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

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ORIGINAL PAPER

DOI: https://doi.org/10.20883/medical.e69

Induction of UDP-glucuronosyltransferase 1A by naturally occurring phytochemicals in human hepatoma cells

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ABSTRACT

UDP-glucuronosyltransferases (UGTs) are important detoxification and drug-metabolizing enzymes, which catalyse the glucuronidation of exogenous and endogenous chemicals. The anti-carcinogenic activity of dietary phytochemicals is partly attributed to the induction of phase II enzymes, including UGT1A. Our earlier study showed that protocatechuic acid increased UGT activity in rat liver. A similar effect was observed for indole-3-carbinol and phenethyl isothiocyanate in rat liver. In this study we assessed the effect of protocatechuic acid, tannic acid, indole-3-carbinol and phenethyl isothiocyanate on the expression and protein level of UGT1A in hepatocellular carcinoma HepG2 cells. Cells were incubated with 2μM and 10μM of protocatechuic acid, tannic acid, or indole-3-carbinol and 1μM and 5μM of phenethyl isothiocyanate for 72 hours. Transcript level was measured by RT-PCR and protein level by the immunoblot assay. Treatment with protocatechuic acid, tannic acid, indole-3-carbinol and phenethyl isothiocyanate induced the expression and protein level of UGT1A. Phenethyl isothiocyanate increased the mRNA of UGT1A to the most extent (0.28–5.7 fold change, $p < 0.05$). Increased expression of UGT1A was accompanied by the enhancement of its protein level, with the exception of protocatechuic acid at the dose of 2μM. Overall, isothiocyanates and indoles were more potent as UGT inducers than phenolic acids. Collectively, the results suggest that the induction of UGT1A could contribute to the hepatoprotective and chemopreventive properties of these phytochemicals.

Keywords: tannic acid, protocatechuic acid, indole-3-carbinol, phenethyl isothiocyanate, UGT1A, HepG2.

Introduction

Uridine diphosphoglucuronosyltransferases (UGTs) are phase II enzymes that conjugate metabolic intermediates with glucuronic acid to form glucuronides, which are characterized by increased solubility in water and therefore are more easily excreted from the body [1]. Two general classes of UGTs exist in humans: UGT1 and UGT2. Both classes contain multiple members that catalyze the glucuronidation of a diverse array of substrates and are differentially expressed in various organs [2]. UGT1A enzymes metabolize both endogenous and exogenous compounds such as bilirubin and drugs, whereas UGT2B enzymes target rather endogenous compounds such as steroid hormones [3].

Several studies demonstrated that diets containing plant foods, particularly cruciferous vegetables induce drug metabolizing enzymes in humans and rodents [4–7]. Glucosinolates present in *Cruciferae* vegetables are hydrolyzed by myrosinase during food processing to biologically active indoles and isothiocyanates. The structure of these phytochemicals is shown in **Figure 1**. In 1997, Taioli et al. [8] reported the induction of UGT activity in humans fed 400mg of indole-3-carbinol for 5 days. Other studies showed that indole-3-carbinol increased UGT activity in male Wistar rats [9]. Phenethyl isothiocyanate increased hepatic UGT activity in male Fisher 344 rats [10]. A similar effect has been observed in the case of phenolic acids, which are prev-

alent among many edible plants. Protocatechuic acid, a simple phenolic acid (**Figure 1**) increased UGT activity in the epidermis of female Swiss mice [11]. On the other hand, tannic acid, which is a mixture of digallic acid esters of glucose, did not significantly change hepatic activity of UGT in Wistar rats [12]. These studies indicate that phytochemicals may increase the activity of UGT in *in vivo* models. Thus, the up-regulation of this enzyme by phenolic acids, indoles and isothiocyanates may also represent one of the cancer preventive mechanisms of these compounds.

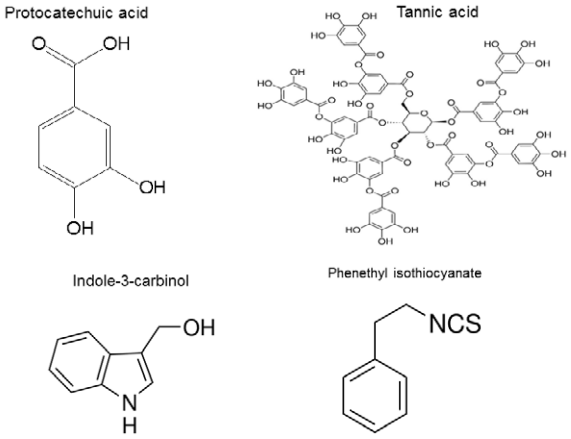

Figure 1. Chemical structure of protocatechuic acid, tannic acid, indole-3-carbinol and phenethyl isothiocyanate

Liver is the major organ for glucuronidation of a variety of xenobiotics. Carcinogens, including benzo(a) pyrene, are conjugated by UGT1A, and increased glucuronidation would result in their increased elimination and potentially decreased toxicity [13]. In this regard, the effective neutralization of chemical mutagens may significantly reduce the risk of cancer initiating events.

Therefore, the objective of this study was to evaluate the effects of phytochemicals such as tannic acid, protocatechuic acid, indole-3-carbinol and phenethyl isothiocyanate on the expression of UGT in human hepatocellular carcinoma HepG2 cells.

Material and methods

Chemicals and antibodies

Antibiotics solution (10^4U penicillin, 10 mg streptomycin, 25µg amphotericin B), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Radio-Immunoprecipitation Assay (RIPA) buffer, Tris were obtained from Sigma-Aldrich (St. Louis, MO, USA). HepG2 (ATCC HB 8065) cells were provided by Prof. Zofia Mazerska from the Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, Poland. All the primers used in PCR reactions were obtained from oligo.pl (Warsaw, Poland). Protease inhibitor cocktail was obtained from Roche Diagnostics GmbH (Penzberg, Germany). Primary and secondary antibodies against UGT and β-actin were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rainbow colored protein molecular weight marker was purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA). All the other chemicals were commercial products of the highest purity available.

Cell culture and treatment

HepG2 cells were maintained in DMEM containing 10% fetal bovine serum and antibiotics. The cells were grown in a humidified incubator at 37°C in the atmosphere of 5% CO_2. To assess the effect of tannic acid, protocatechuic acid, indole-3-carbinol and phenethyl isothiocyanate on the measured parameters, $5x10^5$ cells were seeded per 100mm culture dish. After 24 hours of initial incubation the cells were treated with 2µM and 10µM tannic acid, protocatechuic acid or indole-3-carbinol and 1µM and 5µM phenethyl isothiocyanate or 0.1% vehicle control. The incubation was continued for subsequent 72 hours and cells were harvested.

RNA isolation and total protein lysates preparation

Extraction of total RNA from cells was performed using GeneMatrix Universal DNA/RNA/Protein Purification Kit (EurX, Poland) according to the manufacturer's instructions. Whole cell lysates were prepared using RIPA buffer.

Quantitative PCR

Total RNA was subjected to reverse transcription using RevertAid Kit (Fermentas, Burlington, Canada) followed by quantitative real-time PCR. For real-time analyses the Maxima SYBR Green Kit (Fermentas) and a BioRad Chromo4 thermal cycler were used. The protocol started with 5 minutes enzyme activation at 95°C, followed by 40 cycles of 95°C for 15 s, 54°C for 20 s and 72°C for 40 s and final elongation at 72°C for 5 minutes. Melting curve analysis was used for product verification. The estimation of the expression of *TBP* (TATA box binding protein) and *PBGD* (porphobilinogen deaminase) was used for data normalization. Primer sequences for UGT1A transcript analysis were as follows: 5'-AACGATCTGCTTGGTCAC-3' (forward) and 5'-GAACATTCAGGGTCACTCC-3' (reverse).

Western blot analysis

For the determination of the level of UGT protein the immunoblot assay was used. Protein content in the samples was determined by the Lowry method [14]. All the experiments were repeated three times. Whole cell lysate samples containing 100μg proteins were separated in 10% SDS-PAGE slab gels and proteins were transferred to nitrocellulose membranes. After blocking with 10% skimmed milk, proteins were probed with goat polyclonal UGT and/or rabbit polyclonal β-actin antibodies. Estimation of the level of β-actin was used as an internal control. As the secondary antibodies in the staining reaction, the peroxidase-labeled anti-goat IgG or anti-rabbit IgG were used. Bands were visualized with Advansta Western Bright Quantum Western blotting detection kit. The amount of the immunoreactive product in each lane was determined using the Quantity One software (BioRad Laboratories, Hercules, CA, USA). Values were calculated as relative absorbance units (RQ) per mg protein.

Statistical analysis

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, with $p < 0.05$.

Results

Analysis of the expression of UGT1A

The expression of *UGT1A* was measured by quantitative PCR (**Figure 2**). The expression of *UGT1A* mRNA increased after treatment of HepG2 cells with all the tested compounds. *UGT1A* mRNA level was enhanced by (0.28–5.7-fold) after treatment with either dose of phenethyl isothiocyanate. Indole-3-carbinol in either dose induced about 1.14–1.83-fold higher expression of UGT1A. Tannic acid in either dose increased about 0.48–0.96-fold the expression of UGT1A, while protocatechuic acid increased about 0.27-fold the expression of UGT only in the higher dose.

Evaluation of the protein level of UGT1A

The level of UGT1A protein in HepG2 cells was investigated using the Western blot assay with a specific antibody against the enzyme (**Figure 3**). The content of UGT1A (**Figure 3**) increased by about 28–75% after the treatment with either phenethyl isothiocyanate or indole-3-carbinol in HepG2 cells. The level of UGT1A increased by 26–45% after the treatment with 2 and 10μM tannic acid and 10μM protocatechuic acid in HepG2 cells. No statistically significant changes in the content of the analyzed protein were detected in HepG2 cells as the result of treatment with the protocatechuic acid at the dose of 2μM (**Figure 3**).

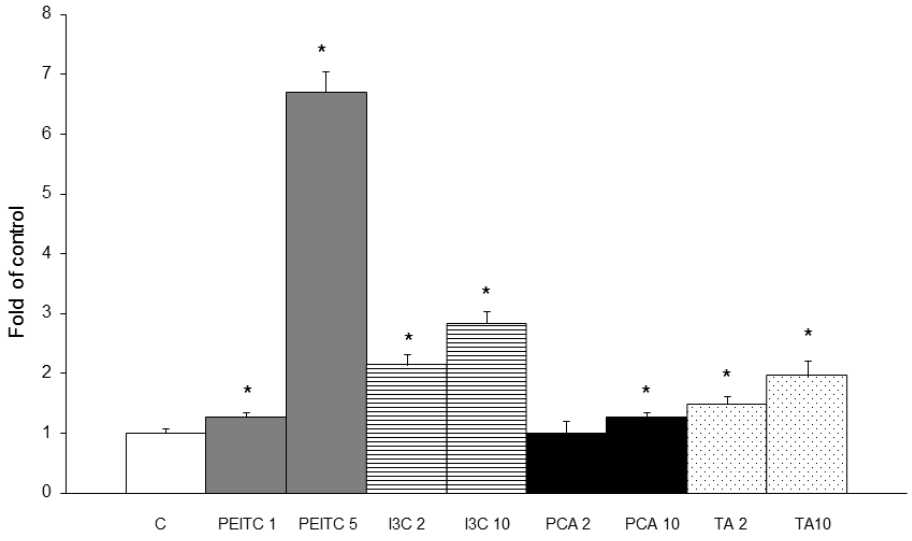

Figure 2. The effect of phenethyl isothiocyanate, indole-3-carbinol, protocatechuic acid and tannic acid on UGT1A transcript level in HepG2 cells. Data (mean±SEM) from three separate experiments are presented, the asterisk above the bar denotes statistically significant differences from the control group, $p < 0.05$
C: vehicle control; PEITC: phenethyl isothiocyanate; I3C: indole-3-carbinol; PCA: protocatechuic acid; TA: tannic acid

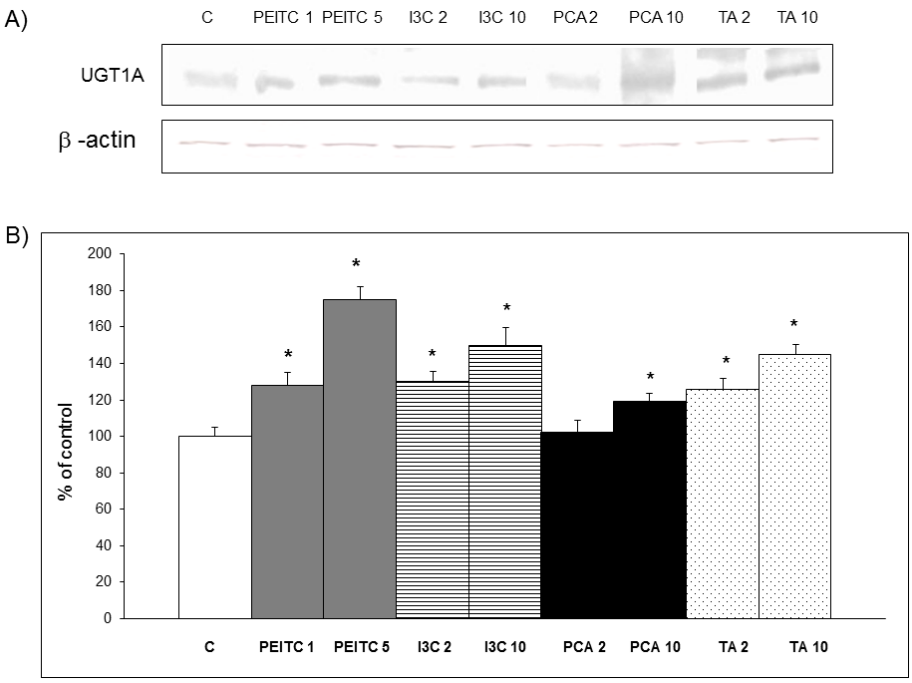

Figure 3. The effect of phenethyl isothiocyanate, indole-3-carbinol, protocatechuic acid and tannic acid on the level of the UGT1A protein in HepG2 cells. (A) Representative immunoblots for the analysis of UGT1A protein; β-actin was used as a normalization control. (B) Results of Western blot analysis of the content of UGT1A protein in comparison to the control group. Data (mean±SEM) from three separate experiments are presented, the asterisk above the bar denotes statistically significant differences from the control group, $p < 0.05$

Discussion

It is well known that dietary factors affect the development of human cancers. The human diet contains a large number of a variety of compounds that may inhibit mutagenesis and/or carcinogenesis [15–18]. These compounds are very diverse in chemical structures and their protective mechanisms are generally unclear. Although the prevention of cancer may be due to multiple mechanisms, one mode of action of anti-carcinogens may be to enhance the carcinogen detoxification systems, such as UGT [19]. UGT can minimize carcinogenicity by conjugation of chemicals with glucuronic acid, which in general results in the generation of biologically less active molecules and increases water-solubility of the conjugated products, which facilitates their excretion from the body via bile or urine [20]. Therefore, the enhancement of the activity of UGT could potentially augment the capacity to withstand the burden of toxic agents and (pre)carcinogens to wchich humans are exposed daily [21]. In this regard, it was shown that UGT1A-deficient rats show reduced capacity to glucuronidate benzo(a)pyrene, a mutagenic polycyclic aromatic hydrocarbon and are more susceptible to formation of DNA adducts [22, 23]. In addition, Berges et al. [24] reported that UGT activity was inversely correlated with the number of preneoplastic liver foci in rats, supporting a role of UGT induction in cancer chemoprevention.

Increased glucuronidation could mediate the protective and antioxidant effects of chemopreventive compounds. Several studies which were performed in animals models indicated that naturally occurring dietary anticarcinogens may be able to elevate UGT activity [25, 26]. In particular, isothiocyanates such as sulforaphane and phenethyl isothiocyanate, that exhibit chemopreventive properties have been shown to induce UGT in animal models [27, 28]. In addition, whole tea, tea extracts, and polyphenols from different types of tea also induce UGT activity and/or expression in animals and cell culture systems [29]. Moreover, our previous study showed that simple phenolic acid, protocatechuic acid increased the activity of UGT in rat liver [30].

In this study, we evaluated the ability of different plant phenolic acids and glucosinolates to modulate the UGT1A expression and protein level in HepG2 hepatoma cell line. Both phenolic acids induced *UGT1A* transcript in HepG2 cells. Tannic acid had a stronger effect on UGT1A than a simple phenolic acid, protocatechuic acid. However, the most potent inducers of UGT1A

were glucosinolates: indole-3-carbinol and phenethyl isothiocyanate. Western blot analysis confirmed that the accumulation of transcript was accompanied by an increase in UGT1A protein level.

Our study showed that phenethyl isothiocyanate induced the expression and protein level of UGT1A to the most extent. A similar effect was observed by Sertzer et al. [9] and van der Loght [12] in rat liver. In contrast to our results, van der Loght et al. [12] observed no effects on hepatic UGT activity after treatment with indole-3-carbinol. This discrepancy may be related to differences in doses and/or species-specific effects.

The inducers of xenobiotic metabolism enzymes can be divided into monofunctional and bifunctional modulators [31]. Isothiocyanates are considered as monofunctional modulators since they induce the activity of only phase II enzymes. In our previous studies we confirmed that phenethyl isothiocyanate was the most effective inducer of phase II enzymes such as NQO1 and GST in rat liver [32]. This study shows that isothiocyanates and indoles are more active as inducers of phase II enzymes than phenolic acids in human hepatoma cells. Collectively, the results suggest that the induction of UGT1A may be one of the factors contributing to the hepatoprotective and chemopreventive properties of phenethyl isothiocyanate, indole-3-carbinol and, to a lesser extent, tannic acid and protocatechuic acid. Further studies are necessary to establish the direct relevance of the studied compounds in providing health benefits for humans.

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Conflicts of interest statement

All the authors declare no conflicts of interest.

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ORIGINAL PAPER

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The influence of diabetic status on the pharmacokinetics of clopidogrel and its metabolites in patients suffered from cardiovascular diseases

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ABSTRACT

Aim. A significant percentage of individuals treated with an anti-platelet agent clopidogrel do not receive the expected therapeutic effect. Clopidogrel resistance is even more prevalent in patients with type 2 diabetes mellitus (DM). An extensive investigation on pharmacokinetics of clopidogrel and its metabolites in patients with type 2 DM suffering from cardiovascular diseases were performed following an administration of 75 mg of the drug.

Material and methods. Plasma concentrations of clopidogrel, its carboxylic metabolite (CLPM) and diastereoisomers of a thiol metabolite (the inactive H3 and the active H4) were determined by a validated HPLC-MS/MS method. The pharmacokinetic parameters of the analytes in diabetic (n = 16) and non-diabetic (n = 28) patients were compared and correlated with platelet aggregation.

Results. DM patients exhibited a slightly higher Cmax of clopidogrel (2.34 ± 2.29 ng/mL) compared with non-diabetic group (1.82 ± 1.86 ng/mL), whereas plasma levels of clopidogrel metabolites were lower in DM than in non-DM patients (2339 ± 989 ng/mL vs. 2662 ± 2090 ng/mL, 4.64 ± 4.79 ng/mL vs. 5.42 ± 4.55 ng/mL and 6.42 ± 4.80 ng/mL vs. 7.44 ± 7.18 ng/mL, respectively for CLPM, H3 and H4). A significant correlation was found between platelet aggregation and the Cmax of the active H4 metabolite in non-diabetic patients.

Conclusions. Pharmacokinetic parameters of clopidogrel, CLPM, H3 and H4 isomers in patients with DM did not differ significantly from those determined in non-diabetic group. Moreover, the antiplatelet response to clopidogrel therapy measured by ADP-stimulated platelet aggregation was similar in both groups of patients.

Keywords: clopidogrel active metabolite, diabetes mellitus, platelet aggregation.

Introduction

Clopidogrel is a pro-drug from the thienopyridine group with an absolute S configuration at carbon 7 [1]. The drug inhibits platelet aggregation and it is widely used in prevention of ischemic events [2]. However, variability of response to clopidogrel treatment associated with an increased risk of death or thrombotic recurrences is present in 5–40% of patients treated with conventional doses of clopidogrel [3]. The etiology behind a reduced efficacy of clopidogrel has not been completely investigated. Multiple studies attempting to characterize this phenomenon have identified genetic polymorphisms of transporters and enzymes participating in clopidogrel absorption and metabolic transformation, and non-genetic factors including co-morbidities, drug-drug interactions and age [4]. Clopidogrel resistance is even more prevalent in patients with type 2 diabetes mellitus (DM)

and leads to a 2- to 4-fold higher risk of developing cardiovascular disease compared to non-diabetic subjects [5]. Evidence suggests that platelets from patients with DM have an increased reactivity and baseline activation compared to healthy controls [6]. The mechanisms involved in platelet dysfunction include hyperglycemia, insulin deficiency and resistance, associated metabolic conditions and other cellular abnormalities, such as increased P-selectin and glycoprotein expression, oxidative stress or increased production of thromboxane [7]. An impaired response to clopidogrel treatment may be also associated with changes in the drug metabolism observed in DM patients [8]. The metabolic transformation of clopidogrel undergoes through two different pathways in the liver. Up to 85% of the absorbed drug might be transformed by carboxyl esterases to an inactive carboxylic acid derivative of clopidogrel (CLPM) [9]. Because the plasma concentrations of the parent drug are very low, the CLPM determination in plasma might be applied to study the pharmacokinetics of clopidogrel in an indirect manner [10]. Only 15% of the absorbed clopidogrel dose is transformed by isoenzymes of cytochrome P450 (CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4) to a thiol metabolite (CTM), which is responsible for the clopidogrel pharmacological effect [11]. CTM selectively and irreversibly blocks ADP binding to a P2Y12 receptor located on the platelet surface and thus inhibits ADP-induced platelet aggregation [12]. It possesses three stereochemical sites but only two isomers named H3 and H4 are present in clinical samples obtained from patients treated with clopidogrel [13]. Moreover, in vitro studies have confirmed, that only the H4 isomer possesses pharmacological activity [1]. An extensive investigation on pharmacokinetics of clopidogrel and its metabolites in patients with DM would greatly contribute to optimization of the clopidogrel therapy in this disease. There is only one paper focused on the pharmacokinetic aspects of the clopidogrel treatment in diabetic patients. Erlinge et al. compared the plasma levels of CTM in DM patients to those determined in non-diabetic group [8]. However, conditions of the applied HPLC-MS/MS method allowed to determine CTM as a mixture of isomers. Such an approach may lead to overestimation of patient exposure to the active metabolite of clopidogrel because only the H4 isomer is clinically relevant. Moreover, the levels of clopidogrel or its main metabolite CLPM were not considered in that study.

According to our best knowledge, investigation on the pharmacokinetics of clopidogrel and its metabolites: the pharmacologically active H4, and the inactive H3 and CLPM have been performed in diabetic patients for the first time. Moreover, a Multiplate analyzer has been applied to determine the platelet reactivity in this group of subjects in order to estimate the pharmacodynamic-pharmacokinetic correlation.

Material and methods

Chemicals

(+)-S clopidogrel bisulphate (purity 99%) and its carboxylic acid metabolite (CLPM; purity 99.6%) were the generous gift of Pharmaceutical Research Institute (Warsaw, Poland). The 3'-methoxyacetophenone derivatives of clopidogrel thiol metabolite H3 (MP-H3) and H4 (MP-H4) isomers were obtained from Sanofi Aventis (Montpellier, France). Piroxicam (PRX, internal standard, IS) was obtained from Jelfa (Jelenia Góra, Poland). The alkylating agent 2-bromo-3'-methoxyacetophenone (MPB) and formic acid (purity >95%) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Acetonitrile (Merck, Darmstadt, Germany) was of HPLC gradient grade. De-ionized water was always used to prepare a mobile phase for HPLC (Simplicity® water purification systems, Merck Millipore, Billerica, USA). Drug free human plasma was obtained from Regional Centre of Blood Donation (Poznań, Poland).

Study population

The study involved 44 patients of Caucasian origin from central Poland undergoing elective coronarography, carotid artery stenting or peripheral artery interventions. Patients received an oral clopidogrel formulation under fasting conditions as a 75 mg maintenance dose for at least 7 days prior to the procedure. In 16 patients taking clopidogrel the concomitant type 2 DM was observed. All diabetic patients were on hypoglycemic treatment (oral anti-diabetic agents or insulin). Exclusion criteria included acute myocardial infarction, malignancies, oral anticoagulation therapy with a coumarin derivatives, treatment with a glycoprotein IIb/IIIa antagonist or other antiplatelet drugs except for aspirin, thrombocytopenia (platelet count < 100 000/μL), chronic liver disease and impaired renal function (serum creatinine level > 2 mg/dL). A detailed characteristics of subjects was presented in **Table 1**. The study protocol was approved by the Ethical Committee at Poznan University of Medical Sciences. All patients gave written informed consent for participation in the study.

Sample collection

Blood samples for pharmacokinetic analysis were collected immediately prior to administration of clopi-

Table 1. Patients' characteristics

	Patients	
	diabetic (n = 16)	non-diabetic (n = 28)
Age [y]	65.5 ± 8.3	61.6 ± 8.6
Body weight [kg]	80.0 ± 12.9	83.0 ± 13.6
BMI [kg/m^2]	29.4 ± 3.8	26.9 ± 6.2
Sex (male/female)	9/7	21/7
Medical history: Hypertension	15	23
Hypercholesterolemia	1	6
Dyslipidemia	5	4
Medical therapy: Proton pump inhibitors	7	11
Statins	15	27
Beta blockers	13	25
ACE inhibitors	10	19

Age, body weight andbody mass index (BMI) are presented as mean ± SD. ACE – angiotensin-converting enzyme

dogrel and at 0.5, 1, 2, 3, 4, 6, 12 and 24 hours post-dosing. An aliquot of 7.5 mL of blood was drawn into collection systems containing K_2EDTA as the anticoagulant (Sarstedt AG&Co., Nűmbrecht, Germany). Due to the limited stability of CTM in human whole blood, 37.5 µL of a 500 mM acetonitrile solution of MPB was added to the systems according to the procedure reported by Takahashi et al. [14]. The blood samples were centrifuged at 1620×g for 10 min and the obtained plasma samples were stored at –25°C until analysis. Following the suggestion of Tuffal et al. [13] the samples with poor signs of the haemolysis after the addition of MPB were considered as not sufficiently stabilized, and the concentrations of both H3 and H4 were not taken into account during the calculation of pharmacokinetic parameters.

Determination of clopidogrel and its metabolites concentrations in plasma

A validated HPLC-MS/MS method [15] was applied for analysis of plasma concentrations of clopidogrel, CLPM and the H3 and H4 isomers in diabetic and non-diabetic patients. The HPLC analysis was performed on a chromatograph Agilent 1200, which was coupled to a triple quadrupole tandem mass spectrometer 6410 B Triple Quad (both from Agilent Technologies, Palo Alto, USA). The analytes were separated in the Zorbax Plus C18 column (100 mm x 2.1 mm, 3.5 µm) (Agilent Technologies, USA) at a column temperature of 40°C. The mobile phase was a mixture of de-ionized water (A) and acetonitrile (B), both containing 0.1% (v/v) formic acid. The gradient was as follows: 0–7 min linear from 42 to 90% B, 7–7.5 min return from 90 to 42% B and the post time of 5 min with 42%B for column equilibration. The mobile phase flow was set at 0.35 ml/min. The eluent from the HPLC column was introduced directly to the MS interface using electrospray ionization in the positive ion mode. The MS parameters were as follows: capillary voltage 4000 V, nebulizer gas (nitrogen) pressure 40 psi (275.8 kPa), desolvation gas (nitrogen) flow 10 L/min and desolvation temperature 300°C. Nitrogen was also used as collision gas. The specific transitions for the analytes were monitored using the multiple reaction monitoring (MRM) mode. Mass transition used for quantitative analysis were as follows: from m/z 322.1 to 212 for clopidogrel, from m/z 504.1 to 155 for the CTM isomers, from m/z 308.1 to 198 for CLPM and from m/z 332.1 to 95 for PRX. An aliquot of 250 µL of plasma was spiked with 25 µL of the IS solution at a concentration of 1 µg/mL. Protein precipitation was performed by adding 450 µL of acetonitrile to each sample. The mixture was vortexed and centrifuged for 10 min at 22570×g at the temperature of 20°C before the supernatant was filtered using Mini Uni Prep filters (Whatman International Ltd., Maidstone, Kent, UK). The resulting filtrate was evaporated under a vacuum at 40°C and the dry residue was reconstituted in 200 µL of a mobile phase. Then, a 25 µL aliquot was injected onto the HPLC-MS/MS system. Calibration curves of the analytes were prepared in concentration ranges of 0.25–5.00 ng/mL for clopidogrel, 50–10000 ng/mL for CLPM and 0.25–50.00 ng/mL for both MP-H3 and MP-H4. The intra- and inter-day accuracy of the method, expressed as the relative error, was ⩽ 16%. The intra- and inter-assay precision, expressed as the relative standard deviation, was ⩽ 19.9%. The analytes were stable in samples stored for 6 h in the autosampler, in plasma samples for 24 h at room temperature and for 3 months at -25°C [15].

Pharmacodynamic assay

Whole blood platelet aggregation was measured in 36 patients using an impedance aggregometer (Multiplate® analyzer, Roche Diagnostics, Mannheim, Germany). Samples for aggregation assay were collected 2–3 hours after clopidogrel administration into the S-Monovette system coated with hirudin (SARSTEDT AG&Co., Nümbrecht, Germany). Whole blood was diluted with 0.9% NaCl solution (1:1, v/v) and stirred in the test cuvettes at 37°C. After addition of 6.4 µmol/L of ADP, the increase in electrical impedance was recorded continuously for 6 min. All materials used for platelet function testing were obtained from Roche Diagnostics (Mannheim, Germany). The platelet aggregation was quantified as arbitrary units (AU) and the area under

the curve of arbitrary units (AU·minute). A cut-off point of 468 AU·min for platelet aggregation in response to ADP was used as the threshold for an increased risk of thrombotic events during clopidogrel therapy according to the recent consensus opinion of Bonello et al. [16].

Pharmacokinetic calculations

The pharmacokinetic parameters of clopidogrel, CLPM, H3 and H4 were calculated with the non-compartmental technique using WinNonlin 6.2 (Pharsight, Mountain View, USA). The maximum plasma concentration (C_{max}) and the time to reach the C_{max} (t_{max}) were directly derived from the observed plasma concentrations. The elimination half-life ($t_{0.5}$) was estimated from $\ln 2/k_{el}$, where k_{el} is the first-order elimination rate constant calculated by the terminal linear segment of the log plasma concentration–time data. The total area under the concentration–time curve $AUC_{0-\infty}$ was estimated by the trapezoidal rule with extrapolation to infinity using C_{last}/k_{el} (C_{last} – the last measurable concentration in plasma).

Statistical analysis

All statistical analyses were performed using Statistica software (version 8.0, StatSoft Inc., Tulsa, USA). Normality was estimated with the Shapiro-Wilk test. The differences between the normally distributed variables were determined with t-Student's test; in the other cases the Mann-Whitney test was applied. Correlations between the parameters were calculated with the Spearman rank correlation coefficient for all the non-normally distributed values. The differences were considered to be significant when $p \leqslant 0.05$.

Results

Pharmacokinetic study

The mean plasma concentration-time profiles of clopidogrel, CLPM, the H3 and H4 isomers of CTM obtained in diabetic and non-diabetic patients following administration of 75 mg clopidogrel were presented in **Figure 1**.

Clopidogrel was rapidly absorbed from the gastrointestinal tract yielding C_{max} values of 2.34 ng/mL and 1.82 ng/mL in plasma of diabetic and non-diabetic patients, respectively. The main clopidogrel metabolite, which is biologically inactive CLPM, reached a C_{max} in plasma thousand-fold greater compared to that of the parent drug (**Figure 1B, Table 2**). The pharmacokinetic profiles of the H3 and H4 isomers of CTM were slightly different in diabetic patients as compared to patients without DM (**Figure 1C, D**) pointing to a higher exposure to H3 and H4 in patients without diabetes. Lower plasma concentrations of the active H4 isomer were noticed in diabetic patients (**Figure 1D**), resulting in lower AUC_{0-t} values (8.7 ng·h/mL vs. 13.5 ng·h/mL in non-diabetic patients) (**Table 2**). Both the H3 and H4 isomers were eliminated rapidly and their concentrations were below limit of quantification at 6 h after clopidogrel administration in most subjects (**Figure 1C, D**). However, in DM patients values of $t_{0.5}$ determined for CTM isomers were higher (0.91 h and 1.15 h, for H3 and H4, respectively) than in non-diabetic group (0.66 h for H3 and 0.77 h for H4 isomer). Lower elimination rate was also observed for CLPM with $t_{0.5}$ of 7.50 h as compared to 7.01 h in non-diabetic patients (**Table 2**). Differences between pharmacokinetic profiles of clopidogrel and its metabolites determined for diabetic and non-diabetic patients were not statistically significant.

Pharmacodynamic study

Platelet aggregation measured in patients treated with 75 mg of clopidogrel ranged between 63–246 AU·min (mean ± SD = 120 ± 66 AU·min, n = 15) in diabetic patients and 43–747 AU·min (180 ± 151 AU·min, n = 21) in patients without DM. Statistical analysis revealed that ADP-induced platelet aggregation was strongly associated with the C_{max} of the active H4 isomer with r = -0.439, p = 0.025 for the overall patient group, and with r = -0.536, p = 0.027 observed in non-diabetic patients. The correlation was less pronounced in patients with DM (p > 0.05).

Table 2. Pharmacokinetic parameters (mean ± SD) of clopidogrel and its metabolites in diabetic and non-diabetic patients

	clopidogrel		H3		H4 (active)		CLPM	
	diabetic (n = 16)	non-diabetic (n = 24)	diabetic (n = 10)	non-diabetic (n = 20)	diabetic (n = 10)	non-diabetic (n = 20)	diabetic (n = 16)	non-diabetic (n = 28)
C_{max} [ng/mL]	2.34 ± 2.29	1.82 ± 1.86	4.64 ± 4.79	5.42 ± 4.55	6.42 ± 4.80	7.44 ± 7.18	2339 ± 989	2662 ± 2090
t_{max} [h]	1.40 ± 0.79	1.39 ± 1.26	1.25 ± 0.84	1.04 ± 0.54	0.94 ± 0.45	1.04 ± 0.54	1.38 ± 0.70	1.35 ± 0.79
$t_{0.5}$ [h]	1.33 ± 0.81	2.05 ± 1.54	0.91 ± 1.06	0.66 ± 0.62	1.15 ± 1.18	0.77 ± 0.65	7.50 ± 3.44	7.01 ± 3.34
AUC_{0-t} [ng·h/mL]	5.01 ± 4.11	4.95 ± 3.70	6.27 ± 4.86	8.61 ± 7.85	8.74 ± 7.03	13.49 ± 13.78	11061 ± 6078	11100 ± 8821
$AUC_{0-\infty}$ [ng·h/mL]	6.07 ± 4.12	6.33 ± 4.32	7.09 ± 4.68	9.11 ± 7.99	9.45 ± 6.73	14.17 ± 13.98	12695 ± 7251	12252 ± 9478

CLPM – carboxylic acid metabolite of clopidogrel; H3, H4 – isomers of clopidogrel thiol metabolite; C_{max} – maximum plasma concentration; t_{max} – time to reach C_{max}; $t_{0.5}$ – elimination half-life; AUC_{0-t} – area under the plasma concentration-time curve from zero to time t; $AUC_{0-\infty}$ – area under the plasma concentration-time curve from zero to infinity

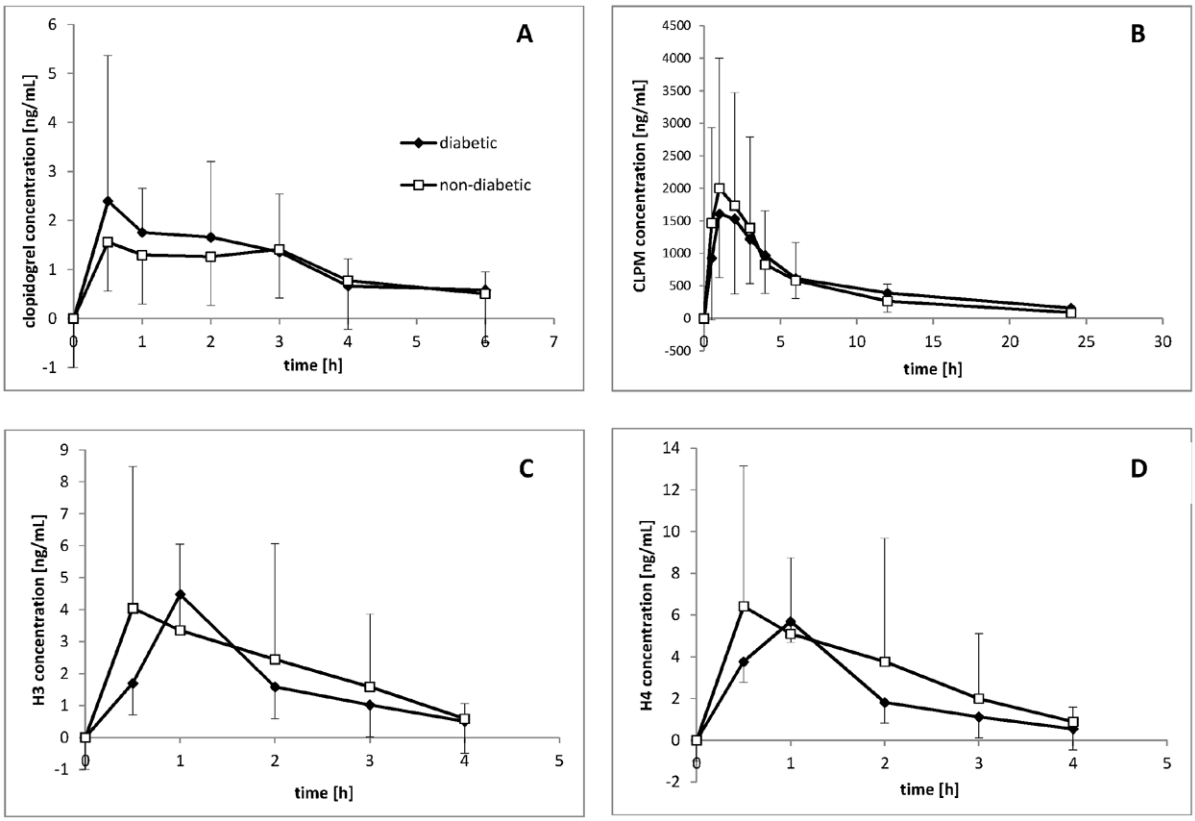

Figure 1. Mean plasma concentrations of clopidogrel (A), its carboxylic metabolite (CLPM) (B) and diastereoisomers of a thiol metabolite, the inactive H3 (C) and the active H4 (D), versus time following administration of 75 mg of clopidogrel to diabetic and non-diabetic patients

Discussion

In the present study, the selective HPLC-MS/MS method [15] permitted to perform the investigation on pharmacokinetics of the clinically relevant H4 isomer along with the parent drug and its non-active metabolites: H3 and CLPM in patients with type 2 DM accompanied by cardiovascular diseases. The values of C_{max} of clopidogrel obtained in the diabetic and non-diabetic patients (**Table 2**) refer to the published data of the C_{max} in healthy volunteers which varied from 0.9 ng/mL [17] to 4.4 ng/mL [18] following administration of 75 mg clopidogrel. There is no available numeric data regarding pharmacokinetics of the parent drug in diabetic patients. However, Park et al. [19] mentioned that they did not observe any differences in clopidogrel levels measured in plasma of Korean patients with and without DM. Some authors speculated on an increased activity of esterases in diabetic patients, which would convert more of the clopidogrel dose into the inactive CLPM [8, 20]. This has been confirmed for aspirin resistance, in which increased activity of plasma esterases hydrolyzed acetylsalicylic acid to a higher extent in patients with type 2 diabetes [21]. In the present study, plasma levels of CLPM were similar in both groups of patients (**Figure 2B**) and did not confirm the above mentioned speculation. Lower plasma concentrations of the active H4 isomer noticed in diabetic patients, resulting in lower AUC_{0-t} values (**Figure 1, Table 2**), may lead to serious clinical consequences. A similar conclusion was drawn by Erlinge et al. [8] who reported lower AUC values of CTM measured as a mixture of the H3 and H4 isomers in patients with DM compared with non-diabetic patients following administration of a loading dose of 600 mg and at day 29 of maintenance therapy with 75 mg of clopidogrel. The authors claimed that one possible reason for lower levels of CTM was reduced gastric motility in diabetic patients leading to slower absorption of the pro-drug [8].

The high inter-subject variability of pharmacokinetic parameters was demonstrated in the studied groups of patients. It requires further explanation with reference to genetic polymorphism of P-glycoprotein, which affects clopidogrel bioavailability, and CYP isoenzymes, responsible for clopidogrel metabolism. Moreover, concomitant use of certain CYP-metabolized drugs may also attenuate pharmacokinetics of clopidogrel and its metabolites, especially in a group of diabetic patients.

Harmsze et al. [22] reported an impaired response to clopidogrel therapy in diabetic patients treated with sulfonylureas as compared to patients without concomitant sulfonylurea treatment. Sulfonylureas are mainly metabolized by the CYP2C9 enzyme, which also plays an important role in the metabolic activation of clopidogrel. Because CYP2C19 is regarded as the main enzyme involved in the clopidogrel metabolism [11], a drug/drug interaction with CYP2C19 inhibitors, such as proton pump inhibitors (PPIs), would be anticipated. However, we did not observe any statistically significant influence of co-administration of PPIs (omeprazole and pantoprazole) on exposure to clopidogrel and its metabolites in the studied groups of patients.

According to the literature data, pharmacodynamic effect of the drug, expressed by inhibition of platelet aggregation was lower in diabetic compared to non-diabetic patients [23, 24]. In our study all diabetic patients had a relatively low platelet aggregation, whereas in non-diabetic group one patient could be characterized as a non-responder with AUC = 747 AU·min. This high value of platelet aggregation coexisting with the low C_{max} of the H4 isomer of 2.5 ng/mL determined in the patient plasma suggests a poor response to clopidogrel treatment associated with higher risk of cardiovascular events. According to the criteria set by Bonello et al., platelet aggregation > 468 AU·min in response to ADP may be used for identification of patients at high risk of thrombotic events. However, patients with DM may have different high on-treatment platelet reactivity cut points as compared to patients without DM [16].

Some studies have demonstrated that estimation of the platelet function may be especially useful in patients with decreased clopidogrel metabolism [25]. Therefore, the routine monitoring of platelet aggregation and the H4 plasma concentrations during clopidogrel treatment may improve the clinical outcomes in patients with cardiovascular diseases accompanied by DM.

Limitations of the study include the limited sample size to evaluate the effect of diabetes on clopidogrel pharmacokinetics in clopidogrel-treated patients. Moreover, the study did not include any genetic polymorphisms that could affect the pharmacokinetics and pharmacodynamics of the drug.

Conclusion

According to our knowledge, this is the first study of the pharmacokinetics of clopidogrel and its main metabolites, the active H4 and inactive H3 and CLPM, in patients with type 2 DM accompanied by cardiovascular diseases. Our results revealed that pharmacokinetic parameters of clopidogrel, CLPM, H3 and H4 in patients with DM did not vary significantly from those determined in non-diabetic group. Moreover, the antiplatelet response to clopidogrel therapy measured by ADP-stimulated platelet aggregation was similar in both groups of patients.

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Assessment of iron intake and the state of knowledge about its disturbances and food sources among students of Poznan universities

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ABSTRACT

Introduction. Iron is one of many essential mineral components necessary for normal functioning of organisms. This microelement takes part in transport of oxygen from the lungs to the tissues, DNA synthesis, and is essential for normal activity of numerous enzymes.
Aim. The aim of the work was the assessment of iron consumption among students of Poznan universities with consideration of the need for this mineral component.
Material and methods. The study was conducted between the months of February and October 2012. Based on the randomly chosen study group, 100 people were analyzed – 66 females and 34 males. In total 25 people from each of the four Poznan universities: Poznan University of Medical Sciences, August Cieszkowski University of Life Sciences, Poznan University of Technology, as well as Eugeniusz Piasecki Academy of Physical Education were included into the studied population. In this study a questionnaire was a tool used in the analysis. Statistical analysis was conducted with the use of computer programs: STATISTICA version 10 and Microsoft Office Excel 2007. Determination of average values of iron, vitamin C, calcium and fiber intake, was performed with the use of DIETETYK 2011.
Results. Satisfaction of the need for iron differs depending on the university and gender. The low iron intake was observed at Poznan University of Medical Sciences, while the high one at the University of Natural Science. Men (25) more often delivered with food adequate amount of iron in comparison to women (3). Increase in iron intake was observed together with the increase in the amount of calories in the diet as well as higher BMI. The study also showed that chronically ill people as well as women heavily bleeding during menses more frequently suffered from anemia. The main source of heme iron for the students was poultry meat, while the source of non-heme iron were mainly whole-wheat products.

Keywords: diet, iron, anemia, demand.

Introduction

Iron is an essential microelement required for normal functioning of living organisms. Iron bound to hemoglobin and myoglobin, takes part in the transport of oxygen. It is also essential on the particular stages of the respiratory chain, regulates the cell cycle, DNA synthesis as well as functioning of enzymes, eg. catalases [1].

The need for iron differs depending on the gender, age as well as the state of the human body. It increases during pregnancy, lactation, during the course of a neoplastic process, in chronic diseases or when vegetarian diet is observed.

Deficiency of iron is the most commonly occurring alimentary deficiency, which results in anemia, causing

among others inhibition of physical and psychological development in children or disturbances in immunological functions. Excessive supply and absorption of iron, on the other hand, can lead to dysfunction of organs as well as an increased risk of developing a neoplasm [2].

Iron in food occurs in the heme form, absorbable in 20%, it's source are for ex. meat, giblets, fish, seafood as well as in the non-heme form, present in green and string vegetables, absorbable only in 5% by the body [3]. Bioavailability of iron is conditioned by the type of food being consumed, among others by the presence of phytates, oxalates, tannins and some macroelements for example calcium [2].

In Poland average consumption of iron amounts to 12.4 mg per day. Men consume on average 15.0 mg of iron per day, women however 10.2 mg per day, which is too low in reference to the consumption norm for women [3].

Familiarity with the symptoms and causes of iron deficiency as well as food sources of this microelement allows for the use of diet with the appropriate iron content and if need be early diagnosis of the disease and implementation of an appropriate treatment.

Aim

The aim of the study was the assessment of iron consumption among students of Poznan universities. This was done with the consideration of the need for this mineral component, as well as the assessment of the state of knowledge on the subject of iron disturbances and their treatment in the studied group.

Material and methods

Selection of the study material

The study was conducted between February and October 2012. Based on the randomly chosen study group, 100 people were analyzed – 66 females and 34 males aged from 19 to 23 years, students of various academic years and departments. Twenty five people from each of the four Poznan universities: Poznan University of Medical Sciences, August Cieszkowski University of Life Sciences, Poznan University of Technology, as well as Eugeniusz Piasecki Academy of Physical Education were included into the studied population.

Methods of survey study

Questionnaire survey composed of 22 closed and open-ended questions was a work tool used in the analysis.

Questions were concerning among others; university name, gender, weight, height, knowledge of the subject of microcytic anemia as well as food sources of iron. Questions about the use of additional supplements/medications, concomitant diseases, volume of menstruation in women, consumption frequency of particular food product groups rich in iron, vitamin C and fiber were included in the survey. Anemia was diagnosed by declaration. Diet diary, which included menu from last three days, comprised second part of the survey.

Methods of statistical calculations and form of result presentation

Statistical analysis was conducted with the use of computer programs: STATISTICA version 10 and Microsoft Office Excel 2007. Determination of average values of iron, vitamin C, calcium and fiber intake, was carried out, with the use of DIETETYK 2011 computer program. Methods used in statistical analysis were: multifactorial analysis of variance (ANOVA), Kruskal-Wallis nonparametric test, correlation analysis and test of significance for Spearman's rank-order correlation coefficient. Statistical *significance was set at* the 0.05 probability *level*.

Results

Excessive consumption of iron appearing in the results below, is defined as a difference between daily consumption and the normal demand for this microelement. For women excessive iron consumption is therefore equal to the iron consumption reduced by 18 mg, however for men – 10 mg was deducted from the iron consumption (according to the norms from 2008). In this way the defined variable allowed for coherent analysis of satisfaction of the demand for iron in both genders. In each case, positive value of this variable signifies meeting of the demand for this microelement, whereas negative value signifies, by what amount the demand for the element was not met.

All figures and tables include university abbreviations, which represent accordingly:

- MU – Poznan University of Medical Sciences,
- ULS – University of Life Sciences,
- PUT – Poznan University of Technology,
- APE – Academy of Physical Education,
- F – female,
- M – male,
- BMI – body mass index.

Survey questionnaire allowed for organization of the studied group according to the BMI. Alltogether

83% of the people examined, possessed normal body mass (59 women, 24 men). Overweight people comprised 9% (1 woman, 8 men) and those with first degree obesity 2% (2 men) of the respondents. In total 6% of people were underweight (6 women).

Amongst the surveyed persons 10 women admitted to have anemia. Additionally among the people studied there were individual people suffering from hypothyroidism, allergies, asthma, epilepsy and irritable bowel syndrome.

Iron consumption dependence on gender and university

The conducted analysis showed that in the studied group, women consumed on average less iron then their demand, while men consumed adequate amount of iron for their demand. Among the women studied, average excessive iron consumption was -8.47 mg, while among men 3.15 mg – **Table 1**.

The lowest excessive iron consumption was observed among the students of MU (average -6.78), the highest one – at ULS (average -0.80) – **Table 2**.

Influence of the diet caloric value on iron consumption

A strong positive correlation was determined between the amount of calories in the diet and the degree of meeting demand for iron. People with larger energy consumption supplied more iron to the body then people who consumed less calories. Rise in diet calories by 1 kcal increased iron consumption on average by 0.0078 mg.

In order to check the gender influence on the dependence between the amount of calories in the diet and iron consumption, correlation coefficients were set for those two variables for women (0.6513) and men (0.7585), separately. In both cases statistical tests showed that the correlation coefficient was significant when the level of significance was 0.05.

Influence of Body Mass Index (BMI) on iron consumption

Analysis of results showed that a positive dependence existed between BMI and adequately met demand for iron. People with higher Body Mass Index, generally consumed more iron than people with lower BMI. The rise in BMI by one unit translated to an increase in average iron consumption by 1.2406 mg.

In order to check the effect of gender on the dependence between BMI and iron consumption, correlation coefficients were set for those two variables, separately for women (-0.2178) and men (0.4580). In both cases the correlation coefficient was statistically different from zero, with significance level of 0.05. In case of men higher BMI, linked in general with a greater iron consumption, in the case of women this was inversely proportional – higher BMI in general translated to a lower iron consumption (**Figure 1**).

Iron consumption in heavily menstruating women

Women were divided into two groups – women with heavy menstruations and those with scant menses. Women who declared that they have heavy menstruations that last longer than 4 days were included to the group of women heavily menstruating.

In the group of 31 heavily menstruating women, average iron consumption totaled 9.79 mg, with stan-

Table 1. The values of excessive iron intake, depending on the university and gender

	The number of cases	Average (mg)	Min (mg)	Max (mg)	Standard deviation
MU	25	-6.78	-12.78	3.84	4.46
ULS	25	-0.80	-11.65	24.42	8.13
PUT	25	-4.21	-12.18	11.79	7.76
APE	25	-6.27	-15.94	4.83	5.63
F	66	-8.47	-15.94	3.84	3.50
M	34	3.15	-4.03	24.42	5.49

Table 2. The impact of chronic use of drugs on the occurrence of anemia

	Anemia	No anemia	Total
People chronically using medications	4 (25%)	12 (75%)	16 (100%)
People not using any medications chronically	6 (7%)	78 (93%)	84 (100%)
Total	10 (10%)	90 (90%)	100 (100%)

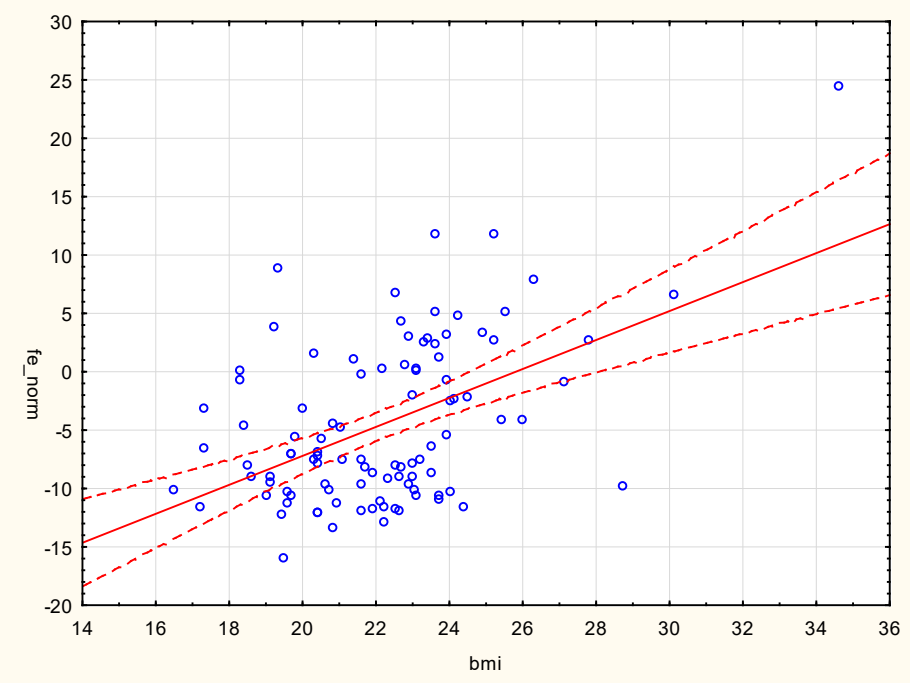

Figure 1. Correlation between iron consumption and BMI

dard deviation of 3.79 mg, while in the group of 35 women with normal menstruations, average iron consumption totaled 9.30 mg, with standard deviation of 3.26 mg.

In the group with heavily menstruating women, iron consumption was consequently slightly higher than in the group of women with normal menses, however this still was an unsatisfactory amount of daily demand.

Chronic diseases and iron deficiency anemia

Among surveyed persons, 16% declared medication use for chronic disease (anti-asthma, anti-allergy, anti-epileptic medications, as well as thyroid hormone replacement drugs).

Among 16 people using medications – 4 individuals (25%) had anemia, whereas among people not taking any medications chronically, 6 individuals suffered from anemia (7.14%) – **Table 3**.

At the significance level of 0.05 it can be stated, that people taking medications because of a chronic illness, statistically speaking suffered from anemia significantly more often.

Correlation between adequate iron consumption and consumption of vitamin C, fiber and calcium

Statistically significant correlation was found between vitamin C as well as fiber consumption and excessive iron consumption. In both cases correlation coefficients were positive, which means, that similarly to higher consumption of vitamin C, higher consumption of fiber was linked to a higher consumption of iron – **Table 4**.

Student knowledge on the subject of iron deficiency anemia

While student knowledge on the subject of iron deficiency anemia was checked, people surveyed were

Table 3. Daily iron, vitamin C, fiber and calcium intake, and correlations between them

	Mean	Standard deviation	r*			
			Fe_norm	Vit C	Fiber	Ca
Excessive iron intake (mg)	3.15	5.49	1.0000	**0.5386** P < 0.05	**0.6078** P < 0.05	0.2371
Vit C (mg)	104.37	90.88	–	1.0000	0.1759	0.2464
Fiber (g)	19.02	7.52	–	–	1.0000	0.1645
Ca (mg)	807.04	336.18	–	–	–	1.0000

** – correlation coefficient*

Table 4. Answers of the surveyed students on the question on a different name of iron deficiency anemia, relative to the university and gender

	MU	ULS	PUT	APE	Females	Males
Microcytic	92%	4%	36%	24%	53%	12%
Megaloblastic	4%	4%	4%	8%	5%	6%
Don't know	4%	92%	60%	68%	42%	82%

asked to pick the correct answer, concerning different name of the same disease entity, from three possibilities: 1) microcytic, 2) megaloblastic, 3) don't know.

The most correct answers (92% – 23 people) were given by questioned students from MU, next from PUT (36% – 9 people) as well as students from APE (24% – 6 people). From ULS as many as 92% (23 people) picked the answer – "don't know." More correct answers were given by women (53% of them) than by men (11.76%).

The next question verifying student's knowledge was to pick five signs/symptoms of anemia, from among nine given choices: brittle nails, constipation, painful tongue, vertebral column pain, hair loss, weakness, vomiting, hand tremor, paleness. To the typical signs/symptoms that can occur with existing anemia, belong: brittle nails, painful tongue, hair loss, weakness, and paleness.

The highest number of correct answers was given by MU students, 64% of them were able to give 4 out of 5 correct signs/symptoms. From APE and ULS majority questioned students were able to give 3 of 5 correct symptoms (52% and 36% respectively). Students from PUT indicated comparably 2 out of 5 and 4 out of 5 correct symptoms (32%, 36%). Not many people questioned were able to indicate all 5 signs/symptoms; respectively MU and PUT (12%), ULS (4%), APE (0%). Women showed higher familiarity of signs/symptoms, because as much as 42% of them were able to give 4 out of 5 signs/symptoms and 9% of them gave all occurring symptoms. Men however mainly were able to indicate 2 out of 5 or 3 out of 5 typical signs/symptoms (41% and 35% respectively). The most frequently chosen signs/symptoms were paleness and weakness.

Summarizing questions concerning student knowledge on the subject of microcytic anemia, we can conclude that students from MU had the greatest knowledge on the subject. It can be also concluded that women possessed the greatest knowledge about anemia. The results are shown in **Table 5**.

Student knowledge on the subject of food sources of heme and non-heme iron

Students were asked to write down at least three products, containing heme iron as well as at least three products, containing non-heme iron. All kinds of meat and meat products for example: beef, pork, poultry, veal, pate, liver, giblets, fish and egg yolk among others, were included as correct answers considering products rich in heme iron, whereas to correct answers considering non-heme iron product sources belonged such products as: grains, whole-wheat goods, oatmeal, bran, as well as beets, green vegetables, string vegetables and cacao.

While working out the results two options were considered; 1) correct answer – when students were able to give 3 correct answers and 2) incorrect answer – when students were unable to give any correct products, or gave only one or two correct food sources.

Correct answer on the subject of sources of heme iron were given by 36% of people surveyed. Among them the most correct answers were given by students from MU (96%), next by students from PUT (36%). As many as 96% of ULS students and 92% of APE students were unable to give correct responses, or gave less than 3 products containing heme iron. Among women the correct answer was given by 48%, whereas among men only 12% gave correct responses.

Table 5. Indication of correct answers on the foods rich in heme and non-heme iron, relative to the university and gender

Heme iron sources	MU	ULS	PUT	APE	Females	Males
The correct answer	96%	4%	36%	8%	48%	12%
Incorrect answer	4%	96%	64%	92%	52%	88%
Non-heme iron sources	MU	ULS	PUT	APE	Females	Males
The correct answer	96%	0%	28%	4%	44%	9%
Incorrect answer	4%	100%	72%	96%	56%	91%

Taking into consideration correct responses about products rich in non-heme iron, MU came out with the best result, because 96% of students were able to give correct answers to this question. 28% of students from PUT and only 4% from APE were able to give at least 3 sources of non-heme iron. No one questioned from ULS was able to give a correct response. The study showed that more questioned women (43%), than men (9%) were able to list at least three products containing non-heme iron fraction of this microelement.

Summarizing the knowledge on the subject of sources of iron, it can be stated that the greatest knowledge of products rich in particular fractions of iron among universities was demonstrated by students from MU whereas with gender consideration, women showed the greatest source of knowledge (**Table 6**).

Iron supplementation formulations

Students were asked about the use of dietary supplements. In the case of a positive answer, they were asked to give the name of the pharmaceutical agent, which gave the possibility of checking if iron was part of the particular preparation.

Majority of students did not use any supplements. The greatest percentage of people that used iron preparations were students from MU (20%) (**Table 7**).

Considering gender, women more frequently than men reached for supplements. Additionally from the conducted studies, the results show that among the 10% of people suffering from anemia – 50% declared the use of iron preparations.

Preparations containing iron in their composition, such as: Sorbifer Durules, Ascofer, Revitaben, Feroplex, Hemofer, Bodymax plus and Centrum, were those which the use of was declared by the surveyed students.

Vitamin C supplementation

In the questionnaire, students were asked about the supplementation of vitamin C, which is one of the factors increasing the absorption of iron from the digestive tract. Most of the students (66%) declared that they do not use any forms of vitamin C preparations.

Supplementation with this vitamin was used by 60% surveyed students from APE. More women, among people asked, consumed vitamin C, as compared to men (**Table 8**).

Results from the conducted studies show that among 10 people declaring consumption of iron containing preparations – 7 people additionally consumed vitamin C.

Consumption of products rich in iron and vitamin C

For the purpose of assessment of frequency of consumed products, a table was created in which students were asked to pick one answer from five choices: 1 = every day, 2 = 1–3 times per week, 3 = >4 times per week, 4 = sporadically, 5 = never, found next to appropriate food group. Taking into consideration iron deficiency anemia, the study mainly focused on the analysis of food groups rich in this mineral component.

Frequency of products consumption, which are the best sources of heme iron, was analyzed. Therefore, the consumption of different types of meats was looked at, such as: pork, beef, poultry, game meat, giblets, fish, seafood. Pork was consumed most frequently 1–3 times per week or sporadically in Poznan universities, where more frequently pork was consumed by men. Beef however was consumed only sporadically and 20% of surveyed declared that it was not part of their diet at all. On the other hand 26% of men declared

Table 6. Consumption of dietary supplements by the responders, depending on the university and gender

	MU	ULS	PUT	APE	Females	Males
Don't take any dietary supplements	68%	80%	92%	68%	73%	85%
Take dietary supplements	20%	4%	0%	16%	14%	3%
Take other supplements	12%	16%	8%	16%	13%	12%

Table 7. Declaration of students about the consumption of vitamin C, depending on the university and gender

	MU	ULS	PUT	APE	Females	Males
Supplementation with vit. C	24%	28%	24%	60%	44%	15%
No supplementation with vit. C	76%	72%	76%	40%	56%	85%

Table 8. Percentage indication of the normal symptoms of anemia, depending on gender

	1/5	2/5	3/5	4/5	5/5
Females	3%	21%	24%	42%	9%
Males	6%	41%	35 %	15%	3%

Table 9. Indication of correct answers on the foods rich in heme and non-heme iron, relative to the university

Heme iron sources	MU	ULS	PUT	APE
The correct answer	96%	4%	36%	8%
Incorrect answer	4%	96%	64%	92%
Non-heme iron sources	MU	ULS	PUT	APE
The correct answer	96%	0%	28%	4%
Incorrect answer	4%	100%	72%	96%

Table 10. Indication of correct answers on the foods rich in heme and non-heme iron, relative to gender

Heme iron sources	Females	Males
The correct answer	48%	12%
Incorrect answer	52%	88%
Non-heme iron sources	Females	Males
The correct answer	44%	9%
Incorrect answer	56%	91%

Table 11. Consumption of dietary supplements by the responders, depending on the university

	MU	ULS	PUT	APE
Don't take any dietary supplements	68%	80%	92%	68%
Take dietary supplements	20%	4%	0%	16%
Take other supplements	12%	16%	8%	16%

Table 12. Consumption of dietary supplements by the responders, depending on gender

	Females	Males
Don't take any dietary supplements	73%	85%
Take dietary supplements	14%	3%
Take other supplements	13%	12%

Table 13. Declaration of students about the consumption of vitamin C, depending on the university

	MU	ULS	PUT	APE
Supplementation with vit. C	24%	28%	24%	60%
No supplementation with vit. C	76%	72%	76%	40%

Table 14. Declaration of students about the consumption of vitamin C, depending on gender

	Females	Males
Supplementation with vit. C	44%	15%
No supplementation with vit. C	56%	85%

that they consume beef even 1–3 times per week. Poultry meat was most frequently consumed from among all the meat types. From 64 to 92% of studied people consumed poultry 1–3 times per week. Game meat could be found on the opposite side of the spectrum. As a meat type it is consumed least frequently by 20–60% of surveyed students or not at all by 72–80% of students. There was an exception with ULS students, 20% of whom declared consumption of game meat 1–3 times per week. Giblets make ULS a meat product group which is consumed by students sporadically – mainly by men. The frequency of fish consumption at the universities varried. Students from MU mostly eat fish 1–3 times per week, while surveyed students from other universities declared sporadic consumption of fish. The least popular was seafood – most students (56–80%) declared that they do not eat them at all. Only women were declaring (34%) that they eat them sporadically.

Consumption frequency of food products containing non-heme iron was also analyzed. These products include: whole-wheat products, string vegetables, group of vegetables such as; spinach, sorrel, beets, rhubarb, cabbage, broccoli, dried fruits, as well as fruit groups such as; bilberry, blackberry, black currents, raspberry, bananas, black grapes. Whole-wheat products were consumed everyday (56–80% surveyed students). Among the questioned people there was no single respondent

who did not consume products from this food group. String vegetables were consumed sporadically – most surveyed women and men (70% and 62% respectively) pointed to their scarce consumption. Products from the vegetable group were most frequently consumed by students from MU – 60% marked that they eat those products 1–3 times per week, while at the other universities the same product group was consumed sporadically – 52% among surveyed students from each university. Dried fruits were also rarely consumed – mainly their consumption was declared by women. The last food group analyzed was the fruit group. People surveyed mainly claimed that they consume them 1–3 times per week, only students from ULS declared that these fruits show sporadically in their diet.

Discussion

In this study the level of consumed iron among Poznan university students was analyzed taking into consideration such factors as: type of university, gender, concomitant diseases. The knowledge on the subject of iron among the respondents was also studied.

Significant differences were observed in the consumption level of iron between universities and between genders. The correct daily iron consumption was observed only in 28% of people surveyed. Excessive iron consumption was seen at ULS (-0.80). Next in order were the following universities: PUT (-4.21), APE (-6.27) and MU (-6.78). It was also shown that excessive iron consumption was more pronounced among men (3.15) then in women (-8.47).

Positive correlation between calorific diet, and iron consumption was found in the analysis. People consuming more calories were supplying their bodies with more iron than people following low calorie diet. Positive correlation was also found between BMI values, and iron consumption. Those surveyed with higher BMI in general consumed larger amounts of iron than people with lower BMI. However, what is interesting is that men with higher BMI consumed more iron, while in women higher BMI translated to its lower consumption, which can be related to adherence to slim diets, as well as to a lower consumption of proteins by females.

Analysis showed that women with heavy menstruations consume a little more iron than women with normal menses.

Studies also showed that chronically ill people suffered more frequently from anemia. In patients with hypothyroidism, development of anemia can be caused by the deficiency of tyrosine, which hinders the synthesis of hemoglobin. Furthermore, medication use alone can be the cause of decreased absorption, not only of iron, but also of folic acid, which predisposes for the development of anemia.

During the study a statistically significant correlation was found between the consumption of vitamin C as well as fiber, and excessive iron consumption. Both alike, higher consumption of fiber as well as vitamin C were linked to a higher consumption of iron in diet. This can be due to the fact that in the student diet besides meat and fish, rich in heme iron, very frequently appeared whole-wheat products, which besides fiber, are a rich source of non-heme iron. Frequently in students' diet also appeared fruits containing this iron fraction and moreover abundant in vitamin C.

Most surveyed (77%) were not taking any dietary supplements, however some (10%) declared consumption of iron containing supplements. Women reached for supplements more frequently, which can be related to their cosmetic or medicinal needs for example, during heavy menstruations. Most students (66%) did not use any vitamin C supplementation. Amongst 10 students consuming iron preparations – 7 people consumed vitamin C.

The greatest knowledge on the subject of microcytic anemia was shown by MU students. They were able to pick the largest correct number of anemia signs/symptoms as well as iron dietary sources. Women possessed the greatest knowledge on the subject of anemia. Nevertheless they supply too little iron with diet.

Analyzing the frequency of consumption of iron rich products, it can be stated that students derive heme iron mainly from poultry meat. This fact can be conditioned by low price of this meat type, easier and faster method of its preparation and gustatory preferences. The least frequently consumed meat is game meat, which can be related to its characteristic taste as well as its scarce market availability.

Non-heme iron is most frequently supplied together with whole-wheat products. Consumption frequency of fruits and vegetables was more or less equal at the particular universities studied. These products are more often consumed by women.

In order to compare the results of this work with other studies, analyses were chosen taking into consideration people studying in secondary schools as well as those in universities.

First of them, conducted by Jeruszka-Bielak et al. included 182 women age 19–26 years old, studying at the Principal School of Rural Farming in Warsaw. The study showed that their nutritional method was inap-

propriate when taking into consideration iron consumption, which was higher in women supplementing this element (11.6 mg/d) than in women not using iron supplementation (9.0 mg/d) [4]. Considering the use of supplements, the study by Sigłowa et al., conducted among 440 students of Warsaw and Tarnów universities showed that 53.1% of student population, mainly women, used vitamin and mineral dietary supplements [5].

In the study of Szczepańska et al., looking at iron consumption among female secondary school students in the Silesia region, inadequate iron consumption (7.2 ± 1.7 mg) as well as low consumption of vitamin C (38 ± 17 mg) was shown in both girls living in cities, as well as those living in the countryside [6].

Differences were also observed in studies by Regulska-Ilow et al., conducted among 198 secondary school students from Oleśnica. Researchers also showed that there was deficient iron consumption among girls (around 10.7 mg/d), while in boys the food ration covered the daily demand for iron [7].

Analysis performed by Marzec et al., on 684 students from Lublin universities, showed a low consumption level of iron in female gender (on average: 9.04 mg at the Medical University, 7.77 mg at the University of Life Sciences and 7.73 mg at the Lublin Catholic University), whereas men consumed iron in quantities satisfying the norm, and sometimes even exceeding it (average: 10.4 mg at the Medical University, 13.1 mg at the University of Life Sciences and 8.87 mg at the Lublin Catholic University). Students taking supplements consumed approximately 3 times more iron and vitamin C in comparison to people not using supplements [8].

Discrepancies in the consumption of iron were observed in the study by Wajszczyk et al. This study included 3304 girls (11–15 years old) and 1855 women (20–23 years old) from Warsaw and vicinity. Higher consumption of iron was noticed as compared to this hereby study – 13 mg in women aged 20–23. It was also found that women living in the city consume more of this element (12.5 mg) than women living in the countryside (11.8 mg) [9].

Adequate level of iron consumption was shown in the study by Socha et al., taking into consideration nutritional methods of female students in Białystok (on average 8.5 mg) [10].

In the study of Bolesławska et al., including 1752 adults of Greater Poland region, it was found that the share of iron in diet was too low in comparison to the recommendation and current norm [11].

Research by Dybkowska et al., was however elaborated differently. The study was conducted on 409 adults living in Warsaw and their average consumption of iron was 10–12.7 mg, which was in accordance with the norm [12].

Analyzing the frequency of consumption of iron rich products, according to Dybkowska et al., products which supplied essential amount of this element included: bread and grain products (30–35%), meat and meat products (25–26%), vegetables (20–21%). The rest of the products supplied around 18–25% of the mineral component [12]. Similar values were also given in the study by Bolesławska et al. [11].

The results of above-mentioned analysis are similar to the results established in this research work, taking into consideration the value of consumed dietary iron. In majority of the studies the level of consumed iron was recognized as inadequate in the student group, especially among women. This can be due to the use of low calorie diets and general women's trend to stay slim. Greater iron consumption among men can be assumed to be conditioned by their greater calorific diet as well as more frequent consumption of meat and meat products. Too little iron consumption is in part caused by inadequate level of knowledge on the subject of anemia.

In Poland programs preventing anemia or population educating campaigns do not exist. Widespread knowledge on this subject would enable rapid recognition of this illness and implementation of appropriate treatment, in particular prophylaxis related to conscious nutrition. A prophylactic action, which can prevent iron deficiency, is the enrichment of such food products as cereal or fruit juices addressed to the groups of increased risk of iron deficiency as well as supplementation with pharmaceutical preparations [2].

Conclusions

1. Majority of the students do not consume adequate amount of iron in reference to the recommended norms for this age group.
2. Men in comparison to women consume more iron derived from the diet, which also stems from men's diet, which is higher in calorific value.
3. Chronically ill patients as well as heavily menstruating women suffer more frequently from anemia than those people who do not take any medications chronically and women with normal menses.
4. Women with heavy menstruations consume a little more iron than those women with normal menses, however these are still inadequate amounts in order to satisfy the demand.

5. Higher consumption of vitamin C and fiber in the diet in general translates to a higher consumption for iron.
6. Students from the Medical University and women have the greatest knowledge on the subject of iron deficiency anemia among the four Poznan universities.

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ORIGINAL PAPER

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Evaluation of dietary supplement use for the improvement of the condition of skin and its appendages

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ABSTRACT

Introduction. The condition of skin, nails and hair depends on a diet. Proper diet ought to include all components necessary to ensure a well-balanced intake of proteins, fats, carbohydrates, vitamins and elements. Dynamic development of the dietary supplement industry offers an expanding range of products aimed to improve the condition of skin, hair and nails.

Aim. To study the issue of using dietary supplements aimed to improve skin and its appendages by men and women (in particular: studying the reason for such supplementation, determining the source of information about the supplements, evaluating the knowledge of the respondents and the effect of the supplements).

Material and methods. The questionnaire-based study was conducted in 2013–2014, in a group of 115 people (65 women and 50 men), aged 16–60 years, who had been using dietary supplements designed to ensure proper appearance of the skin, hair or nails. Statistical analysis was carried out using StatSoft Statistica 10.0 software.

Results. All participants had been using various dietary supplements for at least 4 months prior to the study commencement. 73% respondents declared that their main aim was to improve the condition of the skin, hair, and nails. The main reason for supplementation was the desire to improve the condition of the hair, reported by 48% of women and 64% of men. Both, women (59%) and men (48%) decided to supplement their diet mainly under the influence of the mass media. Considerable improvement in the condition of the skin and its appendages was observed by 79% of the respondents. Adverse effects were reported by 48% of the participants (with 72% concerning gastrointestinal tract).

Conclusions. 1. Information provided by the mass media remains the main cause of purchase and consumption of dietary supplements. 2. Subjective efficiency of supplementation has been confirmed by the majority of the respondents. 3. Approximately half of the studied population reported adverse effects, especially on the gastrointestinal tract.

Keywords: diet, dietary supplement, skin condition.

Introduction

The condition of the skin, nails and hair is largely dependent on a diet. It has been common knowledge for many years that a proper diet ought to include all components necessary to ensure a well-balanced intake of proteins, fats, carbohydrates, vitamins and elements [1–6].

According to the results of a CBOS (Centrum Badania Opinii Społeczneh – Public Opinion Research Center) survey, published in 2010, a vast majority of the Polish population considers themselves to be eating healthy [7]. Meal quality has been the focus of a number of surveys on nutrition and food. Regardless, Polish people

continue to eat large amounts of meat, while fruit and vegetables are still treated as secondary components of everyday diet [8]. Thus, traditional Polish diet does not ensure proper supply of all necessary ingredients [9]. Lack, deficiency or excess or particular elements may result in serious health consequences [10–12].

The mass media have been portraying the feminine and masculine ideal as forever young and beautiful. Thus, an average consumer often associates dietary supplements with health and healthy lifestyle. The Food Act of 25 August, 2006 on food and nutrition safety, defines dietary supplements as "concentrated sources of nutrients or other substances with a nutritional or physiological effect whose purpose is to supplement the normal diet. They are marketed 'in dose' form (...), excluding medicinal products according to pharmaceutical laws and regulations" [13].

Dynamic development of the dietary supplement industry offers an expanding range of products which, according to the producers, improve the condition of the skin, hair and nails [14, 15].

Aim

The aim of the article was to study the reason for using supplements that have beneficial effect on skin and its appendages, to determine the source of information about the supplements, and to evaluate the knowledge of the respondents and the effect of the supplements.

Material and methods

The questionnaire-based study was conducted in 2013–2014, in a group of 115 people (65 women and 50 men), aged 16–60 years, who had been using dietary supplements which, according to manufacturers, have been designed to ensure proper appearance of the skin, hair or nails. The questionnaire was designed especially for the purpose of the study and comprised two parts. The first part allowed to characterize the respondents in terms of age, sex, place of residence, anthropometric indices and diet. The remaining questions referred to their knowledge about dietary supplements, reasons for their use, benefits and safety. The questionnaire mostly consisted of closed questions, with one-choice or multiple-choice answers.

Statistical analysis was carried out using StatSoft Statistica 10.0 software. Crosstabulation tables and Pearson's chi-squared test (with $p = 0.05$ as statistical significance threshold) were used to evaluate statistically significant differences between men's and women's answers.

Results

All participants had been using various dietary supplements for at least 4 months prior to the study commencement. Approximately 73% of the respondents declared that their main aim was to improve the condition of the skin, hair, and nails (**Figure 1**). According to their subjective opinions, the vast majority of the respondents (78%) perceived their diet so far as rational. Therefore, only 9% of the subjects aimed to supplement their diet, while 18% followed recommendations of their doctor, dietician or pharmacist. All respondents reported a pharmacy to be the only place where the supplements were purchased. Use of more than one supplement was declared by 29 (36%) participants.

The main reason for supplementation was the desire to improve the condition of the hair, reported

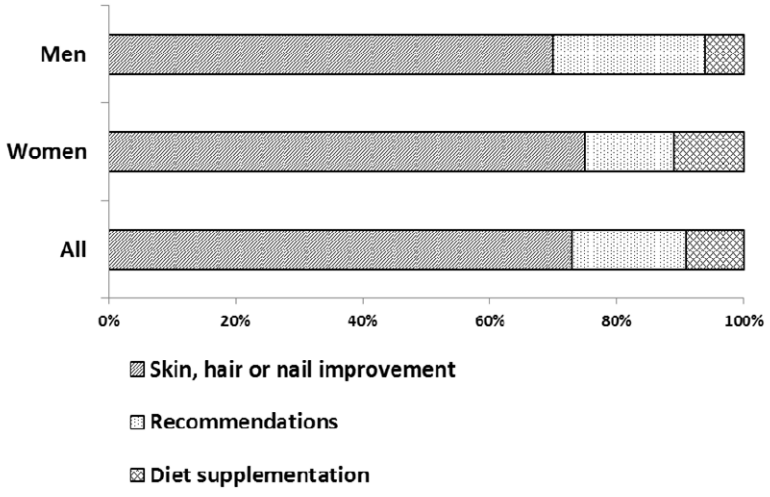

Figure 1. Reasons for supplementation

by 48% of women and 64% of men. The second reason was different in men (improvement of skin – 30% of respondents) and women (improvement of nails – 37%) (**Figure 2**). The differences were statistically significant ($p < 0.001$; **Table 1**).

The respondents used dietary supplements of varying quality and quantity composition. According to the manufacturers, the supplements were combinations of vitamins A, C, E, B_1, B_2 , $B5$, B_6, B_{12}, PP, biotin, folic acid, β-carotene, plant extracts such as bottlebrush, millet, bread wheat, proteins including collagen, elastin, lactoferrin, trace elements: magnesium, iron, selenium, zinc, calcium, iodine, amino acids, mainly cysteine, methionine and fatty acids.

Both, women (59%) and men (48%) decided to supplement their diet mainly under the influence of the mass media: press, radio, TV and Internet. None of the women declared to follow the advice of a dietitian (**Figure 3**).

As far as the definition of a dietary supplement was concerned, 72 (63%) of the respondents were not able to give a correct definition, whereas 37 (32%) did not know it at all. A vast majority of study participants (61%) evaluated their own knowledge about dietary supplements as "average" (**Figure 4**). Women's knowledge appeared to be greater than men's – this difference was statistically significant (< 0.05; **Table 1**).

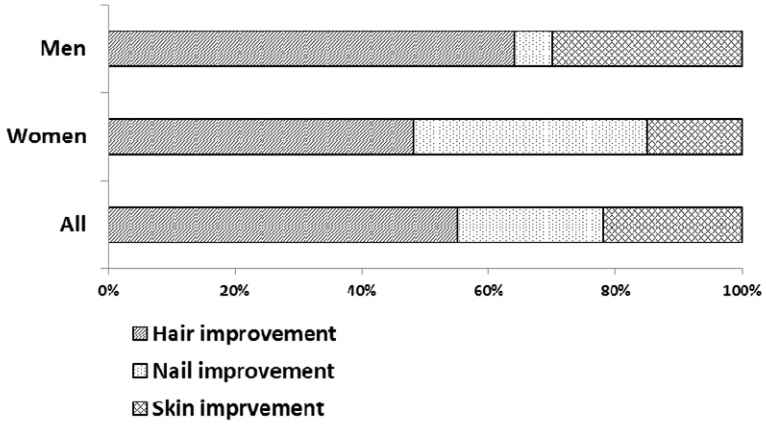

Figure 2. Indications for supplementation

Table 1. Statistical analysis of differences in men's and women's answers

Analyzed parameter	p
Reasons for supplementation	0.2942
Indications for supplementation	0.0004 **
Sources of information	0.0509
Knowledge about the definition	0.0109 *
Effect of dietary supplements	0.4210

*** p < 0.001, * p < 0.05*

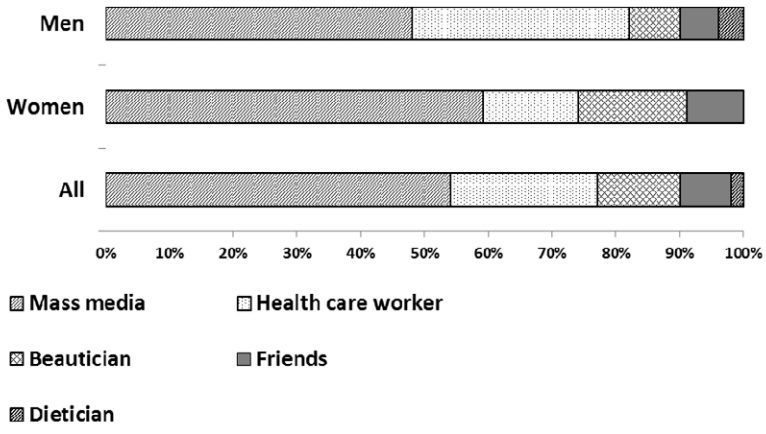

Figure 3. Sources of information that inspired the decision of supplementation

As far as the effects of diet supplementation were concerned, 91 (79%) respondents noticed a considerable improvement in the condition of the skin and its appendages, 20 (17%) found it challenging to assess the efficiency of supplements use, and only 4 (3%) were dissatisfied with these products (**Figure 5**).

The use of dietary supplements according to manufacturer's recommendations was declared by 91 (79%) of the respondents, while 101 (88%) did not exceed the daily recommended dose. A total of 104 (90%) study participants were of the opinion that dietary supplements were entirely safe for their health, basing that view on the conviction that an advertised and nonprescription product cannot possibly constitute a threat to life and health. Thus, only 45 (39%) respondents paid attention to the information in the patient leaflet or the label of dietary supplements. Adverse effects were reported by 55 (48%) participants, with 72% of them reporting gastrointestinal tract complaints (diarrhea, constipation, nausea). Headaches and skin reactions were observed significantly less frequently (7% and 3%, respectively).

Statistical analysis revealed that both men and women gave similar answers. Statistically significant difference was observed only for the question concerning the knowledge about the definition of a dietary supplement (**Table 1**).

Discussion

The range of dietary supplements has been developing dynamically in Poland as well as worldwide. Their diversified composition allows to use the products in different populations – children, seniors or the sick. In 2009 alone, the Chief Sanitary Inspectorate was notified about a record number of market introductions (1900) of dietary supplements [16].

Their development remains to be high, even exceeding the so-called "over-the-counter" medicines. According to experts, ageing society, growing interest

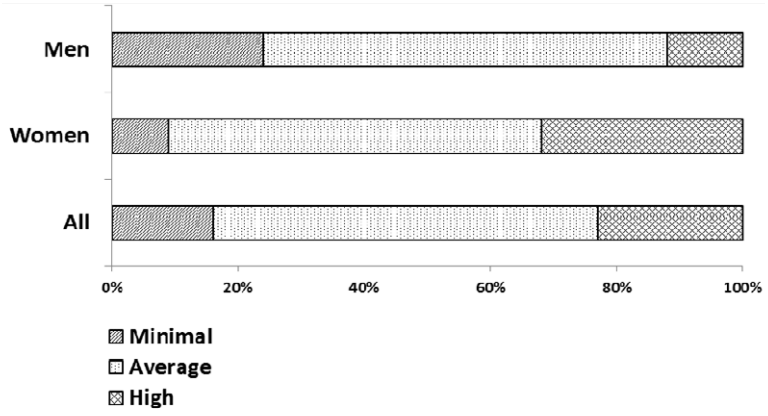

Figure 4. Evaluation of knowledge about the definition of a dietary supplement

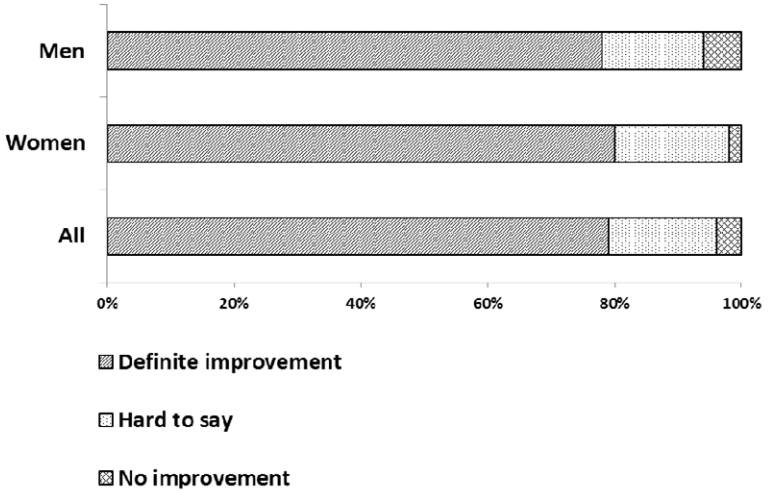

Figure 5. Evaluation of the effect of dietary supplements on the improvement in skin, hair, and nail condition

in the area of self-healing, healthy lifestyle and appearance, as well as ecological and herbal products, will have a positive effect on the sales of dietary supplements [17–19].

An average consumer perceives the pharmaceutical market as a collection of a wide range of products which seem to be medicinal products or are, in fact, medicines. The place where they are purchased remains to be of great importance to the consumers, as well. They consider a pharmacy to be a reliable and safe source, which was confirmed by our findings that over 90% of the respondents found their products to be safe due to the fact they were available in pharmacies. Knowledge and understanding of a definition of a given product was of secondary importance – 32% of the respondents did not know the definition of a dietary supplement, while 63% were unable to recall the correct one, which in no way influenced their self-evaluation of knowledge about the issue. A vast majority (61%) of the subjects subjectively assessed their knowledge about dietary supplements as "average".

Media have played a significant role in shaping attitudes to numerous areas of life, including appearance or eating habits. Press, radio, TV and the Internet remain to be the main source of information, also about medicine-related topics, for many Polish people. Quite often, it is the content of an advertising message that significantly influences the decision whether or not to buy a given product – be it a medicine or dietary supplement. For example, it was confirmed by Conner et al. [20].

Over 50% of study participants identified the mass media as the stimulus behind the decision to use dietary supplements. In light of the fact that dietary supplements are commonly available outside a pharmacy, e.g. in the Internet stores, the process of educating consumers how to distinguish between medicinal products and supplements, or other products, is vital [21].

A great number of people turn to the Internet in search of information on the effectiveness of dietary supplements. Alas, they are limited or scarce when it comes to the possibility of adverse effects. Thus, it is essential to expand the knowledge of the consumers both on the positive and negative consequences of supplementation as well as to educate them about proper food-related behaviors [22].

Statistical analysis showed that for men the most important indicator for starting supplementation was condition of hair and skin while for women – hair and nails. Women also evaluated their knowledge on discussed supplements better than men.

Conclusions

1. Information provided by the mass media remains the main cause of purchase and consumption of dietary supplements aiming to improve the condition of the skin, hair and nails.
2. Subjective efficiency of supplementation has been confirmed by the majority of the respondents.
3. Despite the positive opinion about the safety of dietary supplements, approximately half of the studied population reported adverse effects, especially on the gastrointestinal tract.
4. Statistically significant differences between men's and women's answers were found in issues concerning the reason of supplementation and knowledge on the supplements.

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The use of qualitative approach to detect and assess potential drug-drug interactions which may decrease the effectiveness of oral contraceptives – pilot study and literature review

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ABSTRACT

Aim. The aim of the pilot study was to assess the effectiveness of the tool designed for detecting potential drug-drug interactions of combined oral contraceptives (COCs) with particular emphasis on those which can affect their contraceptive action. A proper study protocol design seems to be essential for further analysis of more data and for establishing correlations between observed interactions and demographic variables.
Material and methods. The cross-sectional descriptive, retrospective study was carried out on Polish females from March to May 2013. Gathered data, including products used concomitantly with contraceptive drugs, were derived electronically by patients and underwent thematic analysis.
Results. Out of 49 respondents who agreed to participate in the study and fit the inclusion criteria only 15 derived qualitative data about other medicinal products they used. However, some of them sent their monthly report more than once, which gave the total of 158 drugs listed in 25 forms gathered during the whole pilot study. Fifty-three potential drug interactions were found, including 13 (24.53%) which could have decreased the effectiveness of contraceptive drugs.
Conclusions. Continuation of the study in accordance with the study protocol will result in identification of common potential drug-related problems, which may enable development of an educational solution for gynecologists, pharmacists and patients increasing their awareness of the potential risk of contraceptive failures and unintended pregnancies.

Keywords: qualitative approach, combined oral contraceptives, drug interactions, contraceptive failures.

Introduction

Combined oral contraceptives (COCs) are one of the most popular methods of preventing unintended pregnancy [1, 2]. Women choose COCs for their easy use, reversibility of fertility, wide access and safety [3–5]. Another important matter is their high effectiveness described with the Pearl index. This ratio was first described by Raymond Pearl in 1933 to present the number of 'failures' observed in groups of contraception users in a one-year time period [6]. Two Pearl indexes are usually defined: one for 'normal use' (contains all pregnancies during the whole time of exposure) and another for 'perfect use' (includes only the pregnancies which occurred during correct and consistent use). For COC use, those ratios are estimated at 8 and 0.3, respectively [7]. Differences in these two values may result from many factors, such as inconsistent use or concomitant use of other drugs. A list of

these substances is very long and includes drugs for a short-term treatment as well as medicines for chronic diseases, and those which are frequent ingredients of commonly used dietary supplements [8]. For this reason special attention should be paid to ensure safety and effectiveness of patients' self-treatment.

Aim

The main study was designed to (i) identify potential drug-drug interactions of COCs, (ii) determine those which can cause a decrease in their effectiveness, and (iii) describe relationships between interactions and different demographic variables, such as age or education level. However, prior to the quantitative analysis, a pilot study was carried out to assess the effectiveness of the designed tool. This publication was prepared to present main results of the initial qualitative assessment and discuss the need for further studies in this area.

Material and methods

Study setting and sample

At the very beginning of the study (December 2012), a paper version of the questionnaire was developed. Four randomly chosen community pharmacies were invited to cooperate in the study. Pharmacy employees were asked to administer the questionnaire with information about the survey directly to those female patients who bought oral contraceptives. To assure respondents' anonymity, an envelope was attached to each questionnaire and a box for filled questionnaires was provided in the pharmacy. Only 23 questionnaires (30.67%) out of 75 prepared ones were distributed to patients and only 3 were filled in and returned, which gave the response rate of 13.04%. That is why the study protocol was revised, and an online method of data collection was designed for the second stage. The appropriate pilot study was carried out from March to May 2013. The sample consisted of females who declared use of oral hormonal preparations for their contraceptive action. Other characteristics, such as age or education level, were used to differentiate respondents within the group. The both study protocols were approved by the Ethical Committee of the Poznan University of Medical Sciences (Poland).

Data collection and analysis

The protocol of the cross-sectional descriptive study involved the use of two questionnaires. Questionnaire A collated sociodemographics and questions related to patients' general health, contraceptive behaviors and sources of knowledge of contraceptive pills. Questionnaire B was designed for respondents to fill in all the products (including drugs, dietary supplements and herbs) which they took during the last month. Prior to the collection of primary data, the literature review was conducted by performing several searches of literature indexed in MEDLINE (PubMed), Web of Science and Google Scholar, to gather information about different types of COC interactions and their clinical significance. Subsquently, data gathered with the use of Questionnaire B underwent thematic analysis and were compiled with those collected during the desk research. Although this pilot study was aimed at assessment of the adequacy of the designed tool and providing qualitative data, collection of a greater number of questionnaires will enable performing also a quantitative analysis and checking the correlation between interactions and different variables, such as education level or number of drugs taken at the same time.

Results

During the 3-month time period of the pilot study, 25 questionnaires B were collected (**Table 1**). However, it should be highlighted that the aim of qualitative

Table 1. General analysis of received Questionnaires B

COC components	Collected questionnaires	Drugs [a]	Potential drug-drug interactions	
			total	affecting COC effectiveness [b]
EE[c], Cyproteronum	2	5	3	1
EE, Desogestrelum	6	36	5	0
EE, Dienogestum	3	34	10	3
EE, Drospirenonum	7	42	16	3
EE, Gestodenum	5	36	17	6
EE, Norgestimatum	2	5	2	0
Total	25	158	53	13

[a] *number of products listed in the questionnaire, including drugs, dietary supplements and herbs, without COC*
[b] *number of drug-drug interactions which can decrease the COC effectiveness*
[c] *ethinyl estradiol*

research was to develop concepts rather than to verify existing theory, that is why statistical representativeness was not considered as a prime requirement [9] and so the number of gathered forms was considered to be sufficient for proper course of this pilot study. Analysis of all potential drug interactions between oral contraceptives and other substances (including drugs, dietary supplements and herbs) was made, focused on those that can decrease the COC effectiveness. A total of 158 drugs was listed in received 25 questionnaires (mean of 6 drugs per questionnaire; range 1–17). Among a total of fifty-three potential drug interactions found (33.54%), 13 are thought to be able to decrease the effectiveness of contraceptive pills (**Table 2**). Another 40 interactions are thought to not affect the COC effectiveness, although they can have other undesirable effects (such as breakthrough bleeding or hyperkalemia) or decrease the effectiveness of treatment of coexisting diseases.

Discussion

The concentration of contraceptive hormones in blood may change due to concomitant use of other drugs. On the other hand, contraceptive hormones may increase or decrease the serum level of other drugs. As mentioned, women using oral contraceptives should be careful about the selection of therapeutic agents (medicines, herbs, dietary supplements), especially when there is a risk of contraceptive failure or other adverse effects [29]. It is also important that each healthcare practitioner involved in designing therapeutic recommendations should have knowledge of other substances taken by the patient.

Pilot study protocol and its limitations

Since potential drug-related problems were detected during the pilot study, the designed tool was considered suitable for identification of drug-drug interactions. However, the study protocol had also a few limitations. First of all, identification of potential interactions did not provide information about their true clinical and/or toxicological consequences. Additionally, even though several potential interactions were detected, investigators could not have reacted in time because (i) the data had been gathered retrospectively, and (ii) investigators had not obtained patients' consents to discuss findings with a respective medical practitioner. That is why another study design, which will take into account ongoing cooperation between a physician, a pharmacist and a patient, is needed. Finally, high differentiation of COC components and the small number of received questionnaires need to be highlighted. Both of these made a valuable statistical analysis impossible. Although pilot study sample seems to be large enough to enable the qualitative exploration of different types of potential drug interactions with COC and reasons of their possible influence increasing risk of contraceptive failures, still obtaining some quantitative results requires gathering and analysis of more plentiful data. Further exploration of data gathered with the use of both Questionnaires A and B

Table 2. Potential drug-drug interactions identified during the study that can decrease the effectiveness of combined oral contraceptives

COC components	Other substances	No. of cases (no. of patients)	Pharmacological effect	Potential therapeutic outcomes
EE*, Gestodenum	spiramycinum	1 (1)	changes in EE enterohepatic circulation [10–13]	decrease in the COC effectiveness in case of high sensitivity of the intestinal flora to antibiotics; diarrhea or vomiting during antibiotic therapy [10–16]
EE, Gestodenum	norfloxacinum	1 (1)		
EE, Drospirenonum	clarithromycinum	1 (1)		
EE, Drospirenonum	azithromycinum	1 (1)		
EE, Cyproteronum	Hypericum herba	1 (1)	low doses – no changes in COC pharmacokinetics [17]; high doses – induction of CYP3A4 and P-glycoprotein transporter; effect on the metabolism of steroid hormones [18–22]	low doses – lack of adverse drug reactions [17]; high doses - breakthrough bleeding, changes in bleeding time [18–22]
EE, Gestodenum	Sennae folium Aloe capensis Frangulae cortex	3 (1)	decrease in EE absorption and shorter time of detectable COC levels in blood [23, 24]	lower COC effectiveness [23, 24]
EE, Dienogestum	dietary fiber	3 (1)	decrease in EE absorption resulting in a lower EE serum concentration; inhibition of enterohepatic circulation; accelerated COC excretion [25–27]	lower COC effectiveness [25–27]
EE, Gestodenum		1 (1)		
EE, Drospirenonum	carbo medicinalis	1 (1)	COC adsorption, interrupted EE enterohepatic circulation, decrease in the EE concentration in blood [28]	lower COC effectiveness [28]

** ethinyl estradiol*

is thought to be essential in order to form the basis for more complex findings such as establishing correlations between observed interactions and different variables like age, education level, comorbidities, duration of COC use or even sources of knowledge of contraceptive pills on which patients rely. In addition, further studies with the use of the study protocol described above will allow to determine the most common drug-related problems for different groups of patients and help avoid them by dissemination of necessary knowledge among healthcare professionals and COC users.

State of art

During the pilot study, 53 potential drug-drug interactions were found. In order to check which ones can affect the COC effectiveness and determine the risk of its clinical importance, a literature review was performed.

Antibiotics

Our study reported patients' use of macrolide antibiotics (spiramycin, clarithromycin, azithromycin) and fluoroquinolones (norfloxacin). The literature contains a lot of controversy about the impact of antibiotics on the effectiveness of combined hormonal contraception. The data provided by Dinger et al. showed that among 1634 women who had an unplanned pregnancy while taking oral contraceptives, every fifth case reported a concomitant use of antibiotics [14]. However, it should be noted that another study, conducted on a group of 578 patients, showed a similar number of contraceptive failures in a group (a) of only women taking oral contraceptives, and (b) receiving concomitantly COCs and antibiotics [30].

Macrolides

Recently conducted studies showed no increase in contraceptive failures in connection with the concomitant use of COCs and macrolide drugs (including clarithromycin and azithromycin) [30–34]. These drugs do not cause a decrease in the effectiveness of a COC compound or even raise the concentration of hormones in blood [10]. Although one case of pregnancy during a COC and minocycline use was documented, the authors concluded that the risk of unintended pregnancy is small but still real [35]. One case of pregnancy have been reported in a COC user who concomitantly received therapy with spiramycin [36].

Fluoroquinolones

Studies on the effects of fluoroquinolone use on low-dose contraceptives showed no change in the concentration of ethinyl estradiol (EE) in blood, neither affecting the effectiveness of the drug. The results for the group treated with antibiotic therapy and placebo were comparable and showed clinically insignificant differences [37–40]. No studies investigating the concomitant use of COCs and norfloxacin were found.

Given the above, the study shows that there are no systemic interactions of macrolides and fluoroquinolones with oral hormonal contraceptives, or lower levels of EE or progesterone in blood. According to the latest guidelines of the World Health Organization (WHO), additional contraceptive methods are not required in case of a concomitant use of combined hormonal contraception with non-liver enzyme-inducing antibiotics [15]. However, the bioavailability of EE as a component of COCs can be lower for some women, which may be due to: elevated steroid hormone metabolism in the liver and intestinal mucosa, changes in the enterohepatic circulation, a borderline low EE level in blood and/or special sensitivity of the intestinal flora to antibiotics [10–13]. This situation, as well as occurrence of adverse effects of antibiotic use – diarrhea and vomiting, may reduce the effectiveness of oral contraceptives, and requires special care [10, 16].

St. John's wort

One case of the use of a formulation containing St. John's wort extract was reported. St. John's wort (Hypericum perforatum L.) is a herbal remedy used mainly to reduce the symptoms of mild depression. Studies and case reports conducted so far showed a decrease in the half-life of EE, and thus a significant reduction in the serum level of contraceptive hormones during concomitant therapy with St. John's wort and COCs. A higher number of breakthrough bleedings during the menstrual cycle, changes in the time of bleeding, and unintended pregnancies were observed among volunteers [18–22, 41]. Researchers agree that St. John's wort extract can affect liver enzymes involved in the metabolism of contraceptive hormones, which leads to a decrease in the serum concentration of COC components and a significant reduction in contraceptive effectiveness [18–22, 41]. Analyzes conducted on patients receiving low doses of St. John's wort extract (500 mg per day) showed no changes in the pharmacokinetics of EE and progestogens, but the study was limited by its small size [17]. Noteworthy, even though Pfrunde et al. showed impact of St. John's wort only on the progestogen component, they observed an increased number of breakthrough bleedings which may result in a higher risk of unintended pregnancies [42].

St John's wort, as an inducer of liver enzymes, exhibits a high degree of interaction with other drugs. According to the British Commission for the Safety of Medicines (CSM) and the Clinical Effectiveness Unit (CEU), women taking oral hormonal contraceptives should avoid the concomitant use of COCs and products containing Hypericum perforatum, and, if necessary, use it with great caution [43].

Laxatives

Anthranoids

Herbal laxative medicines containing anthranoids (Sennae folium, Aloe capensis, Frangulae cortex) irritate nerves and thus stimulate peristaltic movements of the colon [24]. Recent evidence suggests that the use of anthranoid laxatives reduces absorption of EE and decreases the time of detectable COC levels in blood [23, 24].

Our study reported 3 cases of potential interactions with products containing anthranoids. As its pharmacological effect can occur for four hours after taking a laxative preparation, an interval between taking laxatives and COCs is recommended. If not, it is reckoned that a COC pill may not be absorbed completely [44].

Dietary fiber (Glucomannan)

Glucomannan is widely used in medicine and patients' diet, however, it may modify properties of orally administered drugs. Studies on rabbits showed that intake of dietary fiber leads to a decrease in EE absorption and may result in ineffective EE concentrations in blood. However, it also demonstrated some beneficial effects of dosage form (enteric capsules) on EE bioavailability [26, 27]. Another trial proved a reduction of EE enterohepatic recycling in women living on a high-fiber diet [25].

Four cases of the use of a formulation containing dietary fiber was reported. It is recommended to administer COCs 2 hours after or 1.5 hour before consumption of dietary fiber [45].

Adsorbing drugs

Activated charcoal (Carbo medicinalis), as an adsorbent, inhibits estrogen conjugates' absorption which leads to interruption of EE enterohepatic circulation. As a consequence of the above, a decrease in its concentration in blood and lower effectiveness of COCs can be observed [28].

One case of the concomitant use of Carbo medicinalis and a COC was noted in the study. A two-hour delay is recommended between the use of activated charcoal and taking a contraceptive pill [28].

Perspectives

The pilot study confirmed that the designed tool can be effective for identification of some potential drug-related problems. It can be seen that analysis of even a small number of drug lists collected with the use of questionnaire B can provide information about the presence of a potential 'COC-other drug' interaction. Further studies on a higher number of respondents are needed to ensure high-quality statistical data and assess the scope of the problem and to list the most frequent potential interactions.

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Conflict of Interest

None

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ORIGINAL PAPER

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Medical students' awareness of radiation exposure related to radiological imaging procedures

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ABSTRACT

Introduction. Current advancements in fields of medical sciences resulted in an increase of imaging examinations with a use of ionising radiation. Such increase leads to justified concerns about its possible consequences.
Aim. Assessment of awareness and level of knowledge of medicine students in terms of ionising radiation
Material and methods. Statistical analysis of 207 surveys of medical students from University of Medical Sciences in Poznan (UMP), Poland with use of Statistica software
Results. There was no significant difference between the results of the assessment related to gender, year of studies or attendance to radiology classes.
Conclusions. Students level of knowledge about the ionising radiation was unsatisfactory. A change in training program can be a benefit for patients and physicians.

Keywords: medical students, ionising radiation, radiological protection.

Introduction

The advancement and development of imaging procedures involving ionising radiation, growing availability of CT (Computed Tomography) procedures, angiography (DSA – Digital Subtraction Angiography), X-Ray tests resulted in an increase of imaging examinations in everyday clinical practice [1]. Such increase refers especially to CT examinations, the number of which significantly grew in last 10 years [2]. Despite of direct benefits for every patient due to the CT, X-Ray or DSA procedures, unjustified use of these raises concerns and controversy about the possibly higher risk of subsequent neoplasms [2, 3], especially while exposure of the pediatric patients to the ionising radiation is concerned [3]. Patients who undergo series of examinations with the use of X-Ray as a standard part of procedures in various clinical units also belong to a higher risk group, in terms of induced neoplasms.

The influence of the radiation on the tissues leads to a change of their cell structure, particularly when the DNA chain sequence is damaged. It is closely related to the gene or chromosomal mutation, which might result in a multistage development of the neoplasm [4]. It has been proved that the radiation itself might cause a kind of genetic instability in cells, which enhances the risk and rapidity of mutations and other genetic changes after the multiple cellular reproductions. Further proofs indicate the fact that in a cytoplasm exposed to radiation the pace of mutations is significantly increased. Genetic aberrations, including induction in genome mutation and changes of gene expression might also occur in newly created cells that were not directly irradiated [5].

Effective dose characterises the degree of whole body exposure to radiation even upon irradiation of only certain parts of the body. The dose varies and

depends on the examination technique (CT,CR – chest X-Rays, DSA), age of the patient and time of the exposure.

Carcinogenesis is one of the stochastic effects of the radiation with using X- Rays, and appears with a specific probability, which means that it can, but it does not have to occur. There is no trigger dose of ionising radiation, at which the process of carcinogenesis happens. However, the higher is the exposure dose, the higher is also the probability of carcinogenesis, minding the fact that it may occur many years after exposure. It is also thought that the danger of ionising radiation at the early stages of life increases the risk of cancer [2, 4, 6].

Justified concerns related to a high level of radiation exposure, especially increased lifelong risk of cancer encourage to seek solutions on an international stage. This problem was shown to the EURATOM community, which issued a set of recommendations, inter alia including aspects of radiological protection in training program at medical schools [7].

Until today many researches assessing knowledge of clinicians of different specializations about the ionising radiation and its risk of further consequences were conducted. However, the results were unsatisfactory [8–11].

It seems that the majority of physicians, despite completed training, does not have sufficient knowledge about the ionising radiation and doses acquired by the patients who undergo commonly made CT, DSA and X-Ray procedures.

Lack of knowledge about the extent of exposure of the whole organism to the ionizing radiation becomes significantly important, when the number of patients who undergo incorrect or repeated examinations is taken under consideration [8–11].

Although publications referring to the state of medical students' knowledge of radiology already exist [7], we decided that a specific area of awareness among the UMP students concerning doses of radiation related to different imaging examinations and assessing the risk of neoplasm development induced by the radiation requires detailed study.

Aim

The assessment of awareness and knowledge of the students related to exposure to radiation associated with imaging diagnostic procedures and radiation doses acquired by patients undergoing examinations with a use of ionizing radiation.

Material and methods

Students participating in a Radiologic Scientific Club prepared a survey (**Figure 1**) which included one-choice questions about doses of radiation accompanying different radiological procedures. Participants were asked to fill the surveys anonymously and answer every question on their own. The survey consisted of two sections. The first section included basic information about the participants: year of medical studies, gender, attending radiology lecture. The second one was a set of 13 questions prepared to assess the level of both awareness and knowledge about the radiation. Correct answers in the sections were awarded by 1 point for each question, whereas incorrect – by 0 points. The maximum score was 13 points. All participants agreed to take part in a survey. The results were presented regarding gender, studying year, attending radiology lecture.

We compared the accuracy of the answers in the section 2 of the survey between the following research groups: men vs. women, 1 year of students vs. other years, 3 year of studies vs. other years, 4 year of studies vs. other years, 5 year of studies vs. other years, 6 year of studies vs. other years, students before radiology lectures vs. students after attending radiology lectures. We also analysed the correlation between the year of studies and number of correct answers in the test. We examined the correlation between: gender and accuracy of answer to each question, year of studies and accuracy of answer to each question, radiology lecture attendance and accuracy of answer to each question.

Statistical analysis was made with a use of Statistica software. In order to assess the normal distribution a Student's t- test for independent samples was used (assuming the value of p lower than 0.05 as statistically significant). For the trials not showing a normal distribution a U Mann-Whitney test was used. Spearman's rank correlation coefficient was used for correlation tests.

Results

207 students from Poznan University of Medical Sciences took part in a survey research. The group consisted of 141 women and 66 men, radiology lecture attendance was declared by 110 participants, 97 students were before the radiology course. Among the students there were 18 from the first year, 21 from the second, 39 from the third, 69 from the fourth, 39 from the fifth, 21 from the sixth year.

We kindly ask you to complete the following anonymous survey. The results shall verify the awareness of medical students regarding radiation doses associated with diagnostic imaging procedures and radiation protection. They will be the basis of a scientific study, so we count on your honesty. This is not a test for the mark! On behalf of the research team, thank you very much for your time.

1. Gender

F M

2. Field of study.

M.D. program D.D.S program

3. Year of study.

I II III IV V VI

4. Did you have classes in radiology?

Yes No

5. Who bears higher risks associated with ionizing radiation procedures?

a child an adult it is the same in both

6. Who bears higher risks associated with being subjected to radiological examination of the chest?

female male it is the same in both

7. What % of diagnosed tumors per year may be associated with the CT study?

0.1-0.2% 0.5% 1–2% around 5%

8. What is the radiation dose associated with chest radiograph?

0.01 mSv 0.1 mSv 1 mSv 11 mSv 20 mSv

9. For comparison, how many times is the radiation dose higher in the following studies compared to chest radiograph?

Chest CT	7x	50x	70x	100x
Head CT	10x	20x	50x	100x
Abdomen CT	10x	20x	40x	100x
Mammography	1x	7x	20x	50x
Bone scintigraphy	20x	40x	80x	100x
Coronary angiography	50x	80x	100x	200x

10. What is the dose limit to the whole body for the general Polish population (excluding people vocationally exposed to ionizing radiation) ?

1 mSv/year 5 mSv/year 10 mSv/year 20 mSv/year 50 mSv/year

11. What is the dose limit for people vocationally exposed to ionizing radiation classified as A category in Poland?

1 mSv/year 5 mSv/year 10 mSv/year 20 mSv/year 50 mSv/year

12. What is mean dose due to natural background radiation?

0.3 mSv 3 mSv 30 mSv 300 mSV

Figure 1. An anonymous survey assessing the awareness of medical students about doses of radiation related to imaging examinations and radiological protection

The results of statistical analysis were as followed: no significant statistical difference was found in the number of correct answers in terms of gender (p-0.23) (**Figure 2**), as well as attendance to radiology classes (p-0.14) (**Figure 3**). No strong difference was also found between the students from the first year of studies and older ones. There was also no correlation between the year of studies and the average number of points (**Figure 4**).

The least difficult question was this with a number 5 (93.7% of correct answers) – majority of participants pointed to a higher radiation risk among children. Little more than a half of participants answered correctly to a question concerning a higher radiation risk in terms of gender of patient undergoing chest X-ray examination. In question number 7 percentage of correct answers was 25.6. Only 35.7% of students indicated a dose of 0.1 mSv acquired during a chest X-ray and because of that, question number 9 was also troublesome, especially a) point – 94.7% of participants underestimated an effective dose acquired during a chest computed tomography compared to chest X-Ray. Slightly better was the percentage of correct answers for question 9b, 9d and 9e – consequently 30, 38 and 29% of correct answers. Moving onto questions 9c and 9f – related to abdomen computed tomography and coronary angiog-

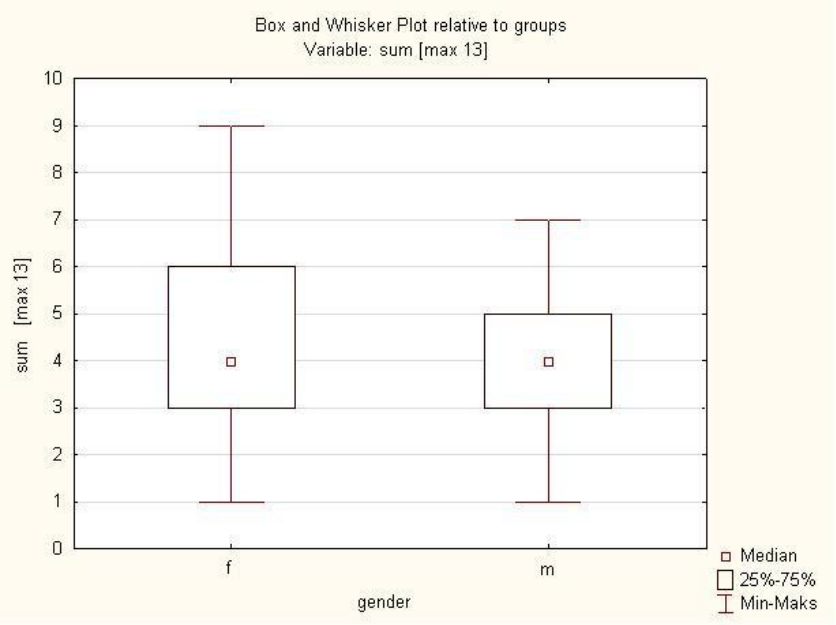

Figure 2. A graph of average number of points depending on gender, with a use of median percentile range (25–75) of minimum and maximum number of acquired points

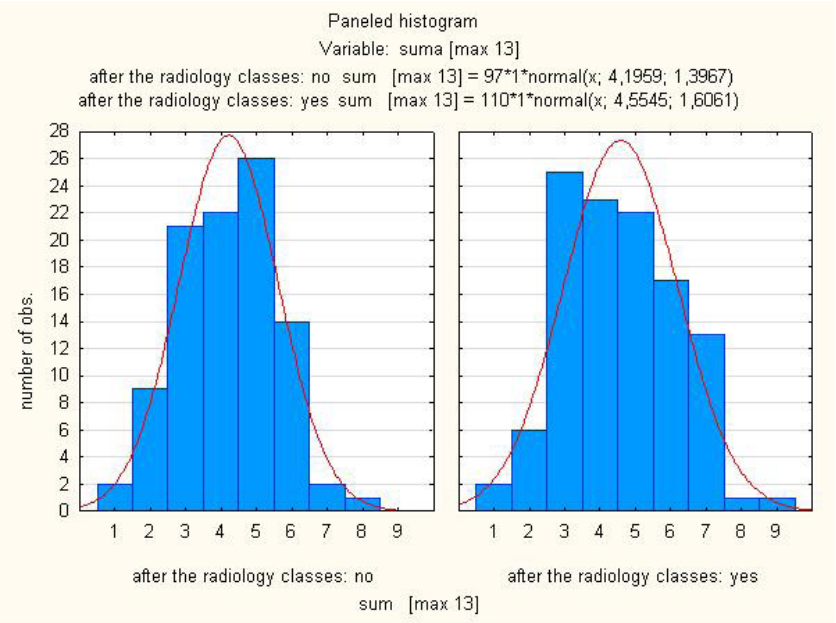

Figure 3. Visualisation of the amount of points obtained in the group before and after radiology and diagnostic imaging classes

raphy, respectively 18.8 and 11.6% of participants gave correct answers. Only 15% of answers where correct, when it comes to question about a trigger dose for total population in Poland, whereas question about such dose for people vocationally exposed to radiation was answered correctly by 27.5% of students. A value of an average natural background radiation was correctly stated by less than 40% of students.

Discussion

The use of ionizing radiation in imaging diagnostics requires experience and decent knowledge about the radiation dose related to all different radiological procedures.

In answer to increasing number of imaging examinations in everyday diagnostics particular impact on

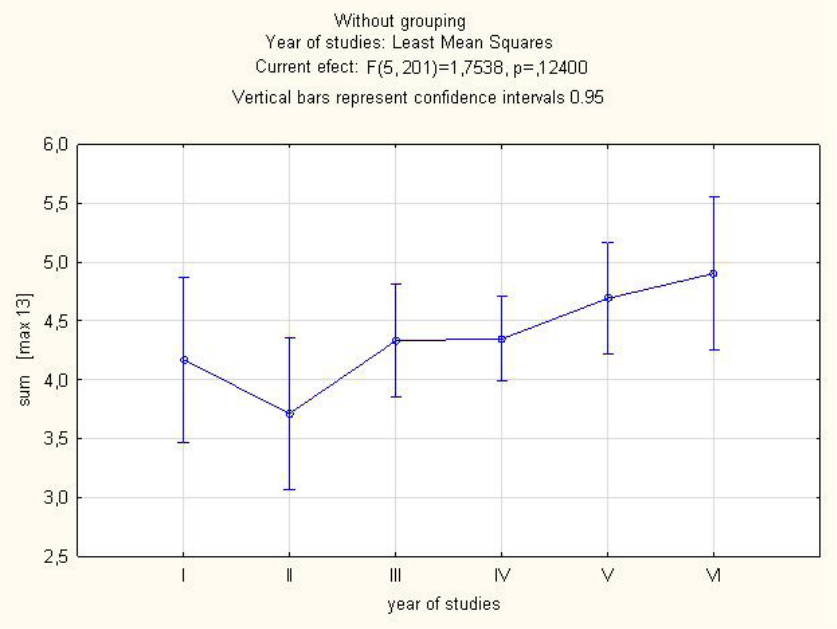

Figure 4. A graph of correlation between the year of studies and the average points scored

acquiring knowledge about the protection against radiation and its law regulations by students of medicine during basic radiology course seems to be reasonable. The level of knowledge expected from students of medicine should represent the current state of cognizance of lecturers. Still, multiple concerns about the knowledge of doctors in this topic already exist.

This gap in knowledge should be taken into consideration while preparing a curriculum for medical studies. Single researches stating a core curriculum for radiology studies has been already made [12]. By providing a clear, reachable curriculum, researches quoted before are a valuable source of information helping in developing education about the radiological protection [12]. The results of our study indicate the fact that the awareness about radiological protection among the medical students should be taught in the first place in order to improve the knowledge of future clinicians.

The fact, that no significant statistic difference in a level of knowledge of students in a group before and after radiology lecture attendance was detected might be related to the amount of material that must be learned by student during the course. According to international data [7, 13] an additional course in radiological protection and radiation risk significantly improves the knowledge of medical students in this topic. Increasing accessibility of medical information for patients and their caretakers as well as law regulations concerning professional liability should be an incentive for teaching students and physicians.

The survey, prepared by students of the fifth year participating in additional radiology courses as a part of Radiological Scientific Circle, was an outcome of a strong will to draw attention to problems due to the escalation in number of imaging examinations performed without proper understanding of the side effects of such procedures.

Conclusions

Our research has shown a poor knowledge of radiation risk among the students of medicine, regardless of gender, year of studies or an already finished course in radiology. Modifying the training program in terms of ionising radiation risk and choice of other rayless imaging techniques might be beneficial for both patients and future physicians.

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REVIEW PAPER

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Long QT in stunned myocardium: unrecognised cause of acquired long QT syndrome

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ABSTRACT

Long QT syndrome (LQTS) is a heart disorder characterized by a prolongation of the QT interval on ECG and a predisposition to ventricular tachyarrhythmias, which may lead to syncope, cardiac arrest or sudden cardiac death. This condition may be inherited or induced by external factors such as drugs, electrolyte imbalances and some acquired cardiac diseases. The review addresses LQTS caused by acute cardiac illnesses which are associated with a large amount of stunned myocardium, i.e. the reperfused myocardial infarction and the group of stress-related cardiomyopathies. In these cases, specific ECG evolutionary changes may be observed, i.e. dynamic deep T-wave inversion and QT interval prolongation which predispose to fatal polymorphic ventricular tachyarrhythmia, i.e. torsade de pointes. However, lethal arrhythmias are relatively rare in these instances and probably concern patients with an underlying predisposition to LQTS. The pathological mechanisms of both repolarization abnormalities and ventricular arrhythmias as well as the practical approach how to interpret electrocardiographic changes and identify high risk patients are discussed in this review.

Keywords: long QT syndrome, myocardial infarction, takotsubo cardiomyopathy, stress related cardiomyopathy, torsade de pointes.

Long QT syndrome (LQTS) is a heart rhythm disorder which is characterised by prolonged ventricular repolarization (reflected by the QT interval prolongation on ECG) predisposing to a high risk of ventricular tachyarrhythmias, syncope and sudden cardiac death [1]. The disease may be an inherited condition, however, it can also be induced by external factors, such as drugs, electrolyte imbalances and some cardiac diseases [1, 2]. The acquired forms of LQTS are much more prevalent. Yet, acute heart diseases are seldom regarded as a potential cause of LQTS since their clinical features and risk of most common complications usually draw more attention than the concomitant QT interval prolongation [1–4]. In this context, it is crucial to be aware that some acute illnesses associated with myocardial stunning may lead to LQTS. Typical examples of such scenarios are the reperfused myocardial infarction and the group of stress-related cardiomyopathies – the latter covers takotsubo cardiomyopathy and transient left ventricular dysfunctions associated with intracranial events (especially subarachnoid haemorrhage), pheochromocytoma, exogenous catecholamine administration or severe acute conditions usually treated in intensive care units (e.g. sepsis) [3–6]. All these diseases have similar cardiac features, i.e. transient contraction abnormalities (presumably stunned myocardium) and specific repolarization ECG changes such as dynamic deep T-wave inversion and QT interval prolongation, which may predispose to fatal polymorphic ventricular tachyarrhythmia, i.e. torsade de pointes (TdP) [3–13]. The described ECG changes, known as Wellens' ECG pattern, were originally reported in the setting of acute coronary syndrome caused by subocclusion of the left anterior descending coronary artery. However, this ECG pattern can also be seen in the aforementioned clinical conditions associated with stunned myocardium (**Figure**

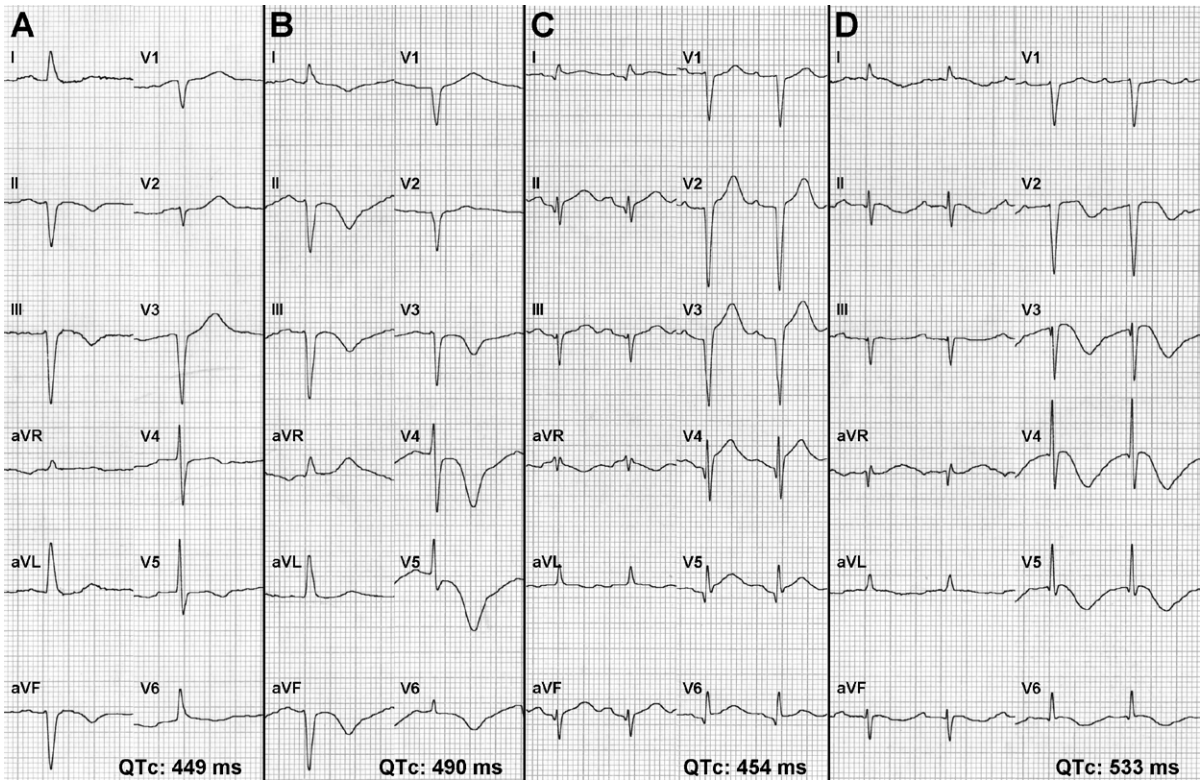

Figure 1. Wellens' ECG patterns in patients with ischemic or non-ischemic heart diseases are shown: ECG recordings taken from a 76-year-old man with a critical left anterior descending coronary artery stenosis, on admission (A) and two days later (B); ECG recordings of a 77-year-old woman with takotsubo cardiomyopathy, on admission (C) and on another day (D). In panels B and D, deep T wave inversions with QT interval prolongations can be seen mainly in precordial leads – such dynamic repolarization changes are called as Wellens pattern. In the both cases, left ventricular contraction abnormalities corresponding to stunned myocardium were observed

1) [14–16]. Some recent reports indicate that myocardial oedema rather than systolic dysfunction is responsible for such repolarization abnormalities (i.e. Wellens' pattern), regardless of ischemic or non-ischemic origin [16, 17]. Studies with cardiac magnetic resonance have demonstrated that repolarization disturbances in these conditions have much closer timely association with myocardial oedema than with contraction abnormalities. It is possible that interstitial oedema may create intramyocardial repolarization inhomogeneity, i.e. either transmural (between endocardium and epicardium) or regional (apico-basal) repolarization gradient, and lead to the QT interval prolongation [16, 17]. Another potential factor which may play a role in the pathogenesis of these electrohysiological changes and ventricular arrhythmias is the sympathetic nervous system activity. Catecholamines can induce early afterdepolarizations and triggered activities in cardiomyocytes which are the arrhythmic stimuli for TdP [18,19]. The catecholamine-mediated myocardial toxicity is regarded as a key causative factor of the ventricular dysfunction in stress-related cardiomyopathies [6,20]. Lastly, an increased catecholamine release can be detected in patients with myocardial infarction [20].

The time course of the ECG evolutionary changes is similar between myocardial infarction and stress cardiomyopathies [7, 15, 21]. The initial ST segment changes usually resolve within 3 days, simultaneously, T waves become inverted and deepen along with QT interval prolongation. Then T waves become shallow for a few days and can deepen again after approximately 2–3 weeks. As the T wave deepens, the QT interval prolongs but it shortens when the T wave becomes shallow – usually after approximately 6 months, the ECG returns to normal in most of the patients (**Figure 2**) [7]. It is difficult to define which pathophysiological mechanism contributes to the second T-wave inversion and QT interval prolongation – a recently postulated concept of a cause-effect relationship between transient myocardial oedema and repolarization abnormalities may not explain this biphasic course of the T-wave and QT interval changes.

Torsade de pointes is, however, relatively rare during the recovery phase after myocardial infarction and in stress cardiomyopathies, and it probably affects patients with some underlying predisposition to LQTS [3–5, 22] – such patients may present so called 'reduced repolarization reserve' [23]. The term 'repolar-

ization reserve' assumes that normal cardiac repolarization depends on the interplay of multiple ion currents and thus there is some redundancy, or 'reserve', to protect against excessive QT interval prolongation by external factors (e.g. drugs). Lesions in these repolarizing mechanisms may be subclinical but they increase the risk of TdP on the exposure of factors lengthening the QT interval [23].

In the study by Halkin et al, 1.8% of patients with acute myocardial infarction developed TdP during electrocardiographic evolution, i.e. when their QT interval prolonged [5]. It is noteworthy that patients with TDP presented significantly longer corrected QT (QTc) interval than those without TdP – on average 558 ms vs. 492 ms [5]. The prevalence of ventricular arrhythmias is also low in stress-related cardiomyopathies – i.e. in 180 cases of takotsubo cardiomyopathy, the incidence of ventricular arrhythmias was only 1–1.5% [22, 24]. However, patients with takotsubo who suffered from TdP presented significantly longer QT intervals than those without TdP [22]. Thus, the excessive QT interval prolongation during the time course of ECG evolutionary changes may be a sign of predisposition to TdP (i.e. reduced repolarization reserve). Therefore, it is reasonable to observe the QT interval in individuals with electrocardiographic evolution and if the QTc prolongs more than 500 ms, measures should be taken to monitor cardiac rhythm and to prevent or appropriately treat TdP [22].

It is also worth noting that a specific feature of T waves may help to identify high risk patients, namely, a double-T-wave appearance, also known as 'notched T waves', T-wave 'humps', or 'pathologic U waves' are associated with a high risk of TdP, especially if the second component of the T wave is higher than the first one (**Figure 3**) [25]. Such giant T-U waves may reflect early afterdepolarizations [26].

To summarise, in our clinical practice it is critical to realize that beyond drugs and electrolyte imbalances also certain acute diseases associated with

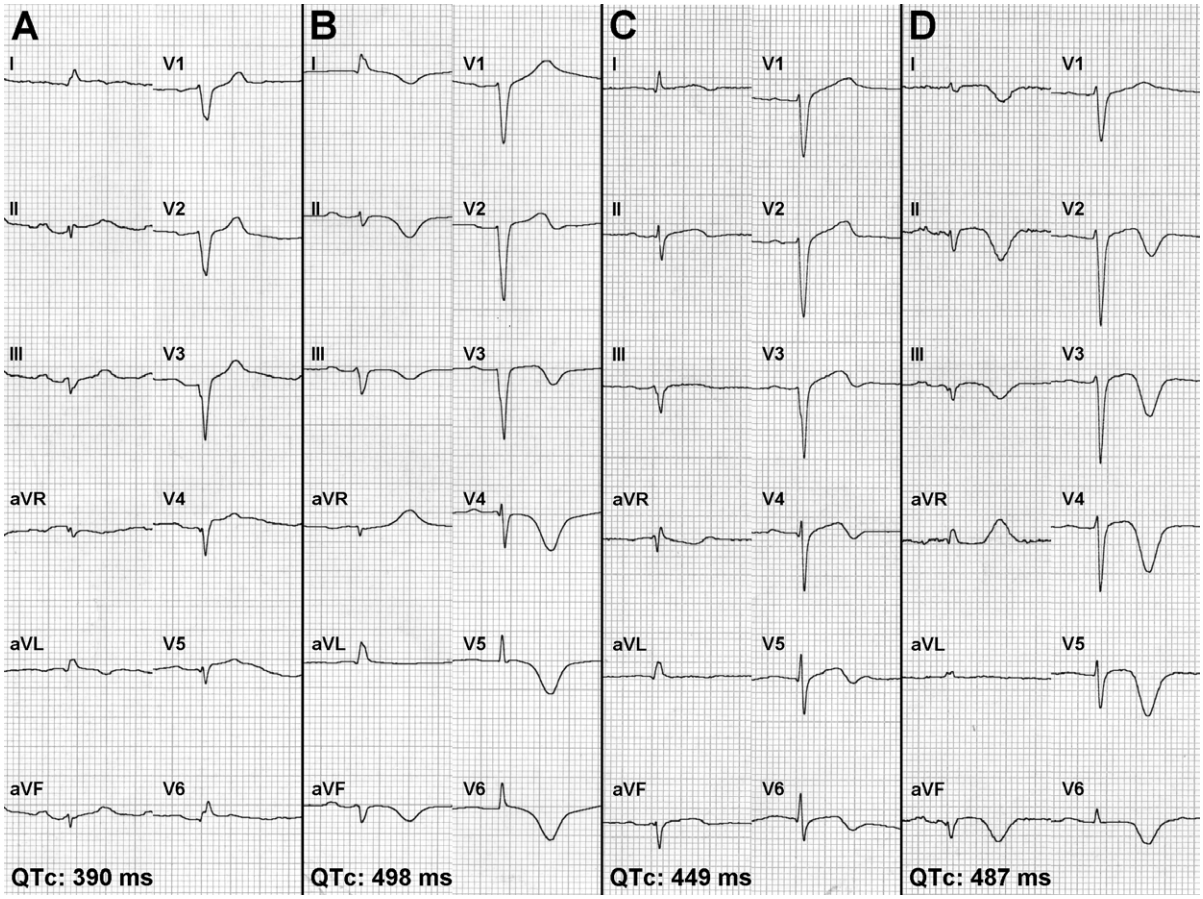

Figure 2. The ECG evolutionary changes in the course of takotsubo cardiomyopathy in a 68-year-old woman are depicted: ECG on admission, minimal R waves with almost no progression in precordial leads V1-V5 (A); ECG after 2 days, deep T waves inversion with QT intervals prolongation (B); ECG after 5 days, partial normalization of the repolarization changes (C); ECG after 13 days, the second T waves inversion along with QT intervals prolongation (D). Myocardial oedema is supposed to be responsible for the first repolarization changes (panel B), however, it is unknown what causes the second abnormalities (panel D)

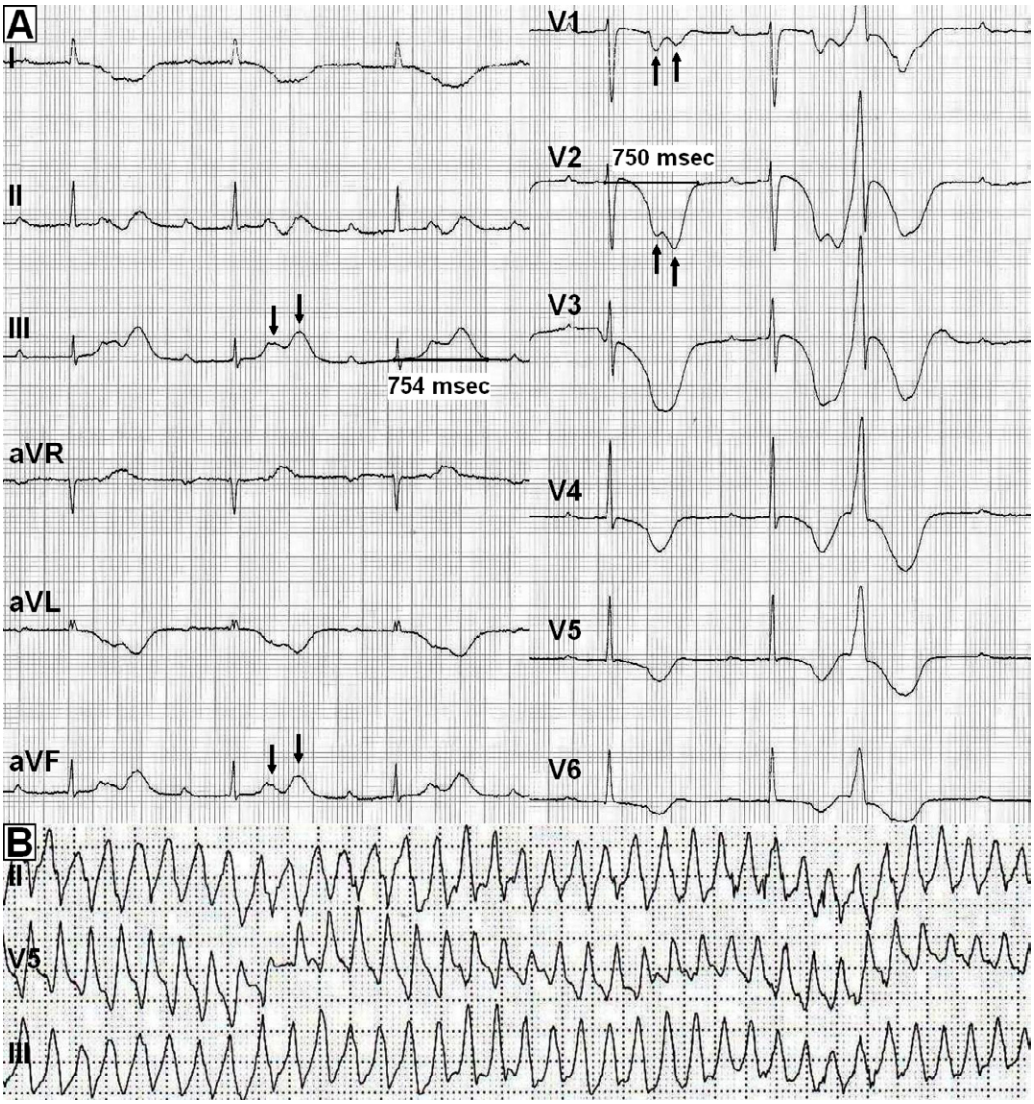

Figure 3. ECG recordings from a 42-year-old woman with a congenital third-degree AV-block and takotsubo cardiomyopathy are presented: giant inverted T waves with large QT intervals prolongation on the second day after the onset of takotsubo cardiomyopathy (arrows indicate double T waves – note that their second component is higher than their first one) (**A**); polymorphic ventricular tachycardia (i.e., torsade de pointes) which occurred while the repolarization abnormalities appeared (**B**). Numbers on the white areas correspond to the QT interval duration. Despite a congenital AV block, the patient had not experienced syncope or any symptoms suggesting lethal arrhythmias until her QT interval was considerably prolonged in the course of takotsubo cardiomyopathy. Reprinted from Sacha et al [4] with permission

large amount of stunned myocardium may cause the acquired long QT syndrome.

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REVIEW PAPER

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Food allergies in children – aspects of epidemiology and diet management

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ABSTRACT

For many years there has been a steady increase in the prevalence of allergic diseases, especially in developed countries. Scientists expect that in the year 2020, atopic diseases will be present in 50% of the population worldwide. Determining the actual scale of the food allergy problem is extremely difficult due to differences in genetic and environmental factors, as well as inconsistent methodology for population and epidemiological research. Treatment of food hypersensitivity is primarily done by excluding a particular food from the diet, while introducing nutritionally equivalent alternatives. An elimination diet should be determined independently for each individual, taking into account the cause, nature, severity and symptoms of the disease. Placing food restrictions on children can interfere with their normal development. The prevailing opinion is that only a properly formulated and followed elimination diet does not endanger the health of the patient. Numerous dietary errors committed, knowingly or unknowingly, by parents can lead to malnutrition and its consequences in their children, such as rickets, anemia, and immune disorders. Therefore, in the long-term treatment of food allergies it is recommended that the caregivers fully cooperate with a doctor, nutritionist and psychotherapist. Due to the ever-increasing incidence of allergic diseases, researchers are looking for effective methods of prevention. Recent scientific reports suggest protective effects from such substances like probiotics, polyunsaturated n-3 fatty acids, vitamin D, antioxidants as well as, certain vegetables and fruits.

Keywords: food allergy, elimination diet, nutrition, childrenIntroduction.

Epidemiology

Food can cause many unwanted symptoms in humans. Abnormal reactions of the body after ingestion of food tend to have a different clinical basis. The European Academy of Allergy and Clinical Immunology (EAACI) classifies any such symptoms as food hypersensitivity within which there are two distinctions: allergic reactions (food allergies) and non-allergic reactions (non-allergic hypersensitivity). By definition, a food allergy is an inappropriate response triggered by the immune system of the body, and repeated each time after the ingestion of a food commonly regarded as "harmless" [1–3].

Over the years there has been an increase in the prevalence of allergic diseases in the world. In 1997–2008, the United States reported an 18% increase in the incidence of food allergies in children up to 18 years old. During that same time, U.S., Canada and the UK observed a three-fold increase in the incidence of allergies to peanuts among young children. It is therefore very probable that, as stated in 1997 in the White Book on Allergy, atopic disorders will affect 50% of the population by the year 2020 [4, 5].

Epidemiological studies on atopic diseases are carried out mainly in developed countries, and the results are not conclusive. It is estimated that the prevalence of food allergies among children is about 4–8% however, there are also reports suggesting that the problem may affect even more than 40% of pediatric patients. The discrepancy in the data is due to genetic and environmental diversity of the populations studied, as well as inconsistent methodology in the research. This makes

it difficult to compare the results of analysis that come from different research centers [6–8].

Looking through the genealogy of families in which allergies to foods are present indicates the importance of genetic factors. Children of healthy parents have a 5–15% chance of manifesting allergies. If one parent suffers from allergies the probability increases to 40%, and if both parents are affected, the chances are as high as 60–80%. The development of an allergy phenotype may also be dependent on industrial pollution, tobacco smoke, food additives and bacterial allergens. Increased exposure to these factors can increase the risk of developing atopic diseases [9, 10].

The prevalence of food hypersensitivity in society is normally determined by questionnaire surveys. This method however, is not very precise and the results are usually overestimated. Many respondents attributed an allergy to various nonspecific symptoms appearing after eating a particular food, which is not always valid. In a study of 20 000 people in the UK, 20% of respondents reported that they have food allergies. In this group, allergy tests were performed that confirmed genuine allergies in only 1.4% of patients. Furthermore, in a Polish survey conducted under the project "EuroPrevall", food hypersensitivity was listed in 42% of children between 7–9 years of age. However, in response to the questionnaire question: "Have you ever had a doctor confirmed that your child has a food allergy?" the answer was "Yes" in only 29.6% of cases [7, 8].

Most patients that experience discomfort after eating food refer to it as a food allergy, and is the reason why society is dominated in the belief that this type of atopy is very common. Horvarth and Dziechciarz suggested that the increase in incidence of food allergies may not actually stem from a legitimate increase in these allergies, but from a temporary overuse of the diagnosis. Therefore, any alleged food allergy should be confirmed by a double-blind, placebo-controlled food challenge (DBPCFC) [6, 11, 12].

Diet management

Food allergies can be the first manifestation of atopic disease. Due to the immaturity of the gastrointestinal tract and immune system, infants and young children get sick more often than adults. With age, the symptoms may disappear completely, can be minimized, or they can change form – referred to as "allergic march". The course of disease is mainly dependent on the type of allergen. Food allergies can be caused by any food ingested, but most often it is caused by one of "the big eight food allergens". These allergens include the protein of milk, egg, soy, peanuts and other nuts, fish, shellfish, and wheat. Studies show that approximately 85% of patients who are allergic to cow's milk, wheat and eggs may begin to tolerate them after some time; however, in allergies to peanuts, hazelnuts and seafood only 15–20% of patients develop a future tolerance to the food. A huge impact on the future health of the body is the proper treatment of an existing food allergy. The success of treatment depends on how strictly diet recommendations are followed and how knowledgeable one is about the rules of elimination diets. A properly created and followed diet plan can prevent any food deficiency related disorders like malnutrition, anemia, osteopenia, rickets, immune disorders and other irregularities [12–17].

Symptoms of food allergies significantly affect the quality of life in patients and can be fatal. The aim of nutritional therapy is controlling immune response, reducing the risk of pseudoallergic reactions, modifying the "allergic march", reducing the permeability of the mucosa barrier, restoring tolerance for food allergens, and recovering intolerance to harmful products, which indicate a healthy individual [14, 18, 19].

An expert consensus in the treatment of food allergies is only consistent in the case of a severe allergic reaction. When the disease is accompanied by milder symptoms, recommendations can be varied: using an elimination diet exclusively, adding in drug therapy, or only treating the symptoms, without any dietary restrictions. The most effective treatment for food allergies is to remove foods that cause adverse reactions from the diet altogether. There are several types of dietary guidelines for the treatment of food allergies. The first of these eliminates one or several types of foods that cause the adverse symptoms. This guideline is recommended in the case of acute allergic reactions, where specific levels of IgE are detected or there is a high probability that nutrient sources are identified to cause clinical symptoms. Most often this recommendation is used in allergies to cow's milk in infants. The second type of guideline is an oligo-antigen diet which is used when the disease symptoms appear after eating various foods. In this case, it is recommended to introduce foods into the diet that rarely cause allergies, such as lamb, rice, corn, cooked apples, broccoli, asparagus, spinach, lettuce, sweet potatoes, salt, sugar, vinegar and olive oil. If after 1–4 weeks of this diet there is no indication of better health, introduction of the third guideline is recommended, which is an elemental

diet based on free amino acids. A common problem encountered is that pediatric patients may not accept the taste of their milk replacement. Most often this is due to the hydrolyzed whey protein or casein used in infants who are allergic to the protein in cow's milk [1, 12, 18, 20, 21].

The duration of treatment using diet is individual and depends on the type of allergen, the clinical form of the disease and the patient's age, but usually should not be less than 9–12 months. Periodically, food challenge tests need to be performed in children up to 3 years of age at least every 6 months, and in older patients every 12 months. A positive test result indicates the need for continued treatment, and a lack of allergic response indicates that treatment can be stopped. The indication of an acquisition of tolerance to the original allergy-inducing food is crucial in avoiding unnecessary dietary restrictions. Reasons for the failure of nutritional therapy may include: exclusion of a food which in reality is not responsible for the symptoms of the disease, a short observation period, conscious or unconscious failure to comply with dietary recommendations, and even coexisting infections that cause symptoms similar to allergies. If the elimination of these errors does not bring the intended benefits, it is necessary to have further allergic or gastroenterological consultations, which should also include the dietician, who will help customize a menu to fit the current needs of the patient and indicate foods that may contain "hidden allergens" [18, 22]. Here is a list of products containing "hidden allergens" of cow's milk protein:

- milk in all forms
- cheese
- sour cream
- yogurt, kefir
- buttermilk
- puddings, mousses, creams
- cereals
- chocolate
- cream fudge
- sweets
- ice cream, sundaes
- some types of bread
- crepes, croquettes, pancakes
- buns, rolls and butter croissant
- cakes, pies
- some sausages
- paté
- some sauces and dressings
- mashed potatoes
- some breaded foods
- spices
- soup stock, soup cubes
- pizza, casseroles, lasagna
- canned fish
- butter
- biscuits, crackers, wafers, waffles
- some margarines [36].

The effectiveness of treatment is assessed on the basis of clinical observations, which include complete resolution or a reduction of symptoms. In children and adolescents it is particularly important to also verify whether by following an elimination diet they still have proper growth and development. It is important that the therapy recommended is not more detrimental to the patient than the symptoms [18, 22].

An elimination diet should be determined individually and adjusted to the cause and nature of the allergy, as well as the type and severity of symptoms. Before introducing dietary restrictions patients should be informed, and in the case of children their parents should be informed, about the necessity and desired outcome of treatment and the possible consequences of not following the diet. Dietary restrictions are often a source of conflict in the family because of altering the food and meals in the house, the introduction of increased discipline and the extra financial costs. The support of a doctor, nutritionist and psychologist is invaluable in this case because they indicate the therapy benefits for both the patient and their family [22, 23].

Risks of elimination diets

Following an elimination diet accurately is vital for infants and young children, whose bodies are in a phase of growth and development. In particular, this group of patients should not be exposed to nutritional deficiencies that may interfere with their normal physical and psychological development [22–24]. Many studies have shown that one of the main causes of child malnutrition is food allergies [25–28]. This is a consequence of inadequate food intake due to decreased appetite, excessive food losses due to persistent diarrhea and vomiting, malabsorption resulting from inflammation in the intestines, and an inappropriate choice of substitutes for the foods excluded in the diet [21, 27]. Opinions on the safety of elimination diets are divided. It is believed that only a properly composed and followed elimination diet does not endanger the health of the patient. In the process of nutritional therapy numerous dietary errors are committed, which result in imbal-

anced feeding of children. It is particularly difficult in balancing the diet after the exclusion of a major group of products, such as milk and dairy products [24]. Adamska et al. found varying degrees of malnutrition in 26.7% of children up to 2 years of age using a dairy elimination diet, while Christine et al. showed that in a group of children treated with this elimination diet as much as 25% consume only 67% of the daily recommended amount of calcium, vitamin D, and E [29, 30]. Kurpińska et al. found that children with a cow's milk protein allergy, who received milk replacements regularly, consumed more calcium than those whose diet was not supplemented [31].

Parents' lack of knowledge concerning the proper composition of elimination diets increases the likelihood of making mistakes. It seems important, therefore, to intensify the education of caregivers of children with food allergies. Parents often unintentionally expose children to allergens. This may be due to inconsistent and inaccurate analysis of food ingredients. The evaluation of the information provided on food labels was carried out in Warsaw among 53 parents of children with celiac disease or an allergy to cow's milk protein and/or gluten. These parents had declared compliance with dietary guidelines, however it was shown that most of them had a problem with the identification of allergens on the basis of ingredients indicated on the packaging of products. A similar problem was observed by Sicherer and Sampson, giving examples of diverse and ambiguous terminology used on the labels of food products [22, 32].

Conclusion

Epidemiological data on food allergies are inconclusive. This indicates a need to standardize the methodology of a study to determine the actual extent of the problem. The only causal treatment of the disease is the use of an elimination diet. However, this may pose a risk of nutritional deficiencies, so balancing menus of pediatric patients should be monitored by a qualified nutritionist. A multitude of dietetic errors identified in children with food allergies indicates a need for greater education for their parents. Long-term use of an elimination diet should be monitored and possible nutrient deficiencies accompanied by the inclusion of vitamin and mineral supplementation.

An increase in the incidence of atopic diseases tends to lead to the implementation of effective preventive measures. Research is still looking for a relationship between the current way of life and an increased incidence of immune-mediated diseases. It is speculated that the most important predisposing factors for allergies are improper eating habits, environmental pollution and sterile conditions for raising children. There is evidence to suggest that certain dietary components, such as probiotics polyunsaturated n-3 fatty acids, vitamin D, antioxidant compounds, and certain vegetables and fruits, have an ability to reduce the risk of allergies, in particular in children with a family history of atopic disease [33–35].

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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CASE STUDY

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Ketoprofen, an emerging photoallergen

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ABSTRACT

Introduction. Ketoprofen, which belongs to the group of non-steroidal anti-inflammatory drugs (NSAIDs), is an emerging photoallergen. Especially its topical use may be a cause of drug-induced photosensitivity.
Material and methods. We report two cases of photoallergic and one case of phototaggravated contact dermatitis due to topical ketoprofen application, confirmed by photopatch testing.
Results. All patients presented positive reactions to ketoprofen at an irradiated site. Only one patient demonstrated a positive reaction to ketoprofen both at an irradiated and non-irradiated site.
Conclusions. Photosensitive reactions due to topical application of ketoprofen being of significant clinical importance need to be properly diagnosed. It is crucial to provide patients with a detailed instruction how to protect photoexposed areas during therapy with ketoprofen.

Keywords: ketoprofen, photoallergic contact dermatitis, photoallergy, photosensitivity, photopatch testing.

Introduction

Ketoprofen belongs to the group of non-steroidal anti-inflammatory drugs (NSAIDs). It is widely used because of its well-known analgetic and anti-inflammatory properties. Photoallergic dermatitis is one of possible side effects of ketoprofen. In fact ketoprofen is considered to be one of the main photocontact allergens. The first case report of photoallergic reaction due to topical ketoprofen was published by a Spanish dermatologist in 1985 [1]. Since then, photoallergy to ketoprofen has been presented mostly by researchers originating from Mediterranean countries [2] as well as from Japan [3, 4], Belgium [5], Sweden [6] and recently also from Poland [7].

In this study, two cases of photoallergic contact dermatitis and one case of phototaggravated contact dermatitis due to topical use of ketoprofen have been presented.

Material and methods

Case 1

A 48-year-old female patient was directed to the Department of Dermatology, Poznan Medical University of Sciences in May 2011, with initially suspected erysipelas. The patient demonstrated itchy, erythemato-papular skin lesions localized within the right calf and erythematous as well as exfoliating skin lesions within the left palm. There was neither fever nor malaise and new eczematous skin lesions on both lower extremities, neck and decollete appeared. Therefore diagnosis of erysipelas has been excluded. The patient reported on applying ketoprofen gel on her legs due to myalgia and previous exposure to the ultraviolet light while working in a garden during sunny weather, wearing short trousers. Our treatment was composed of clemastine 2 mg iv, dexamethasone 8 mg iv, 10 mg oral cetirizine, and topical betamethasone combined with gentamycin

in a cream formulation. Further on 1% hydrocortisone cream has been applied. Within the time period of nine days of the treatment significant improvement has been obtained.

Case 2

A-38-year-old female patient with a history of erythromelalgia related to idiopathic trombocythemia, experienced erythematous and papular eruption on both lower legs, where she had previously applied ketoprofen gel to reduce burning pain associated with erythromelalgia. Further exacerbation of skin lesions has been reported by the patient after the sun exposure.

Case 3

A 22-year-old male developed itchy, erythematous and papular skin lesions with exudation, restricted to the site of application of ketoprofen. It has been prescribed by the general practitioner in order to reduce the contusion-related pain within the neck and left upper thorax. Highly itchy, erythematous and papular skin lesions with significant exudation, restricted to the site of the drug application appeared after 24 hours. A few hours after the first application of the medicine, the patient visited an outdoor swimming-pool.

Patch and photopatch testing

In case of all patients after remission of skin lesions diagnostic testing has been performed. The diagnostic set consisted of standard patch tests (European Standard Patch Test Series, 28 haptens) provided by Chemotechnique Diagnostics. According to the International Contact Dermatitis Research Group recommendations evaluation of results has been performed after 48 and 72 hours.

Minimal erythemal dose (MED) was determined using mono-chromator irradiation simulator test and photopatch tests have been performed according to the European Guideline [8]. Medications tested included: diclofenac 1%, ibuprofen 5%, ketoprofen 2.5%, naproxen 5% (all in white petrolatum, which was also the negative control). Two photopatch series have been applied symmetrically on the back of patients for 48 hours, after which both were removed and one site has been irradiated with UVA at the dose of 5 J/cm^2. Test reactions have been evaluated 24 and 48 hours after irradiation. Results have been assessed according to the recommendations of International Contact Dermatitis Research Group.

Results

In case of all patients response to UV light was normal in MED testing. Patch tests` results with the European Standard Set of contact allergens were all negative. In all cases after 24 hours results of photopatch tests with ketoprofen were positive (erythema and papules). In cases one and two, positive reactions presen-

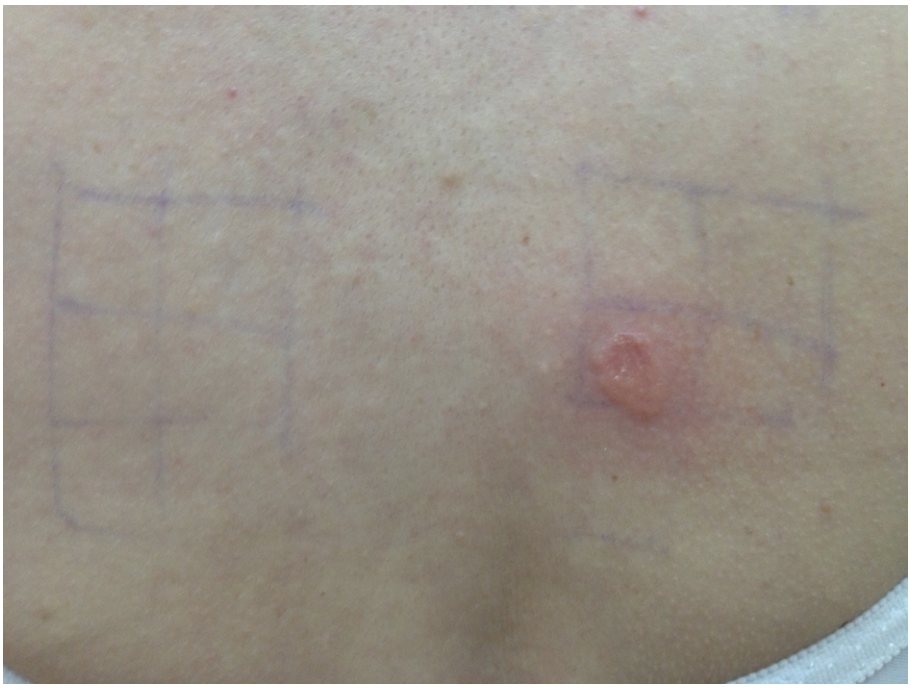

Figure 1. Photopatch testing of a 48-year-old woman

ted the 'crescendo' pattern with an increasing response (erythema, papules and vesicles) 48 hours after irradiation. Patch tests result with ketoprofen (at a non-irradiated site) was positive only in the case of patient three (++) in both readings. Based on results of our diagnostic procedure, patients one and two were diagnosed with photoallergic contact dermatitis and patient number three – as phototaggravated contact dermatitis. Results of photopatch testing have been presented in **Figures 1–3**.

Discussion

The two most common agent groups currently responsible for photoallergic contact dermatitis are organic ultraviolet absorbers in sunscreens and topical NSAIDs

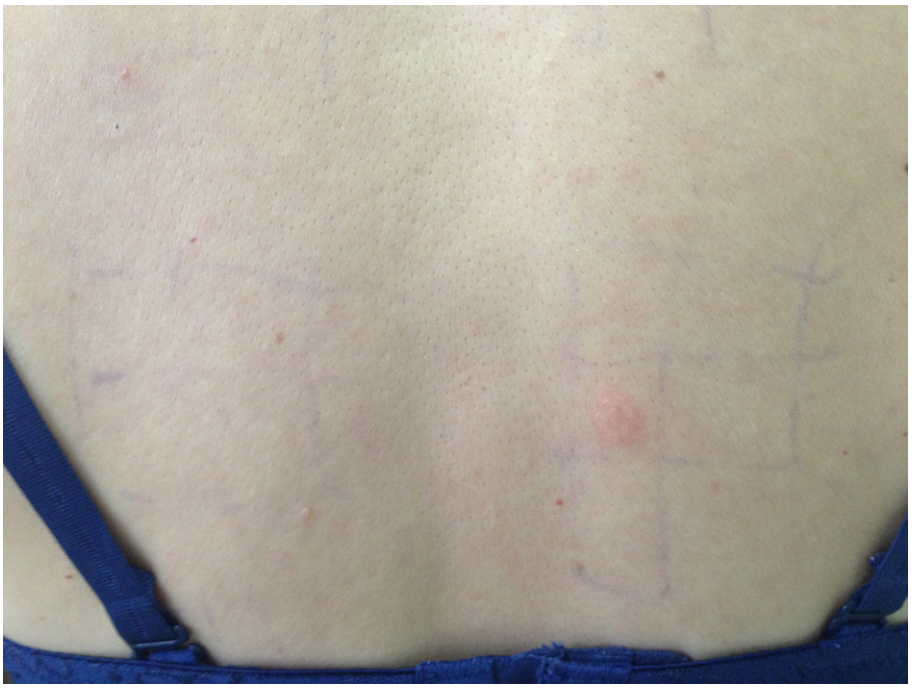

Figure 2. Photopatch testing of a 38-year-old woman

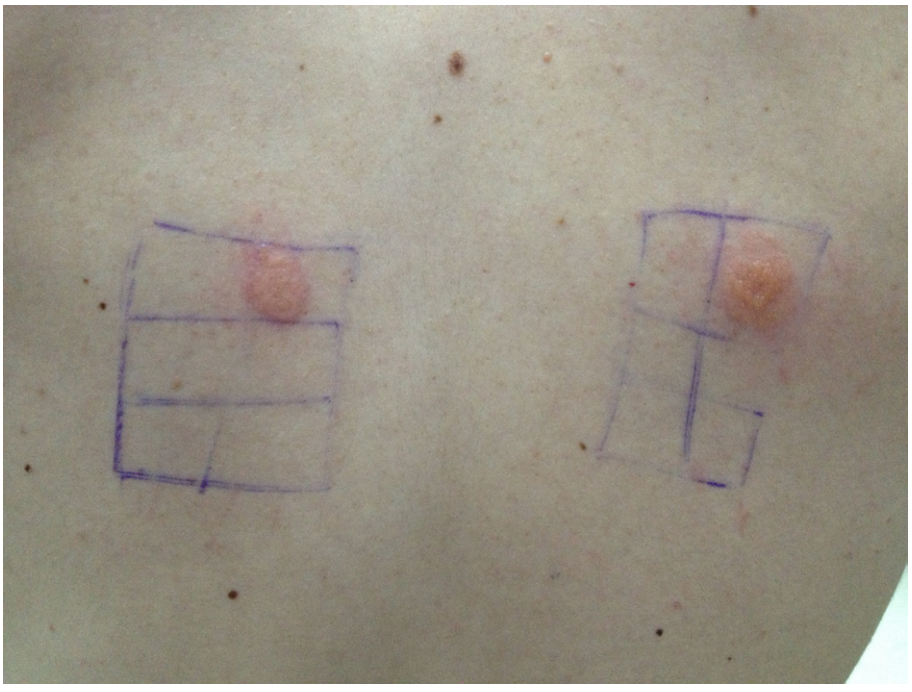

Figure 3. Photopatch testing of a 22-year-old man

[9, 10]. Ketoprofen is considered as the most common cause of NSAIDs-induced photosensitivity. It may promote phototoxic, photoallergic as well as phototaggravated reactions, where photoallergy is much more rare than phototoxic phenomenon [11]. Both topical and oral forms of ketoprofen are commonly prescribed by doctors of all professions.

Foti et al. [12], presented two cases of photodermatitis due to oral ketoprofen in patients with a history of photocontact reactions to topical ketoprofen. Both patients developed eczematous skin lesions after oral intake of ketoprofen and subsequent sun exposure. The authors highlight the risk of photoinduced reactions to systemic ketoprofen in patients with previous sensitisation to topical form of this drug.

Conti et al. [13] reported a case of a patient who developed acute eczematous reaction affecting the right lip commissure, and extending to the upper and lower lip and chin region after administration of oral granulated ketoprofen (OKI) and subsequent sun exposure. The patient never experienced any cutaneous reaction due to systemic ketoprofen, but only when the granules came in contact directly with the perioral skin followed by exposure to the sun. A positive reaction to only photostimulated ketoprofen was observed.

Recently published studies [14, 15] demonstrated that photoallergy to ketoprofen is often associated with photoallergy to octocrylene, and benzophenone-3, which are compounds of sunscreens. Therefore patients, who have experienced a photoallergic reaction to ketoprofen, should be informed about importance of avoiding sun blockers, which contain octocrylene and benzophenone-3.

Although photocross-reactivity to ketoprofen, octocrylene and benzophenone-3 is well-known, such a cross-reactivity between ketoprofen and butyl methoxydibenzoylmethane, a compound of sunscreen preparations, was not reported. Recent research [16] has demonstrated that the addition of a UVA absorber to topical ketoprofen formulations may be effective to reduce the ketoprofen photosensitivity. The authors presented that butyl methoxydibenzoylmethane has a strong potential to reduce ketoprofen photosensitivity. From the viewpoint of cross-reactivity, it is considered that butyl methoxydibenzoylmethane may be one of the best choices as a UVA filter added to topical ketoprofen formulations to prevent photosensitive reactions to ketoprofen.

According to recommendations of the European Medicines Agency's Committee for Medicinal Products for Human Use (CHMP) [17], ketoprofen is not available over the counter in Poland due to significant risk of severe photosensitivity reactions and cross allergy with octocrylene. Medical professionals should inform their patients about photosensitive reactions being a highly possible side effect of ketoprofen. Patients should also protect treated skin areas against UV during topical therapy with ketoprofen and two weeks after termination of the treatment.

On the basis of the results of the European Multicentre Photopatch Test Study [9], and on the presence or absence of photosensitizing agents in consumer products within the European market, a recommended European Baseline photopatch test series has been changed. Ketoprofen, etofenamate, piroxicam, benzydamine, promethazine, and 15 compounds of sunscreens, including octocrylene, and benzophenone-3, are included in the new standard European Baseline photopatch test series [18].

Conclusion

Medical specialists should be aware, that avoiding prescriptions as well as selective use of topical ketoprofen formulations during sunny weather on photoexposed areas may contribute to the reduction of risk of severe photosensitive reactions or cross-sensitizations.

Conflict of interest

The authors declare no conflicts of interest concerning this article.

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Searching for new genes and loci involved in cleft lip and palate in the Polish population – genome-wide association study

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ABSTRACT

The project "Searching for new genes and loci involved in cleft lip and palate in the Polish population – genome-wide association study" is a case-control study in a group of unrelated subjects with non-syndromic cleft lip with or without cleft palate (NSCL/P) and healthy individuals with no family history of clefting or other congenital disorders. The overall goal of this grant proposal is to identify novel genetic factors, which can play a significant role in the pathogenesis of orofacial clefts in the Polish population. To accomplish the proposed aim, a two stage genome-wide association study will be performed. In the first stage, Illumina's HumanOmni Express BeadChips arrays will be used to genotype over 700,000 polymorphisms in NSCL/P patients and controls. In the second stage, SNPs showing the most compelling association with the risk of orofacial clefts will be tested in an independent sample set using standard genotyping methods. This research project is expected to be completed in July 2015.

Keywords: genome wide association study, cleft lip and palate, risk factors, polymorphisms.

General information

The project "Searching for new genes and loci involved in cleft lip and palate in the Polish population – genome-wide association study" was awarded by the Polish National Science Center (NCN) under project number: 2012/07/B/NZ2/00115 (OPUS 7 competition). *The duration of the grant is 24 months, from 2nd July 2013 to 1st July 2015. The contract between NCN and Poznan University of Medical Sciences (PUMS), Poland, was signed on 2 July 2013.*

Management

The Principal Investigator of the grant is Dr Adrianna Mostowska, Assistant Professor in the Department of Biochemistry and Molecular Biology at PUMS. Main Co-Invetigators in the grant are Prof. Paweł P.

Jagodziński from PUMS and Prof. Konrad K. Hozyasz from the Institute of Mother and Child in Warsaw. In addition, Co-Invetigators are: Prof. Piotr Wójcicki (*University Clinic of Medical Academy in Wroclaw* and *Department of Plastic Surgery Specialist Medical Center in Polanica Zdrój*), Dr Barbara Biedziak (PUMS), Prof. Joanna Wesoły (Department of Human Molecular Genetics, Institute of Molecular Biology and Biotechnology in Poznan), Dr Anna Sowińska (PUMS) and MSc Sylwia Matuszewska-Trojan (PUMS).

Ethics

Bioethical Committee at PUMS on 3rd November 2011 accepted all project's protocols and forms, including information for patients form and consent form for participation in a research study (the permission number 891/11).

Finance

The total amount of grant funding is 1,475,140 Polish Zlotys (about 352,000 Euro). The amount of funding in the first year is 1,300,420 Polish Zlotys (about 310,000 Euro). *Grant funds were designed to purchase all reagents,* disposable laboratory equipment and software products for the conduct of the proposed research and analyse received data. In addition, *grant funds were designed to cover personnel costs of project participants and costs associated with* the *dissemination of research results,* i.e., through *publications* in peer reviewed journals and presentations at professional scientific conferences.

Research basic concept and objectives

Orofacial clefts are one of the most common congenital anomalies in humans (OMIM #119530; OMIM #119540). According to the *Polish Registry of Congenital Malformations, the prevalence of these birth defects in Poland ranges from 1/500 to 1/1,000 births* [www.rejestrwad.pl]. Orofacial clefts represent a significant public health problem since their treatment requires comprehensive surgical, orthodontic, phoniatric and psychological management strategies [1]. In addition, long term outcomes of individuals born with these anomalies may include *higher overall death rates*, mental health problems, alternations of childbearing patterns and a higher risk of cancer (particularly breast cancer) in both affected individuals and their family members [2].

The aetiology of non-syndromic cleft lip with or without cleft palate (NSCL/P) is heterogeneous and both genetic and environmental factors affect the risk of this developmental malformation [2]. Research studies of NSCL/P using a wide range of methodological approaches have discovered a number of genes (e.g. *IRF6*, *BMP4*, *FGFR2*, *FOXE1, MSX1* or *MYH9*) and chromosomal regions underlying this structural anomaly [2, 3]. In addition, *four independent genome wide association studies (GWAS) for NSCL/P have successfully identified several loci (8q24.21, 1p22.1, 10q25.3, 17q22 and 20q12) at which nucleotide variants influence the risk of NSCL/P [4–7].* However, all mutations and/or polymorphisms of candidate genes and chromosomal loci characterized so far may *still explain only a fraction of the inherited contribution to NSCL/P aetiology.* This can be due to the locus and allelic heterogeneity among populations (the existence of different candidate genes and many different disease causing alleles at a given locus, respectively), incomplete penetrance, as well as complicated epistasis and gene-environmental interactions. *In addition, in most cases, the functional role of identified polymorphic variants in the pathogenesis of orofacial clefts is unknown.* Therefore, the overall goal of this grant proposal is to identify novel genetic factors, which can influence the risk of NSCL/P in the Polish population. To accomplish the proposed aim, a two stage genome-wide association study will be performed using Illumina's HumanOmni Express *BeadChips* arrays. GWAS is defined by the National Institutes of Health as a study of common genetic variation across the entire genome designed to identify genetic associations with observable traits. GWAS have greater power than linkage studies to detect small to modest effects, even with a strict alpha-level for statistical significance ($\alpha = 5.0 \times 10^{-8}$) [8]. Moreover, by casting a wide net of genetic markers across the entire genome, this approach does not require to select candidate genes for study and examines much of the common variation across the genome. To date, GWAS have detected hundreds of variants associated with a large number of diseases [9]. Many of these findings are novel, since identified SNPs were not previously recognized as disease risk factors.

Research plan

The proposed project will be composed of two stages:
Stage 1

– selection of individuals with orofacial clefts and a suitable comparison group (number of cases =

288, number of controls = 576), DNA isolation and quantification;
- high-throughput genotyping and data review to ensure high genotyping control;
- statistical tests for associations between SNPs passing quality thresholds and orofacial clefts.

Stage 2
- selection of additional individuals with orofacial clefts and a suitable comparison group (number of cases = 400, number of controls = 500), DNA isolation and quantification;
- replication of identified associations in an independent population sample;
- examination of functional implications using various research databases and tools.

Research methodology

Sample collection

Peripheral blood samples from 688 (288 for the first stage and 400 for the second stage of the study) unrelated subjects with NSCL/P will be collected in the Departments of Paediatrics and Paediatric Surgery at the Institute of Mother and Child in Warsaw, as well as in the Department of Plastic Surgery at the Medical Academy in Wroclaw. To avoid sample heterogeneity, individuals with cleft palate only will be excluded from the study prior to genotyping. Also patients with accompanying congenital malformations, chronic medical conditions, or vaguely described dysfunctions (syndromic forms of orofacial clefts) will not be included in the analyses. Eligibility to the patient group will be ascertained from detailed medical records. In addition, 1076 (576 for the first stage and 500 for the second stage of the study) healthy individuals with no family history of clefting or other congenital disorders will be used as controls. Both patients and age- and gender-matched control samples will be Caucasians of Polish origin recruited from the same geographic regions. DNA will be extracted from peripheral blood samples using standard salt extraction procedure. DNA concentrations will be adjusted to 50 ng/µl and verified using PicoGreen dsDNA Quantitation kit (Molecular Probes, Invitrogen, CA, US).

Genome-wide association study

High-throughput genotyping of all samples *will be carried out using* the Illumina's HumanOmni Express *BeadChips* (Illumina, CA, US) according to manufacturer's protocol. *These* 12-sample *BeadChips feature 730,525 strategically selected markers to capture the greatest amount of common SNP variation (> 5% minor allele frequency). The SNPs selection is based on linkage disequilibrium (LD) between nucleotide variants. A high* r^2 *between two SNPs indicates high correlation, making these SNPs good proxies (tag SNPs) to each other. At a maximum* r^2 *of 1, two SNPs are in perfect LD and can serve as pure proxies. Thus, only one SNP needs to be genotyped to know the genotype of the other. Illumina DNA Analysis products offer unparalleled genomic coverage using tag SNPs with the highest average* r^2 *values in the industry (http://www.illumina.com/).*

Statistical analysis of GWAS results

Genotypes for all arrays *will be calculated* using *BeadStudio's genotyping module* (v2.0, *Illumina,* CA, US). Stringent quality control will be applied to the genotyping data in order to exclude experimental errors and low-quality SNPs. *All statistical analyses will be performed using PLINK* version 1.06. The association of SNPs with NSCL/P will be tested by a 1-degree-of-freedom Cochran-Armitage trend test. The p-value below 5×10^{-8} *will be considered as statistically significant* [10]. *Statistical analysis of obtained results will also include the determination of the differences in allele and genotype frequencies between cases and controls using the standard* χ^2 and Fisher exact tests and the calculation of the Odds Ratio (OR) with corresponding 95% confidence intervals (95%CIs). For nucleotide variants showing the strongest associations further computational analyses will be performed in order to determine if these nucleotide variants may be real, etiological polymorphisms involved in NSCL/P aetiology. PolyPhen online database (http://genetics.bwh.harvard.edu/pph/) will be used for prediction of functional consequences of the non-synonymous SNPs on protein structure and function.

Replication study

The SNPs showing the most significant associations with NSCL/P risk in the GWAS will be retested in a subsequent replication study conducted in an independent set of cases and controls (*400 patients z NSCL/P and 500 healthy individuals).* Genotyping will be carried out either by PCR followed by digestion of the amplified products with the appropriate restriction enzyme (PCR-RFLP), high-resolution melting curve analysis *(HRM)* or using TaqMan assays *(Applied Biosystems;* CA, US).

Statistical analysis of replication study results

For each SNP, the *Hardy-Weinberg equilibrium* will be assessed by Pearson's goodness-of-fit Chi-square *statistic*. The differences in allele and genotype frequencies between cases and controls will be determined using standard Chi-square or Fisher tests. SNPs will be tested for association with NSCL/P using the Cochran-Armitage trend test. The OR and associated 95%CI will also be calculated. Haplotype analysis will be performed using the UNPHASED 3.1.5 program. Gene-gene interactions will be evaluated using the nonparametric and genetic model-free Multifactor Dimensionality Reduction (MDR) approach (MDR version 2.0 beta 5).

Expected results

The presented project will allow the identification of novel genes and chromosomal loci, the nucleotide variants of which are significantly associated with the occurrence of NSCL/P in the Polish population. *This will enable a more complete understanding of the highly complex etiology of this common developmental anomaly, which can in the future help to improve the current treatment methods and to design the programs of primary prevention of craniofacial abnormalities.* The results obtained during this project may *also contribute* to *deepening the knowledge of the* molecular mechanisms involved not only in face development, however, also in the development of the whole embryo. Development of the head and face comprises one of the most complex events during embryonic development, coordinated by a network of transcription factors and signalling molecules together with proteins conferring cell polarity and cell–cell interactions [11]. The results of this project should also help us to design our future research plans. Markers selected in the genome-wide association study will be further evaluated in fine mapping and next generation sequencing studies to identify causal variants, and in functional studies to understand the biological mechanism of the observed associations with the non-syndromic orofacial clefts in the Polish population.

Conflict of interest

The authors declare that they have no competing interests.

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