may also be caused by a single nucleotide polymorphism or mutation. The same change was also observed in a healthy sister, which may suggest that this alteration was not responsible for ASD in the proband, or (as in previous reports) the penetrance of this mutation is incomplete.[13]

Deletion and duplication in the 16p11.2 region

Deletion and duplication in the 16p11.2 region are among the most frequent CNVs in patients with ASDs and neurodevelopmental disorders.[7] Several clinical characteristics, including speech articulation abnormalities, limb and trunk hypotonia with hyporeflexia, abnormalities of agility, sacral dimples, macrocephaly, and epilepsy, have been observed in patients with 16p11.2 deletions. Additionally, the 16p11.2 duplication syndrome has been associated with speech articulation abnormalities, hypotonia, abnormalities of agility, sacral dimples, and epilepsy along with action tremor and microcephaly.[7] In both deletion cases in our study, examination of the family members revealed a carrier status in mothers without any clinical features. As in cases of 15q11-13 microaberrations, incomplete penetrance and variable expressivity have been reported in 16p11.2 microdeletions and especially in microduplications.[7]

Deletion and duplication in the 22q13 region

Copy number variants in the 22q13 region are the cause of various neuropsychiatric disorders, including Phelan–McDermid syndrome (PMS), which is the result of a deletion of a critical region with the *SHANK3* gene. A high rate of ASDs (as high as 20–50%) has been reported in children with 22q13 aberrations. Other highly characteristic features include speech and developmental delays.[5]

Microduplications in the 22q13 region, including the *SHANK3* gene, are reported in patients with attention-deficit hyperactivity disorder (ADHD) and schizophrenia, while microdeletions and point mutations of the *SHANK3* gene are present in patients with mild autism without intellectual disability. Incomplete penetrance is also reported in cases of *SHANK3* gene alterations.[5,18] In 1 patient in our study, we found a deletion of 1 probe for exon 15 of the *SHANK3* gene. In this patient, intellectual disability and speech delay suggested fragile X syndrome (Table 1). Because of the severity of symptoms observed in our patient, it is highly unlikely that the microdeletion detected in the 22q13 region is responsible for his clinical features.

Conclusions

The MPLA analysis is an effective and low-cost technique for screening genetic causes in ASD patients. It allows patients to be selected for further diagnostic consideration with more expensive methods. Because deletions and amplifications in the 15q11-13, 16p11.2 and 22q13 regions have also been described in healthy subjects, confirmation of the results of the MLPA analysis is recommended using another MLPA probe mix or another diagnostic method, e.g., FISH or aCGH. The importance of any detected changes should always be interpreted in relation to clinical data. Abnormal results of an MLPA analysis can also arise from the presence of a point mutation or a single nucleotide polymorphism within the sequence analyzed. Therefore, if sequencing is not applied, the parents of the patient should be examined in order to determine the origin of the change (inherited/de novo).[18] Our analysis revealed that although MLPA can be an initial test in ASD patients, the majority of them will need further investigations, such as aCGH or WES.

Unraveling the genetic underpinnings of ASDs is an imperative not only for the patients but also for the whole family. It provides a basis for genetic counseling and an explanation of observed neurodevelopmental and behavioral characteristics. Finally, it facilitates comprehensive medical care for various comorbidities that might appear due to specific genetic alterations.

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Analysis of the rRNA methylation complex components in pediatric B-cell precursor acute lymphoblastic leukemia: A pilot study

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Abstract

Background. Dysregulation of ribosome biogenesis and alteration of ribosome composition, including alteration in ribosomal RNA (rRNA) 2′-O-ribose methylation, can play a role in malignant transformation and cancer progression. Several studies recently reported that components of the rRNA methylation complex are associated with leukemogenesis. However, no study ever investigated the alteration of ribosome biogenesis factors in the most common pediatric malignancy – B-cell precursor acute lymphoblastic leukemia (BCP-ALL).

Objectives. The objective of this study was to examine the expression of factors building the rRNA methylation complex, either the protein components (1 methyl-transferase (*FBL*), *NOP56*, *NOP58*, *NHP2L1*) or some RNA components (box C/D snoRNAs: *SNORD35B*, *SNORD65*, *SNORD46*, *SNORD50A*, *SNORD38B*), as well as *CMYC*, and nucleolin (NCL) – a protein involved in rRNA synthesis. Clinical effects in children with BCP-ALL were also investigated.

Material and methods. The factors involved in ribosome biogenesis were studied in 28 children with BCP-ALL with the use of real-time polymerase chain reaction (RT-PCR) using the BioMark HD System (Fluidigm, San Francisco, USA) in cDNA prepared from the bone marrow samples collected at diagnosis.

Results. Strong correlations were observed between *NOP56*, *NOP58* and *NCL*, and multiple weaker correlations were observed in the box C/D snoRNA category, and between box C/D snoRNA and transcripts coding proteins of the rRNA methylation complex. The expression of analyzed transcripts did not correlate with the initial white blood cells count (WBC) or with bone marrow blast percentage. Ribosome biogenesis upregulation with overexpression of *FBL* and *NOP56*, and *CMYC* was found in patients who subsequently relapsed and the upregulation signature was not associated with known risk predictors.

Conclusions. This is the first report on the clinical aspect of ribosome biogenesis in pediatric BCP-ALL, and it shows that overexpression of *CMYC* and C/D box nucleoproteins *FBL* and *NOP56* is an antecedent event in patients who subsequently relapse. The dysregulation pattern is different from the previous reports in acute myeloid leukemia (AML), suggesting that dysregulation of ribosome biogenesis is specific for BCP-ALL.

Key words: pediatric, methylation, relapse, acute lymphoblastic leukemia (ALL), ribosome

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood with an incidence of 46.7 cases per million children under 15 years of age per year.[1] The ALL is a group of various subtypes with the most common being the B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Although the majority of children with ALL are cured with chemotherapy, up to 20% of patients succumb to the disease mostly due to resistant or relapsing leukemia.[2] From a clinical perspective, pediatric ALL is a highly heterogeneous entity and early identification of patients with suboptimal response is necessary for therapy intensification.

Among the genes associated with leukemogenesis and clinical response in leukemia, those involved in ribosome biogenesis are promising and only now beginning to be studied.[3–8] In addition, several studies reported that the high proliferation rate of tumor cells is sustained by an increased ribosome biogenesis due to hyperactivation of RNA polymerase I (RNA pol I) associated with an increase in protein synthesis.[9] This view is sustained by the recent discovery that inhibiting RNA pol I activity specifically kills cancer cells in lymphoma without damaging non-cancer cells.[10]

Ribosomes are essential machinery in all cells, as they translate mRNA into protein. Due to their conserved sequence, their analysis was helpful in establishing evolutionary relationships between different organisms.[11] Moreover, ribosomes showed a conserved structure through evolution, which is the basis of the conserved intrinsic activity of the ribosome in the tree of life. In eukaryotes, the ribosomes consist of a small subunit containing one 18S ribosomal RNA (rRNA) and 33 ribosomal proteins (RPS), and a large subunit comprised of the 28S, 5.8S and 5S rRNAs as well as 47 ribosomal proteins (RPL). Production of such complex ribonucleoprotein machinery takes place in the nucleolus and up to 4,500 nucleolar factors have been identified so far.[12] Ribosome biogenesis requires fine regulation of rRNA synthesis using RNA pol I to give rise to a pre-rRNA, a step that is regulated by several factors, including nucleolin (NCL). A recent study reports that NCL is a marker of prognosis in acute myeloid leukemia (AML).[5] The pre-rRNA is then subjected to numerous cleavages, specific folding and extensive nucleotide modifications, which are necessary to maintain the conserved ribosome structure and thus its intrinsic translational activity.[13] However, recent findings showed that alterations of rRNA chemical modifications occur during tumor initiation and tumor progression, directly affecting the translation of some mRNAs coding oncogenes or tumor suppressors.[14–18] Among rRNA chemical modifications, the 2'-O-ribose methylation of rRNA is the most abundant, representing more than 50% of rRNA chemical modifications. They are catalyzed by a box C/D small nucleolar ribonucleoproteins (snoRNPs) complex that contains 3 proteins involved in snoRNP structuration and assembly (NOP56, NOP58, NHP2L1), 1 methyl-transferase (FBL) and 1 box C/D small nucleolar RNA (snoRNA or SNORD), which pairs with the target RNAs determining the specific nucleotide to modification.[18–21] About 250 different box C/D snoRNAs that are necessary to methylate the 106 rRNA sites at their 2'-O ribose residues have been identified. Several box C/D snoRNAs have been shown to promote leukemogenesis, including SNORD50 in T-ALL or SNORD35 in AML.[3,6] Although many snoRNAs and factors involved in ribosome biogenesis have been identified to be associated with leukemogenesis or clinical outcome in different leukemia types, no study has ever investigated their alteration of expression and their clinical value in pediatric BCP-ALL. Here, we have investigated the significance of factors involved in ribosome biogenesis in the proliferative capacity and relapse propensity.

Material and methods

Human samples

The study group consisted of 28 consecutive children diagnosed with BCP-ALL treated at Department of Pediatric Hematology and Oncology (Wroclaw Medical University, Poland). The clinical characteristics of the analyzed group are presented in Table 1. The patients were treated within the ALL IC-BFM 2009 study and according to biological and clinical factors were stratified into 3 chemotherapy arms – standard risk group (SRG), intermediate risk group (IRG) and high risk group (HRG).[22] The response to the initial 7-day long therapy with glucocorticosteroids, defined

Table 1. Patient characteristics

Category	Parameter	Value
Sex	male	22
	female	6
Age at diagnosis in months	median	40.5
	range	7.3–198.1
Initial peripheral blast count per µL	median	8,456
	range	0–18,7680
Initial bone marrow blast percentage	median	56
	range	0–95
Prednisone response	prednisone good response	25
	prednisone poor response	3
Risk group	standard	7
	intermediate	16
	high	5
Relapse	yes	3
	no	25
Follow-up in months	median	61
	range	43–84

as good (prednisone good response (PGR)) with the blast count below 1,000/μL or poor (prednisone poor response (PPR)) with blast count ≥1,000/μL was used as a stratification factor. During the observation period, 3 patients showed bone marrow relapse, and none of the studied patients died. The parents gave their written informed consent for the treatment and analysis of clinical data and the study was approved by the Bioethical Committee at Wroclaw Medical University.

Quantification of RNA using medium throughput RT-qPCR

Initial diagnostic bone marrow samples were collected and suspended in Trizol Reagent (Thermo Fisher Scientific, Waltham, USA) for frozen conservation. Ribonucleic acid was then purified by adding chloroform to separate nucleic acids from proteins, before it was precipitated using isopropanol and 70% ethanol wash. Reverse transcription was performed in duplicate using the PrimeScript RT kit (Takara, Kusatsu, Japan). Briefly, 200 ng of total RNA were retrotranscribed into cDNA using a mix of 2 kind of primers: 2.5 M oligo dT primers for mRNAs, which contain polyA at their 3'-end; and 5 μM random 6-mer primers, which allow reverse transcription of all RNAs, containing or not a poly-A, including mRNAs and snoRNAs. RNA expression levels of genes of interest were finally quantified in triplicate with real-time quantitative polymerase chain reaction (RT-qPCR) using the microfluidic medium-throughput BioMark HD System (Fluidigm, San Francisco, USA). Utilizing the 48.48 Dynamic Array™ IFC (Fluidigm), this system allowed us to perform RT-qPCR on 48 samples by using up to 48 couples of primers. As recommended by the supplier, cDNA was subjected to an initial step of pre-amplification for 10 cycles using the PreAmp Master Mix (Fluidigm) and a mix of all the primers of interest to increase the quantity of initial cDNA matrices since each well of the 48.48 Dynamic Array™ IFC corresponds to only 6 nL. Residues of primers were then degraded by incubation with the Exonuclease I (New England Biolabs, Ipswich, USA). Pre-amplified cDNAs were then 1:10 diluted in 1X TE buffer prior to the addition of 1X GE Sample Loading Reagent (Fluidigm) and 1X SsoFast™ EvaGreen® Supermix with Low Rox (BioRad, Hercules, USA). In parallel, 2.5 M of each couple of primers was mixed with 1X Assay Loading Reagent (Fluidigm) and 1X DNA Suspension Buffer. After the priming of the 48.48 Dynamic Array™ IFC in the IFC controller to prepare the microfluidic system to receive fluids, samples and assays were loaded on the chip by the IFC controller using controlled pressure, this loading method improving the technical reproducibility of the RT-qPCR. The loaded 48.48 Dynamic Array™ IFC was then introduced in the BioMark system (Fluidigm) to measure fluorescent EvaGreen at each end of PCR cycle. Raw data were analyzed using the Fluidigm Real-Time PCR Analysis software (Fluidigm).

Each couple of primers was introduced in triplicate to obtain 3 cycle thresholds (CT) values per sample. In addition to *CMYC*, 10 genes involved in the ribosome biogenesis were quantified: the 4 genes coding proteins involved in rRNA 2'-O-ribose methylation complex (*FBL*, *NOP56, NOP58*, *NHP2L1*); 5 C/D snoRNAs or SNORDs (*SNORD35B*, *SNORD65*, *SNORD46*, *SNORD50A*, *SNORD38B*); and 1 gene involved in rRNA synthesis (*NCL*). Quantification of RNA was first normalized using mean of 6 housekeeping genes (*ACTIN*, *GAPDH*, *HPRT1*, *PPIA*, *PGK1*, *RNU6B*) then using Human XpressRef Universal Total RNA (Qiagen, Hilden, Germany) for inter- and intra-run normalization, respectively, to calculate the relative fold-change using the 2-ΔΔCt. Expression of ribosome biogenesis factors and snoRNAs was presented as mRNA relative levels.

Statistical analysis

Statistical analysis and data presentation were performed using GraphPad Prism computer software (GraphPad Prism v. 6.07 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Associations between the mRNA relative levels were analyzed using Spearman's rank correlation. The Mann–Whitney U test was applied for the comparison of 2 groups, and Kruskal–Wallis test for 3 or more groups. Survival curves were estimated using the Kaplan–Meier method and the differences in leukemia-free survival (LFS), defined as time from diagnosis to relapse, were analyzed between subgroups using log-rank test. A p-value less than 0.05 was considered significant.

Results

The values of the mRNA relative levels were first tested for correlation with peripheral blood absolute blast count and with bone marrow blast percentage, but no statistically significant results were observed. Results are presented in Table 2. The RNA levels of all analyzed genes were analyzed and several two-by-two correlations were identified (Table 3). To determine whether correlations occur between the different gene categories (i.e., box C/D snoRNAs, proteins involved in the rRNA methylation complex or rRNA synthesis), graphical representation of correlation data was drawn (Fig. 1). Correlations were observed within the box C/D snoRNA category, and separately in the group containing the mRNA transcripts of the genes encoding the C/D box snoRNPs and *NCL*. The strongest correlations were observed between *NOP56*, *NOP58* and *NCL*. In addition, multiple weaker correlations were observed in the box C/D snoRNA category, and between box C/D snoRNA and transcripts coding proteins of the rRNA methylation complex. For example, the *SNORD35B* expression correlated with *NOP56*/*NOP58*/*NCL* expression,

Table 2. Results of Spearman's rank correlation coefficient between mRNA relative levels and blast count in peripheral blood (PB) or bone marrow (BM)

Variable		*FBL*	*Nop56*	*Nop58*	*NHP2L1*	*NCL*	*CMYC*	*SNORD35B*	*SNORD65*	*SNORD46*	*SNORD50A*	*SNORD38B*
PB blast count vs RNA levels	Spearman's r	−0.34	−0.11	0.067	0.21	0.27	−0.36	0.14	−0.13	−0.081	0.062	−0.073
	95% CI	−0.64 to 0.051	−0.48 to 0.28	−0.32 to 0.44	−0.19 to 0.55	−0.12 to 0.59	−0.65 to 0.027	−0.26 to 0.49	−0.49 to 0.27	−0.45 to 0.31	−0.33 to 0.43	−0.44 to 0.32
	p-value (two-tailed)	0.0778	0.5673	0.7365	0.2882	0.1612	0.06	0.489	0.5057	0.6818	0.7544	0.7125
	p-value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
BM blast percentage vs RNA levels	Spearman's r	−0.26	−0.22	−0.21	0.029	−0.19	−0.039	−0.2	0.12	−0.028	−0.23	−0.057
	95% CI	−0.58 to 0.14	−0.56 to 0.18	−0.55 to 0.19	−0.36 to 0.41	−0.54 to 0.20	−0.42 to 0.35	−0.54 to 0.19	−0.27 to 0.48	−0.41 to 0.36	−0.56 to 0.17	−0.43 to 0.33
	p-value (two-tailed)	0.1879	0.2608	0.2817	0.8832	0.3219	0.8446	0.2965	0.5389	0.8888	0.2483	0.7715
	p-value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

95% CI – 95% confidence interval.

Table 3. Results of Spearman's rank correlation coefficient between mRNA relative levels of the analyzed transcripts

Gene	*FBL*	*NOP56*	*NOP58*	*NHP2L1*	*NCL*	*CMYC*	*SNORD35B*	*SNORD65*	*SNORD46*	*SNORD50A*	*SNORD38B*
FBL		p = 0.008	p = 0.21	p = 0.15	p = 0.4	p = 0.1	p = 0.36	p = 0.087	p = 0.23	p = 0.98	p = 0.59
NOP56	**0.49***		p < 0.001	p = 0.04	p < 0.001	p = 0.7	p < 0.001	p = 0.51	p = 0.25	p = 0.65	p = 0.21
NOP58	0.24	**0.68***		p = 0.02	p < 0.001	p = 0.67	p < 0.001	p = 0.86	p = 0.06	p = 0.45	p = 0.027
NHP2L1	0.28	**0.38***	**0.42***		p = 0.007	p = 0.7	p = 0.29	p = 0.25	p = 0.3	p = 0.12	p = 0.42
NCL	0.17	**0.74***	**0.65***	**0.5***		p = 0.31	p = 0.002	p = 0.8	p = 0.82	p = 0.11	p = 0.88
CMYC	0.31	0.07	−0.08	−0.07	−0.2		p = 0.75	p = 0.46	p = 0.88	p = 0.69	p = 0.96
SNORD35B	0.18	**0.65***	**0.67***	0.21	**0.55***	−0.06		p = 0.37	p = 0.02	p = 0.14	p = 0.009
SNORD65	−0.32	−0.13	−0.03	−0.22	−0.05	−0.14	−0.18		p = 0.26	p = 0.4	p = 0.38
SNORD46	0.23	0.22	0.35	0.2	−0.04	0.03	**0.43***	−0.22		p = 0.007	p = 0.012
SNORD50A	0	−0.1	0.15	−0.38	−0.31	−0.08	0.28	−0.16	**0.5***		p < 0.001
SNORD38B	0.1	0.24	**0.42***	−0.16	−0.03	−0.01	**0.48***	−0.17	**0.47***	**0.61***	

Lower part of the table shows Spearman's rho and upper part the p-values. Statistically significant results are shown in bold and marked with an asterisk.

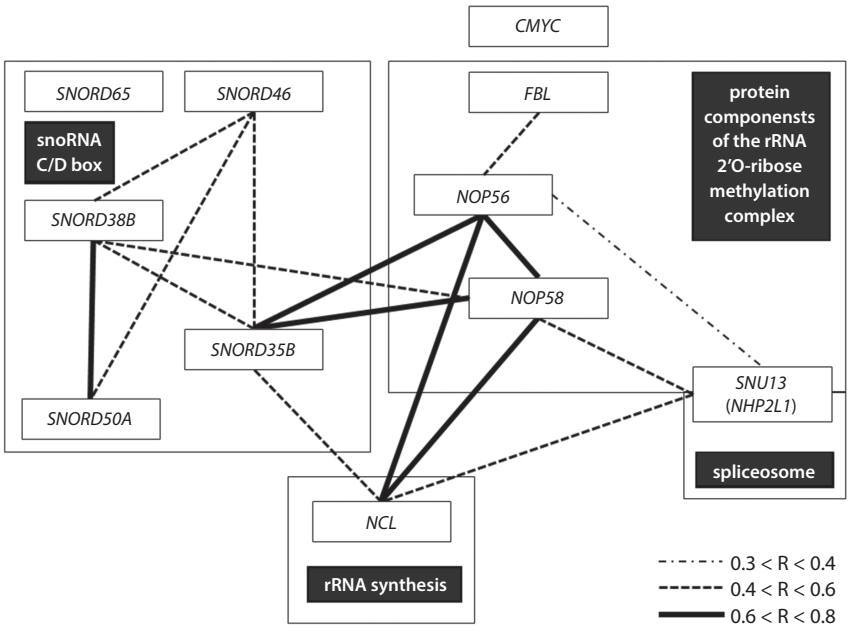

Fig. 1. The correlations between mRNA relative levels of analyzed transcripts. The lines represent the values of Spearman's rank correlation coefficient

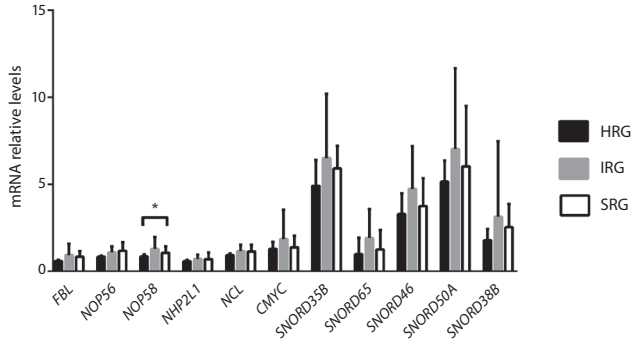

Fig. 2. The mRNA relative levels of analyzed transcripts among patients within different risk stratification groups (high risk group (HRG), intermediate risk group (IRG) and standard risk group (SRG))

Bars and whiskers on the plot represent mean and standard deviation (SD). * $p < 0.05$.

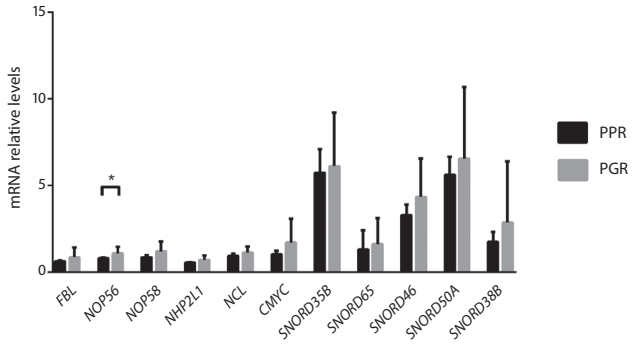

Fig. 3. The RNA relative levels in patients with prednisone good response (PGR) and prednisone poor response (PPR)

Bars and whiskers on the plot represent mean and standard deviation (SD). * $p < 0.05$.

while the *SNORD46*/*SNORD38B* correlated in turn with *SNORD35B*. RNA relative levels of all transcripts were analyzed for correlation with initial peripheral blast count, initial marrow blast percentage and factors used to establish the different risk stratification groups (HRG, IRG and SRG) (Fig. 2). The RNA levels of the *NOP58* were associated with risk stratification ($p = 0.04$). The response to the initial steroid therapy was assessed as PGR in 25 children and PPR in 3 children. The mRNA relative levels for all tested genes were compared between groups with PGR and PPR (Fig. 3). In the PGR group, only the *NOP56* RNA levels were lower ($p = 0.03$). During the observation period, 3 patients relapsed, all in the IRG, and all showed PGR. We then evaluated the association of expression of ribosome biogenesis factors with the later stage of the BCP-ALL disease. The groups of patients who either remained in complete remission (CR) or relapsed were compared for the mRNA relative levels of analyzed transcripts (Fig. 4). Initial RNA levels of *FBL*, *NOP56*, *CMYC*, *SNORD35B*, and *SNORD46* were significantly higher in the patients who subsequently experienced the leukemia relapse than in those who remained in CR. For each factor identified as significant (*FBL*, *NOP56*, *CMYC*, *SNORD35B*, and *SNORD46*), the patients

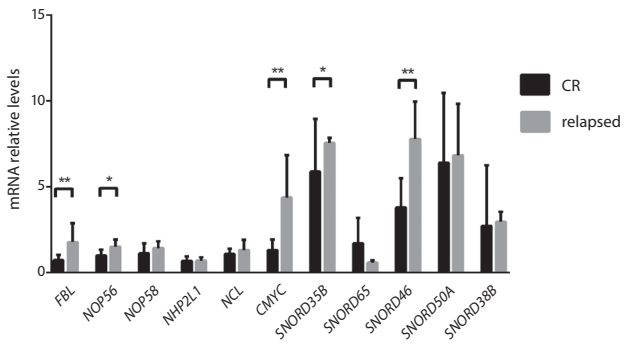

Fig. 4. The mRNA relative levels at first diagnosis of analyzed transcripts between patients who maintained complete remission (CR) and those who relapsed

Bars and whiskers on the plot represent mean and standard deviation (SD). * $p < 0.05$; ** $p < 0.005$.

were grouped based on the RNA levels – 1st group 1–3Q – below the 3rd quartile, and the 2nd – 4Q – above the 3rd quartile (Fig. 5). The survival analysis revealed inferior probability of LFS (pLFS) in the *FBL*, *CMYC*, *SNORD35B*, and *SNORD46* 4Q groups (1–3Q vs 4Q, 100% vs 57%, $p = 0.0011$), and in all relapsing patients the analyzed RNA levels were above the 3rd quartile threshold.

Overall, these data show that expression of some ribosome biogenesis factors at diagnosis might be associated with patient relapse.

Discussion

The association between ribosomal dysregulation and tumorigenesis is indisputable, as proven by increased carcinogenesis risk in constitutional diseases caused by ribosomal gene mutations.[23] In T-cell acute lymphoblastic leukemia (T-ALL), the ribosome function is indeed deregulated through mutations in RPL genes, leading to impaired ribosome biogenesis and translational fidelity.[24–26] Homeostasis of ribosomal machinery thus appears crucial for the preservation of steadfast protein synthesis and there are observations that during tumorigenesis, including leukemogenesis, these mechanisms are flawed.

Our research on expression of different ribosomal biogenesis factors in the analyzed BCP-ALL group showed multiple positive correlations between mRNA expression levels. The correlation of *NOP56*/*NOP58*/*NCL* points to common regulation mechanisms responsible for ribosome biogenesis dysregulation in leukemic cells. The upregulation of box C/D snoRNP components can be caused by the dysregulation of the intracellular genetic homeostasis enacted by upstream regulators like *TP53* and *CMYC*, both of them reported as strong regulators of box C/D snoRNAs, and other proteins involved in the rRNA methylation complex.[6,16,27,28]

Interestingly, we observed the signature of ribosome biogenesis upregulation at initial diagnosis in BCP-ALL

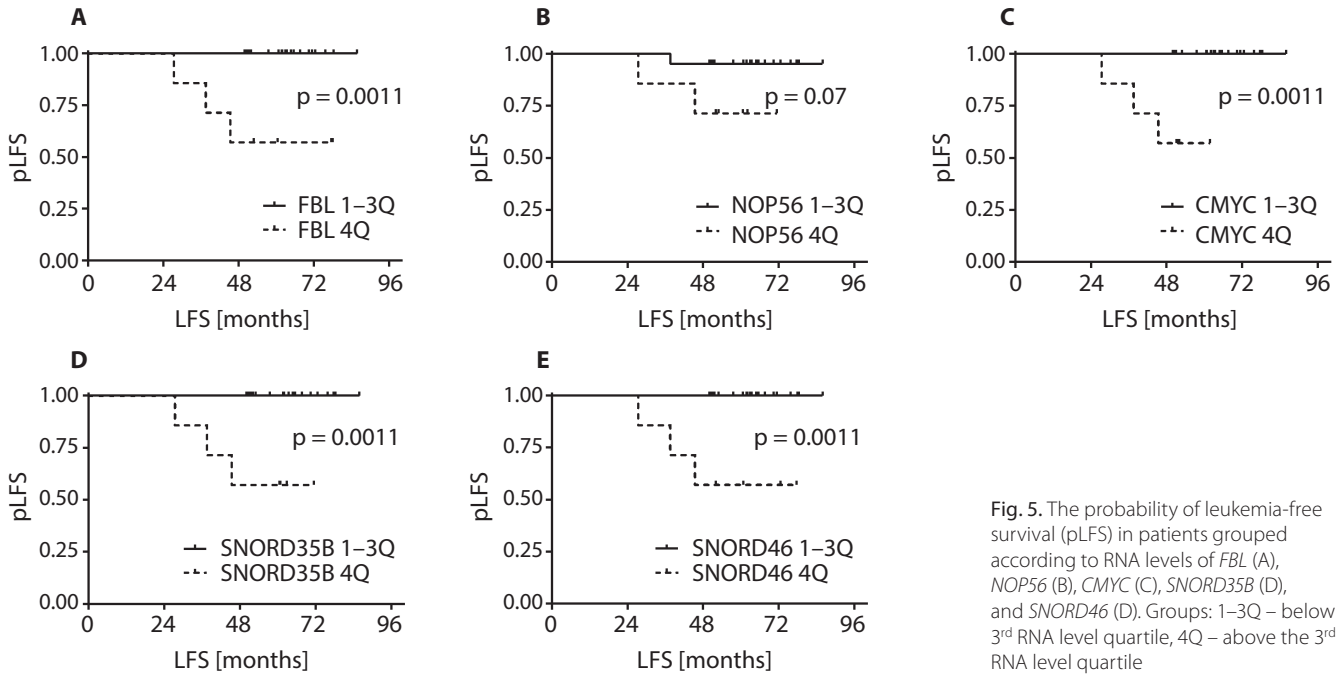

Fig. 5. The probability of leukemia-free survival (pLFS) in patients grouped according to RNA levels of *FBL* (A), *NOP56* (B), *CMYC* (C), *SNORD35B* (D), and *SNORD46* (D). Groups: 1–3Q – below 3rd RNA level quartile, 4Q – above the 3rd RNA level quartile

patients who subsequently relapsed. The increased expression levels of *CMYC* and the components of the rRNA methylation complex, such as *FBL* and *NOP56*, were identified in patients, even though leukemic clone in 3 out of 3 children did not show PPR or resistance to chemotherapy with delayed remission. The role of *FBL* and *NOP56* proteins can be pivotal in the enhancement of ribosomal biogenesis. Animal studies showed that *FBL* regulates stem cell pluripotency, and gene knockdown led to significant delays in rRNA processing, growth inhibition and apoptosis.[29] Thus, the upregulation of *FBL* can protect leukemic cells from self-elimination and sustains mechanism leading to further evolution. The *FBL* was shown to be responsible for the decrease in translational quality control in response to p53 inhibition.[16] The *FBL* overexpression promoted in vitro MCF7 breast cancer cells proliferation and protected cells from doxorubicin, and was associated with poor survival in tumor patients.[16] The lack of correlation of RNA expression with peripheral blast count and tumor burden suggests that the leukemic cells do not require the upregulation of protein synthesis machinery to maintain massive proliferation. The increased initial peripheral blast count in ALL is considered an unfavorable prognostic factor and is included into the stratification of therapeutic protocol with a chance of chemotherapy intensification. In the analyzed cohort, the *NCL* gene – direct enhancer of ribosomal production – was not overexpressed. The normal expression of NCL-1 in *Caenorhabditis elegans* (*C. elegans*) is correlated with the repression of rRNA synthesis and decreased cell, and loss of function mutations in the nucleolin gene leads to increased protein and rRNA production with increased nematodes body size.[30] In AML, the mutations of *NPM1* lead to loss of interaction with NCL protein contributing to leukemogenesis.[31] Nucleolin was found to be overexpressed in AML blasts and a high *NCL* mRNA expression level was associated with poor overall survival, particularly in elderly patients.[5] The prognostic role of the *NCL* expression was not identified in our cohort and the *NCL* upregulation did not identify the patients at risk of relapse, suggesting that the intensified chemotherapy in pediatric ALL can compensate for this effect.

Only the *NOP56* RNA levels were significantly higher in the PPR subgroup, but *NOP56* RNA level did not affect LFS. Steroid resistance in ALL is only partially elucidated by the presence of complex mechanisms related to intracellular expression of the glucocorticosteroid receptor itself, signaling pathways modulating its function and enzymes modifying the availability of its ligands.[32–34] This effect emphasizes the complexity of leukemic transformation with multiple dysregulated mechanisms of cellular homeostasis.

Conclusions

This is the first report on the clinical aspect of ribosome biogenesis in pediatric BCP-ALL identifying the overexpression of the rRNA methylation complex components, *FBL*, *NOP56*, *CMYC*, and *SNORD46* as an antecedent event in patients who subsequently relapse. The results may suggest that the analyzed ribosome biogenesis factor expression levels are not related to known initial risk predictors. Due to the low number of analyzed patients, the study lacks the power to identify all factors associated with leukemia relapse. In order to validate the presented

findings and to determine the value of ribosomal profiling as potential new risk factors, studies in larger groups are necessary. In conclusion, the observed dysregulation pattern in BCP-ALL is different from the reports in AML, suggesting a specificity in ribosome biogenesis defects in BCP-ALL compared to other leukemias.

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Plasma tau protein and Aβ42 level as markers of cognitive impairment in patients with Parkinson's disease

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Abstract

Background. Parkinson's disease (PD) is a progressive neurodegenerative disorder with a characteristic clinical picture. Apart from classical movement disorders, a significant role is also played by non-motor symptoms, in particular cognitive impairments, which have a significant impact on the quality of life of the patients. Tau protein and amyloid beta are well-known non-specific biomarkers in Alzheimer's disease (AD).

Objectives. The study assessed the practical value of determining tau protein and amyloid beta (Aβ42) in the blood serum of patients with PD and their relationship with cognitive impairments, radiographic image and the used dose of L-DOPA.

Material and methods. The neuropsychological assessment was carried for 64 patients with PD. The levels of amyloid beta 1-42 (Aβ42) and tau proteins in serum were also measured.

Results. The Aβ42 level in the serum was statistically higher in patients with longer duration of the disease ($p < 0.05$) and those who were taking a higher dose of L-DOPA ($p < 0.05$). The average level of tau protein in the serum was slightly lower in the study groups than in the control group and showed no statistical significance. No correlation was found between the levels of tau protein and Aβ42 and the results of neuropsychological tests. Tau protein correlated with hippocampal atrophy ($p < 0.05$).

Conclusions. Serum levels of Aβ42 and tau protein in PD may be a useful marker for the assessment of cognitive impairments. The role of L-DOPA in the process of dementia in PD remains unclear.

Key words: Parkinson's disease, dementia, amyloid β-protein, tau protein

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease with a characteristic clinical picture.[1] An important role in PD is played by non-motor symptoms.[2] Special significance is given to cognitive disturbances, which can evolve to mild cognitive impairment (MCI) or dementia. Dementia in PD occurs in about 40% of patients and the risk of cognitive disorders in this group is about 6 times greater than in the general population and increases with the duration of the disease.[3] The search continues for specific biomarkers for PD in body fluids. Currently, tests include determination of the levels of alpha-synuclein (ASN), uric acid, DJ-1, amyloid beta, tau protein, epidermal growth factor (EGF), and glutathione.[4] In the case of Alzheimer's disease (AD), amyloid beta and tau protein are well-established biomarkers. A change in their levels permits a diagnosis of AD with high sensitivity and specificity (90–95%), also during the preclinical period.[5]

This study aimed to assess the practical value of the levels of tau protein and amyloid beta (Aβ42) in the blood serum of PD patients with symptoms of cognitive disorders of varying degrees of severity, including the duration of the disease and the applied dose of L-DOPA, and to determine whether these proteins can be a markers of neurodegenerative changes in PD.

Methods

Subjects

The study group (group I) comprised 64 patients (29 women, 35 men) with an established diagnosis of idiopathic PD according to the United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) criteria, divided into 2 subgroups according to the disease duration: up to 5 years (group IA) and over 5 years (group IB). The majority of the patients (42 cases) were hospitalized at the Clinic of Neurology of the University Hospital in Wrocław, Poland, whereas the other subjects consisted of outpatients. All patients provided written informed consent for participation in the study. The control group (group C) was matched by sex and age with the patients and consisted of 30 healthy volunteers without evidence of cognitive impairment, extrapyramidal syndrome or neurological deficit in neurological examination.

Measures of clinical symptoms

The following data was collected for all patients: demographic data, general medical history, history of the disease, history of previous treatment, history of comorbidities, and assessment of the neurological status. In PD patients, the total applied dose of L-DOPA was calculated. The main purpose of this study required the use of only the five-point Hoehn and Yahr scale (H–Y) to assess the degree of motor disability in the PD group.[1,6]

Neuropsychological assessment

Cognitive functions were examined with the use of neuropsychological tests: Mini-Mental State Examination (MMSE) and the Clock Drawing Test (CDT) which is recognized worldwide as a neuropsychological screening tool. One of the most frequently used methods of CDT scoring is the Sunderland et al. version. A part of the Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS-cog test) assessing the ability to remember a list of words ("word recall"), and a verbal fluency test (Controlled Oral Word Association Test – COWAT) in the alphabet-phonemic category (COWATf) and the semantic category (COWATs) consisting of "fruit and vegetables." The obtained results were referred to the standards set for each age group and gender of the studied patients. Depressive disorders were excluded in all subjects examined using the Beck Depression Inventory (BDI).

Neuroradiological assessment

Seven PD patients were evaluated neuroradiologically using computed tomography (CT) because of contraindications to magnetic resonance imaging (MRI). The remaining patients underwent MRI. The degree of hippocampal atrophy was assessed using the Scheltens rating scale between 0 and 4 to evaluate atrophy of the hippocampus, parahippocampal gyrus, entorhinal cortex, and surrounding cerebrospinal fluid (CSF) on coronal reconstructions,[7,8] where the following degrees of the scale meant:

0 – no atrophy,
1 – only widening of choroid fissure,
2 – also widening of temporal horn of lateral ventricle,
3 – moderate loss of hippocampal volume (decrease in height), and
4 – severe volume loss of hippocampal volume.

Cortical atrophy was assessed with respect to frontal, frontotemporal, parietal, and occipital lobe regions, and within the brainstem using the visual rating scale scoring between 0 and 3,[7,8] where the following degrees of the scale meant:

0 – no cortical atrophy,
1 – mild atrophy,
2 – moderate atrophy, and
3 – severe atrophy.

Assessment of subcortical atrophy included the presence of a widened ventricular system and vasogenic lesions (on a scale of 0–3, where 0 means no lesions of this type).

Plasma tau and Aβ42 measurements

Ten milliliters of native peripheral blood samples were collected from each PD patient and each

subject in group C; the samples were then centrifuged at 2,000 g for 10 min. Pure serum was drained and frozen in test tubes at –80°C. The samples were thawed just before determination of the levels of tau protein and Aβ42. Tau protein and Aβ42 levels were assessed using the High Sensitivity Human Amyloid β42 ELISA kit (Merck Millipore, Burlington, USA) and the Invitrogen Human Tau (Total) ELISA kit (Invitrogen, Carlsbad, USA). The substance levels were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Both kits used specific monoclonal antibodies that recognized a particular epitope within tau protein or amyloid beta. In the next stage of the reaction, streptavidin conjugated with horseradish peroxidase was added, marking appropriate, specific antibody-antigen complexes. After the tracer solution was added, the contents of the sample changed color if tau protein or amyloid beta were present. The process of color reaction was inhibited by the addition of sulfuric acid, which was expressed by another color change. The color intensity of the solution was measured in a spectrophotometer at 450 nm. The sensitivity threshold was 16 pg/mL in the case of Aβ42 and 12 pg/mL in the analysis of tau protein.

Statistical analysis

All statistical analyses were performed with STATISTICA v. 10 PL software (StatSoft Inc., Tulsa, USA). We first assessed the normality assumption of all variables by using the Kolmogorov–Smirnov test. If normal distribution was confirmed, the data was presented as mean with standard deviation (SD). The results in both groups (I vs C) were then compared using Student's t-test. Correlation was assessed using Pearson's correlation coefficient. Analysis of variance (ANOVA) with Scheffe's post hoc test was used to compare more than 2 group means (IA vs IB vs C). In the absence of normal distribution, medians and percentiles were calculated and group comparisons were made using the Kruskal–Wallis test, while Spearman's rank correlation coefficient was used to determine whether correlations exist. The significance threshold was set at $p < 0.05$.

Results

Table 1 shows the demographic characteristics, mean levels of Aβ42 and tau protein, and results obtained in neuropsychological tests and BDI in the study groups.

Table 1. Demographic and clinical characteristics and levels of Aβ42 and tau in patients with PD and controls

Variable	Group I (n = 64)	Group IA (n = 32)	Group IB (n = 32)	Control group (C) (n = 30)	I vs C P (t-test)	IA vs IB vs C P (ANOVA)
Age	68.3 ±9.8 (44–87)	69.0 ±10.0	67.6 ±9.7	64.8 ±9.5	0.1 (NS)	0.24
Sex (M/F)	35/29	11/21	24/8	15/15	0.67 (χ^2 test)	0.005 (χ^2 test)
Duration of illness [years]	6.2 ±4.6	2.7 ±1.6	9.6 ±3.9	–	–	–
H–Y scale	2.8 ±0.8	2.5 ±0.8	3.1 ±0.8	–	–	0.002 (IA vs IB)
MMSE	27.2 ±2.3	27.2 ±2.2	27.2 ±2.4	28.5 ±1.7	0.01	0.03
MMSE men	27.2 ±2.6 (n = 35)	26.2 ±2.4 (n = 11)	26.9 ±2.4 (n = 24)	28.6 ±1.5 (n = 15)		0.03 (IA vs C)
MMSE cor	27.2 ±2.1	27.3 ±2.0	27.0 ±2.2	28.2 ±1.6	0.02	0.05 (NS)
CDT	8.0 ±2.0	8.1 ±1.8 *	7.9 ±2.1 *	9.3 ±1.4	0.001	0.004
CDT men	8.2 ±2.1 (n = 35)	7.6 ±2.2 (n = 11)	7.8 ±2.1 (n = 24)	9.3 ±1.4 (n = 15)	–	0.03 (IB vs C)
COWATs	16.1 ±5.7	16.8 ±5.4	15.3 ±6.0	18.6 ±5.7	0.049	0.08
COWATs men	16.0 ±6.2 (n = 35)	14.3 ±5.2 (n = 11)	14.7 ±6.2 (n = 24)	19.4 ±5.4 (n = 15)	–	0.03 (IB vs C)
COWATf	12.0 ±4.7	11.7 ±4.9	12.3 ±4.5	11.3 ±4.3	0.5	0.71
ADAS-cog	16.4 ±5.1	16.8 ±3.7	16.0 ±6.1	18.1 ±4.4	0.12	0.23
Beck	8.1 ±2.2	7.9 ±2.3 *	8.4 ±2.1 *	5.9 ±3.5	0.001	0.001
Beck men	6.9 ±2.9 (n = 35)	7.0 ±2.6 (n = 11)	8.25 ±2.1 (n = 24)	4.9 ±3.4 (n = 15)	–	0.002 (IB vs C)
Aβ42 [pg/mL]	12.1 ±12.6	8.5 ±10.9	15.8 ±13.3**	26.0 ±64.4	0.56 (Scheffe's test)	0.006 (IB vs C)
Tau [pg/mL]	20.1 ±15.7	20.9 ±19.9	19.2 ±10.1	21.4 ±13.3	0.82	–

Values expressed as mean ±SD. * analyzed group vs control <0.05 (Scheffe's test); ** group IB vs control $p < 0.05$ (according to the Kruskal–Wallis test); NS – nonsignificant; ANOVA – analysis of variance; M – male; F – female; H–Y scale – Hoehn and Yahr scale; MMSE – Mini-Mental State Examination; MMSE cor – correlation of Mini-Mental State Examination; CDT – Clock Drawing Test; COWATf – Controlled Oral Word Association Test in the alphabetic-phonemic category; COWATs – Controlled Oral Word Association Test in the semantic category; ADAS-cog – Alzheimer's Disease Assessment Scale–Cognitive Subscale; Aβ42 – amyloid beta 42.

There were no differences between the groups in terms of age ($p > 0.05$). Patients with longer disease duration had statistically significantly higher scores on the H–Y scale. The mean dose of the used L-DOPA was 673 mg/day in the whole group, 463 mg/day in the IA group, and 884 mg/day in the IB group.

A statistically significant difference was identified in MMSE and its adjusted value between group I and group C ($p < 0.05$). Also, a statistically significant MMSE difference was identified between the group of male patients with shorter disease duration and the males from group C. No significant correlation was found between the groups IA, IB, and group C (Scheffe's test). In the CDT test, the mean score for the whole group I was statistically significantly lower compared to group C. Scheffe's comparison test also showed statistical significance between the groups IA, IB, and group C. The scores obtained in the verbal fluency test in the semantic category were statistically significantly lower ($p < 0.05$) in group I compared to group C. Male patients with longer disease duration obtained statistically lower scores in the semantic category of the verbal fluency test then the other males. No statistical significance was found in the other groups. There were no significant differences in verbal fluency results in the phonetic category or the ADAS-cog test (all $p > 0.05$). Statistically significantly higher scores on the BDI scale were obtained in the whole group of PD patients (group I) and in the Scheffe's comparative test between the study subgroups (IA and IB). Also, male patients with longer disease duration had statistically significantly higher scores on the BDI scale than the other males. No differences were observed between females.

The median test showed differences indicating a statistically higher level of Aβ42 in the IB group; 75% of the value was greater than the total median ($p < 0.05$). The mean level of tau protein in the blood serum in the study groups was slightly lower than in group C and showed no statistical significance. Fifteen patients in group I showed an elevated level of tau protein and a decreased level of Aβ42 as in the case of AD.

Table 2 shows Spearman's rank correlation coefficient, which revealed no significant correlations between the level of Aβ42; the mean scores of MMSE, MMSE correlation (MMSE cor), CTD, COWATf, COWATs, and ADAS-cog; and the mean score on the BDI scale (all $p > 0.05$). In the group of patients with a higher mean dose of L-DOPA, there was a significantly higher level of Aβ42 ($r = 0.043$; $p = 0.013$). In the case of tau protein, no correlation was found with neuropsychological tests, the BDI scale, and the used mean dose of L-DOPA in Spearman's rank correlation (all $p > 0.05$).

Atrophy of the hippocampus (changes ≥1 on the Scheltens's scale) was found in 46 patients (71%), predominantly on the right side. In the IA subgroup, hippocampal atrophy occurred in 21 patients (69%), with a slight prevalence on the right side. In the IB subgroup, it occurred in 25 patients (78%) and manifested symmetrically. Cortical atrophy affected mostly frontal and temporal lobes

Table 2. Correlation between neuropsychological tests and Aβ42 and tau protein level

Aβ42						
Variable	Group I		Group IA		Group IB	
	Spearman's r	p-value	Spearman's r	p-value	Spearman's r	p-value
MMSE	0.0262	0.8367	−0.0438	0.8115	0.0984	0.5920
MMSE cor	−0.0374	0.7691	0.0068	0.9702	0.0043	0.9811
CDT	0.0250	0.8441	0.1157	0.5282	−0.1302	0.4773
COWATf	0.0201	0.8745	0.0714	0.6975	−0.2192	0.2279
COWATs	0.0596	0.6395	0.1795	0.3254	−0.0063	0.9724
ADAS-cog	−0.0179	0.8882	0.2051	0.2599	−0.1336	0.4658
Beck	−0.1504	0.2353	−0.2345	0.1963	−0.1355	0.4594
Tau protein						
MMSE	0.0581	0.648	−0.0047	0.980	0.184	0.313
MMSE cor	0.106	0.404	0.0425	0.818	0.2317	0.202
CDT	0.1366	0.282	0.1833	0.315	0.0788	0.668
COWATf	−0.0235	0.854	−0.1265	0.490	0.2043	0.262
COWATs	0.0655	0.607	−0.0105	0.954	0.1923	0.291
ADAS-cog	−0.087	0.494	−0.184	0.313	−0.0136	0.941
Beck	−0.1219	0.337	−0.1988	0.275	0.049	0.790

Values expressed as mean ±SD. * analyzed group vs control <0.05 (Scheffe's test); ** group IB vs control $p < 0.05$ (according to the Kruskal–Wallis test); NS – nonsignificant; MMSE – Mini-Mental State Examination; MMSE cor – correlation of Mini-Mental State Examination; CDT – Clock Drawing Test; COWATf – Controlled Oral Word Association Test in the alphabetic-phonemic category; COWATs – Controlled Oral Word Association Test in the semantic category; ADAS cog – Alzheimer's Disease Assessment Scale–Cognitive Subscale.

Table 3. The location of brain atrophy and vascular changes in PD

Group IA						
Score	FA	TA	PA	BA	SA	VCh
0	13	8	13	29	14	17
1	16	22	17	3	12	10
2	3	2	2	0	6	3
3	0	0	0	0	0	2
Group IB						
0	9	4	7	23	12	16
1	14	23	21	8	10	11
2	7	4	4	1	10	4
3	2	1	0	0	0	1

PD – Parkinson's disease; FA – frontal atrophy; TA – temporal atrophy; PA – parietal atrophy; BA – brainstem atrophy; SA – subcortical atrophy; Vch – vascular change.

and had a low degree of severity (mostly grade 1 atrophy). Cerebellar atrophy was reported in 30 patients and brainstem atrophy was found in 12 patients. There were no cases with identified atrophy of the occipital area. There were 38 patients with grade 1 and 16 patients with grade 2 (moderate) subcortical atrophy. Vascular changes were present in 31 PD patients (48%). The results are presented in Table 3. The level of Aβ42 in the serum did not correlate with any of the evaluated parameters in neuroimaging studies ($p > 0.05$). The level of tau protein only correlated with hippocampal atrophy ($r = -0.3096$, $p = 0.013$).

Discussion

In the conducted study, the mean level of Aβ42 protein was significantly higher in the blood serum in the group with longer disease duration (IB) than in the IA group and the control group. The level of tau protein was lower than in group C and showed no statistical significance in the respective groups. The level of Aβ42 in the serum did not correlate with any of the evaluated parameters in neuroimaging studies. The level of tau protein only correlated with hippocampal atrophy. No correlation was found between, on the one hand, tau protein and Aβ42 and, on the other hand, the results of neuropsychological tests and BDI. Only in 15 patients from the entire group I the absolute levels of Aβ42 and tau protein behaved as in the CSF in AD.[5]

Literature data mainly concerns determination of Aβ42 and tau protein in body fluids of AD patients, although attempts are being made to determine both those substances in PD patients. Authors of studies on the levels of Aβ42 and tau protein in the CSF hold different opinions on the applicability of their research to PD and PD with dementia (PD-D). Some of the authors found no differences in the levels of Aβ42 and tau protein in the CSF of PD patients compared to controls. Montine et al.[9] determined the levels of Aβ42, tau protein, and its phosphorylated form phospho-tau (P181-Tau) in the CSF in patients with PD and PD-D, noting a lowered level of amyloid beta with normal or low levels of tau protein. Alves and Shi[10,11] showed decreased levels of tau protein and its phosphorylated form in the CSF. A study by Leverenz et al.[12] involving a group of 22 patients with PD without cognitive disorders, analyzed results of neuropsychological tests and the levels of Aβ42, tau protein, and brain-derived neurotrophic factor (BDNF) in the CSF. A relationship was found between the levels of Aβ42, BDNF, and Aβ42/t-tau and the mean scores in neuropsychological tests. No correlation was found with the level of tau protein. The authors suggest the coexistence of the AD mechanism underlying dementia in PD. Similar results were obtained in a prospective study of 45 patients, which showed a link between a decreased level of Aβ42 in the CSF and the progression of cognitive decline without finding such relationship in the case of tau protein and its phosphorylated form.[13] A study of 48 patients with PD of varying degrees of severity found an elevated level of tau protein and tau/Aβ42 ratio in the CSF.[14] The Aβ42 and tau protein determined in the CSF are not unique to AD; they can also be markers of cognitive decline and clinical progression in PD-D.

Data relating to the determination of Aβ42 and tau protein in the serum is limited. The Aβ42 level in the serum shows diverse and ambiguous behavior. Alzheimer's disease shows normal, increased, or decreased levels of Aβ42.[15–17] The observed differences in peripheral blood may be due to external factors, including the collection time and storage conditions of the blood samples.[18] The level of Aβ42 may depend on the drugs used and the degree of atrophy of medial structures of the temporal lobe. An elevated level of Aβ42 in the serum was found in cases of a genetically determined, familial form of AD, associated with mutations in the presenilin or *APP* genes and in the case of trisomy of chromosome 21.[19] Drugs that may affect the levels of Aβ40 and Aβ42 include, among others, calcium channel blockers, digitalis preparations, anticoagulants, antipsychotics, insulin, and other hypoglycemic agents. It is believed that L-DOPA used in the treatment of PD may also have an effect. Our research has demonstrated that the mean daily dose of L-DOPA has a significant impact on the increased level of Aβ42 in the serum. In the case of levodopa, the route of administration of the drug may be important. L-DOPA is absorbed after oral administration in the small intestine by active transportation along the pathway of macromolecular neutral amino acids. Its absorption is subject to fluctuations because of competitive displacement by amino acids from foods, such as phenylalanine, leucine and valine. Other possible influences include disorders of peristalsis, changes in the movement of material from the stomach to the small intestine, intestinal barrier impairment, or activation of immune mechanisms. Blasko et al.,[20] while analyzing the levels of Aβ42 in a group of 526 people over the age of 75 without cognitive impairment, found a correlation between the levels of Aβ42

and age. Similar results were obtained in the assessment of the levels of Aβ40 and Aβ42 in the serum depending on the age in AD, MCI and PD.[21] The Framingham Heart Study[22] found a link between lower levels of Aβ42 in the serum and a higher risk of developing AD in more than 2,000 patients over 60 years of age without dementia. No such relationship was found in the case of Aβ40. Different results were obtained in the Cardiovascular Health Study,[23] where the levels of Aβ40 and 42 in the serum increased with age in 274 people over 70 years of age. However, no relationship was found between the level of Aβ in the serum and the risk of developing AD. Studies by Hansson et al. and van Oijen et al.[24,25] showed that an increased level of Aβ40, a decreased level of Aβ42, and a decreased level of the Aβ42:Aβ40 ratio were associated with a higher risk of dementia in people over 70 years of age. A meta-analysis of 13 studies found that a reduction of the Aβ42:Aβ40 ratio in the serum was associated with a higher risk of developing AD.[26] A change in the levels of Aβ42 and Aβ40 in the serum and the CSF was reported in the case of depressive disorders. Patients with diagnosed depression exhibit a lower level of Aβ42 in the CSF and the serum and an increase in the Aβ 40:Aβ 42 ratio in the serum.[27] Researchers also noted elevated levels of Aβ42 in the serum of patients with chronic kidney disease, correlating with eGFR.[28] These observations seem to indicate that chronic renal failure may be an independent risk factor for the development of dementia.[29] Tau protein levels in the serum are characterized by high variability and range from <10 pg/mL to >1,000 pg/mL while the gradient of tau protein between the CSF and the serum is 10:1.[30] The level of tau protein in the serum may be significantly lower than in the CSF. Higher concentration of the analyzed material may increase its detectability. The level of detection of tau protein in the serum depends on the sensitivity of the ELISA method and the used monoclonal antibodies. The level of tau protein may also be affected by enzymatic modifications leading to separation of epitopes detected with the used monoclonal antibody. Detectability of tau protein may be weakened by the serum presence of proteins that are homogeneous in relation to tau protein (e.g., MAP4 protein). Tau protein may also come from dorsal root ganglia, which has no clinical significance.[31] Hattori et al. determined the level of tau protein in the oral epithelium in AD patients and concluded that it was higher than in the control group.[32] Ingelson et al. found no significant difference between the levels of tau protein in the serum of PD patients and the control group.[33]

Our research has shown that levels of Aβ42 and tau protein in in the serum of PD patients are highly variable and do not correlate with the mean scores in the tests used to evaluate the severity of cognitive disorders and cannot be markers of neurodegenerative changes in PD with cognitive impairment. The dose of the used L-DOPA may have a negative impact on the level of Aβ42 in PD, but its role in the process of PD dementia remains unclear.

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Long-term follow-up of implantable cardioverter-defibrillators in children: Indications and outcomes

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Abstract

Background. Validation data of the use of implantable cardioverter-defibrillators (ICD) in the pediatric population is insufficient, with limited follow-up periods.

Objectives. The aim of the study was to report on 17 years of experience with implantable cardioverter-defibrillator (ICD) therapy in children and young adults.

Materials and methods. This retrospective review included patients below the age of 18 years at the time of ICD implantation between May 2000 and December 2017. For the statistical analysis, the sample was divided into groups by gender and the type of indications for ICD implantation (primary vs secondary prevention).

Results. The study group included 20 children (8 female, 12 male) who underwent ICD implantation for primary or secondary prevention of sudden cardiac death (SCD). The average age at the time of the initial procedure was 15.6 years (range: 3.8–17.7 years). Primary electrical disease (PED) was present in 9 patients, cardiomyopathy (CMP) in 9 and 2 others had congenital heart defects (CHDs). The median follow-up time was 6.7 years (range: 0.4–12.5 years). The outcomes of ICD therapy were analyzed. No differences between the sexes were found in terms of treatment strategy effectiveness ($p > 0.05$). The girls were more often treated as primary prevention ($p = 0.009$). After implantation, all the patients were on optimal pharmacotherapy. Alltogether there were 126 ICD interventions in 11 patients, including 23 inadequate interventions (IA) in 2 children (18.2%).Three children (15%) died due to electrical storms. In the per-procedure analysis, the overall freedom rate from ICD lead replacement was 90%, 80%, and 57% at 1, 5 and 10 years of observation, respectively.

Conclusions. Implantable cardioverter-defibrillator implantation indications in children are more heterogeneous in comparison to adult population. In the pediatric population undergoing ICD implantation, the treatment strategy is influenced by gender. The rate of inappropriate ICD discharges (IA) in our group of pediatric patients was low. Rigorous pharmacotherapy and individual ICD programming seemed of paramount importance. Lead malfunctions LF constituted the most prevalent complication observed.

Key words: sudden cardiac death, implantable cardioverter-defibrillator, pediatric cardiology

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Introduction

The authors present their own experience in the of use of implantable cardioverter-defibrillator (ICD) therapy in children, with one of the longest follow-up periods conducted in a single center from pediatrics to adulthood. Validation data for ICD use in pediatric populations is insufficient, with limited follow-up periods, and is primarily based on isolated clinical cases, single-center studies, registries, and a few comparison studies with ICD use in adults. Therefore, this paper is of high educational value to young scientists who at the beginning of their scientific and medical carrier need to deepen their medical knowledge and stay up-to-date with current guidelines and trends in medicine worldwide.

The implementation of ICD therapy has significantly decreased the number of sudden cardiac deaths (SCD) in the adult population.[1–3] According to the American Heart Association (AHA) and American College of Cardiology (ACC) recommendations, the secondary prevention indications for ICD therapy are the same in children as in adults. The main indications for ICD implantation in the pediatric population are primary electrical heart disease (PED), cardiomyopathy (CMP), congenital heart defect (CHD) post-surgical correction, ventricular tachycardia (VT), and idiopathic ventricular fibrillation (VF) in patients with an anatomically normal heart.[4,5]

As there are no randomized pediatric ICD studies and because experience in the pediatric population is limited to small retrospective case series with short follow-up periods only, statistically significant results are difficult to obtain in this field. We hypothesized that single-center observations of a relatively large non-uniform pediatric group of patients with one of the longest-term follow-ups could contribute to better care of young patients.

The aim of the study was to analyze the indications for ICD implantation, programming issues and treatment options for any arrhythmic and device complications (failure rate and extractability of ICD leads implanted in patients below 18 years of age) in a follow-up period of up to 17 years, which in turn could also improve our understanding of the related issues.

Material and methods

The study was designed as a single-center retrospective analysis of all patients aged below 18 years who underwent ICD implantation between May 2000 and December 2017. The study was approved by the institutional review board and fully complies with the declaration of Helsinki (protocol No. NKBBN/7/2017).

Demographic information, diagnoses, indications for ICD implantation, age and weight at implantation, as well as device and lead information and the patients' course were collected. The indications for ICD implantation for SCD were established according to the guidelines and recommendations.[4,6–8] Primary prevention indications were determined either according to the respective guidelines and recommendations or on an individual basis with special reference to the underlying cardiac condition.[9–13] At the time of implantation, all the ICD systems were tested according to standard clinical practice and contemporary guidelines.[14,15] Follow-up data included in-house follow-up as well as examinations by collaborating specialists. The patient follow-up started at the time of the 1st implantation and lasted until death by any cause, or the date of the last documented device interrogation, or the end of the study in December 2017. The initial ICD check took place within the first days post implantation, after which follow-ups were performed at 4–6 weeks, 12 weeks and every 6 months thereafter, or more often if needed. Follow-up visits took place in the outpatient ICD clinic according to the standard protocol, and consisted of interrogation and retrieval of all data stored since the previous visit and routine measurements of pacing and sensing parameters. In addition, some patients were subject to remote monitoring of their ICD systems.

The follow-up data was evaluated for the presence of both appropriate (AI) and inappropriate shocks (IA), ICD programming information, any history of supraventricular tachycardia (SVT), and the use of antiarrhythmic (AA) medications. Inappropriate shocks were defined as shocks delivered for reasons other than VT or VF meeting programmed detection criteria. The details of the ICD programming were recorded at the time of either IA or the most recent follow-up visit if no IA occurred. The detection rate was recorded in beats per minute (bpm). The detection duration was recorded in either the number of beats or the number of seconds, depending on the device programming/manufacturer. For comparison purposes, detection duration in seconds was converted into duration in beats by using the programmed upper detection rate and the detection duration.

Ineffectiveness of the combined pharmacological therapy and ICD implantation was defined as no impact on the mortality rate in this specific pediatric group.

The technical outcome of ICD therapy was also analyzed retrospectively, and included lead malfunctions and complications. We identified implantation procedure complications and late follow-up complications as a composite of the following: mechanical complications caused by an ICD system, infection and inflammatory reaction due to the presence of the cardiac device or the implantation procedure, iatrogenic pneumothorax, accidental puncture or laceration, post-operative hemorrhage or hematoma, and other complications described in the literature.

Statistical analysis

Statistical analyses were performed using Wizard Pro v. 1.9.22 software (Evan Miller, Chicago, USA). Categorical variables were expressed as numbers (n) and percentages. Continuous variables were expressed as means ± standard deviation (SD) or medians (minimum–maximum),

depending on the distribution. The normality of distribution was tested using the Shapiro–Wilk test. The paired Student t-test and Wilcoxon signed-rank test were used for repeated measures, while the unpaired Student t-test and Mann–Whitney test were used for independent samples. For multiple comparisons, proper analysis of variance (ANOVA) or Friedman's test were used. Categorical data was compared using the χ^2 test. A p-value <0.05 was considered significant for all tests.

For the statistical analysis, the sample was divided into groups by gender and the type of indications for ICD implantation (primary vs secondary prevention).

Results

Patient characteristics/demographics

The clinical and demographic characteristics of the study group, including indications for ICD implantation, are presented in Tables 1 and 2. Two patients moved to another town and are under cardiological follow-up in another center. The distribution of primary and secondary prevention indications and types of cardiovascular disease (CVD) in the study group are presented in detail in Fig. 1.

Initial indications for ICD implantation

The indication for ICD implantation was resuscitated sudden cardiac arrest (SCA) in 12 patients (60%, secondary prevention), while ICDs for primary prevention were implanted in 8 patients (40%) (Table 2). The main symptoms leading to ICD implantation in the whole study group were transient loss of consciousness (TLOC) in 5 children (25%) and palpitations in 2 (10%). The other 13 patients (65%) were asymptomatic. In 5 of the 7 symptomatic children echocardiographic (ECG) examinations revealed left ventricle ejection fraction (LVEF) below 20% and Holter ECG recordings showed non-sustained VT (nsVT). In 3 cases, electrophysiological studies were performed before the implantation procedure due unknown causes of SCA. Cardiac magnetic resonance imaging (MRI) was performed in 4 patients: 2 with CMP and 2 after resuscitated SCA. In 2 of these patients, there were no abnormalities; in one patient, a diagnosis of non-compact left ventricle (NCLV) was confirmed; in the other, non-ischemic dilated cardiomyopathy (DCM) was eventually diagnosed.

Table 1. Clinical and demographic characteristics of the study group

Characteristic	Value
Number of patients	20
Median age of implantation [years]	15.6 (3.8–17.7)
Gender	8 F (40%)/12 M (60%)
Body weight at initial implantation [kg]	52.1 ±18.6
Indications for ICD implantation primary/secondary	6 (30%)/14 (70%)
Mean follow-up period [years]	6.5 ±4.8
Deaths	3 (15%)
Heart transplantation	1 (5%)

F – female; M – male; ICD – implantable cardioverter-defibrillator.

Anti-arrhythmic therapy

Before ICD implantation, 14 children (70%) were on AA medication, and after implantation, all 20 children (100%) were. The most common therapy was beta-blockade (nadolol, metoprolol), either alone or in combination with class III AA drugs (amiodaron, sotalol), as shown in Tables 2 and 3. The optimal treatment for heart failure (HF) according to current guidelines and recommendations was used before ICD implantation in all the children with CMP.[12,16]

Table 2. Clinical data, indications for ICD implantation, therapy and outcomes in terms of gender

Variable	Female	Male	p-value
Number of patients	8 (40%)	12 (60%)	0.371
Age at 1st intervention [years]	16.2	16.4	0.905
ICD indication primary secondary	 6 (75%) 2 (25%)	 2 (17%) 10 (83%)	0.009
Diagnosis PED CMP CHD	 3 (38%) 5 (63%) 0 (0%)	 6 (50%) 4 (33%) 2 (17%)	0.300
Symptoms TLOC palpitations asymptomatic	 2 (25%) 2 (25%) 4 (50%)	 3 (25%) 0 (0%) 9 (75%)	0.049
Pharmacotherapy before ICD implantation post ICD implantation	 8 (100%) all	 6 (50%) all	0.017
Pharmacotherapy II III AA therapy + HF	 3 (38%) 1 (13%) 4 (50%)	 6 (50%) 0 (0%) 6 (50%)	0.435
Deaths	1 (13%)	2 (17%)	0.761
HTX	1 (13%)	0 (0%)	0.209
ICD interventions AI IA both none	 3 (38%) 1 (12%) 1 (12%) 3 (38%)	 5 (42%) 1 (8%) 0 (0%) 6 (50%)	0.621
Time to first AI [days]	361	163	1.000
Time to first IA [days]	1,664	350	0.667
Therapy effectiveness effective non-effective	 4 (50%) 4 (50%)	 5 (42%) 7 (58%)	0.714

ICD – implantable cardioverter-defibrillators; CMP – cardiomyopathy; CHD – congenital heart defect; PED – primary electrical disease; TLOC – transient loss of consciousness; AA therapy – antiarrhythmic therapy; HF – heart failure; HTX – heart transplantation; AI – appropriate interventions; IA – inappropriate interventions.

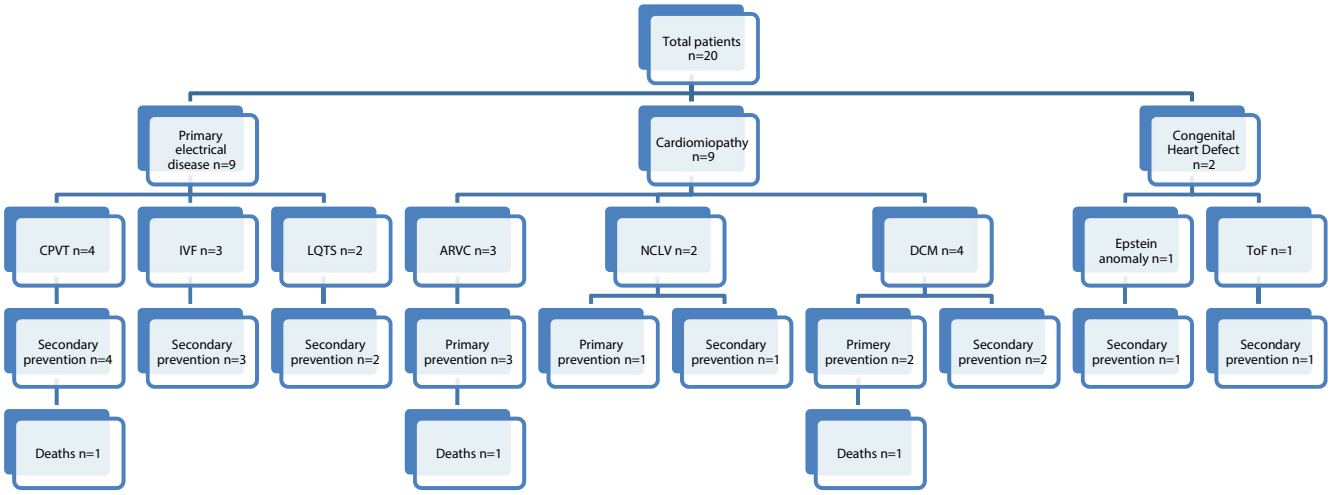

Fig. 1. Indications for implantable cardioverter-defibrillator (ICD) implantation and cardiovascular disease (CVD) in the study group

Technical aspects of ICD implantation

All the initial procedures were performed under general anesthesia. The most common initial approach for ICD implantation was transvenous (used in 19 children). Among the implanted systems there were 14 single-chamber and 5 dual-chamber ICDs. A biventricular device (cardiac resynchronization) was placed in 1 patient. Single-coil leads were placed in 17 children and dual-coil leads in 3.

In 3 children, a single-chamber ICD was placed in the abdominal region and an active-fixation ICD lead was simultaneously implanted through the subclavian vein. The incision was made parallel to the linea and then to the tendon of the rectus abdominalis, and the ICD was placed under that muscle in a subrectus pocket. Then, the ICD lead was tunneled subcutaneously to the subclavian region and was placed through the cephalic vein in the apex of the right ventricle. The ICD "active can" was connected to the lead (or leads), all electrical parameters were measured again and a defibrillation test was performed.

A defibrillation threshold test (DFT) was performed according to current guidelines. The mean DFT result at primary implantation was 15 J (range: 6–20 J). The VF detection duration was programmed initially between "18 out of 24" and "75 out of 100" based on the reason for ICD implantation. The initial R wave amplitude was 8.9 ±4 mV. In our only patient with an epicardial lead, the DFT was 10 J. All shocks were programmed to the maximum energy output of the devices.

There were no acute intraprocedural or early post-procedural complications at the initial ICD implantations.

The effects of gender

To check whether gender had any impact, we examined potential differences between females and males in this study (Table 2). Loss of consciousness or family history were significantly more frequent reasons for establishing the diagnosis and qualification for SCD prevention in boys (p = 0.042) than in girls, while palpitations were significantly more frequent reasons among girls. Girls were more likely to receive pharmacotherapy prior to ICD implantation (p = 0.017) and were more often treated in primary prevention (p = 0.009). No differences (p > 0.05) between the sexes as to the effectiveness of treatment with ICD or the effectiveness of pharmacotherapy were found.

Primary vs secondary prevention of SCD

Patients were also divided into groups depending on the type of indication for ICD implantation – primary vs secondary prevention (Table 3), and our comparative

Table 3. Clinical data, etiology, arrhythmia occurrence, EF and results of the treatment used in the study group in terms of ICD implantation for secondary vs. primary prevention

Prevention of SCD	Primary	Secondary	p-value
Number of patients	8	12	0.371
Sex			
F	2 (17%)	6 (75%)	0.009
M	10 (83%)	2 (25%)	
Diagnosis			
CHD	0 (0%)	2 (17%)	0.007
CMP	7 (88%)	2 (17%)	
PED	1 (13%)	8 (67%)	
EF			
>50%	2 (25%)	12 (100%)	0.001
<50%	6 (75%)	0 (0%)	
Pharmacotherapy			
I	0	0	0.043
II	1 (13%)	8 (67%)	
III	1 (13%)	0 (0%)	
AA therapy + HF	6 (75%)	4 (33%)	
Therapy effectiveness			
effective	6 (75%)	3 (25%)	0.028
non-effective	2 (25%)	9 (75%)	

ICD – implantable cardioverter-defibrillators; SCD – sudden cardiac death; CMP – cardiomyopathy; CHD – congenital heart defect; PED – primary electrical disease; EF – ejection fraction; AA therapy – anti-arrhythmic therapy; HF – heart failure.

analysis showed several statistically significant differences between these groups. As mentioned earlier, ICDs were implanted in girls for primary prevention significantly more often than for secondary (p = 0.009). Also, there were significant differences (p < 0.001) between the reasons for reporting to a cardiologist among the patients treated in primary prevention (with no history of SAC) compared to those in secondary prevention (with a history of resuscitated SAC). Additionally, in primary prevention, patients with CMP were treated more often compared to patients with normal heart anatomy (p = 0.009) or those with CHD (tetralogy of Fallot, Ebstein's anomaly) (p = 0.039).

Patients treated for primary prevention had significantly higher (p = 0.011) incidence of nonspecific intraventricular conduction delays (Fig. 2) compared to those treated for secondary prevention, who more often had right bundle branch blocks (RBBBs) or incomplete right bundle branch blocks (IRBBBs).[17] One child had a left bundle branch block (LBBB). Ventricular arrhythmias of at least 3 morphologies (p < 0.001) and documented episodes of VF were more frequent among the patients qualified for secondary prevention (p = 0.006). Patients with reduced left ventral contractility (LVEF < 50%) more frequently had ICDs implanted as primary prevention (p < 0.001).

If the primary cause of the referral to a cardiologist was TLOC, treatment (ICD and pharmacotherapy combined) was more effective (p = 0.035); if the reason for the referral was family history, it was ineffective (p < 0.001). Other reasons for referrals were not found to affect the effectiveness of the combined treatment. The type of prevention (primary or secondary) was also a factor influencing the effectiveness of the treatment (p = 0.028).

It was also found that in secondary prevention, arrhythmia was more complex: nsVT/VT/VF, as opposed to VPB in primary prevention).

Appropriate and inappropriate device discharge

An analysis of ICD intervention in terms of AI and IA or a lack of intervention was carried out (Table 4). There were 126 ICD interventions in 11 patients (55%): 102 (81%)

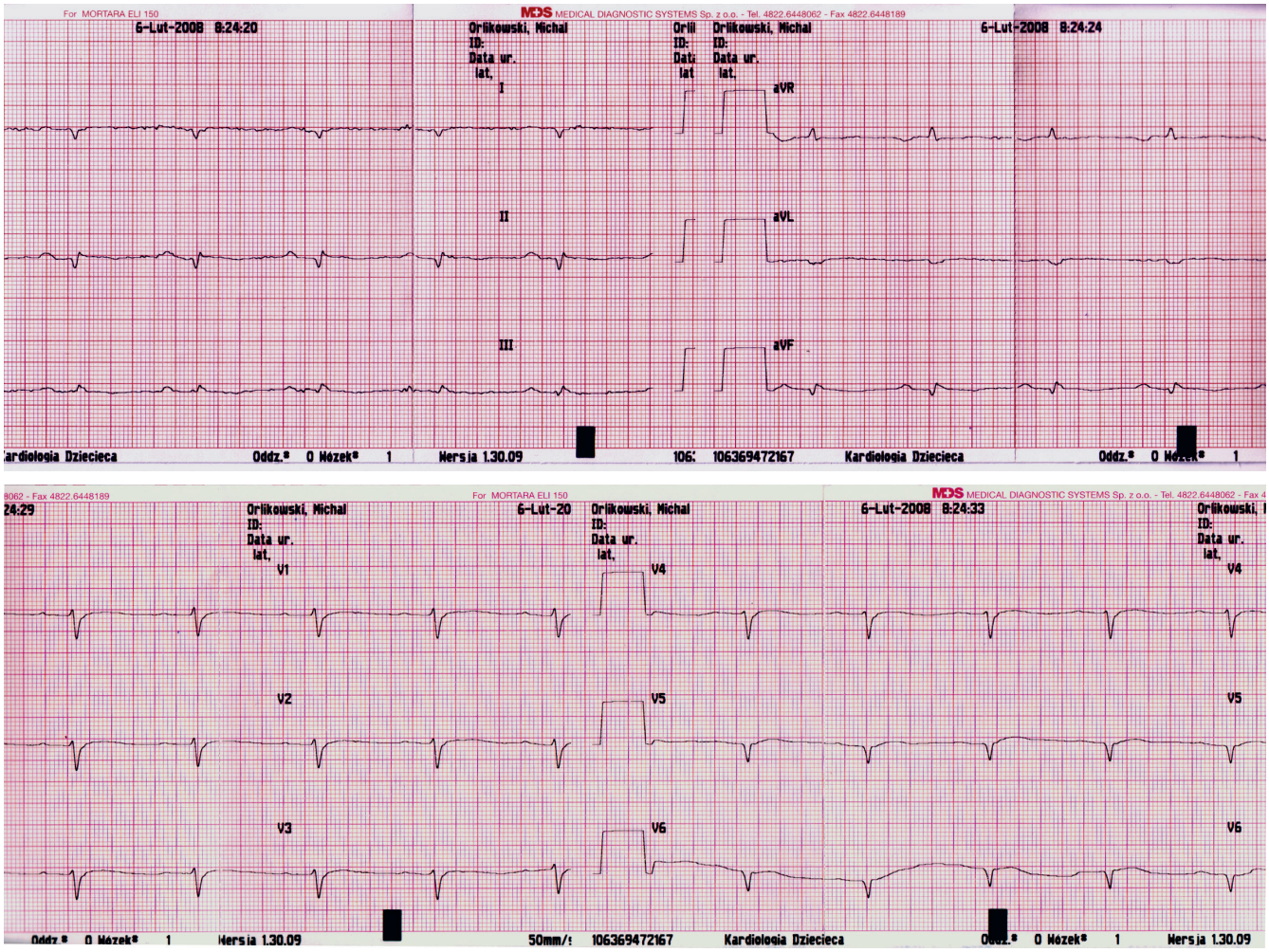

Fig. 2. ECG demonstrates intraventricular non-specific conduction block in a 6-year old patient qualified for the primary implantable cardioverter-defibrillator (ICD) implantation due to dilated cardiomyopathy (DCM). Interventricular conduction delay (IVCD) is determined on the based on the current recommendation[17]

Table 4. Appropriate and inappropriate device discharges

Variable	Total number of patients having any interventions	AI	IA	Both interventions (AI & IA)	p-value
Number of patients	11	8	2	1	0.020
Sex female male	 5 (63%) 6 (50%)	 3 (38%) 5 (42%)	 1 (13%) 1 (8%)	 1 (13%) 0 (0%)	0.621
ICD indication primary secondary	 5 (63%) 6 (49%)	 4 (50%) 4 (33%)	 1 (13%) 1 (8%)	 0 (0%) 1 (8%)	0.632
Diagnosis PED CMP CHD	 4 (44%) 5 (55%) 2 (100%)	 2 (22%) 4 (44%) 2 (100%)	 1 (11%) 1 (11%) 0 (0%)	 1 (11%) 0 (0%) 0 (0%)	0.528
Time to the 1st intervention [days] (range)	350 (6–2,412)	163 (6–2,412)	637 (350–2,691)	82	

ICD – implantable cardioverter-defibrillator; PED – primary electrical disease; CMP – cardiomyopathy; CHD – congenital heart defect; AA therapy – anti-arrhythmic therapy; AI – appropriate interventions; IA – inappropriate interventions.

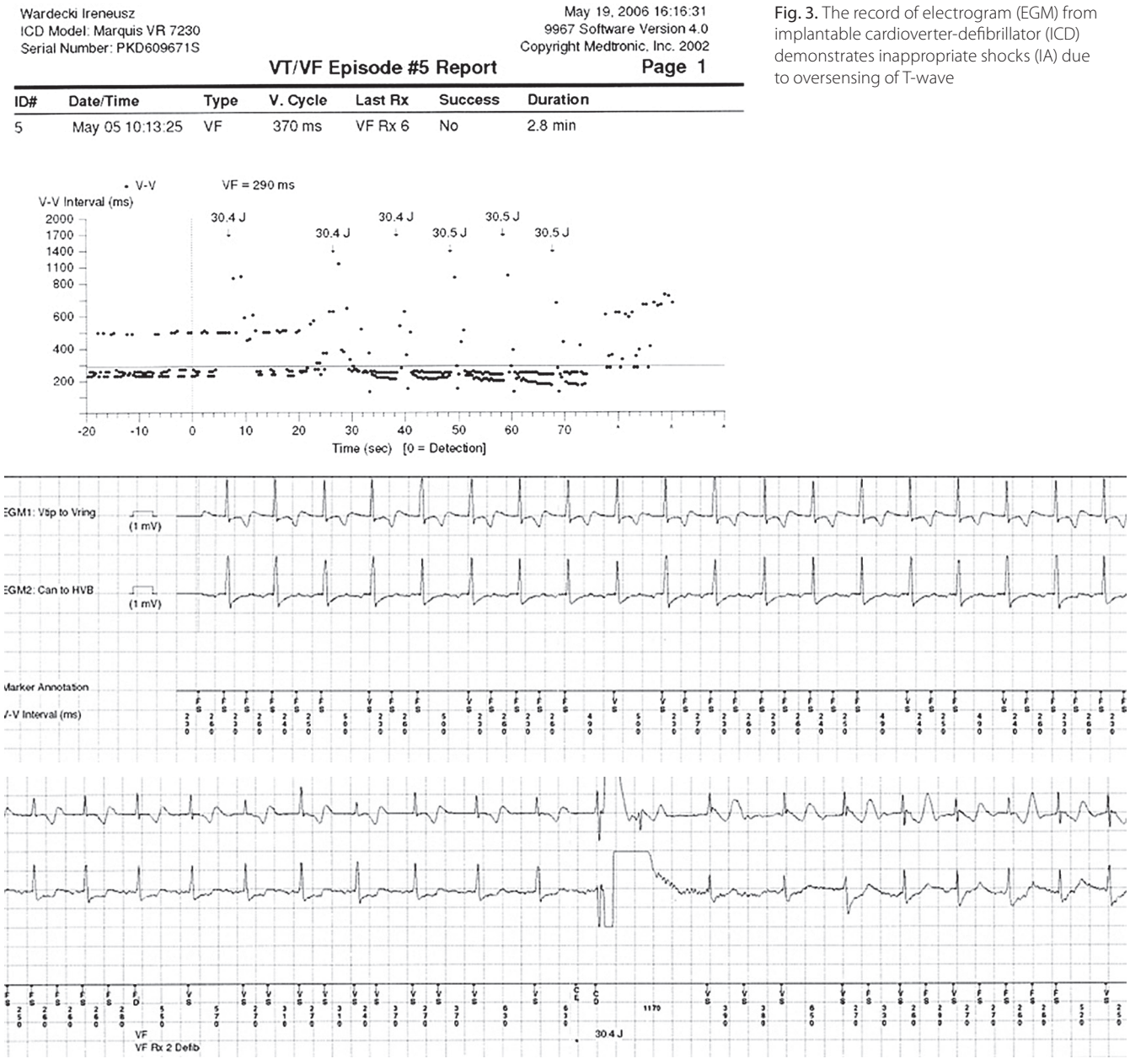

Fig. 3. The record of electrogram (EGM) from implantable cardioverter-defibrillator (ICD) demonstrates inappropriate shocks (IA) due to oversensing of T-wave

AIs in 8 children (40%) and 22 (18.2%) IA in 2 patients (10%); 1 teenager had 1 AI (0.8%) and 1 IA (0.8%) intervention (Fig. 3). The rate of IA due to non-optimal programming of the device was 5%; oversensing of the T-wave caused 10%; pace/sense lead dislocations were the cause of 5%; and HV lead failure (LF) caused 10% of IAs. There were no interventions at all in 9 patients (45%). The median time before the 1st intervention (of any kind) was 350 days (range: 6–2,412 days).

ICD-system related complications and actions taken to solve them

During the follow-up period, 1 girl with arrhythmogenic right ventricle CMP (ARVC) underwent heart transplantation 6 months after ICD implantation, at the age of 17. A patient with an ICD implanted for primary prevention of SCD gave a birth to a healthy neonate 7 years after the ICD implantation. None of the patients in our study group had the ICD removed permanently, and there were no infections of the implanted ICD systems during the follow-up period. In 2 cases, remote monitoring was used; the devices documented atrial fibrillation in 1 case and nsVT in the other. In both cases, the pharmacological AA therapy was then modified.

During the follow-up period 3 of the patients died. The 1st patient was an adolescent with catecholaminergic polymorphic ventricle tachycardia (CPVT), who received his ICD in 2000 (the 1st patient in our study) at the age of 15; he died 3 years after ICD implantation due to therapy-resistant VF. He was on maximal pharmacotherapy at that time (a combination of class II and class III AA drugs). The ICD in this case was a Medtronic Micro Jewel II, model 7223 Cx (Medtronic plc, Dublin, Ireland), implanted in a left abdominal pocket with the lead tunneled from the infraclavicular region to the pocket. The implant defibrillation threshold (DFT) was 15 J measured using a biphasic waveform. The DFT was rechecked within 2.5 months after implantation and remained unchanged. The lead impedance at implant was 45 Ω. The 1st AI was delivered just 1 week after implantation. The device was programmed for the VT and VF zones, and 34 J shocks. At the time of the patient's therapy-resistant VF, the VF detection rate was 240 bpm, the detection number count was 24 out of 32, and the VT zone was 200 bpm and 75 out of 100.

The 2nd patient that died, a girl with DCM who received her ICD as a bridge to a heart transplant at the age of 16, died 6 months after implantation due to therapy-resistant VF (an electrical storm).

The 3rd patient that died, a boy with ARVC, underwent ICD implantation at the age of 6 as a bridge to a transplant. He died 4 months after implantation due to HF resistant to pharmacotherapy. The 1st AI in this case (ATP due to FVT) was documented 56 days post implantation.

Data concerning ICD-system related complications and actions taken to solve them is presented in Table 5. Six patients (30%) experienced LF, 2 patients (10%) experienced failures of pace/sense leads and 5 patients (25%) experienced failures of high-voltage (HV) leads. In 2 patients, more than 1 LF took place. In total, 7 re-interventions were performed from 0.1 to 11.3 years (median: 7.4 years) from the initial system implantation; 6 of them were related to malfunctions of Sprint Fidelis HV leads (Medtronic plc). In the per-procedure analysis, the overall freedom from HV lead replacement was 90%, 80%, and 57% during 1, 5 and 10 years of observation, respectively.

During the follow-up period, the whole ICD system was replaced in 4 cases using Cook lead extraction tools (Cook Medical LLC, Bloomington, USA).

Table 5. Complications due to HV lead dislocation or failure

Patient ID	Year of initial surgery	Re-intervention		
		Time to [years]	Cause	Procedure performed
2	2005	11.3	HV LF – Sprint Fidelis (6949) (high threshold on FU visit)	Extraction with Cook, new HV Lead implanted (Biotronic Linox Smart S65)
3	2005	0.1 10.4	HV lead dislocation – Sprint Fidelis (6949) HV LF – Sprint Fidelis (6949) (high threshold on FU visit)	Extraction with traction, new HV lead implanted (Medtronic 6949) Extraction with Cook of old leads, new leads implanted (Biotronic Protego ProMRI and Medtronic CapSureFix Novus 5076-52 cm)
4	2005 2006	0.5 9.7	HV lead dislocation – Sprint Fidelis (6949) HV LF – Sprint Fidelis (6949) (high threshold on FU visit)	Extraction with traction, new HV lead implanted (Medtronic 6949) Extraction with Cook, new HV lead implanted (Biotronic Linox S65) Complication: embolisation of subclavian vein
9	2007	1.8	HV LF – Sprint Fidelis (6931) (IA shocks due to HV lead fracture)	Extraction with traction, new HV lead implanted (Biotronic Linox S65)
12	2008	7.4	HV LF – Biotronic Linox S65 (high threshold on FU visit)	Extraction with Cook Evolution, implantation of new leads (Biotronic Linox and Medtronic 5076-52 cm)

HV – high voltage; LF – lead failure; IA – inappropriate intervention.

Discussion

The present data from a long-term single-center registry of children and adolescents undergoing ICD implantation offers one of the longest follow-up periods under the same supervisor in the same center in the available literature. Furthermore, this patient population presents a remarkable incidence of appropriate therapy delivery and displays the long-term course of children with ICDs in detail, particularly regarding device complications.

Effects of gender

We divided our study cohort according to gender. The groups did not differ significantly in terms of age or diagnosis (both organic heart disease and electrical pathology); however, in a more detailed statistical analysis, there were several significant differences between the groups. This may suggest that girls pay more attention to the feeling of heart "palpitations", while boys get medical care only after incidents of unconsciousness. As a secondary result of this difference, girls were more likely to receive pharmacotherapy prior to ICD implantation and were more often treated for primary prevention. In the literature, we did not find any studies that analyzed this aspect. Interestingly, no differences between the sexes were identified in our study in terms of the effectiveness of either ICD treatment or pharmacotherapy. There are only few available studies concerning this subject.[18] In their study, Sears et al. investigated differences between male and female pediatric ICD patients in terms of quality of life (QOL), and their conclusion was that "the observed differences in lower psychosocial, physical, and cardiac-specific QOL between female and male patients suggest that female patients feel particularly limited across these domains of functioning".[19]

Secondary vs primary prevention of SCD

Implantable cardioverter-defibrillator implantation techniques have been simplified considerably in recent years thanks to significant reductions in the dimensions of modern generators and the use of transvenous leads.[9,10,13,14,20] In the past, ICD implantation was mainly performed for secondary prevention of SCD, but nowadays ICDs are often implanted for primary prevention, although this is still a field of great controversy.[4,8,13,21,22] We studied several factors to determine the effectiveness of primary vs secondary prevention of SCD.

With regard to pharmacotherapy, the 2 analyzed groups differed significantly. Those treated for primary prevention were more likely to receive pharmacotherapy before implantation ($p = 0.017$), and they more often received AA drugs (class III) or combination therapy: class III and optimal HF treatment according to current guidelines and recommendations. The patients in the secondary prevention group primarily had β-blocker therapy.[23] This was mainly related to differences in the underlying diseases.

Comparing the groups in which the treatment was assessed as effective or ineffective, it was found that they differed in terms of certain characteristics. Firstly, in the group characterized by ineffective treatment, patients with CMP were more frequent, while in the effective treatment group, more patients with congenital malformations or PED with normal heart anatomy were found ($p = 0.025$). To some extent, this observation might have been influenced by the guidelines and how the patients were qualified for treatment.

Secondly, the type of prevention (primary or secondary) was a factor determining the effectiveness of the treatment ($p = 0.028$). It was revealed that in secondary prevention of SCD, complex arrhythmia was diagnosed more frequently than in primary prevention.

The statistical analysis also showed that ventricular arrhythmia morphology was a factor determining the effectiveness of ICD interventions and combination therapy ($p = 0.004$). The treatment used was significantly less effective in the presence of 2 ventricular beat morphologies (both LBBB and RBBB). In addition, reduced LVEF was a significant ($p = 0.002$) parameter determining overall treatment efficacy (pharmacological treatment and ICD interventions). Apart from the above, no other factors related to the effectiveness of therapy were found.

Appropriate and inappropriate device therapy

Implantable cardioverter-defibrillator shocks delivered for reasons other than life-threatening ventricular arrhythmias, known as IA, occur frequently in ICD-equipped patients.[1,24–26] Pediatric patients and patients with CHD have a particularly high rate of IAs, with the largest studies reporting up to 20% of patients receiving them, mainly due to higher heart rates in younger patients, a higher risk of lead malfunction due to more active lifestyles and longer follow-up periods.[26–30] That is why in our center, since the very first case, we have been programming devices individually, based on the patients' individual characteristics, in order to achieve the best results.[1,7,31]

In our study, 3 patients (15%) suffered from a total of 23 IAs. Specifically, the rate of IAs due to non-optimal programming of the device was 5%; due to T-wave oversensing 10%; due to pace/sense lead dislocations 5%; and due to HV LF in 10% of the incidents. Some of the children had more than 1 cause of IA intervention. Some of the children had more than 1 cause of IA. Using the experience in the present study, we were able to demonstrate that individualized ICD programming, such as higher VT detection rates, detection restricted to the VF zone and longer detection intervals, was probably crucial to keeping the rate of IAs low.[10,24,32]

There is more and more evidence that defibrillator shocks can cause myocardial damage, and the shocks have

been associated with increased mortality.[5,33] In the pediatric population, reductions in the number of IA and the associated significant reductions in total IA energy delivered to the myocardium may have resulted in diminished myocardial damage and lower mortality in this particular group of patients. The time period prior to the 1st intervention (AI or IA) was 350 days (range: 6–2,412 days); and it did not correlate with any other of the factors studied; in particular, it was not an independent variable of SCD/SCA.

There were 3 deaths during the present study, all of them in the pioneer era of ICD implantation in children, and detailed subgroup analyses of the relationship between device programming and death have limited power. The findings from randomized trials and our own experience support the ongoing evolution of ICD therapy for primary prevention, in parallel with programming approaches reducing inappropriate therapies and increasing survival among patients with ICDs.[10,24]

ICD-system related complications and actions taken to solve them

In 1980, Mirowski published an article about the use of ICDs in patients who had undergone successful resuscitation.[34] The external cardioverter-defibrillator had been a recognized therapeutic tool for decades, but Mirowski was the first to introduce a fully automatic implantable device, comparable to implantable pacemakers.

The 1st report of ICD implantation in a young patient was published in 1988 by Kral et al.[14] The 1st ICD implantation in a teenager in our center was performed in 2000. In that young teenager with CPVT, we initially programmed the VF detection rate at 240 bpm and the detection counter at 24 out of 32; the VT zone was from 200 bpm. Unfortunately, despite optimal pharmacotherapy, therapy-resistant VF occurred. Because of the recurrent VF, despite repeated shocks, we decided to delay the therapy, switching the detection counter to 75 out of 100. That was meant to reduce the number of shocks and thus to reduce adrenergic stimulation, which may be effective in some patients. Nonetheless, the arrhythmia could not be suppressed in any way, and the patient died. Since that time, we have programmed the detection counter to 75 out of 100 in all 3 of our subsequent patients with CPVT. None of them experienced electrical storms during their follow-up periods (which lasted 13, 12 and 10 years, respectively). One of them, who is subject to regular cardiological follow-up every 6–12 months, now has a 5-year old daughter.

Lead-related complications are the most common adverse events during follow-up.[8,10,27] Lewandowski et al. reported a 21% rate of complications requiring surgical intervention.[26] In our study, considering all 23 IA (18.2%), more than half of them took place because of LF (17/23; 74%). Despite the significant technological progress that has been made in recent years in the field, ICD implantation is still more complicated in children than in adults.[8,9,35–38] There are no dedicated leads for small vessel diameters. Existing ICD devices are not adapted to the small body surface and weight of a pediatric patient, and abdominal implantation of the power generator may be necessary.[20,28,29,39,40] Lead failure is an important issue to all physicians taking care of patients with ICDs because of its serious consequences. Unfortunately, its incidence is difficult to determine, due to several factors. During the past 2 decades, several recalled leads have appeared on the market.[37] The recalled Medtronic Sprint Fidelis series serves as an example of conductor and insulation failure. Recently, there was a recall of SJM devices. According to data from the literature, the failure rate for Sprint Fidelis leads is about 20% at 10 years. Our management of children with functioning recalled leads was very individual. In all cases, we focused on intensifying the monitoring plan, and followed the programming strategy recommended by the manufacturer.[37] In cases of patients with externalized cables, but with no electrical abnormalities, we reprogrammed the devices, and performed extraction with the insertion of a new ICD lead during a planned device exchange. Since 2010 lead extraction has been performed at our center with surgical backup.

Study limitations

This study has several limitations. The study design was a retrospective cohort analysis, and underestimation of LF cannot be excluded. The indications for ICD implantation are more heterogenic than in an adult population, where ischemic origins predominate. The programming details of anti-tachycardia therapy could not be systematically assessed, as it was very heterogeneous over the long study period and frequently changed even in the same individual. Patients presenting with abnormal lead parameters underwent standard radiography to assess whether any structural damage was present. The exact mechanisms of LF could not be confirmed in every case. Based on clinical decisions whether to extract or abandon the lead, not all the leads were visually examined, and were not available for a returned product analysis by the manufacturer.

Conclusions

The ICD implantation indications in children are more heterogeneous than in the adult population. In the pediatric population undergoing ICD implantation, the treatment strategy is influenced by gender. The rate of IAs in our group of pediatric patients was low. Rigorous pharmacotherapy and individual ICD programming seemed to be of paramount importance. Lead malfunctions were the most prevalent complications observed, and most of them were related to Sprint Fidelis leads.

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Vitexin improves neuron apoptosis and memory impairment induced by isoflurane via regulation of miR-409 expression

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Abstract

Background. Anesthetics, such as isoflurane, sevoflurane, ketamine, and desflurane, are commonly used in clinics. Specifically, isoflurane is one of the most commonly used inhalational anesthetics, which can be used in surgery patients of all ages, including children.

Objectives. The aim of the study was to investigate the mechanisms of vitexin against isoflurane-induced neurotoxicity.

Material and methods. Reference memory testing was performed for 5 days (4 trials, 2 per day) before anesthesia. Reversal testing was performed on the 3rd day after anesthesia. The cell viability and apoptosis of PC-12 cells were detected using MTT and TUNEL assays, respectively. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), glutathione (GSH), and superoxide dismutase (SOD) concentrations. The concentration of reactive oxygen species (ROS) was detected using ROS measurement. Expression of miR-409 was determined using quantitative reverse-transcription polymerase chain reaction (qPT-PCR). Protein expression levels were detected using western blotting.

Results. Rats treated with isoflurane showed significant increases in the escape latency periods (ELP) and the apoptosis of hippocampus neuron cells; this effect was reversed by 3 mg/kg or 10 mg/kg of vitexin ($p < 0.05$). Further testing showed that isoflurane could significantly decrease the cell viability and increase the apoptosis of PC-12, the expression of inflammatory cytokines (TNF-α and IL-6) and ROS ($p < 0.05$). However, these results were reversed by 10/100 μM of vitexin. In addition, vitexin could significantly increase the expression of miR-409 ($p < 0.05$). Further studies showed that overexpression of miR-409 could significantly promote the effect of vitexin on isoflurane-induced neurotoxicity ($p < 0.05$). Finally, overexpression miR-409 could significantly increase the expression of p-AMPK/t-AMPK and p-GSK3β/t-GSK3β.

Conclusions. Vitexin has protective effects against isoflurane-induced neurotoxicity by targeting miR-409 and the AMPK/GSK3β pathway.

Key words: neurotoxicity, isoflurane, vitexin, miR-409, AMPK/GSK3β signaling pathway

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Each year, more than 2 billion people around the world need surgical treatment. Anesthetics such as isoflurane, sevoflurane, ketamine, and desflurane are commonly used in clinics. Specifically, isoflurane is one of the most commonly used inhalational anesthetics, which can be used in surgery patients of all ages, including children. However, recent studies have shown that isoflurane has a correlation with postoperative cognitive dysfunction. Further experimental results demonstrate that isoflurane is neurotoxic, with effects like increasing apoptotic neurons, reducing neurogenesis and changing the ultrastructure of synapses in the central nervous system, including the hippocampus, thalamus, cortex, and spinal cord of rats in the developmental period.[1] Aged neurons are particularly vulnerable to isoflurane.[2,3] It has also been confirmed that isoflurane can cause cognitive dysfunction persisting for several weeks after treatment in adult and aged rats.[4,5] Researchers have demonstrated that general anesthesia might contribute to cognitive deficits after surgery, especially in elderly patients.[2,6] It has also been found that isoflurane anesthesia can cause long-term behavioral changes in the developmental period. These studies indicate that the neurotoxic effects of isoflurane anesthesia might be a risk factor increasing the likelihood that children will develop learning disabilities or deviant behavior in later life.[7] For all these reasons, it is particularly important to seek drugs that can effectively prevent or eliminate these neurotoxicity effects.

It has been reported that flavonoids extracted from hawthorn can lower blood pressure and lipid peroxidation, and increase coronary flow, resulting in protective effects on myocardial ischemic injury. In addition, it has also been revealed that flavonoids have beneficial effects on memory and learning.[8] In 2000, Commenges et al. showed that flavonoids play an important role in preventing human dementia.[9] It has also been reported that flavonoids inhibit acetylcholinesterase activity,[10,11] which can promote neuron development and nerve regeneration. It has been shown that flavonoids perform a regulative role through selective actions at different signaling cascades, such as PI3 kinase (PI3K)/Akt, protein kinase C (PKC), tyrosine kinase and mitogen-activated protein kinase (MAP kinase) signaling pathways.[12–15] There is also some evidence that flavonoids interact with the genes involved in mitogen-activated protein kinase (MAPK) signaling pathways,[14,16] which have also been confirmed to be involved in mediating neuronal survival, regeneration and apoptosis.[17]

Vitexin, a flavone glycoside isolated from hawthorn leaves, is attributed with various medicinal properties, such as lowering blood pressure, reducing inflammation and inhibiting tumors.[18] It has been reported that hawthorn leaves may possess cardiotonic, antiarrhythmic, antianginal, and antioxidative functions, and that it may reduce the symptoms of acute myocardial ischemia.[19] In the present study, the apoptosis of hippocampus neuron and memory deficits in rats were investigated. Meanwhile, in vitro explorations were also conducted to reveal the role of miR-409 in neuronal apoptosis. PC-12 cells are a clonal cell line derived from rat pheochromocytoma. They have been widely used as models of both adrenal chromaffin cells and sympathetic neurons.[20] These cells are also critical to in vitro explorations of neural cell behavior due to their reversible adoption of several neuronal characteristics upon exposure to nerve growth factor (NGF).[21] In addition, these cells are vulnerable to anesthetic-induced neurotoxicity.[22] Therefore, we chose PC-12 cells as the experimental model. Our study might provide new insights into therapeutic targets for the treatment of isoflurane-induced neurotoxicity.

Material and methods

Animals

A total of 50 male Sprague Dawley rats (200–250 g) were obtained from the Experimental Animal Center of the Central Hospital of Cangzhou, China, and were housed in groups of 4 per cage under standard laboratory conditions. They were maintained at constant room temperature (21 ±2°C) under a normal 12 h light/12 h dark (12L:12D) regime with free access to food and water. All the animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) to minimize the number of animals and their suffering.

Experimental procedure

The rats were randomly divided into 5 groups of 10 animals each: group 1 – the control group; group 2 – an isoflurane-treated group; group 3 – a 1 mg/kg vitexin-treated group; group 4 – a 3 mg/kg vitexin-treated group; and group 5 – a 100 mg/kg vitexin-treated group. The isoflurane- and vitexin-treated groups were exposed to 1.4% isoflurane (Sigma-Aldrich/Merck Millipore, Darmstadt, Germany) in a 100% oxygen environment for 2 h. Following isoflurane treatment, the vitexin-treated groups additionally received 1 mg/kg, 3 mg/kg and 10 mg/kg vitexin (Sigma-Aldrich/Merck Millipore) for 30 min.

Learning and memory testing

Before and after exposure to the anesthetics, learning and memory tests were performed on the rats in a Morris water maze (MWM). The MWM was 150 cm in diameter and was filled with opacified water (22 ±1°C) to the height of 1.5 cm above the top of a movable 15 cm diameter platform. The rats were tracked with a video camera mounted above the pool connected to a computer running IMAQ PCI-1407 software. The time between trials was at least 60 min. Reference memory tests were performed for 5 days (4 trials, 2 per day) before anesthesia. In accordance with an earlier study,[23] reversal tests were performed on the 3rd day after anesthesia.

Reference memory test

For all trials, the platform was placed in the target quadrant and the rats started in a random quadrant. The maximum swimming time was 120 s; if rats failed to find the hidden platform in 120 s, they were gently guided to the platform and remained on the platform for 10–15 s. The time to reach the platform (escape latency period (ELP)), and the swimming speed were recorded for each trial.

Reversal testing

The platform was moved to the opposite quadrant of the pool but all distal visual cues remained consistent. Preceding the start of the test, the animals were placed on the platform for 30 s, then removed from the pool. For the test they were placed in the pool and swam to locate the platform in the new target quadrant. The maximum swimming time was set at 120 s for each of 3 trials. Escape latency period and the swimming speed were recorded for each trial. Reversal learning measured how quickly an animal is able to erase their initial learning of the platform's position and acquire a direct path to the new location.[24]

Following these tests, the rats were euthanized by decapitation under anesthesia.

Cell culture

Human PC-12 pheochromocytoma neurosecretory cells were induced to differentiate by NGF and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone; GE Healthcare Life Sciences, Logan, USA) containing 9% heat-inactivated fetal calf serum (Invitrogen/Thermo Fisher Scientific Inc., Carlsbad, USA), 100 µg/mL streptomycin, 100 U/mL penicillin and 2 mM L-glutamine (Thermo Fisher Scientific Inc.), and were maintained at 37°C in 5% CO_2 with 95% humidity.

Cell treatment and viability analysis

Cells were seeded at a density of 1×10^4 cells per well in 96-well plates before being exposed to 2% isoflurane for 12 h and then treated with 1 µM, 10 µM and 100 µM vitexin for 24 h. In accordance with an earlier study,[25] MTT solution (Beyotime Institute of Biotechnology, Haimen, China) was added into each well at a final concentration of 0.5 mg/mL, and cells were subsequently incubated at 37°C for 4 h. Dimethyl sulfoxide solution (98%; 150 µL; Sangon Biotech Co., Ltd., Shanghai, China) was then added to each well. Optical density (OD) was read at 570 nm using a Universal Microplate Reader (Elx800; BioTek Instruments, Inc., Winooski, USA).

Transfection

For the miR-409 functional analysis, miR-409 mimic, negative control (NC) mimics, miR-409 inhibitor, or NC inhibitor (Genecopoeia, Rockville, USA) were transfected into PC-12 cells using Lipofectamine 2000 (Life Technologies Corp., Carlsbad, USA) according to the manufacturer's instructions.

Enzyme linked immunosorbent assay

PC-12 cells were seeded at a density of 1×10^4 cells per well in 96-well plates before being exposed to 2% isoflurane for 12 h, and then cultured with 1 µM, 10 µM and 100 µM vitexin for 24 h. The PC-12 cells were immediately collected and centrifuged at 4,000 g for 10 min. Enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to measure serum concentrations of tumor necrosis factor α (TNF-α; catalog No. R0 19), interleukin 6 (IL-6; catalog No. R0 16), glutathione (GSH; catalog No. A005), and superoxide dismutase (SOD; catalog No. A001-1).

Reactive oxygen species measurement

PC-12 cells were seeded at a density of 1×10^4 cells per well in 96-well plates before being exposed to 2% isoflurane for 12 h and then cultured with 1 µM, 10 µM and 100 µM vitexin for 24 h. The PC-12 cells were treated with 2',7'-dichlorofluorescein diacetate for 6 h, then incubated with cell lysis buffer (OxiSelect ROS assay kit; Cell Biolabs, Inc., San Diego, USA) for 5 min at 25°C. The OD was read at 480/530 nm using the aforementioned microplate reader.

Measurement of apoptotic cells with TUNEL assay

A TUNEL assay was performed with an in situ cell-death detection kit according to the manufacturer's instructions.[26] Briefly, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After being washed with phosphate-buffered saline (PBS), the cells were permeabilized with 0.2% Triton-X 100 in methanol for 2 min at 4°C. The cells were then incubated with TUNEL assay solution at 37°C for 60 min. Finally, the cells were washed with PBS and mounted with fluorescent mounting medium. The number of TUNEL-positive cells was obtained by counting cells in 6 randomly selected microscopic fields from each coverslip with a ×20 objective. The percentage of TUNEL-positive cells in the total number of cells was calculated and averaged.

Quantitative real-time PCR

The miRNA samples were extracted using a commercial miRNA isolation kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. The miRNA reverse transcription was performed using miRcute miRNA First-strand cDNA Synthesis kits (Tiangen, Beijing, China) according to the manufacturer's instructions. The quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of miR-409 was performed with a mirVana RT-qPCR miRNA Detection Kit (Ambion, Austin, USA), with each miRNA-specific primer. The miRNA level was presented as a level relative to U6 small RNA (taken as an internal control) using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein from the hippocampus was extracted with protein lysis buffer (20 mmol/L Tris-HCL, pH 7.6, 150 mmol/L NaCl, 1% NP-40) containing a protease inhibitor cocktail. Lysates were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, USA). The blots were incubated with rabbit anti-APP (Abcam Inc., Cambridge, UK) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4°C. Then the blots were incubated with secondary antibodies conjugated with horseradish peroxidase (1:3,500 dilution, Santa Cruz Biotechnology) for 2 h, shaking at room temperature. The signals were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

Statistical analysis

The statistical analysis was carried out with SPSS v. 13.0 software for Windows (SPSS Inc., Chicago, USA). Values are expressed as means ± standard deviation (SD). Normal distribution of data was tested with the one-sample Kolmogorov–Smirnov test. A one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) test, were used to compare the measurement data of the 5 groups. Statistical significance was set at $p < 0.05$.

Results

Effects of vitexin on learning and memory functions, cell apoptosis and expression of miR-409 in animal models

To investigate the neuroprotective effect of vitexin on the hippocampus of rats, isoflurane anesthesia was used to induce brain damage, and then the effects on neuron apoptosis and memory function were investigated. Before exposure to the anesthetic, reference memory testing showed no differences between the groups in ELP (Fig. 1A), but after exposure to isoflurane, the rats took significantly longer to reach the platform during the reversal test ($p < 0.05$). In addition, rats treated with 1 μM vitexin showed no decrease in the ELP. No differences were found between the isoflurane and 1 μM vitexin + isoflurane groups. However, the 10 μM and 100 μM vitexin groups showed significant decreases in the ELP ($p < 0.05$) and the effect was dose-dependent (Fig. 1B). Moreover, 3 μM and 10 μM vitexin could significantly decrease the apoptosis of hippocampal neurons and increase the expression of miR-409 ($p < 0.05$) (Fig. 1C,D). These results indicated that vitexin could protect hippocampal neurons from damage induced by isoflurane by regulating miR-409 expression.

The effects of vitexin on cell viability, cell apoptosis, inflammatory cytokine expression, oxidative stress, and miR-409 expression in PC-12 cells

To further study the protective mechanisms of vitexin on neurons, cell viability, the apoptosis and expression of inflammatory factors in PC-12 cells were investigated. As shown in Fig. 2A, isoflurane could significantly decrease the cell viability of PC-12 compared with the control group ($p < 0.05$), while this effect could be reversed by 10/100 μM vitexin. The apoptosis of PC-12 was significantly increased in the isoflurane group compared with the control group ($p < 0.05$), while this effect was also reversed by 10/100 μM vitexin. Expression levels of TNF-α, IL-6 and ROS showed the same trends with regard to the apoptosis of PC-12 cells (Fig. 2C,D,G). Levels of GSH, SOD (Fig. 2E,F) and miR-409 expression showed the same trends with regard to PC-12 cell viability (Fig. 2H).These results were consistent with those obtained in the animal experiments.

The effects of miR-409 inhibitor on cell viability, cell apoptosis, inflammatory cytokines expression, and oxidative stress in PC-12 cells

To evaluate the effect of miR-409 on isoflurane-induced neuron damage, cell viability, apoptosis, expression of inflammatory cytokines, and oxidative stress factors were assessed in PC-12 after transfection with miR-409 inhibitor. As shown in Fig. 3A, the expression level of miR-409 in the miR-409 inhibitor group was significantly decreased in comparison with the control group. As shown in Fig. 3B–H, knockdown miR-409 reversed the neuroprotective effects of vitexin. Cell viability and the levels of GSH and SOD in the vitexin + isoflurane + miR-409 inhibitor group were significantly decreased compared with the vitexin + isoflurane + NC group ($p < 0.05$). Meanwhile, cell apoptosis and the expression levels of TNF-α, IL-6 and ROS in the vitexin + isoflurane + miR-409 inhibitor group were significantly increased compared with the vitexin + isoflurane + NC group ($p < 0.05$).

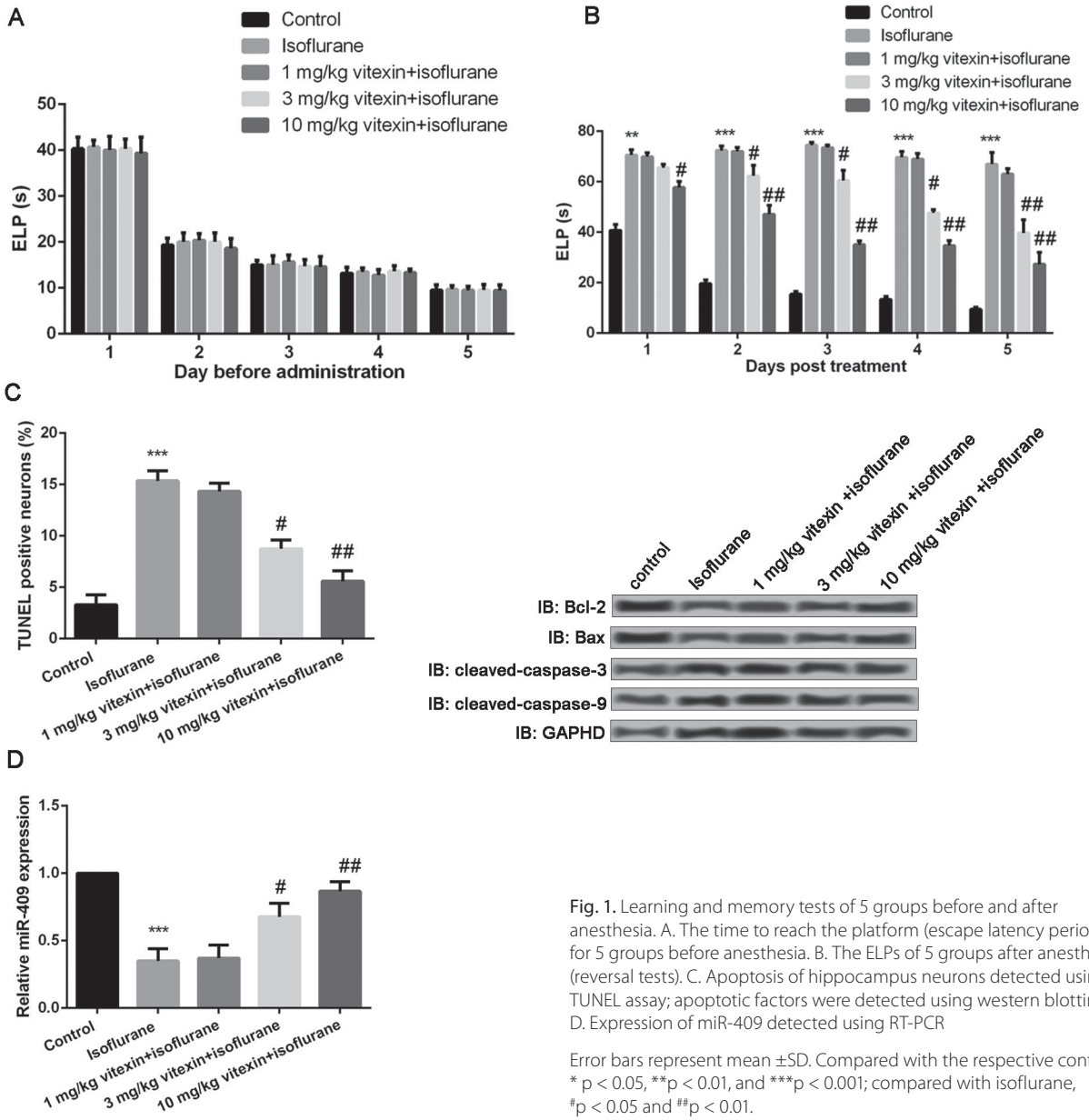

Fig. 1. Learning and memory tests of 5 groups before and after anesthesia. A. The time to reach the platform (escape latency period (ELP)) for 5 groups before anesthesia. B. The ELPs of 5 groups after anesthesia (reversal tests). C. Apoptosis of hippocampus neurons detected using TUNEL assay; apoptotic factors were detected using western blotting. D. Expression of miR-409 detected using RT-PCR

Error bars represent mean ±SD. Compared with the respective controls, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; compared with isoflurane, # $p < 0.05$ and ## $p < 0.01$.

The effects of miR-409 mimic on cell viability, cell apoptosis, inflammatory cytokines expression, and oxidative stress in PC-12 cells

Following the investigations described above, cell viability, apoptosis and the expression levels of inflammatory cytokines and oxidative stress factors in PC-12 cells were measured after transfection with miR-409 mimic. As shown in Fig. 4A, the expression level of miR-409 in the miR-409 mimic group was significantly increased compared with the control group. As shown in Fig. 4B–H, overexpression of miR-409 showed the opposite trend, with miR-409 knockdown in PC-12 cells, which enhanced the neuroprotective effect of vitexin. Cell viability and the levels of GSH and SOD in the vitexin + isoflurane + miR-409 mimic group were significantly increased compared with the vitexin + isoflurane + NC group ($p < 0.05$). Meanwhile, cell apoptosis and the expression levels of TNF-α, IL-6 and ROS in the vitexin + isoflurane + miR-409 mimic group were significantly decreased compared with the vitexin + isoflurane + NC group ($p < 0.05$).

The effects of miR-409 on the AMPK/GSK3β pathway in PC-12 cells

It has been reported that the AMPK signaling pathway plays an important role in nerve protection.[27] Therefore, this study assessed the expression levels of AMPK pathway-related proteins. The results showed that isoflurane could significantly decrease the expression levels of p-AMPK and p-GSK3β, while vitexin could significantly increase the expression levels of those proteins – an effect which was finally reversed by miR-409 inhibitor (Fig. 5A,B, $p < 0.05$).

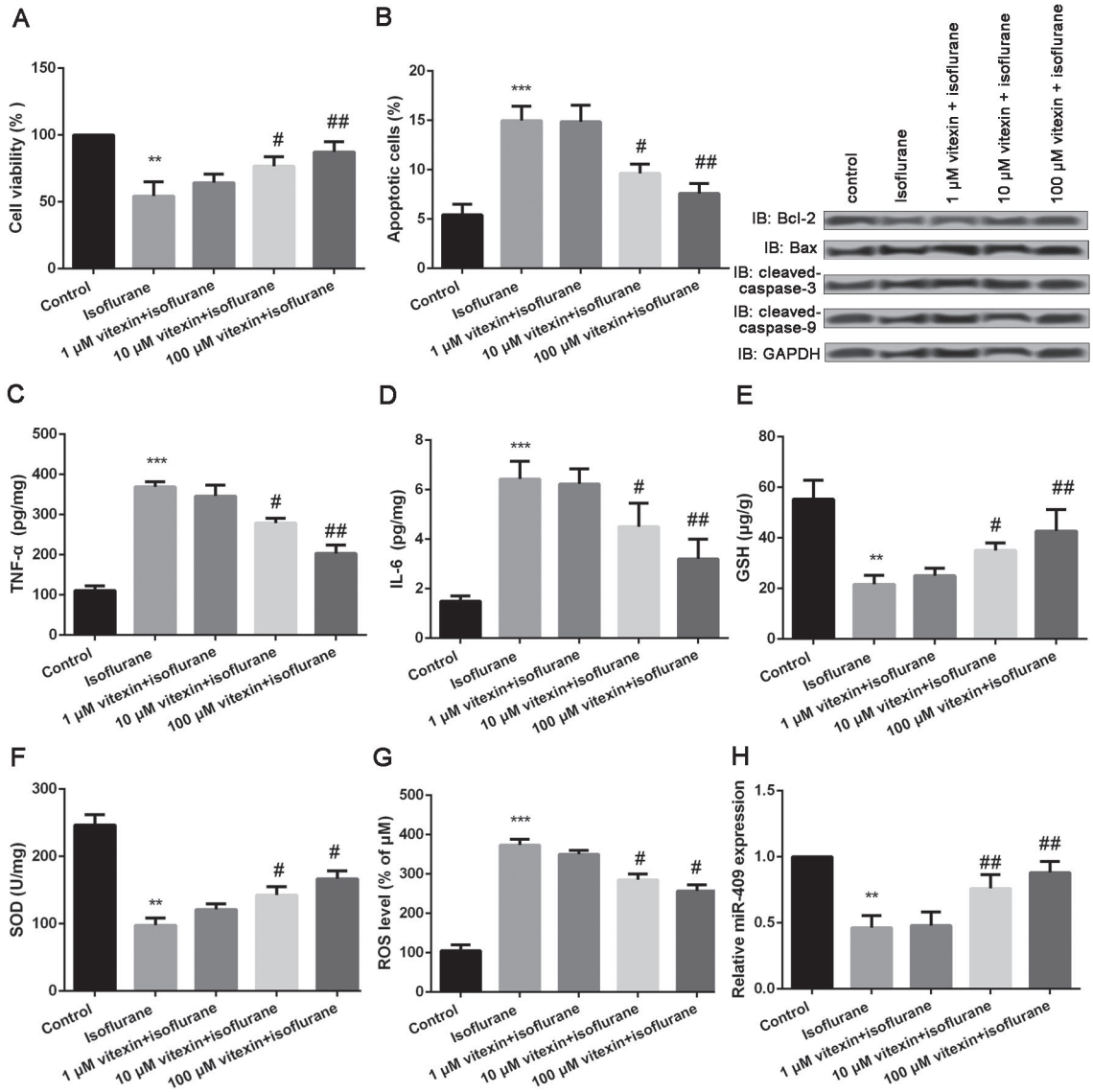

Fig. 2. The effects of vitexin on cell viability, apoptosis, inflammatory response and oxidative stress in PC-12 cells after anesthesia. A. Cell viability detected using MTT. B. Apoptosis of hippocampus neurons detected using TUNEL assay; the apoptotic factors were detected using western blotting. C–F. Concentrations of TNF-α, IL-6, GSH, and SOD detected using ELISA kits. G. The concentration of ROS detected using ROS measurement. H. The expression of miR-409 detected using RT-PCR

Error bars represent mean ±SD. Compared with the respective controls, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; compared with isoflurane, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

However, compared with miR-409 inhibitor, miR-409 mimic showed the opposite trend, enhancing the effect of vitexin on the AMPK pathway (Fig. 5A,B). These results indicated that vitexin protected neurons from isoflurane-induced cell damage by upregulating the expression of miR-409, thus activating the AMPK signaling pathway.

Discussion

Previous studies have shown that isoflurane is potentially neurotoxic, inducing dose- and time-dependent damage to the nervous system (i.e., hippocampal slices, primary cortical and striatal neurons and neurosecretory PC-12 cells).[1,28–30] Previous work in animal models had shown that isoflurane could induce neuronal apoptosis throughout brain, including the hippocampus and cerebral cortex.[31,32] The present study showed that isoflurane could significantly increase apoptosis in the hippocampus neuron. It has also been reported that isoflurane can regulate the central cholinergic system, such as cholinergic receptor insensitivity and affinity, especially in the hippocampus,[33] which is related to spatial learning and memory impairment.[33,34] Our results support other findings which demonstrated that isoflurane exposure could impair learning and memory abilities in rats.[35]

Vitexin has been shown to reverse scopolamine-induced memory impairment at 100 μM.[36] Our study, consistently

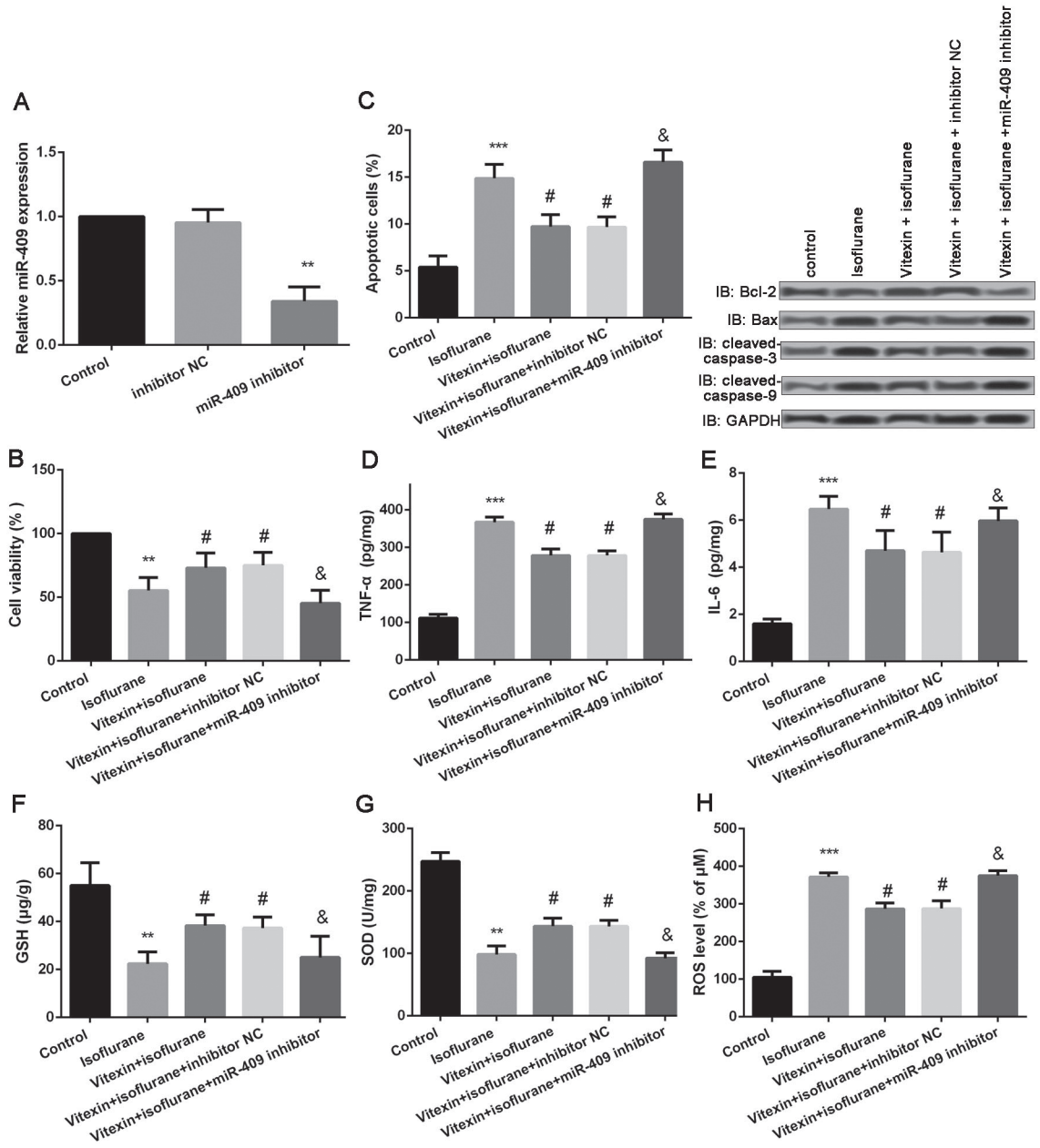

Fig. 3. The effects of miR-409 inhibitor on cell viability, apoptosis, inflammatory response, and oxidative stress in PC-12 cells after anesthesia. A. Expression of miR-409 detected using RT-PCR. B. Cell viability detected using MTT. C. Apoptosis of hippocampus neurons detected using TUNEL assay; apoptotic factors were detected using western blotting. D–G. The concentrations of TNF-α, IL-6, GSH, and SOD detected using ELISA kits. H. The concentration of ROS detected using ROS measurement

Error bars represent mean ±SD. Compared with the respective controls, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; compared with isoflurane, $^{\#}p < 0.05$; compared with the vitexin + isoflurane + inhibitor NC group, $^{\&}p < 0.05$.

with Chen et al.,[18] showed that 10/100 μM vitexin significantly increased the cell viability and decreased the apoptosis of PC-12, and reduced the expression levels of TNF-α, IL-6 and ROS in isoflurane-treated PC-12 cells. Oxidative stress increases pro-inflammatory gene expression, which triggers overproduction of ROS and results in a vicious cycle, provoking the occurrence and development of various diseases including nerve cell injury.[37,38] Yang et al. suggested that vitexin protects PC-12 cells against 20 h of reoxygenation-induced injury by reducing the expression of ROS.[36] In an animal model, Dong et al. found that vitexin protects against myocardial ischemia/reperfusion injury by inhibiting the inflammatory response.[37] Furthermore, Min et al. demonstrated that vitexin can reduce hypoxia-ischemia neuronal brain injury.[38] In addition, we found that vitexin could increase the expression of miR-409.

Increasing evidence indicates that the majority of miRNAs are expressed in the central nervous system[42] and play important roles in brain development and nervous system diseases.[43–45] Hence, miRNAs might participate in isoflurane-induced learning and memory impairment.

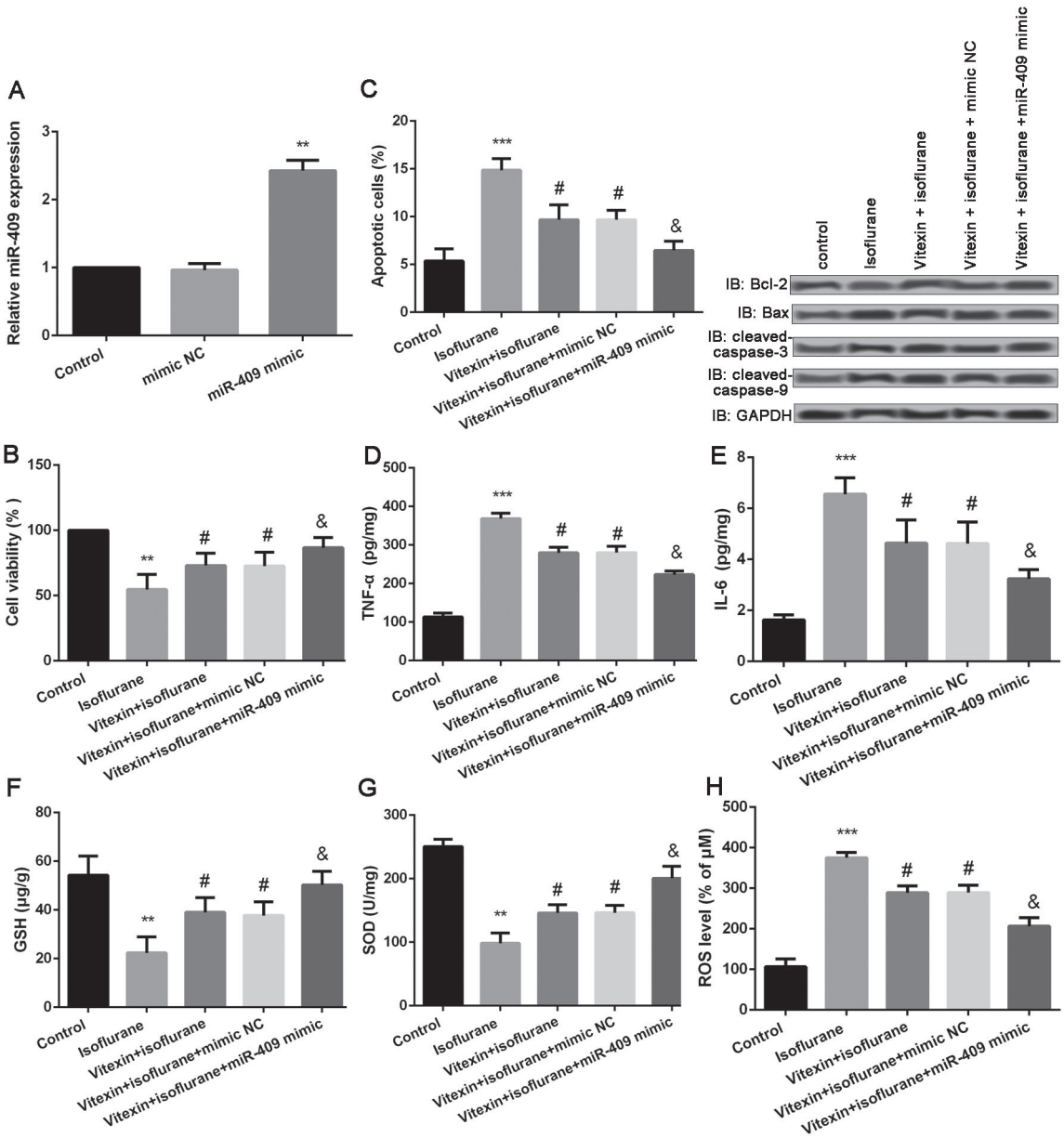

Fig. 4. The effects of miR-409 mimic on cell viability, apoptosis, inflammatory response, and oxidative stress in PC-12 cells after anesthesia. A. Expression of miR-409 detected using RT-PCR. B. Cell viability detected using MTT. C. Apoptosis of hippocampus neurons detected using TUNEL assay; apoptotic factors were detected using western blotting. D–G. The concentrations of TNF-α, IL-6, GSH, and SOD detected using ELISA kits. H. The concentration of ROS detected using ROS measurement

Error bars represent mean ±SD. Compared with the respective controls, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; compared with isoflurane, # $p < 0.05$; compared with the vitexin + isoflurane + inhibitor NC group, & $p < 0.05$.

Yan et al. observed that isoflurane induces cytotoxicity and neuronal cell death by downregulating miR-214.[29] Using a rat pup model, Luo et al. demonstrated that let-7d miRNA plays an important role in isoflurane-induced learning and memory impairment.[32] In the present study, overexpression of miR409 could decrease the apoptosis of PC-12 induced by isoflurane. Simultaneously, miR409 overexpression reduced the inflammatory response and oxidative stress. It has been reported that ROS is involved in isoflurane-induced neurotoxicity, and increasing ROS increases the neurotoxic effect of isoflurane. The present study showed that isoflurane increased the expression of ROS, and that this was reversed by overexpression of miR-409. These results indicated that miR-409 could reduce isoflurane-induced neurotoxicity.

There is increasing evidence suggesting that the MAPK family, such as ERK1/2 and JNK, may be involved in the signaling of neuronal survival, regeneration and death.[27] ERK1/2 is generally associated with pro-survival signaling that can activate cAMP-response-element-binding protein and upregulation of Bcl-2.[49] c-Jun N-terminal kinases, on the other hand, participates in the apoptotic signaling

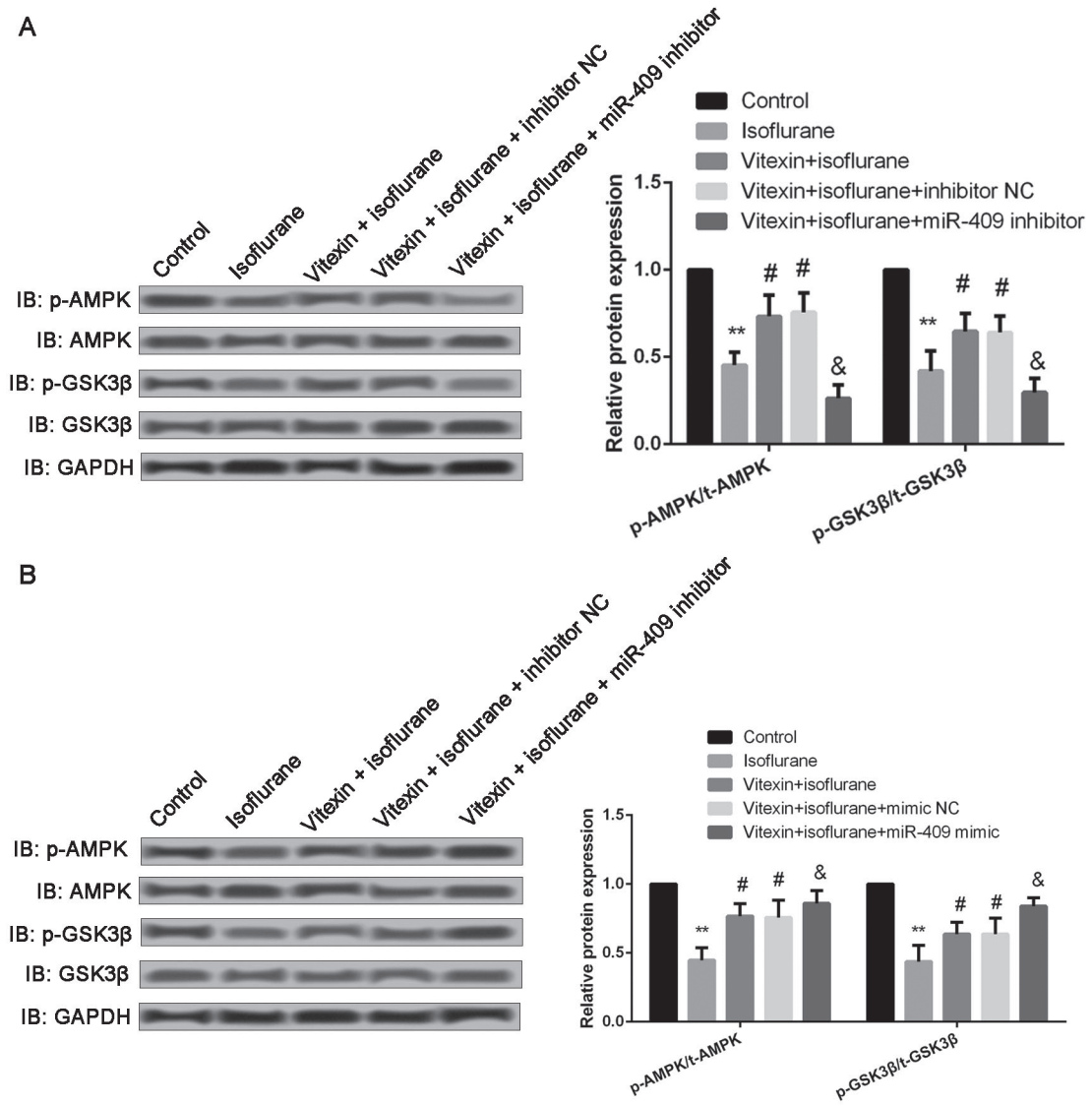

Fig. 5. The effect of miR-409 on the AMPK/GSK3β pathway. miR-409 knockdown (A) inhibits the AMPK pathway, while (B) over-expression of miR-409 activates the AMPK pathway; the expression levels of p-AMPK and p-GSK3β detected using western blotting

Error bars represent mean ±SD. Compared with the respective controls, *$p < 0.05$ and **$p < 0.01$; compared with isoflurane, $^{\#}p < 0.05$; compared with the vitexin + isoflurane + inhibitor NC group, $^{\&}p < 0.05$.

pathway.[17,50] In the present study, we found that vitexin could activate the AMPK/GSK3β pathway by upregulating miR-409, thus protecting neurons against isoflurane-induced injury. AMPK, as a key energy sensor of cellular metabolism, involves in metabolic stress, such as neurodegeneration, inflammation and oxidative stress.[51] It has been reported that *Akebiae caulis* extract can inhibit oxidative stress through the AMPK/GSK3β pathway.[47] Su reported that xanthohumol protects against LPS-induced acute lung injury, oxidative stress and inflammation damage by activating the AMPK/GSK3β signaling pathway.[48] Wang et al. demonstrated that esculentoside A protects the liver against acetaminophen toxicity through the AMPK/GSK3β pathway.[49] Furthermore, in the mature brain, post-mitotic neurons utilize MAP kinase and PI3K cascades in the regulation of key functions, such as synaptic plasticity and memory formation.[50] These findings suggested that vitexin protects hippocampus neurons against isoflurane-induced oxidative stress and inflammation damage by activating the AMPK/GSK3β pathway in rats.

Conclusions

In conclusion, isoflurane impaired hippocampus-dependent learning and memory in rats, and 10/100 μM vitexin could significantly reduce the isoflurane-induced injury by upregulating the expression of miR-409 to activate the AMPK/GSK3β pathway. These results suggest that vitexin might be a promising candidate in neurotoxicity drug treatment. However, further studies are required.

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Predictors of mortality in emergency department patients with chest pain without cardiovascular emergencies

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

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Abstract

Background. Chest pain is one of the most frequent symptoms in patients seeking treatment at emergency departments (ED). These patients differ according to the cause of their reported symptoms and resultant mortality.

Objectives. Evaluation of the influence of hospitalization and biochemical parameters on mortality rates in patients admitted to the ED with chest pain, in whom no cardiovascular emergencies were established.

Material and methods. The study group consisted of 243 patients with chest pain admitted to the ED in the Wroclaw Medical University Clinical Hospital, Poland, between January 1 and March 31, 2015, in whom no specific diagnosis was made at discharge. A retrospective analysis was carried out based on medical documentation, and 60-day and 1-year survival was assessed.

Results. In the study group, the 60-day mortality rate was 0.8% (2 persons) while the 1-year mortality rate was 6.6% (16 persons). The stepwise multivariable logistic regression analysis revealed that 1-year mortality was related to increased level of D-dimer (odds ratio (OR) = 8.5, 95% confidence interval (95% CI) = 21.9–37.5, $p < 0.005$), age (OR (per year) = 1.10, 95% CI = 1.03–1.18, $p < 0.03$) and lower than 12 g/dL hemoglobin concentration (OR = 18.5, 95% CI = 4.2–80.4, $p < 0.001$). Troponin I (TNI) levels and hospitalization were not related independently to mortality when other clinical factors were considered.

Conclusions. Hospitalization of patients with chest pain who were not diagnosed with cardiac emergencies is not related with better survival than of those discharged home from the ED. The 60-day mortality is very low and occurs in older patients with numerous comorbidities. In multivariate analysis, survival of the 1-year period depends on the patient's age, hemoglobin levels and D-dimer levels. Risk of death in patients admitted to the ED due to chest pain in whom the cause of the chest pain was not due to cardiovascular emergencies depends on the presence of old age and comorbidities.

Key words: hospitalization, emergency department, D-dimer, chest pain, troponin I

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Introduction

Chest pain is one of the most frequent symptoms in patients seeking treatment at the emergency department (ED). These patients form a heterogeneous group. They differ regarding the cause of reported symptoms and resultant mortality. Diagnosis in the ED should make it possible to quickly determine patients at highest risk, requiring a rapid introduction of treatment and longer hospitalization. Chest pain can result from coronary artery disease (CAD), pulmonary embolism (PE) and acute aortic syndrome (AAS) as well as non-cardiac causes. A key role in diagnosing acute coronary syndrome (ACS) is played by diagnostic algorithms based on electrocardiography (ECG) testing and troponin concentration as well as D-dimer and imaging studies in the cases of suspected PE or AAS.[1–5] Patients with the same pathomechanism of pain may vary greatly with regard to the risk of death.

Patients who present with ACS also form a heterogeneous group. The risk of death among patients with myocardial infarction with a persistent ST segment elevation (STEMI) is significantly higher than among patients with myocardial infarction without a persistent ST segment elevation (NSTEMI). These differences become less significant over longer observation. After 2 years, the mortality rate between these groups does not differ significantly, which most likely results from the differences in age and comorbidities in both groups. Patients with NSTEMI tend to be older and with more comorbidities than patients with STEMI.

Patients who present with unstable angina (UA) are characterized by the lowest mortality rate[6–8]; however, a lack of increased concentration of myocardial injury markers in this group may be a cause for underestimation of the risk of death, especially in patients with an atypical presentation of the disease. The differences in the risk of death lead to different diagnostic and therapeutic strategies. It is doubtless that patients with STEMI should undergo revascularization as quickly as possible. The guidelines for patients with NSTEMI and UA are likewise clear. Patients who cannot be qualified into any of these groups even after chest pain diagnostic algorithms have been followed, pose particular difficulties. This relates to patients with stable angina whose risk of death is significantly lower compared to patients with ACS, but significantly greater compared to patients with non-cardiac causes of chest pain such as intercostal neuralgia, degenerative spinal changes or dyspeptic symptoms. The introduction of highly sensitive methods of troponin level marking has significantly quickened the diagnosis of ACS but at the same time reduced its accuracy.

Difficulties also arise in patients whose troponin levels are only negligibly increased and remain stable in subsequent tests. The abovementioned biochemical picture in addition to an atypical angina history are not characteristic for ACS; however, they indicate a chronic heart injury and are associated with a worse long-term prognosis.[9–13] An atypical course of the disease as well as difficulties in identifying unequivocal symptoms reported by a patient pose additional diagnostic challenges, often leading to an underestimation of the risk of death among these patients. In the population of patients diagnosed in the ED, there is a large group of patients in whom ACS as well as other conditions posing an immediate threat to the patient's life have been excluded but no definitive cause of their symptoms has been established. In such cases, an outpatient follow-up is typically recommended. Taking into consideration the significant differences in these patients' characteristics, i.e., CAD diagnosed prior to admission or a lack thereof, or the presence of atherosclerosis risk factors or other comorbidities, the diagnostic strategy should be more personalized and clearly outlined.

Difficulties in accessing outpatient specialist care constitute an additional obstacle and may lead to delays in proper identification of patients with a greater risk of death, who would benefit from rapid diagnosis. Patients with chest pain, who despite an initial exclusion of cardiogenic causes of that chest pain in the ED are qualified for admission to different departments for further diagnosis and treatment, form a separate group. This usually concerns patients with a higher cardiovascular risk and more comorbidities. It is not clear, however, that patients benefit, especially when a longer hospitalization does not lead to verification of the initial diagnosis and qualification to invasive diagnostics of CAD and revascularization of the heart muscle.

Objectives

The objective of this study was to evaluate the influence of hospitalization on mortality rates in patients admitted to the ED with chest pain, in whom no serious cause of pain like ACS, PE, AAS, or decompensated congestive heart failure (CHF) was established either prior to discharge from the ED or in the department they were referred to from the ED.

The second objective of this study was to determine the significance of biochemical parameters tested in the ED as predictive factors in the total 60-day and 1-year mortality.

Material and methods

The parameters presented herein were assessed in 283 patients, in whom 2 measurements of troponin I (TNI) were taken during their hospitalization and in whom no myocardial infarction was diagnosed, and no non-cardiac causes of chest pain were definitively diagnosed. The group consisted of 123 male patients aged 61.2 ±16.0 years and 160 female patients aged 71.9 ±13.8 years. Data on the status of 272 of the patients (deceased or living) was obtained

from the Polish Ministry of Digital Affairs. Owing to the discrepancies in this data pertaining patient's name or surname, 11 patients were eventually disqualified from the study. Out of this group, 186 patients were discharged home and 86 patients were admitted to other departments of the hospital.

The patients admitted to other hospital departments were diagnosed with cardiovascular emergency (29 patients) or cardiovascular emegency was excluded (57 patients). However, they were admitted for further evaluation and treatment. Cardiovascular emergency was defined as ACS, decompensated CHF or PE.

Finally, the group studied consisted of 243 patients. The flow diagram of the study is presented in Fig. 1. A retrospective analysis was carried out based on an analysis of the following metrics from the medical documentation: age, gender, presence of comorbidities such as CAD, hypertension, diabetes, past stroke, TNI levels at admission (TNI1) and after 3 h (TNI2), sodium levels, potassium and glucose levels, as well as D-dimer levels if available. A 12-lead ECG was analyzed with a 7-step interpretation algorithm. Baseline rhythm, electric axis, conduction disorders, enlargement and hypertrophy of the heart cavities, ischemia, and arrhythmias were assessed. None of patients had a implanted pacemaker.[14] Ischemia was found if T wave inversion was present in leads other than aVR, V1 or III, or when ST segment depression was present. The final diagnosis of the hospitalized patients was assessed on the basis of discharge diagnoses. After establishing a database, the life status of the patients was determined based on data from the Polish Ministry of Digital Affairs.

Statistical analysis

Continuous variables were presented as means and standard deviations (SD) while discrete variables were presented as numbers and percentages. The continuous variables were analyzed as raw data as well as after dichotomization, taking into account the values of the reference range.

Age was analyzed as a continuous variable and the cut-off point resulted from receiver operating characteristic (ROC) curves. Troponin I values were analyzed as a continuous variable, as well as normal or elevated values throughout the duration of the study. The norm of TNI level established was 0–0.029 ng/mL. D-dimer levels were analyzed as a continuous variable as well as normal or elevated values, with the normal values accepted as 0.5 + age <50/100. Hemoglobin levels were analyzed as a continuous variable and after dichotomization, on the basis of ROC curve analysis results.

Comparisons of the variables were made using the Student's t-test, the Mann–Whitney U test and the χ^2 test depending on the distribution of the variables where $p < 0.05$ was considered statistically significant. The 60-day and 1-year survivals were assessed as numbers and percentages. The ROC curves were constructed and area under the curve (AUC) was constructed to find cut-off points for the age and biochemical parameters which differed between survivors and non-survivors. The AUC and p-values were evaluated. If AUC was 1, the cut-off point was perfect, if >0.8 it was good, if 0.6–0.8 it was moderate, and if <0.6 it was poor. The determined cut-off points were regarded as significant where AUC was at least 0.6 and the p-value was less than 0.05. A multivariable analysis was performed taking significant biochemical and clinical parameters as dependent variables which varied depending on the group, with $p < 0.15$.

To assess the presence of multicollinearity, the Spearman's rank coefficients were calculated. The multivariable analysis was carried out using the stepwise multivariate logistic regression analysis as well as the Classification and Regression Trees (CART) analysis. The results of stepwise multivariable logistic regression analysis were presented as odds ratios (OR) and 95% confidence intervals (95% CI). The CART analysis divides a large heterogeneous population into smaller, more homogeneous populations which

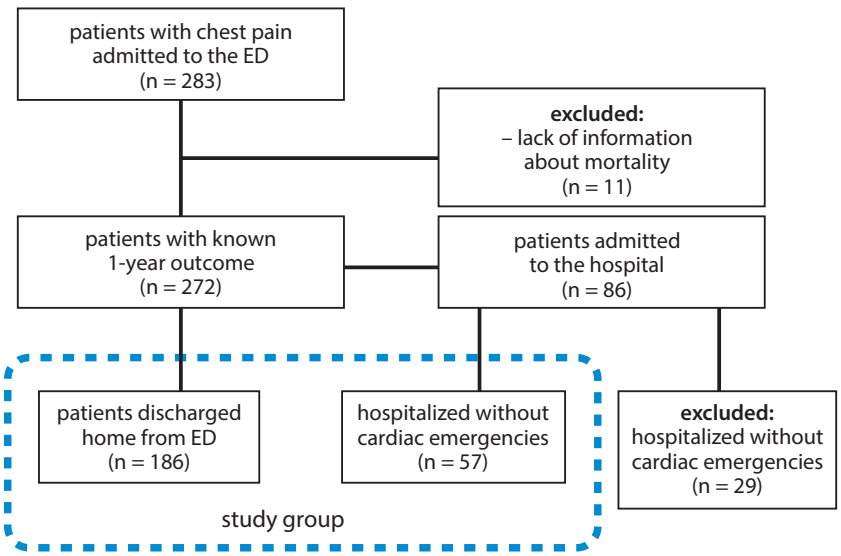

Fig. 1. Flow diagram of the study. The study group consists of 243 patients, 186 patients discharged home from the ED and 57 patients hospitalized without cardiovascular emergencies (ACS, PE, significant coronary stenosis, decompensated CHF, AAS). The boxes of patients qualified and included in the study group are circled with a dashed line

may differ in term of outcomes, e.g., mortality, as in our study. The number in the upper right corner depicts whether in the given subgroup the mortality rate is lower than in the total population (0) or is higher than in the total population (1).

The accuracy of a global 10-fold cost validation (CV) was presented as global cost and its SD. The age was correlated with other variables (Spearman's rank correlation coefficient up to 0.40) – therefore, 2 models were built, with and without age. The p-value less than 0.05 was considered statistically significant.

Results

The study group consisted of 243 patients aged 66.3 ±16.0 years: 139 women aged 71.2 ±14.1 years and 104 males aged 59.8 ±16.2 years. A total of 57 (23.5%) patients were hospitalized further and 186 (76.5%) patients were discharged home from ED.

In Table 1, demographic and clinical characteristics as well as biochemical test results are presented for both groups of patients: those who were hospitalized without cardiovascular emergencies and those discharged home.

Hospitalized patients were older, had higher rates of diabetes or past stroke, were more likely to be diagnosed with CAD, as well as presented with higher levels of TNI at admission and after 3 h, higher levels of D-dimers, lower levels of hemoglobin, and higher levels of C-reactive protein (CRP) and creatinine. In the study group, the 60-day mortality rate was 0.8% (2 persons) while the 1-year mortality rate was 6.6% (16 persons). Of the 2 persons who died within 60 days of admission to the ED, 1 patient was hospitalized in another department and 1 patient was discharged home. The mean age of the 2 patients who died was 86 years. Of the 16 persons who died within 1 year after admission to the ED, 8 patients (50%) were hospitalized without recognized cardiovascular emergencies and 8 patients (50%) were discharged home from the ED (Table 2).

Because the 60-day mortality was very low, no further statistical analysis was performed. The 1-year survivors were younger, had lower TNI levels at admission and after 3 h, lower D-dimer and potassium levels, and higher sodium and hemoglobin concentrations (Table 3).

Table 1. Demographic and clinical characteristics and biochemical test results of patients hospitalized without serious diagnosis and patients discharged home

Characteristics*	Hospitalized without cardiovascular emergencies diagnosis (n = 57)	Discharged home from ED (n = 186)	p-value
Male gender, n (%)	19 (33.3)	85 (45.7)	0.10
Age [years]	73.2 ±13.1	64.2 ±16.3	0.002
Hypertension, n (%)	42 (73.7)	107 (57.5)	0.07
Diabetes, n (%)	17 (29.8)	24 (12.9)	0.003
Coronary artery disease, n (%) (n = 240)	29 (53.7)	59 (31.7)	0.003
Past stroke, n (%)	8 (14.4)	12 (6.5)	0.09
Systolic blood pressure [mm Hg]	139.2 ±21.2	136.4 ±20.5	0.36
HR [bpm]	79.1 ±15.8	77.9 ±14.3	0.58
TNI at admission [ng/mL]	0.30 ±0.36	0.01 ±0.03	<0.001
TNI at admission above normal levels, n (%)	17 (29.8)	12 (6.5)	<0.001
TNI after 3 h [ng/mL]	0.49 ±1.6	0.02 ±0.08	<0.001
TNI after 3 h above normal levels, n (%)	31 (54.4)	21 (11.3)	<0.001
TNI after 3 h higher than at admission, n (%)	28 (49.1)	26 (14.0)	<0.001
Hemoglobin [g/dL]	13.1 ±2.4	13.8 ±1.7	0.006
Sodium [mEq/L]	138.5 ±4.4	138.7 ±3.9	0.85
Potassium [mEq/L]	3.8 ±0.5	4.0 ±0.6	0.16
Glucose [mg%] (n = 229)	152.1 ±52.6	123.5 ±36.3	<0.001
Creatinine [mg%]	1.03 ±0.28	1.00 ±0.62	0.68
D-dimer [µg/mL] (n = 215)	1.35 ±1.58	0.58 ±0.61	<0.001
D-dimer above normal levels, n (%) (n = 215)	24 (48.0)	40 (24.2)	<0.001
CRP [mg%] (n = 226)	9.6 ±12.9	7.2 ±17.7	0.33
Magnesium [mg%] (n = 188)	1.91 ±0.36	1.98 ±0.23	0.12
ECG results normal, n (%)	26 (45.6)	38 (20.4)	<0.001

* In cases where no results for a specific patient were available, the n value for persons whose results were available was used. ED – emergency department; HR – heart rate; TNI – troponin I; CRP – C-reactive protein; ECG – electrocardiography.

Table 2. Comparison of clinical and biochemical parameters of patients who survived 60 days as well as patients who died within this timeframe

Characteristics*	Patients who died within 60 days (n = 2)	Patients who survived 60+ days (n = 241)	p-value
Male gender, n (%)	0 (0)	104 (43)	0.22
Age [years]	86 (14)	66 (16)	0.08
Hypertension, n (%)	2 (100)	147 (61)	0.26
Diabetes, n (%)	2 (100)	39 (16)	0.001
Coronary artery disease, n (%) (n = 240)	1 (50)	87 (37)	0.69
Past stroke, n (%)	0 (0)	20 (8.3)	0.67
Systolic blood pressure [mm Hg]	156 ±27	137 ±21	0.19
HR [bpm]	87 ±5	78 ±15	0.42
TNI at admission [ng/mL]	0.02 ±0.03	0.06 ±0.55	0.90
TNI at admission above normal levels, n (%)	1 (50)	28 (11.6)	0.10
TNI after 3 h [ng/mL]	0.06 ±0.004	0.13 ±0.78	0.91
TNI after 3 h above normal levels, n (%)	2 (100)	50 (21)	0.007
TNI after 3 h higher than at admission, n (%)	2 (100)	52 (22)	0.008
Hemoglobin [g/dL] (n = 243)	10.8 ±1.2	13.7 ±1.9	0.02
Sodium [mEq/L]	128.5 ±17.6	138.7 ±3.7	0.001
Potassium [mEq/L]	4.6 ±0.2	3.9 ±0.6	0.13
Glucose [mg%] (n = 229)	222 ±128	123 ±42	0.002
Creatinine [mg%]	1.12 ±0.28	1.00 ±0.56	0.48
D-dimer [µg/mL] (n = 215)	1.25 ±1.01	0.75 ±0.98	0.48
D-dimer above normal levels, n (%) (n = 215)	1 (50)	63 (30)	0.53
CRP [mg%] (n = 226)	2.7 ±2.0	7.8 ±16.2	0.65
Magnesium [mg%] (n = 188)	1.5 ±0.1	2.0 ±0.3	0.02
ECG results normal, n (%)	1 (50)	63 (30)	0.53
Hospitalization, n (%)	1 (50)	56 (23)	0.37

* In cases where no results for a specific patient were available, the n value for persons whose results were available was used. HR – heart rate; TNI – troponin I; CRP – C-reactive protein; ECG – electrocardiography.

The ROC curve analysis revealed that among the biochemical parameters, only D-dimer level, and potassium and hemoglobin concentrations had significant cut-off points which could differentiate survivors and non-survivors (Fig. 2–4). The cut-off point for age was 75 years ($p < 0.001$, AUC = 0.77). There was no significant cut-off point for TNI either at admission (Fig. 5) or after 3 h.

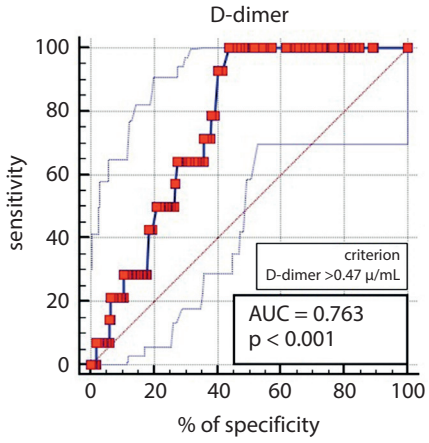

Fig. 2. Receiver operating characteristic (ROC) curve. D-dimer level for prediction of 1-year survival

AUC – area under curve.

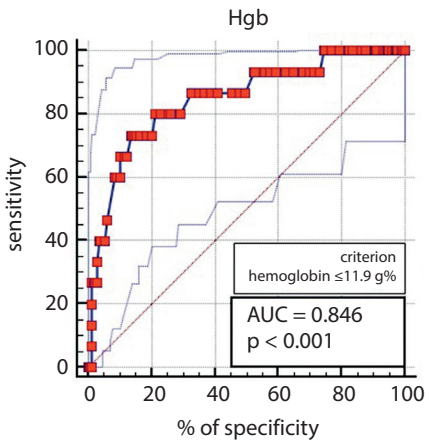

Fig. 3. Receiver operating characteristic (ROC) curve. Hemoglobin concentration for prediction of 1-year survival

AUC – area under curve.

Table 3. Comparison of clinical and biochemical parameters of patients who survived 365 days as well as patients who died during this timeframe

Characteristics*	Patients who died within 365 days (n = 16)	Patients who survived 365+ days (n = 227)	p-value
Male gender, n (%)	5 (31)	9 (44)	0.34
Age [years]	79.6 ±11.2	65.4 ±15.9	<0.001
Hypertension, n (%)	9 (56.3)	140 (61.7)	0.67
Diabetes, n (%)	8 (50)	33 (15)	<0.001
Coronary artery disease, n (%) (n = 240)	7 (47)	81 (36)	0.41
Past stroke, n (%)	2 (12.5)	18 (7.9)	0.52
Systolic blood pressure [mm Hg]	140.1 ±18.4	136.8 ±20.8	0.54
HR [bpm]	81.4 ±14.3	77.9 ±14.7	0.35
TNI at admission [ng/mL]	0.41 ±1.6	0.04 ±0.34	0.006
TNI at admission above normal levels, n (%)	15 (51.7)	26 (12.2)	<0.001
TNI after 3 h [ng/mL]	0.48 ±1.8	0.10 ±0.7	0.06
TNI after 3 h above normal levels, n (%)	7 (43.8)	45 (19.8)	0.024
TNI after 3 h higher than at admission, n (%)	8 (50)	46 (20)	0.006
Hemoglobin [g/dL]	11.3 ±1.9	13.8 ±1.8	<0.001
Sodium [mEq/L]	135.7 ±7.1	138.8 ±3.6	0.003
Potassium [mEq/L]	4.4 ±0.5	3.9 ±0.6	0.003
Glucose [mg%] (n = 229)	132.7 ±70.9	129.9 ±40.8	0.81
Creatinine [mg%]	1.16 ±0.26	0.99 ±0.58	0.25
D-dimer [µg/mL] (n = 215)	1.2 ±1.0	0.7 ±1.0	0.07
D-dimer above normal levels, n (%) (n = 215)	9 (64)	55 (27)	0.003
CRP [mg%] (n = 226)	12.2 ±14.2	7.5 ±16.3	0.27
Magnesium [mg%] (n = 188)	2.0 ±0.5	2.0 ±0.3	0.63
ECG results normal, n (%)	5 (31.3)	59 (26)	0.64
Hospitalization, n (%)	8 (50.0)	59 (21.6)	0.009

* In cases where no results for a specific patient were available, the n value for persons whose results were available was used. HR – heart rate; TNI – troponin I; CRP – C-reactive protein; ECG – electrocardiography.

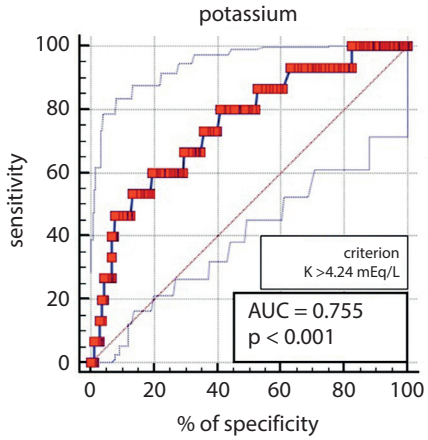

Fig. 4. Receiver operating characteristic (ROC) curve. Potassium level for prediction of 1-year survival

AUC – area under curve.

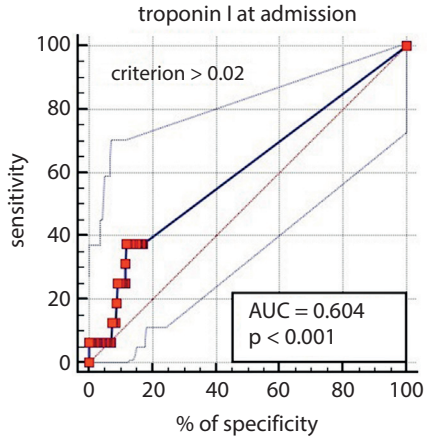

Fig. 5. Receiver operating characteristic (ROC) curve. Troponin I level for prediction of 1-year survival

AUC – area under curve.

Multivariable analysis

The stepwise multivariable logistic regression analysis revealed that 1-year mortality was related to an increased level of D-dimer (OR = 8.5, 95% CI = 21.9–37.5, p < 0.005), age (OR (per year) = 1.10, 95% CI = 1.03–1.18, p < 0.03) and lower than 12 g% hemoglobin concentration (OR = 18.5, 95% CI = 4.2–80.4, p < 0.001).

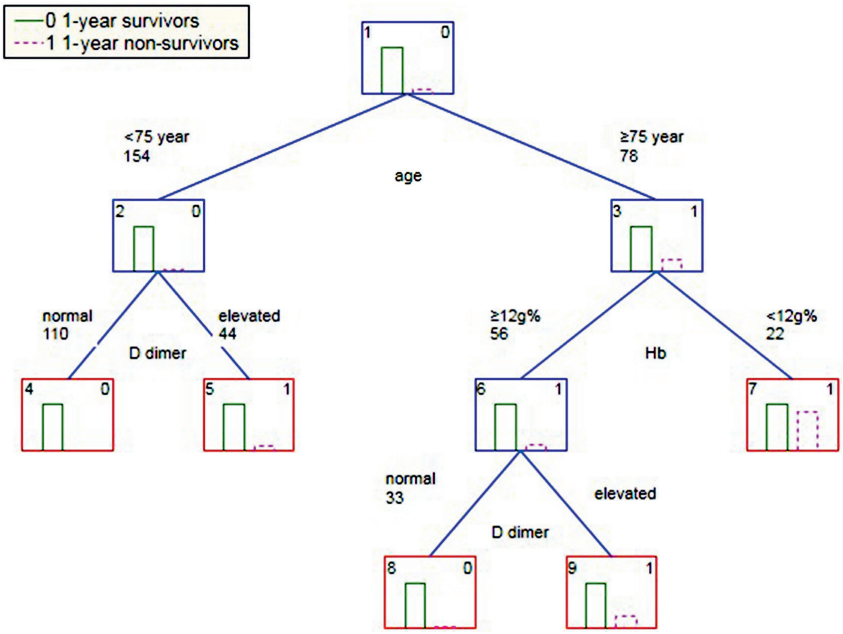

Fig. 6. Factors related to 1-year survival. Global cost = 0.23; Standard deviation (SD) = 0.06. Model with demographic and clinical data

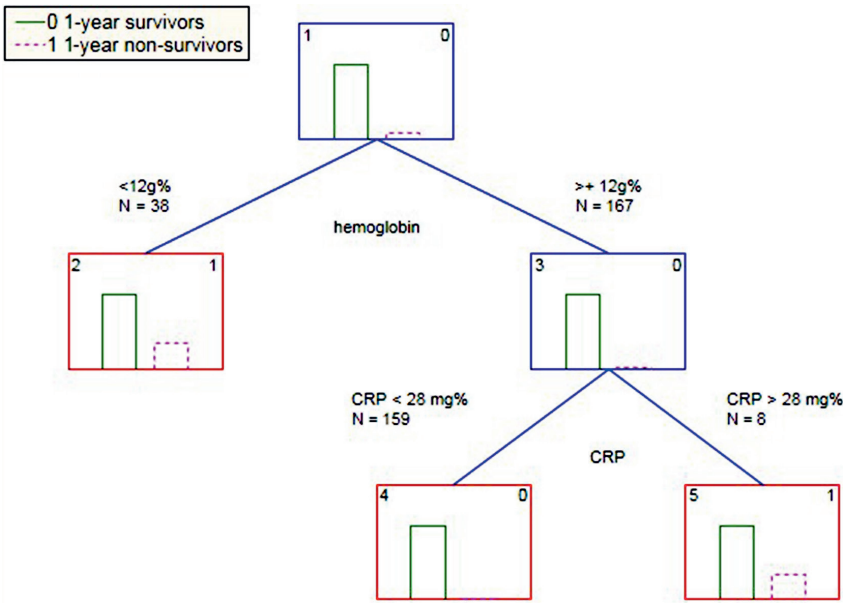

Fig. 7. Factors related to 1-year survival. Global cost = 0.20; Standard deviation (SD) = 0.05. Model with clinical data, age not included in the analysis

In the model without age, the stepwise logistic regression analysis revealed that 1-year mortality was related to an increased level of D-dimer, lower than 12 g/dL hemoglobin concentration and the presence of diabetes. Hospitalization, which was correlated to age (R-Spearman's coefficient = 0.25 $p < 0.01$), was not a predictor of survival in either model.

The result of the nonlinear analysis carried out with the use of the CART method confirmed this result and revealed that a patient's death within 1 year was related with lower hemoglobin level, older age and increased D-dimer level (Fig. 6). After excluding age, the factors related to death were anemia and higher CRP level (Fig. 7). Troponin I levels and hospitalization were not related independently to mortality when other clinical factors were considered.

Discussion

The study group included patients reporting to the ED due to chest pain in whom no cardiovascular emergencies were found after initial diagnostic testing or during hospitalization in other departments. The studied patients formed a heterogeneous group regarding TNI concentration and D-dimer levels as well as age and present comorbidities.

The main finding of the study was that the survival of patients in the study group did not depend on hospitalization and in multivariate analysis it was found that factors other than TNI are related to poor prognosis. These factors were age, low hemoglobin level, increased D-dimer level, and increased CRP level. Increased troponin level

is not itself a poor prognostic factor – it becomes one when there is a serious cardiovascular disease which presents with increased troponin level, like ACS, decompensated CHF or PE. The introduction of high-sensitivity cardiac troponin assays is associated with increased sensitivity and lower specificity. It has been demonstrated that in the population of patients with chest pain in whom no cardiovascular emergencies were found, higher troponin levels depended on the male sex, older age and impaired renal function.[15]

Other factors which may cause troponin increase are cardiac arrhythmias, CHF, hyperthyroidism, and chronic renal failure. Hospitalization may have little influence on the course of these diseases. Moreover, their impact on 1-year survival may be too low to be related with worse 1-year prognosis. Finally, other comorbidities, not defined at discharge, presenting with anemia or elevated CRP and D-dimer, may lead to increased 1-year mortality.

We presume that hospitalization had no influence on survival because these patients had no interventions which could have impact on long-term survival like coronary revascularization, cardioverter-defibrillator implantation, or treatment of acute heart failure or PE.

In published studies, a correlation between stable increased levels of troponin and mortality rate were proven even when ACS was not diagnosed. However, the groups assessed in those studies were much more homogeneous and comprised of a greater number of patients. Most often they were patients without a significant prior medical history.[9–13] The authors know that chronic stable increased troponin concentration may result from a chronic heart injury and be associated with a worse prognosis as well as, for instance, with decreased renal function which constitutes a factor for atherosclerosis and death per se. One publication even showed a correlation between observable troponin concentration in a highly sensitive test of 5–9 ng/L, remaining below the 99th percentile, and a greater 3–5-year risk of death due to cardiovascular as well as non-cardiovascular causes.[13] In that study, similarly to our study, the studied group was not so homogeneous in relation to comorbidities; however, it included a considerably greater number of patients. In our study, a higher troponin concentration was correlated with a higher mortality rate. This indicates a significantly worse prognosis in patients with chronic heart injury, e.g., in the course of CHF.

The 60-day mortality in our group was very low and only 2 patients, with a mean age of 86 years, died during that period. One of the patients was hospitalized and the other one was discharged home from the ED.

The duration of the observation of patients after hospital discharge varied from 30 days to 1 year. The longer the observational period, the higher probability of death due to causes other than the factor which was responsible for chest pain.

A significantly greater mortality rate in both 60-day and 1-year observation groups, despite the lack of cardiac emergencies subjecting a patient to immediate hospitalization, indicates the significance of clinical parameters not included in the strict diagnostic algorithms of the above diseases and highlights the importance of an individual diagnostic approach to every patient.

Limitations

The main limitation of the study is that we do not know whether the modification of the treatment during hospitalization had an impact on the prognosis. However, such modifications may also be performed in the patients discharged home from the ED.

Conclusions

Hospitalization of patients with chest pain who were not diagnosed with ACS, PE, significant coronary stenosis, or decompensated CHF is not related with better survival and they can be safely discharged home from ED.

Sixty-day mortality is very low and occurs in older patients with numerous comorbidities.

Patients who died within 1 year had significantly higher rates of increased TNI concentration at admission and after 3 h than patients who survived this period; however, there were no cut-off points to distinguish these 2 populations.

In multivariate analysis, survival of the 1-year period depends on the patient's age, hemoglobin levels and D-dimer levels.

Our results indicate that the risk of death in patients admitted to the ED due to chest pain in whom the cause of the chest pain was not due to cardiovascular emergencies depends on the presence of old age and comorbidities.

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Palmoplantar pustulosis: Factors causing and influencing the course of the disease

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Abstract

Palmoplantar pustulosis (PPP) is a chronic inflammatory disease, most often occurring in middle-aged women. In the course of the condition, painful skin lesions appear on the hands and feet, i.e., areas that are extremely important in everyday life. Therefore, the disease significantly reduces quality of life. The pathogenesis of this disease is poorly understood, although it is known that genetic, immunological and environmental factors play a role in its development. Clinical observations confirm the role of nicotine and contact allergens in the development of the lesions. The skin lesions can also occur as a side effect of certain medications. In some cases, PPP coexists with other diseases, i.e., seronegative arthropathies, as well as celiac and thyroid diseases. There is also a connection between the disease and infectious bacterial foci. Exacerbation of the skin lesions is triggered by stress. Therefore, patients require multidirectional tests, since finding the cause of the disease is essential to administering effective treatment.

Key words: etiopathogenesis, palmoplantar pustulosis, exacerbating factors

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Palmoplantar pustulosis (PPP) is a chronic inflammatory disease, the pathogenesis of which is poorly understood. It is classified either as a variant of psoriasis or as a separate entity.[1] The distinct nature of PPP as opposed to psoriasis is indicated by an absence of psoriasis in the family, a late onset of the disease, a lack of typical psoriatic lesions, and increased incidence of contact allergy to metals.[2,3] Palmoplantar pustulosis is more common in women than men, and most often occurs in middle age; it results in impairment of daily functioning and fulfilling social roles. Lesions are found on the palms and soles, usually symmetrically.[1] Sterile pustules are formed on an erythematous base and subsequently dry within a few days. Desquamation and linear fissures can be observed. The disease becomes periodically exacerbated with eruptions of new pustules. Hyperkeratosis of the nails is frequently observed, and pustules may form beneath the nail plates (Fig. 1).[1] Patients experience pain, itching or burning.

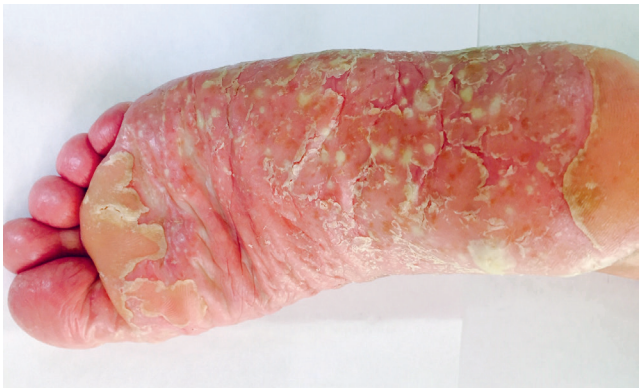

Fig. 1. Pustulosis localized on the sole

Lesions in the course of PPP develop in areas that are rich in eccrine sweat glands, which probably play a role in the pathogenesis of the disease. The results of recent research indicate that acrosyringium, the intraepidermal part of the excretory sweat gland ducts, is the main location where pustules form in the course of PPP.[4]

Biopsies of the skin lesions reveal changes similar to those seen in psoriasis: parakeratosis, a loss of the granular layer and spongiosis. Sterile pustules found in the upper layers of the epidermis are filled with neutrophils and eosinophils. Scattered, mixed infiltrations consisting of lymphocytes, neutrophils, eosinophils, and mast cells are found in the upper dermis and perivascularly. T lymphocytes predominate ($CD3^+$ expression).[5,6]

Contact dermatitis, pityriasis rubra pilaris, dyshidrotic eczema, and tinea should be taken into consideration in the differential diagnosis of PPP.

Etiopathogenesis of palmoplantar pustulosis

Genetic, immunological and environmental factors play a role in the development of PPP (Table 1).[1]

Table 1. Postulated pathogenic mechanisms in PPP development

Postulated pathogenic mechanisms in PPP development	References
Genetic background	
HLA-B27 antigen in 32% of patients with PPP associated by joints pain in the anterior thorax, hip or peripheral joints	Szanto and Linse (1991)[9]
Gene *ATG16L1* SNPs: rs2241880G and rs2241879A	Douroudis et al. (2011)[10]
Mutation of *IL-36RN* gene antagonist	Wang et al. (2016)[11]
Immunological background	
Increased blood levels: TNF-α, IL-17, IL-22, and IFN-γ	Murakami et al. (2011)[14]
Increased blood level and expression in skin lesions of IL-6	Croxford et al. (2014)[16]
Increased expression of IL-17 in the PPP lesions, without concomitant IL-12 and IL-23 elevation	Bissonnette et al. (2014)[17]
Dysregulation of IL-36 function	Marrakchi et al. (2011)[30] Carrier et al. (2011)[32]
Environmental factors	
Cigarette smoking – nicotine activates macrophages, keratinocytes and lymphocytes T as well as increases keratosis of the excretory ducts – PPP lesion regression after cessation of smoking	Hagforsen et al. (2010)[5] Elahmed (2013)[34]
Contact allergy – 25.2% of patients with PPP positive in patch test	Caca-Biljanovska et al. (2005)[35]
Drug side effect – PPP observed as side effect of TNF inhibitors	Shmidt et al. (2012)[39] Collamer et al. (2008)[40]
Focal bacterial infections – tonsillectomy or effective treatment of focal bacterial infections led to significant improvement or resolution of PPP	Takahara (2011)[44]
Chlamydia – chlamydial antibody titer ≥1/16 in 53% of patients with PPP	Jansen et al. (1980)[46]

PPP – palmoplantar pustulosis; SNPs – single nucleotide polymorphisms; IL – interleukin; IFN-γ – interferon gamma; TNF – tumor necrosis factor.

Genetic background

The genetic background of PPP is not fully understood. Some authors consider PPP a variant of psoriasis, and studies comparing the prevalence of certain genes in these 2 diseases have been published. Genes coding for HLA-Cw6, WWCC HCR and CDSN5, located in the locus PSORS1 (6p21) on chromosome 6, are considered the main genetic basis of psoriasis.[7] On the other hand, studies conducted on Swedish and British populations have shown that none of the abovementioned alleles are related to the occurrence of PPP. The frequency of occurrence of HLA-Cw6 is similar among patients with PPP (19–20%) and healthy subjects

(15%). Similarly, WWCC HCR occurs in 50% of patients with PPP and 40% of healthy individuals.[8,9]

Another important genetic factor playing a role in the pathogenesis of psoriasis is the mutation of the *ATG16L1* gene located on chromosome 2. *ATG16L1* participates in the immunological response, and mutation of this gene leads to decreased production of antimicrobial peptides and enhanced production of pro-inflammatory cytokines interleukin 1 (IL-1) and IL-18, propagating systemic inflammation. More frequent occurrence of this mutation has been identified in inflammatory diseases such as psoriasis and Crohn's disease. More frequent occurrence of rs2241880 G and rs2241879A single nucleotide polymorphisms (SNPs) of the *ATG16L1* gene has been observed among patients with PPP compared to healthy individuals.[10]

Due to the possible coexistence of PPP with arthritis and arthralgia in some patients, the genetic base of PPP has been compared with that of psoriatic arthritis. A correlation was found between the HLA-B27 antigen, characteristic of psoriatic arthritis, and PPP. The HLA-B27 antigen was identified in 32% of patients with PPP associated with arthralgia in the area of the anterior thorax, hip or peripheral joints.[9]

Recent studies also indicate the role of antagonist receptor IL-36 (IL-36RN) gene mutation in the development of PPP, as well as in various forms of psoriasis: the plaque type, the generalized pustular type and the pustular palmoplantar type.[11] This mutation is known to be the genetic basis of deficiency of IL-36 receptor antagonist (DITRA), a rare inherited autosomal recessive disorder characterized by disseminated pustular lesions resembling pustular psoriasis or acute generalized exanthematous pustulosis (AGEP).[12]

Immunological background

Immunological processes in the course of PPP lead to accumulation of large numbers of granulocytes in the area of the sweat gland excretion tract, the acrosyringium.[5,13] Cytokines regulating immunological processes play a key role in the pathogenesis of the disorder. As in psoriasis, in PPP, increases in blood levels of pro-inflammatory interleukins such as tumor necrosis factor alpha (TNF-α), IL-17, IL-22, and interferon gamma (IFN-γ) are observed.[14] However, psoriatic and PPP lesions have been found to be different. In psoriatic lesions, there are large amounts of cytokines produced by the Th17 lymphocytes (IL-12, IL-17 and IL-23), while a significant increase in the expression of IL-17 alone has been noted in PPP lesions, without concomitant IL-12 and IL-23 elevation. Therefore, isolated increases in IL-17 levels in PPP indicate that neutrophils, not Th-17 lymphocytes, are the main source of this response.[15–17] A high level of IL-17 found locally in the epidermis stimulates keratinocytes to produce IL-6, which activates neutrophils and monocytes and exerts a chemotactic effect, attracting granulocytes to the epidermis, which leads to the formation of pustules. Interleukin 6 probably plays a crucial role in the formation of lesions in PPP, and increased levels of this cytokine have been found within the skin lesions as well as in the blood of patients with this disease.[16] Interleukin 6 is a pro-inflammatory cytokine and belongs to the gp130 cytokine family.[18,19] It activates receptor kinase JAK1/JAK2 and Tyk2, and regulates the STAT1/STAT3 SHP2-MAPK signaling duct.[19–21] Interleukin 6 stimulates synthesis of acute phase proteins, differentiation of B cells into mature plasma cells, differentiation and activation of T cells, and activation of Th17 lymphocytes and other non-immunological cells such as keratinocytes and fibroblasts.[19,22,23] Moreover, IL-6 stimulates production of chemokines, e.g., IL-8 and monocyte chemotactic protein 1 (MCP-1), via macrophages and adhesion molecules in the vascular endothelium. This leads to increased migration of granulocytes and the formation of pustules.[23–25] Thus, IL-6 may be an attractive new target in the treatment of PPP.[20] Interleukin 36, produced by activated keratinocytes, is another cytokine that plays a role in the pathogenesis of psoriasis and PPP. This cytokine directly affects immune system cells and stimulates production of IL-1, IL-6, IL-23, TNF-α, and IFNγ,[26–28] which reciprocatively induces keratinocytes to produce IL-6, IL-8 and antibacterial peptides S 100 A7 and A15. Peptides S 100 A7 and A15, like other pro-inflammatory cytokines, enhance granulocyte migration.[29–32] Recent genetics research indicates that IL-36 has a crucial role in the pathogenesis of PPP. As mentioned above, the IL-36RN gene mutation encoding the IL-36Ra protein, the antagonist of the IL-36 receptor, has been demonstrated in patients with localized and generalized forms of pustulosis, occurring in families and sporadically. As a result of this mutation, the function of IL-36 is dysregulated, resulting in increased production of IL-1, IL-6 and IL-8, and the development of an inflammatory reaction.[30,31,33]

Relationship between palmoplantar pustulosis and smoking

Palmoplantar pustulosis is very common in smokers. There are many reports of lesion regression in patients with PPP after they quit smoking.[34] Smoking causes oxidative stress and leads to an accumulation of inflammatory cells in the epidermis. Newly created free radicals stimulate cellular signaling ducts, as in psoriasis. Consequently, protein kinases are activated, which is also triggered by bacterial antigens. Moreover, nicotine triggers macrophages and keratinocytes to release cytokines, and activates T lymphocytes, which sustains the chronic inflammatory process.[5,13] Recent studies indicate that acrosyringium plays a significant role in the development of PPP symptoms. The sweat glands are innervated by the cholinergic system, where acetylcholine (Ach) is the major inductor of sweating. Acetylcholine acts through 2 types of receptors: muscarinic (mAchR) and nicotinic (nAchR). The level of Ach

is regulated by nicotinic Ach transferase, and can affect the functioning of the sweat glands. It has been shown that the level of Ach is lower in the lower layers of the acrosyringium, because the large quantities of esterase present there decompose the neurotransmitter. In the absence of Ach, nicotine binds to the nAch receptors. It is believed that nicotine Ach receptors, activated by nicotine and not Ach, may play a role in the pathogenesis of PPP and lead to the accumulation of neutrophils and eosinophils, and to the formation of pustules. Nicotine also affects keratinocytes around the sweat glands and causes increased keratosis of the excretory ducts.[5,13]

Impact of contact allergens on skin lesions in palmoplantar pustulosis

Based on clinical observations, contact hypersensitivity plays a role in the background of PPP. Clinical studies have shown that contact allergies to one or more substances occurred in 25.2% patients diagnosed with PPP, whereas in a control group comprising people diagnosed with psoriasis vulgaris, only in 11% of patients had contact allergies.

Significantly more women than men suffering from PPP presented positive patch tests. The following allergens were the most common: nickel, rubber additives, Peru balsam, chromium, mercury, and various fragrances. Therefore, performing patch tests in PPP patients who do not respond to treatment can be helpful in further medical care. In some centers, patch test are performed routinely in the course of diagnosing PPP, and avoidance of contact allergens is an important element of therapy.[35,36]

Palmoplantar pustulosis as a side effect of drugs

Palmoplantar pustulosis primarily affects adults and the elderly. Many patients with PPP are on long-term medication due to the coexistence of other chronic diseases, and skin lesions may appear as a side effect of these drugs.[37] Hagforsen et al. documented that in a group of patients with PPP, 30% were treated with ß-blockers, angiotensin-converting-enzyme (ACE) inhibitors or calcium channel blockers, 13% with anti-diabetic drugs, 30% with hormonal therapy, and 15% received antidepressants.[38] Recently, widely used biological agents bring huge therapeutic benefits to many groups of patients. Although TNF inhibitors (adalimumab, infliximab and etanercept) have high efficacy in the treatment of psoriasis, their side effects include formation of psoriatic lesions. During TNF inhibitor therapy, exacerbation of psoriasis and the development of inverse, pustular and erythrodermic forms of the disease have been observed.[39] Some reports consider palmoplantar pustular psoriasis the most common type of psoriasis induced by TNF inhibitors.[39,40] The mechanism of the formation of psoriatic lesions as a side effect of TNF inhibitors is not fully understood. It has been suggested that blocking the crucial functions of pro-inflammatory cytokines, such as TNF, triggers alternative paths of T lymphocyte action that result in the occurrence of psoriatic lesions in predisposed patients.[41]

Relationship between palmoplantar pustulosis and bacterial infections

Clinical observations indicate an association between some inflammatory skin diseases and focal bacterial infections.[42] Many case reports have described PPP associated with tonsillitis, chronic sinusitis or odontogenic infection. Tonsillectomy or effective treatment of focal bacterial infections led to significant improvement or resolution of PPP.[43,44] In a group of 116 patients with PPP, 109 showed improvement after tonsillectomies.[44] The association between PPP and tonsillitis has also been proved by the results of immunological and molecular tests. Increased expression of class II activation markers, such as CD25 and skin homing receptors (CLA and CCR6), on T cells in the palatine tonsils and in peripheral blood in patients with pustulosis has been observed. Moreover, there are ligands for these receptors, such as E-selectin and ligand 20, in psoriatic skin lesions. Excessive stimulation of T lymphocyte migration to the skin by bacteria colonizing the palatine tonsils probably plays a very important role in the pathogenesis of PPP as a focal disease.[44] Furthermore, increased expression of the inducible co-stimulatory molecule (ICOS) receptor on tonsillar tissue T cells in patients with PPP also confirms the relationship between PPP and infectious foci. The ICOS receptor, like CD28, belongs to the co-stimulatory receptor family and is not present on resting lymphocytes. Its expression indicates stimulation of the immunological cells and plays a role in triggering T lymphocyte response.[42]

Other microbiological factors than tonsillar and dental infections are also regarded as cause or aggravating factors in PPP. Erythematous-infiltrative skin lesions with eruptions resembling PPP are observed in some patients with Reiter's syndrome and classified as a spondyloarthropathy reactive arthritis. The classic triad of Reiter's syndrome symptoms consists of urethritis, arthritis and conjunctivitis. Some patients also suffer from dermal and mucosal lesions, such as balanitis circinata located on the penis glans, keratoderma blennorrhagicum located on the hands and feet, aphthous ulcers within the oral mucosa, and nail disorders. Skin lesions are mainly observed coexisting with *Chlamydia trachomatis* (*Ch. trachomatis*) infections.[45] It has been postulated that *Ch. trachomatis* infections may be a potential cause of isolated pustulosis. This theory is supported by the observation of high antibody titers (equal to or higher than 1/64) against *Chlamydia* more frequently in patients with PPP (38%) than in psoriatic patients (13%), patients with eczema or urticaria (12%) and a control group of healthy individuals (3%). *Chlamydia* antibody titers greater than or equal to 1/16 were observed in 53% of patients with PPP.[46]

Anecdotal case reports also indicate *Helicobacter pylori* as a causative agent in PPP.[47]

Stress as a possible factor aggravating the course of palmoplantar pustulosis

Psychological factors, particularly stress, can play a significant role in the pathogenesis of certain dermatological diseases, including PPP. About 90% of patients with PPP report exacerbations of the disease in association with stress. Psychological tests have confirmed these clinical observations. A study based on the Eysenck Personality Questionnaire (EPQ), which measures 3 personality factors (extraversion–introversion, neuroticism and psychoticism), showed that fear, anxiety and psychosomatic disorders occur in 43% of patients with PPP, compared to 19% of people in the control group.[48] Patients with PPP have also been subjected to the Inventory of Situations and Responses to Anxiety (ISRA). Fear and activation of the autonomic nervous system, manifested by tachycardia, dry mouth and sweating, was observed in 85% of patients with PPP, compared to 19% in the control group.[48]

Relationship between palmoplantar pustulosis and gluten intolerance

It is suspected that gluten intolerance might play a role in the pathogenesis of PPP, but the results of published studies are inconclusive. In a population of Swedish patients with PPP, the presence of IgA anti-gliadin antibodies was demonstrated in 18% and of anti-transglutaminase tissue in 7–10% of the subjects who did not report gastrointestinal symptoms. However, the duodenal mucosa biopsy in a group of patients with anti-gliadin antibodies demonstrated intestinal villi atrophy. In most patients, adherence to a gluten-free diet resulted in regression of the skin lesions.[38,49] Different results were obtained in patients from Germany; neither anti-gliadin antibodies nor anti-transglutaminase tissue were detected in patients with PPP. The discrepancies may be related to many factors, such as ethnic differences between the populations surveyed.[50]

Coexistence of palmoplantar pustulosis with other diseases

Seronegative arthropathies coexisting with palmoplantar pustulosis

Palmoplantar pustulosis is one of the symptoms of a group of syndromes classified as seronegative spondyloarthropathies. These disorders include synovitis/acne/pustulosis/hyperostosis/osteitis (SAPHO) syndrome, Sonozaki syndrome and Reiter's syndrome.

The SAPHO syndrome was described in 1987 as the coexistence of synovitis, acne, PPP, hyperostosis, and osteitis.[51] Skin lesions can precede, occur simultaneously with or follow osteoarticular symptoms. The timespan between lesion formation and osteoarticular symptoms does not exceed 2 years.[52] The etiopathogenesis of SAPHO syndrome is unknown. The adverse effect of retinoids among patients treated for severe acne was considered as a possible cause, but this theory was not confirmed, since a significant number of patients with SAPHO syndrome had not been treated with retinoids.[53] Infectious agents have also been suspected as part of the etiology of SAPHO syndrome. In some patients, *Corynebacterium* has been isolated from the sternoclavicular joints. However, the role of this pathogen is questionable.[53,54]

Pustulotic arthro-osteitis (PAO) was first described by Sonozaki in 1979 as symmetrical erythematous and pustular lesions on the hands and feet accompanied by sternoclavicular joint pain. Recently, it has been suggested that Sonozaki syndrome should be classified as a spectrum of SAPHO syndrome. However, SAPHO syndrome and other spondylopathies, like psoriatic arthritis and Reiter's syndrome, are related to the HLA B27 antigen, while in Sonozaki syndrome, the prevalence of this antigen is low.[53]

Thyroid diseases in patients with palmoplantar pustulosis

Thyroid diseases have been observed more frequently in women with PPP than in a healthy population. Thyroid dysfunction coexists in 25% of studied women suffering from PPP. These disorders include hypothyroidism, hyperthyroidism, struma nodosa, and any thyroid surgery. Due to the fact that PPP coexists with thyroid diseases, it is advisable to gather more data concerning PPP patients and conduct screening of their thyroid functions.[55]

Summary

Palmoplantar pustulosis is a chronic skin disease resistant to treatment. It causes significant impairment of the patients' functioning and adversely affects social interactions. Since the pathogenesis of PPP involves genetic, immunological and environmental factors, patient examinations should be omnidirectional. The key to diagnosis is to collect a detailed medical history, especially concerning comorbidities, medications, exacerbating factors, smoking, and intensity of response to stress in everyday life. The coexistence of bacterial infections as factors provoking eruptions or exacerbations of pustular skin lesions should be excluded. It is advisable to conduct patch tests in order to detect contact allergy. It seems that in the future, modern molecular techniques could be helpful in detecting family predisposition to PPP. Determining the cause of the disease is extremely important for the effective treatment of patients with PPP.

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Do nutritional behaviors depend on biological sex and cultural gender?

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Abstract

Conventional knowledge, resulting from observations and experience, maintains the conviction that there are gender differences in the acquisition, preparation and consumption of food. This review shows differences between the sexes in eating behavior, food choice and nutritional strategy which were conditioned by evolution and by intra-individual (biological or psychological) and extra-individual (socioeconomic and cultural) factors. Women manifest a more pronounced trust in healthy nutrition, greater engagement in controlling body weight, a higher tendency to eat in a group and in stressful situations, and they frequently experience frustration due to their own nutritional behaviors, which reflects higher social pressure and their attempts to reduce eating-related pleasure. On the other hand, men prefer fatty meals with a strong taste, and are directed mainly by the pleasure of consumption; they more frequently furtively eat sweet foods while watching television, use more dietary supplements and more frequently visit fast food restaurants. Nutritional behavior, styles of nutrition, dietary profiles, approach to nourishment, approach to the place of meal consumption, and the sources of nutritional knowledge all demonstrate associations with gender. Reciprocal interactions between gender and diet are conditioned by physiological, psychological and sociocultural factors. This system of reciprocal interactions includes feedback: biological sex and cultural gender shape one's diet and, reciprocally, one's diet affects the deepening or flattening of gender differences. The analysis of reciprocally interacting factors entangled in the formation of a nutritional model may also represent an important element of pro-health prophylaxis and should be used in medical and dietary practice. Males in particular should be informed and educated about health-promoting diets.

Key words: eating habits, food intake, nutritional strategy, female subjects, male subjects

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Introduction

Humans need energy to expend on physiological processes and physical activity. The energetic requirements are covered by supplying the human body with an appropriate amount of qualitatively adequate food, containing suitable proportions of specific nutritional components. Changes in metabolic requirements and energy expenditure, and, consequently dietary changes, have played a very important role in the process of hominids' evolution, especially when it comes to the development of the large human brain.[1] The manner of nutrition should satisfy not only the energetic requirements of the body, but also normal psychophysical development and maintenance of good health and well-being. Human nutritional behaviors depend on individual (biological and psychological) and external (geophysical, socioeconomic and cultural) factors.[2]

Food choice is determined by biological variables, directed at satisfying physiological needs (first of all at appeasing hunger) – by compensating for a deficit in nutritional components until satiation is reached – and at satisfying needs linked to specific sensations of taste, smell and sight, which shape our nutritional habits. In turn, psychological factors, like stress level, emotional excitation and mood influence the manner in which we satisfy our appetites through the motivation to ingest the food, as well as influencing its quantity and quality. The geophysical factors which influence diet selection include the availability of specific food types, determined by natural conditions – the local topography, soil, climate, and the degree of development in transport and trade. The socioeconomic factors which shape our manner of nutrition include socioeconomic status, educational level, occupation, income, family status, and nutritional education, while tradition, national and regional culinary inheritance, beliefs, religious commands or prohibitions, and culinary culture are classified as cultural factors.[3,4] The manner in which human alimentary needs are satisfied varies in time (historical aspect) and in space (geographical aspect). The key changes over time were connected with evolution from a nomadic lifestyle, with hunting and gathering as the means of acquiring food, to a settled way of life in the Neolithic age, which is associated with animal husbandry and agriculture. Spatial variability in satisfying alimentary needs can be illustrated by specific diets (e.g., Scandinavian or Mediterranean) or by national and regional cuisines, which differ in culinary art and the manner in which dishes are served, as well as in the principles governing their consumption.[2,3]

Popular knowledge, resulting from observation and experience, includes a conviction that there are gender differences in the acquisition, preparation and consumption of food. By pre-Neolithic times, these gender differences were noted: women played the role of gatherers while men hunted. Among contemporary hunter-gatherers – for example, the Hadza population in north-central Tanzania – gender differences can be found in the frequency of consuming different food types. Hadza women spend less time foraging than men and are able to eat far more frequently, while the men eat a higher quality diet than the women.[5] Perhaps modern men inherited from hunters their higher tendency to consume high-energy and high-fat foods. Women, on the other hand, inherited from their ancestresses a preference for carbohydrates and, at the same time, being under constant social pressure, they pay more attention to maintaining a healthy body weight, therefore following weight-loss diets more often than men.

The multitude of the abovementioned factors, shaping the manner of satisfying nutritional needs, provides the basis for formulating a thesis on the differences between women and men and for distinguishing male and female nutritional strategies.

The aim of this review of current scientific reports is to attempt to provide an answer to the question of whether – and if so, why – there are gender differences in nutritional behaviors.

Cultural and biological factors affecting food habits and the human body

The concept of a biological sex, based on anatomical, physiological and morphological traits, differentiates human beings into men and women, while the terms gender or cultural sex is related to the characteristics, roles, behaviors, activities, and attributes that society, culture, religion and tradition define as masculine or feminine.[6]

Scientific studies on the gender differentiation of diet, nutritional styles and any behaviors related to the satisfaction of alimentary needs – particularly in the context of genderism – do not have an exceptionally long history, but their results seem to prove gender-determined choices of nutritional strategy.[7] The summary written by Beckmann indicates that the differentiation of nutritional styles between women and men reflects to a significant degree the model of organization in an individual's personal and family life, their education, the occupational and family functions they fulfill, the duties they perform, and the manner in which they spend their free time. Cultural norms, including patterns propagated by mass media, also represent a significant influential factor. Nutrition, as one of the most important vital functions of a human being, provides a broad area in which the typical traits of sexual dissimilarities, resulting from the abovementioned conditioning, are manifested.[8]

Biological sex, understood as the sum of morphofunctional differences, affects the choice of diet and of nutritional strategy. The larger energy demand in males is the result of genetically determined larger body height

and weight in men. The modification of nutritional needs and their fulfillment in women during pregnancy is the result of hormonal changes taking place. In turn, the model of nourishment one adopts reciprocally affects the functioning and morphological traits of the human body. The percent of fat in body composition is higher on average in females and obesity is observed more frequently in women than in men. These findings reflect the significant gender metabolism of energetic substrates: female sex is characterized by a higher index of lipid synthesis (lipoproteins and unsaturated lipids) due to a pronounced effect of estrogens on the accumulation of lipids, while male sex manifests as a higher index of protein metabolism. However, advancing age in women is associated with a decrease in lipid biosynthesis, while advancing age in men promotes an increase in lipid synthesis over protein biosynthesis.[9] In addition, higher concentrations on average of ghrelin (the hormone of hunger) are noted in women than in men, in whom the concentrations additionally continue to decrease with advancing age and falling testosterone level.[10–12] In a similar way, the level of leptin (the hormone of satiety) – due to the action of estrogens – remains 2–3 times higher in women than in men, in whom testosterone reduces leptin synthesis. Treatment with testosterone decreases leptin concentration and, therefore, reduces the mass of lipids.[13–15]

The differences in the frequency of obesity in women and men may have more than a strictly biological background: on a global scale and in poor nations, the prevalence of female obesity is one and a half or even twice higher than the prevalence of male obesity, although in highly developed countries the proportions are reversed, e.g., in 19 European Union member countries in 2008, 37–56% of women and as many as 69.3% of men were overweight or obese.[16,17] In view of the estrogen hypothesis of obesity suggested by Grantham and Henneberg,[18] the gender differences in the incidence of obesity in highly developed countries, which are unfavorable for men, most likely reflect the action of the significant amounts of estrogen drugs metabolism and xenoestrogens released to environment, as they disturb hormonal metabolism. These endocrinally active compounds penetrate the human body through direct consumption, like phytoestrogens, contained in soy products (particularly common in the USA) or through water and biological liquids, as in the case of di-2-ethyl-hexyl phthalate (DEHP), which is found in solvents of glues, varnishes and resins and in polyvinyl chloride – a common ingredient of plastic packaging.[19]

Models of nutritional behavior

The models of nutritional behavior differ between women and men both in the quantity and quality (composition) of diet, as well as in the frequency of meals, the time and place of consumption and in pro-health behaviors, which are linked to food habits and modifications of food habits.

A study on the mechanism of gender differences in food choice conducted on 19,298 students from 23 countries demonstrated that women more frequently than men give up high-fat foods, restrict their use of salt in the kitchen and more eagerly select meals with high content of dietary fiber, particularly fruits and vegetables. Gender differences in food choice – as much as 40% – can be explained by health beliefs: women have a more pronounced conviction regarding the benefits gained from eating healthy food and are more engaged in controlling their body weight. Finally, the distinct views of women and men related to dietary behaviors and health beliefs are responsible for 50% of gender-specific food choice.[20]

Differences are also observed in men's and women's approach to the consumption of sugary foods. Studies conducted by Grogan et al. on a group of 129 British students found significant gender differences in the amount and frequency of sweet snacks consumed. The study evaluated intentional and real attitudes and behaviors towards eating sugary snacks, the motivation to follow appropriate dietary recommendations and the inclination to take pleasure from consuming such snacks. Compared to men, women significantly more often declared an intention to avoid sweet foods, which probably reflected the fact that men's consumption of sugary foods was affected by less social pressure. Moreover, the females in the study presented a contradictory approach to consuming sweet snacks to a greater extent: in their minds, the attraction of taste prevailed over their awareness of the unfavorable effects of sugary snacks on their health.[21] In addition, empirical studies on the sociocultural conditioning of consuming sugary foods, conducted on a representative group of 1,000 Polish adults, demonstrated that social situations promote their consumption differently for men and for women: women were found to eat sugary snacks more frequently during social meetings while men did so while watching television.[22]

Apart from the quantity and quality of meals consumed, the important elements shaping the models of nutritional behaviors also significantly depend on the frequency and place of their consumption. In a study group of 259 American college students, it was observed that twice as many women as men never visit fast food restaurants. In contrast, a higher percentage of men than women (74% vs 60%) eat meals in this type of restaurant 1–3 times per week and in most cases, when selecting from the menu, they take into account the energy value of the food. Although a higher proportion of women (55%) declared that "the nutritive value of food is important to me" ($p < 0.0001$), they are much less effective in choosing nutritional food than men.[23]

Using the three-factor Eating Behavior Scale (EBS), Horiguchi et al. analyzed the eating behaviors of young Japanese adults; they also detected significant gender

differences.[24] In a group of 404 men and 390 women under the age of 30 years, the consumption of meals by women was found to be more frequently stimulated by external factors ("extrinsic eating"):

– eating when others eat or
– eating when feeling irritated.

On the other hand, in the group of men the prevailing motivating factor was "a strong taste," promoting :

– selection of meals with a clear-cut taste and
– consumption of greasy foods.

There were no gender differences in "eating quickly":

– rate of eating meals and
– the habit of insufficiently chewing the food.

Healthy eating

According to an all-European sample survey (14,331 respondents), a normal or healthy diet should first of all contain more fruits and vegetables, but a detailed analysis demonstrated differences between individual countries as well as a variability depending on the age, educational level and gender of the examined individuals. Women more frequently than men indicated that a healthy diet should contain more vegetables or less fat and that the diet should be balanced, but the highest contradiction between women and men was related to fruit and vegetable consumption.[25]

According to a study of 309 Brazilian adult respondents, the main gender difference in healthy eating, and thus the choice of healthy food, consisted of different interpretations of healthiness as a motivation for food choices. The men preferred food that they believed maintains their health, while women focused on the nutritional value of food products.[26]

An analysis of the eating habits of 682 Polish university students showed that 53.4% of them evaluated their nutrition to be abnormal due to a lack of time (63%), poor organization of classes (45.9%), a lack of appetite (11.5%), an attempt to lose weight (9.2%), and financial problems (5.9%). As with earlier research, this study showed that women report following the principles of proper nutrition, but no statistical significance could be demonstrated in this variable.[27]

The dietary behaviors of men are manifested in their use of various dietary supplements. According to Lieberman et al.,[28] they take supplements significantly more often and more numerously than women do (17.4 vs 8.6), a finding from their studies on 1,248 students of 5 American universities. Men used protein preparations and amino acids more often in order to improve their muscular strength and more frequently consumed drinks and gels designed for athletes and purported steroid analogues in order to increase energy, efficiency and resistance of the body. On the other hand, multivitamins and mineral supplements for improving overall health were used by women significantly more often (22.7 vs 31.9). As a result, men spend twice as much money on dietary supplements per month than women do (24 USD vs 12 USD).

Moreover, men to a greater extent than women link a healthy lifestyle with the inclusion of regular physical activity in everyday duties rather than with the modification of their model of nutrition.[29] Thus, physical activity may represent for them the first step toward altering nutritional behavior expressed as a reduced caloric intake and an improved nutritional quality of meals. Furthermore, individuals practicing sport to a greater extent care about their profile and attempt to balance their menu in respect to both macro- and microelements in order to gain better endurance results. Reduced concentrations of neuropeptides controlling the sensation of hunger (cocaine- and amphetamine-mediated transcript – CART) resulting from physical exercise may also significantly shape nutritional habits.[30]

A summary of numerous studies related to the selection and consumption of food by females in Western societies published by Arganini et al. indicates that they tend to select healthier food and are much more concerned than men with choosing appropriate food and nutritional behaviors which are important for maintaining good physical condition.[31]

Gender differences in changes to the perception of the importance of a well-balanced diet and its impact on health were studied in a Norwegian population.[32] It was found that women are more aware of the relationship between diet and health, are more likely to change their views and more often implement a healthy, well-balanced diet than men. These gender differences result from greater health consciousness among women; consequently, they more often change their diet in relation to dietary recommendations and have more knowledge about health. Similarly, girls have a greater tendency to eat healthy food, such as vegetables, than boys, according to a study on 11-year-old Finnish children.[33] Moreover, these girls had more of a preference for eating vegetables than boys 1 year after their parents intervened in order to encourage them to consume vegetables every day. The authors suggest that intervention aimed at increasing vegetable consumption among boys should consist of education that reduces their reluctance to eat vegetables and shows the benefits of eating them.

It seems that the gender differences in perception, inclinations and dietary barriers are already present in childhood and are reduced or deepened by the family. This is confirmed by numerous studies, including one on low-income American families.[34] It was found that the perception of the home environment in terms of nutritional factors, especially fruit and vegetable diet, held by children and their parents were similar. This similarity of parents' and children's perceptions of the home nutritional environment should be taken into account when designing intervention measures to change nutrition towards a well-balanced diet, for example by reducing the volume of meals

and increasing the number of them, and increasing children's consumption of fruit and vegetables.

According to Menozzi et al., in their study of 751 Italian students, women showed more rational behavior to a vegetable diet in light of the theory of planned behavior application.[35] Females showed significantly greater perceived behavior control, attitudes toward behavior and subjective norms, and consequently greater intention to more frequently eating and serving vegetables than males. These results suggest that efforts to increase intention by targeting attitudes, subjective norms and perceived behavior control could have the effect of increasing the consumption of vegetables among the male population.

Own eating habits and body weight

Davy et al. demonstrated that the perception of one's own food habits differs significantly between women and men.[36] Women much more frequently than men judged that they eat too many carbohydrates (59.7% vs 41.9%) and expressed the need to reduce this consumption in their diet more frequently than men did (46.4% vs 27.6%). Moreover, they noted more frequently than men the need to reduce the amount of fat in their diet (71.7% vs 52.4%) and the need to reduce their body weight (57.4% vs 28.6%). Despite these gender differences, as many as 94.4% of all participants in the study agreed that one should consume more vegetables and fruits, and that a healthy diet which assures good health is a diversified diet. Following such a diet was reported by 66% of respondents, even if twice as many women (over 20%) as men (over 10%) simultaneously expressed a deep conviction of the need to reduce their body weight.[36] Similar results had been obtained by Timperio et al., who confirmed that women are more engaged than men in maintaining correct body weight by avoiding body weight gain (30.2% vs 23.3%) and by weight loss attempts (22.2% vs 12.6%).[37]

A significant factor affecting the analysis of one's own eating habits and adherence to dietary recommendations is the body weight of the individual. In multiple studies, individuals with overweight and obesity reported a higher motivation to observe dietary recommendations, irrespective of their gender. This was also confirmed by the positive correlation found between body mass index (BMI) and implementation of nutritional recommendation in both genders; however, women more frequently than men reduced consumption and, therefore, women more frequently than men were underweight (24.4% vs 15.5%).[38]

Abnormal body weight and adjustment of food habits

Markey and Markey proved that people who are dissatisfied with their body weight and who aimed to look leaner were more inclined to engage in pro-health behaviors and exhibited a higher tendency to engage in risky nutritional behaviors. On the other hand, a reciprocal relationship was detected between satisfaction with one's body weight and healthy and risky nutritional models.[39]

Sex/gender also significantly affects the results of treatment in obese individuals. A study by Presnell et al. aimed at analyzing differences in the behaviors of men and women during a program of obesity treatment.[40] The investigators evaluated factors such as self-control of body weight, attacks of unrestrained appetite and symptoms of depression, which were found to be potential predictors of a loss in body weight. Men were found to manifest a much higher level of self-control in their aspiration for effective treatment only at the beginning of the therapy. On the other hand, women demonstrated a higher tendency to overeat and much more frequently manifested symptoms of depression.[40]

Statistically significant gender differences were demonstrated by Leblanc et al. in a group of 123 individuals subjected to a 12-week nutritional intervention (Mediterranean diet) based on the self-determination theory (SDT).[41] In an analysis of changes to eating habits, nutritional behaviors, anthropometric data, and metabolic parameters, the group of men manifested a significantly more pronounced reduction than women in the consumption of fried red meat and an increase in the consumption of fruits, vegetables and dietary fiber. Moreover, the male population showed a reduction in the intake of polyunsaturated fatty acids in their diet. In both women and men, an improvement was noted in lipid panels (TC:HDL-C, TG and TG:HDL-C), but the changes were more accentuated in the group of men. The observations indicated that nutritional interventions based on personal motivation bring about more favorable long-term effects among men.[41]

Eating behavior and social environment

The effect of social environment on the differences observed in the nutritional behaviors of women and men are also significant: in a study by Lin et al.,[38] a reciprocal relationship was demonstrated between an individual's nutritional behaviors and social functioning. Eating habits and physical activity, aside from the support of family and friends, represented significant factors influencing the psychological sphere including the feeeling of social acceptance. On the other hand, the personal nutritional model was influenced by an individual's current body weight, fear of it increasing and their general emotional condition. The study also stressed the role of psychological and behavioral factors in implementing nutritional intervention programs and, in particular, programs for reducing body weight. The social pressure which is unfavorable for

women and which promotes a slim figure results in more pronounced nutritional restrictions and less favorable emotional states for women. Nutritional restrictions frequently provide grounds for developing deficits in nutritive components, reflecting drastic dietary self-restraint and following an unbalanced diet. Moreover, in certain women, the restrictive diet accentuate emotional disturbances, which aggravate improper nutritional behaviors. Even if obesity is found in highly urbanized societies more frequently in men than in women, men encounter less pronounced social discrimination than women do, because body weight in women represents an important variable which affects the implementation of their ambitions and occupational plans.[38]

Ahmed et al. defined the effect of stress on nutritional behaviors.[42] In a group of 407 students from Kuwait, stress was demonstrated to determine changes in nutritional behaviors. Among the groups of both women and men, stressful situations resulted in statistically more frequent consumption of high-calorie, high-fat meals and in an increased intake of high-calorie drinks. On the other hand, in the group of women, the clearly increased consumption was related to sweet snacks and coffee and tea.[42]

In their studies on the relationships between the level of stress and dietary restrictions and preferences, Habhab et al. showed that marginally stressed women preferred sweets and high-energy food, while less stressed women consumed more low-energy food.[43] The experiment remains consistent with the results of a previously conducted study in which people on a low-carbohydrate diet showed a decrease in positive emotions and an increase in low moods, exhaustion and tiredness.[44]

In a study by Leblanc et al.[45] on groups of 64 men and 59 women, gender differences were demonstrated in their nutritional habits and in their level of motivation towards nutrition. The male diet contained a higher overall energy value and a higher value per meal, with a higher percentage of energy in the daily ration originating from fats than from carbohydrates. The women had a better dietary profile than the men: a healthier diet, lower caloric density and a better metabolic profile. As compared to the men, the female nutritional model was dictated by their higher emotional sensitivity and higher value of Self-Determination Index (SDI). The value of this index showed a negative correlation with the energy content of their meals.[45]

Sources of nutritional knowledge

A significant effect on modification of dietetic behavior is exerted by the range of sources from which women and men draw information on nutrition. According to Davy et al.,[36] the sources of information on diet most frequently employed by respondents over the age of 25 years (irrespective of gender) were as follows: television (72%), journals (58%), newspapers (33%), radio (18%), family and/or friends (17%), books (14%), the internet (13%), a physician (12%), work (4%), and school (4%). The analysis by gender indicated that women significantly more frequently than men took advantage of the knowledge transmitted by family and friends (58.0% vs 40.0%), newspapers, magazines, and books (43.1% vs 30.5%). On the other hand, men searched for information on diet mainly in school.[36] Similar results were obtained by Morse and Driskell[23] among 259 American college students. In general, more women (60%) than men (49%) learned about nourishment from family and a significantly higher percentage of women than men drew their knowledge on nutrition from friends (39% vs 25%), from newspapers and from magazines (54% vs 36%). In turn, according to results obtained by Lieberman et al.,[28] there are also gender differences in finding information on dietary supplements. Family, healthcare providers and television constitute significantly more frequent sources of information for women, while men obtain their information more frequently from friends, trainers, instructors, newspapers and magazines, educational websites, or salesmen. The highest percentage (45.1%) of men receive information on dietary supplements from the internet, while the highest percentage of women (43.3%) get it from their families.[28] Additional education, conducted among the male population in particular, as well as a solid and essentially correct manner of transmitting such information might provide an additional element of prohealth policy, leading to improved knowledge on correct nutritional habits. The effect of knowledge obtained from the popular media cannot be neglected as well.

Summary and conclusions

This review of scientific reports justifies the statement that nutritional behavior, styles of nutrition, dietary profile, approach to nourishment and to the place of consuming meals, and the sources of knowledge on nutrition demonstrate associations with gender. Half of the gender-related differences in food choice can be explained by nutritional behaviors and health convictions. Women demonstrate more trust in healthy nutrition and a greater engagement in controlling body weight, and they select healthier food than men do. Moreover, women manifest a higher tendency to eat in a group setting, including the consumption of snacks when other people eat, and in stressful situations. Besides, they choose the pleasure of tasting sugary foods over the consequences of their adverse effects on weight loss and overall health. On the other hand, men prefer fatty meals with strong flavors and they more frequently eat sugary snacks furtively while watching television. Men also more frequently than women use dietary supplements and visit fast food restaurants. Moreover, men more frequently than women practice regular physical activity and modify their diet in order to reduce their body weight and

to improve their physical condition, whereas women more frequently experience frustration due to their own nutritional behaviors, which reflects higher social pressure and their attitude towards reducing eating-associated pleasure. On the other hand, in men, who are guided mainly by taste, the pleasure of consumption prevails.

The type and place of consuming food, any dietary challenges undertaken and fixed nutritional behaviors might represent an additional element of pro-health policy, leading to wider knowledge about proper eating habits despite the anatomical and physiological differences between genders. Likewise, the dissemination of knowledge about the nutritional strategies accepted by men and women, which differ in terms of the quantity and quality of food consumed, nutritional style and habits linked to the preparation and consumption of food as well as sociocultural factors (eg. education, professional and economic status, tradition, religion). The reciprocal effects of sex/gender (in the biological and cultural sense) on diet – and, more strictly, on the entirety of nutritional behaviors – and the reciprocal effect of diet on the psychophysical and cultural traits of gender remain conditioned by physiological and sociocultural factors. This system of reciprocal interactions creates a feedback loop: sex and gender exert an influence on diet and, reciprocally, diet affects the deepening or flattening of sex/gender differences (Fig. 1).

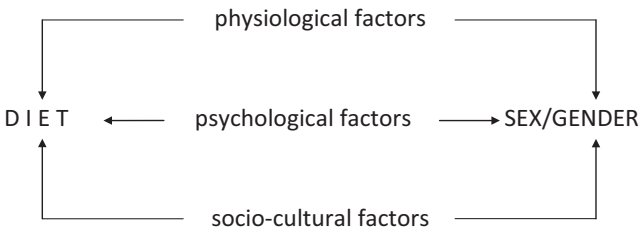

Fig. 1. Factors which modify sex/gender ←→ dietary feedback

There is a clear need to design interventions to improve the health and wellbeing of populations by insisting on increasing the number of health-promoting dietary choices and discouraging food choices that adversely affect health. An analysis of reciprocally interacting factors entangled in the formation of a nutritional model may also represent an important element of pro-health prophylaxis and should be used in the nutritional recommendations suggested by physicians and dieticians. Both parents and children should be informed and educated about proper nutrition, a well-balanced diet and the resulting health benefits. Boys and adult men in particular should be subjected to nutritional education, as research has revealed gender differences which put them at a disadvantage.

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