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Contents

Original papers

Reviews

Blocking MET receptor signaling in multiple myeloma cells in vitro and in vivo

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

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Abstract

Background. Numerous studies have shown a role of the hepatocyte growth factor (HGF) as a ligand for the MET receptor in promoting aggressiveness in myeloma cells.

Objectives. The aim of this study was to confirm the presence of the MET receptor in myeloma cell lines, to establish a stable lentiviral construct directed against MET receptor mRNA and then to evaluate the effect of blocking MET receptor expression both in vitro and in vivo.

Material and methods. The U266 and INA6 cells were transduced using a lentiviral vector carrying siRNA to achieve the reduction of MET receptor expression. The ocular sinus of NOD/SCID mice was injected with wt-U266, shMET-U266 and shLacZ-U266 cells.

Results. MET receptor expression was demonstrated in all tested myeloma cell lines. Blocking the HGF/MET axis did not affect the growth of transduced U266 and INA6 cell lines. The inoculation of NOD/SCID mice with myeloma cells with reduced expression of MET led to increased survival of the animals.

Conclusions. MET receptor expression was constituently expressed in all tested myeloma cell lines. A lentiviral construct can effectively reduce the expression of the MET receptor in myeloma cells. Further studies are necessary to evaluate the effect of the reduction of MET receptor expression in multiple myeloma, focusing on animal models with a larger test group size.

Key words: hepatocyte growth factor, transduction, U266, INA6

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Multiple myeloma (MM) is a plasma cell malignancy characterized by uncontrolled proliferation of clonal plasma cells manifested clinically by increased secretion of monoclonal immunoglobulins or light chains. Over the past decade, new therapeutic agents have been incorporated into the treatment algorithm for patients with MM, increasing their survival rates.[1–3] Further research into new treatments for MM is essential in order to improve the survival of patients. One potential target for MM therapy is the hepatocyte growth factor (HGF)/mesenchymal-epithelial transition (MET) axis.[4]

HGF belongs to the plasminogen protein family. Under physiological conditions, HGF can be produced by fibroblasts, fat collecting liver cells, bone marrow stromal cells, endothelial, and epithelial cells. HGF is the only known ligand of the MET receptor. The MET receptor is a tyrosine kinase receptor. It is composed of an extracellular 50 kD α chain and a transmembrane 140 kD β chain. HGF binding to the MET receptor results in tyrosine phosphorylation of the c-terminal portion, leading to the recruitment of adaptive and signaling proteins, and the activation of multiple signal transduction pathways. This activation results in the migration, mitosis and morphogenesis of multiple cell lines, and excessive activation has been implicated in the pathogenesis of many cancers.[5,6]

In patients with MM who showed elevated levels of HGF, there is a direct correlation between serum HGF levels and aggressiveness of the disease.[4,7] The prognosis of patients with MM is usually worse in the presence of high levels of HGF.[8–10] Among the many factors activating myeloma cells, HGF is one of the main factors and the presence of the MET receptor is a common finding in myeloma cells, both at the mRNA and protein levels.[11,12] HGF is secreted by cells of the bone marrow stroma, thus allowing for autocrine and paracrine regulation of tumor cells, stimulates proliferation and inhibits apoptosis of myeloma cells.[11] HGF-induced IL-11 secretion from osteoblastic cells may contribute to osteolysis seen in a majority of patients with MM.[13]

Numerous studies have shown a role of HGF/MET axis in promoting aggressiveness in myeloma cells. The aim of our study was to confirm the presence of the MET receptor in myeloma cell lines and to establish a stable lentiviral construct directed against MET receptor mRNA. An additional goal was to determine whether silencing of the MET receptor would have an impact on the growth of myeloma cells in vitro and life expectancy of mice inoculated with transduced myeloma cells in vivo.

Material and methods

Myeloma cell lines

Myeloma cell lines INA6 and U266 (American Type Culture Collection, Manassas, USA) were cultured in RPMI 1640 medium (Gibco BRL, ThermoFisher Scientific, Waltham, USA) and supplemented with fetal bovine serum (FBS, 10% and 15%, respectively) (PAA Laboratories, GE Healthcare Bio-Sciences Austria GmbH, Pasching, Austria), 2 mmol L-glutamine, and 100 IU/mL penicillin and 10 µg/mL streptomycin (Gibco BRL), at 37°C in an atmosphere of 5% CO_2 with 95% humidity. Additionally, INA6 required the presence of IL-6 at the concentration of 2 ng/mL.

Assessment of gene expression by real-time polymerase chain reaction

RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, USA). The concentration and purity of the obtained RNA was assessed by measuring absorbance at a wavelength of 260 and 280 nm, using a DU 640B spectrophotometer (Beckman Coulter, Fullerton, USA). RNA was transcribed into cDNA using MMLV reverse transcriptase (Promega, Madison, USA) and non-specific primers, called random primers, (Promega). The analysis of gene expression was performed by quantitative PCR in real time (qRT-PCR) based on specific TaqMan probes (Applied Biosystems, Foster City, USA), using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). The components of the reaction mixture used in the qRT-PCR were: TaqMan PCR Master Mix 25 µL, cDNA 100 ng, 20× probe 2.5 µL, water added until final volume was 50 µL. Probes used for qRT-PCR were manufactured by Applied Biosystems accordingly: TaqMan MET Hs01565589_m1 and GAPDH Hs99999905_m1. To calculate results, we analyzed relative gene expression using a ΔΔCt calculation, based on the comparison values of Ct for the test and control gene. We used mRNA isolated from umbilical cord blood mononuclear cells as a negative control.

Determination of the MET receptor presence

Immunohistochemical staining was used to determine the presence of the MET receptor by means of cytospin preparations. Preparations were stained using monoclonal anti-MET antibodies (1:100 dilution) visualized with the DAKO LSAB2 visualization system and kit (DakoCytomation, Glostrup, Denmark). In order to visualize the antigen-antibody reaction, 1 drop of streptavidin was added, and then chromogen was used as an activating agent. The obtained preparations were stained with hematoxylin and embedded in glycerol gel. Samples were evaluated using a light microscope produced by Olympus (Tokyo, Japan).

Transfection of myeloma cell lines U266 and INA6

Separate test tubes were prepared according to the following specifications: Lipofectamine 2000 in a solution of 50 mL OptiMEM I medium (Invitrogen, ThermoFisher Scientific, Waltham, USA) and 1 µg siRNA in 50 mL

OptiMEM I medium without FBS or antibiotics. These agents were combined and allowed to stand for 20 min to form 2000 siRNA-lipofectamine complexes.

Cell lines U266 and INA6 were transduced using a lentiviral vector carrying siRNA directed against MET to achieve the reduction of gene expression, and LacZ as a control to confirm proper action of the receptor. The lentiviral vector used for the experiments was based on a 19-nucleotide sequence of siRNA directed against MET 5'-CCG AGA AGU AUG UGA UGA ATT-3'. Cell lines U266 and INA6 were seeded at a concentration of 3 × 104, and converted using lentiviral transduction. Ten µL viral particles suspended in 1 mL RPMI with 10% FBS was added to the cells in the presence of 6 mg/mL hexadimethrine bromide (Sigma-Aldrich Co., St. Louis, USA). After 48 h, blasticidin was introduced (10 µg/mL for the U266 line and 4 µg/mL for the INA6 line). After 12 days, dead or apoptotic MM cells not resistant to blasticidin were observed. We established a naming convention for these cells by adding wt (wild type, input cell lines), shMET or shLacZ for the transduced cell lines. Transduced and wild type cell lines U266 and INA6 cells were plated in complete RPMI medium onto 6-well culture plates at a concentration of 2 × 104/well, and then counted in specified time intervals to judge growth. The number of cells after 24 h was used as the baseline and compared to the number of cells after 48, 72 and 96 h of incubation.

Analysis of a mute MET receptor in a mouse model using transduced U266 cell lines

For in vivo studies, 15 non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice purchased from Jackson Laboratories, Bar Harbor, USA were used. This study was approved by the local ethics committee. The NOD/SCID mice were irradiated at 300 cGy using a GammaCell irradiator. The mice were divided into 3 subgroups, 5 in each group, and after 24 h, the ocular sinus was injected with 5 × 106 cells of the following cell lines: wt-U266, shMET-U266 and shLacZ-U266. After the death of the mice, their long bones were isolated, fixed in paraformaldehyde, and then prepared in paraffin blocks for slide mounting immunohistochemical staining.

Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, USA). Survival analysis of the mice was performed using the Kaplan-Meier analysis. The statistical significance of differences between groups was tested using the Student's t-test or one-way ANOVA at a significance level of $p < 0.05$.

Results

We observed MET receptor expression in myeloma cell lines both at the mRNA and protein levels. In order to investigate the presence of MET receptor mRNA in myeloma cell lines, 3 independent experiments were performed using RT-PCR. There was no presence of MET receptor expression in blood mononuclear cells isolated from the umbilical cord as a negative control (Fig. 1).

A

Line	Control cells	U266	INA6
MET expression	–	+	+

B

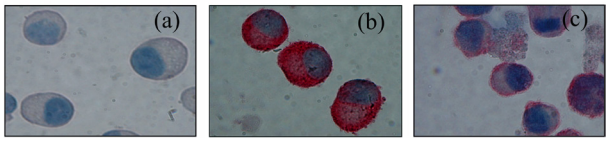

Fig. 1. A – immunohistochemical staining of multiple myeloma cell lines for the presence of the MET receptor; B – (a) control cells, (b) U266 cells, (c) INA6 cells. Representative pictures, microscope magnification x200

Characteristics of transduced U266 and INA6 myeloma cell lines

The line shMET-U266 showed a significant decrease in mRNA expression for the *MET* gene compared to wt-U266 (0.38 vs 8.63 au), and shMET-INA6 showed a significant decrease in mRNA expression for the *MET* gene compared to wt-INA6 (0.36 vs 6.0 au) (Fig. 2A). The mRNA expression for the *MET* gene in shLacZ-U266/INA6 confirms proper acting of the lentiviral construct. The decrease in MET mRNA was reflected in decreased expression of the MET receptor (Fig. 2B). Western blotting detected a significant decrease in the amount of protein compared to MET

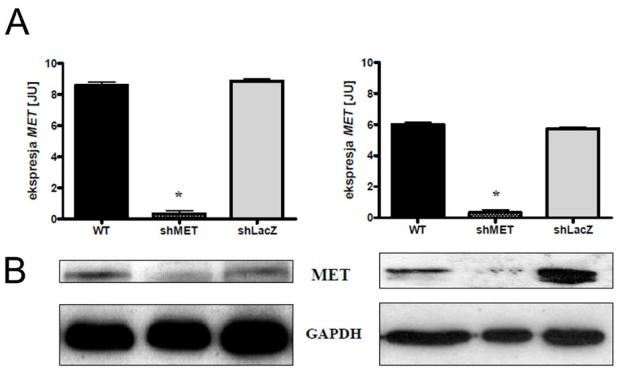

Fig. 2. shMET-U266 showed a significant decrease in mRNA expression for the *MET* gene compared to wt-U266 (0.38 vs 8.63 au), and shMET-INA6 showed a significant decrease in mRNA expression for the *MET* gene compared to wt-INA6 (0.36 vs 6.0 au)

A – mRNA expression for the *MET* gene in shLacZ-U266/INA6 confirms proper acting of the lentiviral construct; B – the decrease in MET mRNA was reflected in decreased expression of the MET receptor.

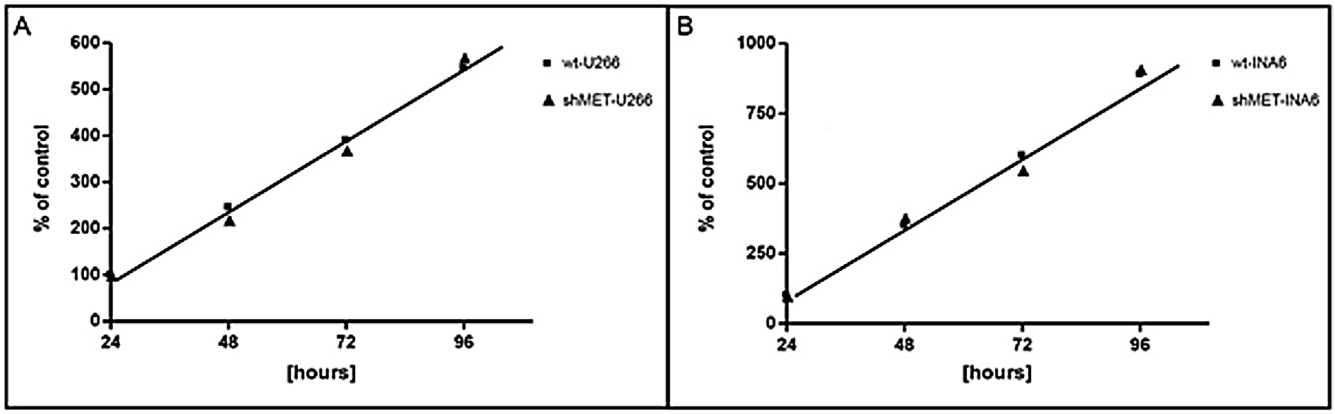

Fig. 3. Comparison of the growth rate of wild type cells and cells transduced with a lentiviral vector

A – comparison of cell lines wt-U266 and shMET-U266; B – comparison of lines wt-INA6 and shMET-INA6.

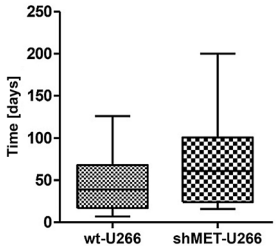

Fig. 4. Effect of reducing the expression of MET in U266 cells on the survival of NOD/SCID mice

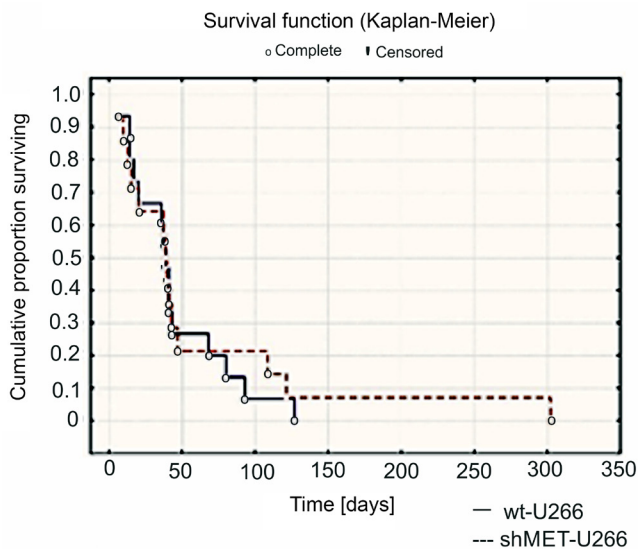

Fig. 5. Survival expectancy of NOD/SCID mice – murine myeloma model

output lines. The lentiviral construct directed against MET receptor mRNAs, can effectively reduce the MET receptor.

Blocking the HGF/MET axis did not affect the growth of cell lines U266 and INA6.

There was no difference in growth between wt-U266 and shMET-U266, or between wt-INA6 and shMET-INA6 (Fig. 3). Median survival rates were as follows: wt-U266 – 40 days, shMET-U266 – 62 days (Fig. 4). Blocking the HGF/MET axis increased the survival of NOD/SCID mice, but the observed changes were not statistically significant (Fig. 5).

Discussion

The HGF/MET axis is involved in many physiological processes including growth, repression of intercellular adhesion, cell migration, epithelial-mesenchymal transition (EMT), inhibition of apoptosis, proliferation and morphogenesis as well as in wound healing and tissue regeneration.[5,7,14–16] This axis is also important in oncogenesis and plays a significant role in the migration, proliferation and adhesion of various neoplastic cells. Overexpression or excessive activation of HGF/MET has been demonstrated in mesenchymal and epithelial tumors such as breast cancer, ovarian cancer, gastrointestinal tract cancer, lung cancer, prostate cancer, glioblastoma, sarcomas, and MM.[17] MET is also partly responsible for the metastasis of tumor cells by increasing their migration, secretion of proteolytic enzymes, ability to survive in the blood vessels, and ability to remain in the capillary bed. Together with other mitogenic factors, such as the SDF-1-CXCR4 axis, it is involved in the colonization of distant tissues by tumor cells, and stimulates their growth in microenvironments normally foreign to them.[4]

In this study, we observed the expression of MET mRNA and MET protein in myeloma cell lines. Reports on the role of HGF/MET axis encourage research on the use of inhibitors of this axis in the treatment of various neoplastic diseases, including multiple myeloma. With respect to the methods of inhibiting the axis of HGF/MET, one could consider inhibiting the attachment of HGF to the MET receptor, the prevention of dimerization of the MET receptor, the inhibition of the MET receptor tyrosine kinase, or the inhibition of the expression of HGF or MET.[17] Blocking the interaction between the receptor and the ligand may occur after the application of competitive antagonists of HGF or antibodies directed against HGF or MET. NK4 is a truncated form of HGF and competes with it for binding at the MET receptor, and it does

not have the effect of receptor activation. In a study by Brockmann et al., it was demonstrated that NK4 inhibits glioblastoma growth in mice with implanted tumor cells via pro-apoptotic and anti-mitogenic mechanisms.[17] NK4 exhibited anti-cancer effects mainly due to the inhibition of invasion and metastasis as well as the inhibition of angiogenesis-dependent tumor growth.[18] Du et al. showed reduced growth of myeloma cells in mice treated with NK4.[19] Martens et al. demonstrated growth inhibition using anti-HGF neutralizing antibodies in a mouse model of glioblastoma.[20] Vigna et al. also applied anti-MET antibodies, which resulted in the inhibition of the growth of epithelial cancers.[21] Recent studies have demonstrated that the use of angiotensin IV analog, norleual as an antagonist of HGF/MET, not only blocked its dimerization, but also inhibited HGF-dependent MET activation and had anticancer activity.[22] The semaphorin domain is necessary for MET receptor dimerization. Kong-Beltran et al. demonstrated that recombinant soluble semaphorin domains block phosphorylation of the MET receptor, regardless of the presence or absence of HGF.[23] To inhibit the MET tyrosine kinase and its signaling activity, low molecular weight inhibitors, such as geldanamycin, K252a, indolinone, and its analogs (17-AAG, 17-DMAG) or PHA665752 were used.[25–28] Hov et al., using PHA665752 small molecule acting directly on the receptor MET, observed the inhibition of cell proliferation, migration and adhesion in ANBL6 myeloma cell lines.[24] To reduce the expression of MET and HGF, nonsense RNA or ribozymes can be used. Nonsense RNA or single-stranded DNA is a molecule consisting of a 15–25 nucleotide sequence, which can be used to induce degradation of mRNA or block its translation. Blocking the *MET* gene causes a decrease in expression of the MET receptor, thus inhibiting the growth of tumor cells.[26] Abounader et al. demonstrated that ribozymes, naturally occurring RNA molecules, can catalyze the specific cleavage of mRNA and reduce the expression of HGF and MET, resulting in the inhibition of activation of the MET receptor, and consequently the inhibition of colony formation and migration of tumor cells in vitro.[28] Que et al. revealed that the down-regulation of MET inhibits the proliferation and invasion of U266 myeloma cells, and increases their chemosensitivity to doxorubicin and bortezomib.[29–31]

Posttranscriptional gene silencing, a technique that is based on the phenomenon of RNA interference (RNAi) through siRNA, uses the natural process of gene expression dependent on double-stranded RNA. Translation of the mRNA is blocked by the introduction of siRNA with a sequence complementary to the target RNA. The use of an adenoviral vector encoding siRNA against MET in gastric cancer, prostate cancer and glioma cell lines resulted in decreased mitogenic activity. *MET* gene silencing resulted in the induction of apoptosis.[32,33] In vitro siRNA can be delivered directly to the cell or in the form of vectors expressing siRNA.[34,35] The use of viral systems allows for long-term silencing of the receptor and high reproducibility of results.

In our study, in order to reduce the expression of mRNA for MET, a lentiviral model was used. Transduction of U266 and INA6 cells using siRNA test vectors effectively silenced the expression of MET at both the mRNA and protein levels. Taulli et al. examined rhabdomyosarcoma cells transduced with an siRNA lentiviral vector directed against the MET receptor, and observed not only inhibition of migration, but also decreased cell proliferation.[36] In our study, we observed that the transduced U266 and INA6 cell lines showed no differences in the rate of in vitro growth compared to the wt. Differences previously reported probably occurred due to the use of different experimental designs and different tumor types. Taulli et al. used a lentiviral vector in rhabdomyosarcoma cell lines induced by the presence of doxycycline in assessing the impact of the MET receptor inhibition over 5 days of RNAi induction.[36] In the present study, we used myeloma cell lines constitutively reduced in the expression of the MET receptor, and experiments were performed at least 3 weeks after the introduction of the vector. Hypothetically, prolonged exposure to siRNA may allow cells to develop a mechanism to avoid apoptosis.

The present study evaluated whether blocking the HGF/MET axis using lentiviral vectors may be a potential therapy to treat multiple myeloma. HGF is a potent chemoattractant for cells expressing the MET receptor on their surface. As a result of the increasing gradient of HGF, bloodborne myeloma cells settle in the bone marrow.[37] A study by Teoh et al. demonstrated in U266 cells cocultured with mesenchymal stromal cells transfected with IL-6 siRNA a significant inhibition of cell growth, IL-6 synthesis, and suggested potential use of RNA interference-mediated therapy for multiple myeloma.[38] Impaired response to the increased HGF gradient, caused by the reduction of the expression of the MET receptor, may influence the ability of tumor cells to migrate toward the bone marrow, which could hypothetically decrease neoplastic aggressiveness. Our experiments demonstrate that mice inoculated with myeloma cells with reduced expression of MET were characterized by higher median survival time than animals treated with wild type myeloma cell lines. The observed changes were not statistically significant; however, it should be noted that the test group of mice was not particularly large, and the observed difference in number of survival days, 40 vs 62, was evident. This gives us hope that a larger study group could lead to statistically significant results.

In conclusion, MET receptor expression is typical for myeloma cell lines. The lentiviral construct directed against the MET receptor mRNAs can effectively reduce expression of the MET receptor in myeloma cells. Further studies are necessary to evaluate the effect of the reduction of MET receptor expression in multiple myeloma, focusing on animal models with a larger test group size.

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Urine nerve growth factor (NGF) level, bladder nerve staining and symptom/problem scores in patients with interstitial cystitis

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Abstract

Background. Interstitial cystitis (IC)/bladder pain syndrome (BPS) is a challenging disease, affecting thousands of people all around the world, especially women. Although there have been numerous theories regarding IC/BPS etiology, the physiopathology of the disease still remains unclear and there is a lack of certain treatment.

Objectives. The aim of the study was to assess the role of nerve fibers and nerve growth factor (NGF) in the etiopathogenesis of IC/BPS symptoms by demonstrating if there is a correlation between urine NGF levels, amount of peripheral nerves in bladder mucosa and symptom severity.

Material and methods. A prospective clinical study was conducted with 15 IC/BPS patients and 18 controls. Urine NGF levels were measured by enzyme-linked immunosorbent assay (ELISA). Bladder punch biopsies were obtained from 15 IC/BPS patients and 9 controls. Immunohistochemistry was performed for S-100 to highlight peripheral nerve twigs in bladder mucosa. The O'Leary-Sant Interstitial Cystitis Symptom and Problem Index (OSICSPI) was used to assess symptom severity and effects of the disease on the patients' life.

Results. NGF normalized to urine creatinine (NGF/Cr) levels in IC/BPS patients were significantly higher than in controls, 0.34 ±0.22 and 0.09 ±0.08 pg/mL: mg/dL, respectively ($p < 0.001$). The mean symptom score in IC patients was 12.27 ±2.4 (median: 12) and the mean problem score was 10.9 ±2.3 (median: 12). The mean mucosal nerve (S-100 stained) area in the IC/BPS group was significantly higher than in the controls, 2.53 ±1.90 vs 1.0 ±0.70, respectively ($p = 0.018$). In correlation analyses, the NGF/Cr level in IC/BPS patients was found significantly correlated with the O'Leary-Sant IC Symptom and Problem Index scores independently ($p = 0.001$ and $p = 0.028$, respectively).

Conclusions. NGF seems to be a promising biomarker in IC/BPS. It may help clinicians in diagnoses and patient follow-up. Thus, unnecessary, expensive and invasive tests, interventions and treatments might be avoided.

Key words: nerve growth factor, bladder pain syndrome/interstitial cystitis, nerve staining

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Introduction

Interstitial cystitis (IC)/bladder pain syndrome (BPS) is a challenging disease that affects thousands of people worldwide, especially women. IC/BPS is characterized by pelvic pain, pressure and discomfort perceived to be related to the urinary bladder, associated with lower urinary tract symptoms (LUTS) in the absence of urinary infection and other obvious pathology.[1]

Although there are numerous theories in regard to IC/BPS etiology, its physiopathology still remains unclear and there is a lack of certain treatment.[2]

Various diagnostic tests and attempts at treatment lead to a substantial economic burden as well as patient distress. Thus, using biomarkers in diagnosis has garnered greater interest in the last decade.[3] Nerve growth factor (NGF) is one of these.

In the present study, we aimed to assess the role of nerve fibers and nerve growth factor (NGF) in the etiopathogenesis of IC/BPS symptoms by demonstrating if there is a correlation between urine NGF levels, amount of peripheral nerves in bladder mucosa and symptom severity. This might open a new horizon for disease diagnosis and yield novel treatment alternatives.

Material and methods

After local ethics committee approval, a prospective clinical study was conducted between March, 2014 and February, 2015 with 15 patients diagnosed with IC/BPS according to AUA 2011 criteria[1] and 18 controls. Written informed consent was obtained from patients and controls. Previous intake of oral medications or intravesical instillations for the treatment of IC/BPS was not exclusionary. The sole inclusion criterion for the patient group was to be symptomatic. The control group consisted of patients who underwent cystoscopy for previous bladder tumor surveillance or LUTS. Urinary tract infections were excluded by means of urine culture in all cases. The O'Leary-Sant Interstitial Cystitis Symptom and Problem Index (OSICSPI) was used to assess the symptom severity and effects of the disease on the patient's life.[4]

Urine samples and bladder biopsies were obtained from all patients with IC/BPS. Although urine samples were obtained from all controls, bladder biopsies could be obtained from 9 controls. Among these 9 controls, 5 were on follow-up for bladder tumor (with no evidence of disease for at least 1 year) and 4 were the patients who underwent cystoscopy for LUTS.

Midstream clean catch urine specimens were collected from all patients and controls. Urine samples were put on ice and promptly transferred to a biochemistry laboratory for NGF and creatinine (Cr) measurement. The samples were centrifuged at 1500 rpm for 20 min at 4°C. The supernatant was taken into Eppendorf tubes and stored at –80°C until NGF measurement after 3 mL was separated for urinary Cr measurement.

The urine NGF level was measured by the enzyme-linked immunosorbent assay (ELISA) method using a NGF specific ELISA kit (Cloud-Clone Corp., Houston, USA). Assays were performed in accordance with the manufacturer's instructions. All samples were run in duplicate, and then the results were averaged. Afterwards, urine NGF levels were normalized via urine creatinine concentration (NGF/Cr).

Bladder punch biopsies were obtained by means of cystoscopy under local or general anesthesia from the lesion sites or suspicious areas of the bladder, if present, otherwise from the lateral wall in IC/BPS and from the normal mucosa in controls. Biopsy specimens were put in 4% formaldehyde solution for fixation and submitted to a pathology laboratory. After routine overnight tissue processing, paraffin blocks were prepared and 5 µm sections were obtained for hematoxylin and eosin (H&E) staining and microscopic evaluation. Immunohistochemistry was performed for S-100 to highlight peripheral nerve twigs in bladder mucosa. Immunohistochemical staining was carried out according to standard procedures using a polymer detection system (Cat. No. DS9800, Leica Biosystems, Wetzlar, Germany) and a BOND-MAX automated immunostainer. S-100 primary antibody (Dako, Glostrup, Denmark) was applied at a dilution of 1/400.

Evaluation of nerve density using S-100 immunohistochemistry

Visual analysis

The amount of peripheral nerve twigs in bladder mucosa was evaluated and scored semiquantitatively by a pathologist (DEB) under the microscope on a range of 0–3 as follows: 0 = <1 mean nerve twig (NT) per high power field (hpf)$^{-1}$; 1 = 1–2 NTs/hpf^{-1}; 2 = 3–4 NTs/hpf^{-1}, and 3 = ≥ 5NTs/hpf^{-1} at various thickness (Fig. 1). Subjects were divided into 3 groups – no staining, mild staining and obvious staining – according to the aforementioned scores (no staining = score 0; mild staining = score 1; obvious staining = score 2 or 3).

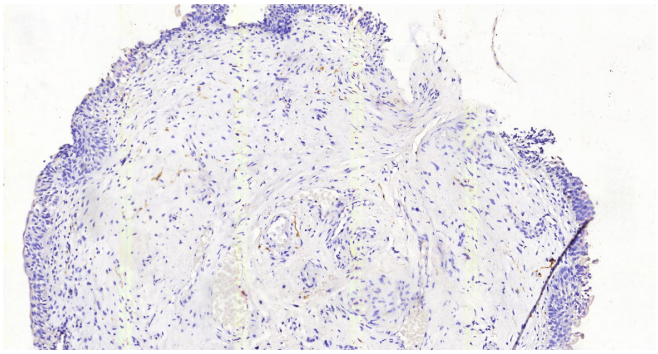

Fig. 1. Microscopic examination of urinary bladder specimen with no nerve staining at magnification ×10 with H&E and S-100 stains

Image analyzer

The strength of the immunohistochemical staining of urinary bladder was assessed using a computer-assisted image analyzer (Pannoramic250 Flash II; 3DHistech, Budapest, Hungary) and the density of nerve fibers was determined automatically.

Statistical analysis

Mean ±SD and median values were used to express quantitative measurements. Numbers and percentages were given for qualitative measurements. The Shapiro-Wilk test was used to determine whether the measurements distributed normally or not. The Mann-Whitney U test was used to compare numerical values between independent groups. For the comparison of the qualitative characteristics of the 2 groups, cross table analysis, χ^2 Fisher's exact test and the Fisher-Freeman-Halton test were applied. Statistical analysis was performed via IBM SPSS statistics v. 21 (IBM Corp., Armonk, USA) and $p < 0.05$ was considered to indicate statistical significance.

Results

A total of 33 patients were included in the study, of whom 15 were patients with IC/BPS and 18 were controls. All patients with IC/BPS were females, whereas among the 18 controls, 12 were males and 6 were females. The mean age of the IC/BPS patients was 52 ±9.1 years, while that of the control group was 46.9 ±19.2 years. There was no statistically significant difference between IC/BPS patients and controls in terms of age ($p > 0.5$). Patient demographics are given in Table 1.

The mean urine NGF level normalized to the urine Cr level (NGF/Cr) in IC/BPS patients was significantly higher than in controls, 0.34 ±0.22 pg/mL: mg/dL and 0.09 ±0.08 pg/mL: mg/dL, respectively ($p < 0.001$). The mean symptom score in IC/BPS patients was 12.27 ±2.4 (median: 12) and the mean problem score was 10.9 ±2.3 (median: 12) (Table 1).

The biopsies of the controls displayed only a few nerve twigs in bladder mucosa in general (Fig. 1). On the other hand, the majority of IC/BPS patients (93.3%) had an increased number of mucosal nerve twigs (Fig. 2), visually scored as 2 or 3 in S-100 stained slides (Table 2). This difference between the control and study groups was statistically significant ($p < 0.05$).

Image analysis showed similar results concerning the difference in the amounts of nerve twigs between the 2 groups (Table 1). The mean mucosal nerve (S-100 stained) area in the IC/BPS group was significantly higher than in the controls, 2.53 ±1.90 vs 1.0 ±0.70, respectively ($p = 0.018$).

In correlation analyses (Table 3), the normalized NGF level (NGF/Cr) in IC/BPS patients was found significantly and

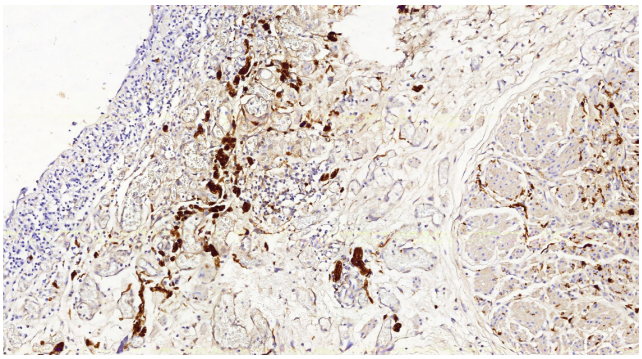

Fig. 2. Microscopic examination of urinary bladder specimen with obvious nerve staining at magnification ×10 with H&E and S-100 stains

Table 1. Patient age, NGF, automated nerve density and questionnaire scores

Variable	Group		p-value
	IC/BPS (n = 15)	Control (n = 18)	
Age (mean ±SD) [years]	52 ±9.1	46.9 ±19.2	>0.5
NGF/Cr (mean ±SD) [pg/mL : mg/dL]	0.34 ±0.2	0.09 ±0.08	<0.001
Automated nerve density (mean S100 (+) area %)	2.53 ±1.9	1.0 ±0.7*	0.018
O'Leary-Sant IC Symptom Index Score	12.2 ±2.4	–	–
O'Leary-Sant IC Problem Index Score	10.9 ±2.3	–	–

* n+9.

Table 2. Distribution of patients according to nerve staining

Visual nerve staining density groups	Group		Total
	IC/BPS	control	
Group 1. Number of patients with no staining	1	6	7
Group 2. Number of patients with mild staining	10	3	13
Group 3. Number of patients with obvious staining	4	0	4
Total	15	9	24

Table 3. Correlation analyses

Variable	Spearman's rho	O'Leary-Sant IC Symptom Index Score	O'Leary-Sant IC Problem Index Score	NGF/Cr level
NGF/Cr level	correlation coefficient	0.777	0.567	–
	significance (2-tailed)	0.001	0.028	–
Visual nerve density	correlation coefficient	0.577	0.640	0.325
	significance (2-tailed)	0.024	0.010	0.237
Automated nerve density	correlation coefficient	0.420	0.650	0.265
	significance (2-tailed)	0.119	0.009	0.341

independently correlated with the O'Leary-Sant IC Symptom and Problem Index scores (p = 0.001 and p = 0.028, respectively). Visually analyzed mucosal nerve density and image analyzed nerve density were found to be correlated with the O'Leary-Sant IC Problem Index (p = 0.01 and p = 0.009, respectively). However, image analyzed nerve density was not correlated with the O'Leary-Sant IC Symptom Index (p = 0.119), whereas visually analyzed nerve density was (p = 0.024). Furthermore, no correlation was observed between nerve density and NGF/Cr levels (p = 0.34).

Discussion

IC/BPS is a debilitating, chronic disease characterized by pelvic pain, discomfort and pressure perceived to be related to the urinary bladder and accompanied by at least one LUTS-like urgency or frequency.[5] The prevalence of IC/BPS is higher in females compared to males, 52–500/100,000 vs 8–41/100,000 and its incidence is estimated to be 1.2/100,000.[6]

IC/BPS etiology is still unknown which leads to inaccurate diagnostic tests and treatment modalities. It has been thought to be associated with several conditions such as chronic fatigue syndrome, fibromyalgia, irritable bowel syndrome, temporomandibular junction disorder, chronic pelvic pain syndrome, vulvodynia, migraine, sicca syndrome, allergies, asthma, and depression.[7] Many theories have been suggested in respect of IC/BPS pathogenesis, which consists of increased urinary bladder permeability, abnormal neuronal function, mast cell activation, autoimmunity, glycosaminoglycan (GAG) layer defect, infection, and urinary toxic and anti-proliferative agents.[6,8–11]

The first study which focused on the nerve fibers in IC/BPS was conducted by Hand in 1949. In that study, he found submucosal nerve fiber increase in the urinary bladders of IC/BPS patients and concluded that nerve fibers within closed mast cells might have a role in disease pathogenesis.[12] In concordance with Hand, Christmas et al. also observed nerve fiber proliferation in the suburothelium and detrusor muscle layer of the urinary bladder of IC/BPS patients compared to controls and other patients with chronic cystitis.[13] Pang et al. showed an increase in nerve fibers containing substance-P in the submucosa of IC/BPS patients but not in the detrusor. They suggested that substance-P, a neuropeptide secreted from sensorial nerve endings, might act in pain pathophysiology as well as triggering mast cell secretion.[14]

Hofmeister et al. also attempted to establish a diagnostic algorithm for IC/BPS by using quantitative image analysis and morphometry. They suggested that the neuroimmune process or mediation might be a part of the disease pathogenesis and a count of either mast cells or nerve fibers could be used in IC/BPS diagnosis.[15]

In concordance with the aforementioned studies, we found the mucosa of the urinary bladder of IC/BPS patients to have significantly increased nerve fiber proliferation, either visually or image analyzed. None of the patients in the control group demonstrated obvious nerve staining in the urinary bladder in contrast to IC/BPS patients, which may support the role of a neural process in IC/BPS etiopathogenesis.

Studies conducted in recent years have shown increased levels of NGF in the urothelium, where the sensorial nerve fibers end, in patients with IC/BPS, idiopathic urgency and chronic cystitis. NGF was thought to be responsible for hyperalgesia in the absence of inflammation. NGF was assumed to act via a direct effect on sensorial nerve fiber endings or increasing sensorial neuropeptides, substance-P or calcitonin-gene-related peptide.[16] In another study, NGF mRNA was found significantly increased in bladder tissues of IC/BPS patients compared to controls, and decreased to normal levels as controls after botulinum toxin A injection.[17] A similar study based on urinary NGF again showed that a decrease in the NGF level could be correlated with treatment response in some IC/BPS patients.[18] Many studies have presented a significant increase of NGF levels in IC/BPS and neurogenic overactive bladder, but stressed that further studies were mandatory to determine its role in the pathogenesis.[19]

Some researchers investigated both serum and urine NGF in IC/BPS patients, but no correlation was found between urine and serum NGF levels. Moreover, no association was observed between an elevated serum NGF level and disease severity. It was suggested that an elevated serum NGF level might be a consequence of comorbidities

of IC/BPS patients rather than the cause of IC/BPS. Elevated urinary NGF levels were deemed to support the role of chronic inflammation in the disease pathogenesis.[20]

In a recent study, inflammation intensity in the urinary bladder was shown to be significantly correlated with suburothelial NGF levels and transient receptor potential cation channel subfamily V member 1 (TRPV1)-immunoreactive nerve fibers. Nerve fiber density was also significantly correlated with pain and urgency scores.[21]

In a meta-analysis, NGF and NGF/Cr levels in the urine of patients with IC/PBS were found higher than in controls and lower than in overactive bladder syndrome patients. NGF is thought to be a potentially useful biomarker in IC/BPS diagnosis and differential diagnosis. NGF was also proposed to be a predictor of specific treatment modalities.[3] Antibodies against NGF have emerged as a promising treatment choice for IC/BPS patients.[22,23]

In our study, urinary NGF levels in the urine of IC/BPS patients were significantly higher than those of the controls. Furthermore, the normalized urine NGF level (NGF/Cr) in IC/BPS patients was found significantly correlated with the O'Leary-Sant IC Symptom and Problem Index scores independently. This raises the possibility of using NGF to establish disease alleviation or progression on patient follow-up without the need of an invasive test.

Unfortunately, no statistically significant association was observed between nerve staining and NGF levels, which we believe to be the consequence of the small patient population. However, NGF biology and production might also be considered. It has been shown that, aside from nerve cells, many different cell types, such as vascular and smooth muscle cells, produce NGF in the human body.[24]

Our study has some limitations. As previously mentioned, our patient population was small and, whereas all the IC/BPS patients were female, some of the controls were males. But the results were similar if we excluded the male controls. Some of the control patients had a history of bladder tumor, so we preferred patients with no evidence of tumor for at least 1 year to prevent the possible effect of the bladder tumor on bladder nerve staining and urine NGF levels.

Conclusions

NGF appears to be a promising biomarker in patients with IC/BPS. The correlation of NGF and disease symptom/problem scores might help clinicians in diagnosis and patient follow-up, so that unnecessary, expensive and invasive tests, interventions and treatments might be avoided. Additional randomized controlled studies with larger patient populations are mandatory to confirm these results.

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Splenectomy delays fracture healing by affecting the level of tumor necrosis factor alpha, interleukin 6 and bone morphogenetic protein

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Abstract

Background. Abdominal injuries combined with bone fractures are increasing. Splenectomies are often required, but have prolonged healing time for bone fracture.

Objectives. The aim of the study was to explore the molecular mechanism for splenectomy delaying fracture healing.

Material and methods. Eighty-four patients (42 received splenectomy) who received hip fractures operations were recruited in our hospital. One-year follow-up analysis was performed. To ensure the results, an animal model was established. Sprague-Dawley (SD) rats were randomly divided into 5 groups: group A: experimental group, femoral fractures + splenectomy; group B: femoral fractures; group C: splenectomy; group D: femoral fracture + sham splenectomy; group E: sham fracture. After the femoral fracture surgery, the callus status was evaluated by X-ray.

Results. After 1-year follow-up, the healing index and bone quality was higher in the fracture-operated-only group than in the splenectomy group. In contrast, the rate of healing complications was lower in the fracture-operated-only group than in the splenectomy group. Biomarker analysis showed that the serum levels of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and bone morphogenetic protein (BMP) were higher in the fracture-operated-only group than in the splenectomy group. No difference of the callus status was found among the rats in groups B, D and E ($p > 0.05$), while there were significant differences of the callus status of the rats in groups A and C at different stages ($p < 0.05$). On the other hand, the levels of TNF-α, IL-6 and BMP increased, reached peak after 7-day splenectomy surgery, and then decreased significantly in groups A and C ($p > 0.05$).

Conclusions. Splenectomy delays fracture healing by affecting the levels of TNF-α, IL-6 and BMP.

Key words: immune function, splenectomy, fracture, tumor necrosis factor-alpha, bone morphogenetic protein

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Introduction

Abdominal injuries combined with bone fractures caused by accident are increasing.[1] Spleen rupture accounts for 20–40% of blunt abdominal trauma cases. In many cases, splenectomies are required to save the patients' life. Follow-ups have found that patients with splenectomy have prolonged healing time for bone fractures.

During the bone formation process, there is a balance between osteoblast activity and osteoclast activity. The role of osteoblasts is to synthesize the bone matrix, and they are differentiated from the mesenchymal stem cells; the role of osteoclasts is to degrade the bone matrix, and they are differentiated from bone marrow mononuclear macrophages. The fracture healing process requires a lot of new osteoblasts,which help speed up bone synthesis and increase bone volume and density.[2]

Bone fracture healing is a complex process of bone regeneration, in which immune factors may play important roles.[3,4] Cytokines, a group of proteins known to regulate hemopoietic and immune activities, have been found to be involved in fracture healing.[5] The interleukin 6 (IL-6) polymorphism has been found to be associated with fracture risk. Meta-analysis shows that the IL-6-174 C/G polymorphism is related to the risk of wrist and osteoporotic fracture.[6] Multiple studies show that TNF-α, in combination with the host reservoir of perifracture mesenchymal stem cells, is associated with bone healing.[7] Bone morphogenetic protein (BMP) is a kind of multi-functional growth factor which belongs to the transforming growth factor beta (TGF-β) superfamily. High-level expression of BMP improves bone callus formation and shortens the time of bone fracture healing.[8] In this study, delayed bone healing was found in the fracture-operated patients who also received splenectomy. Furthermore, an experimental animal splenectomy model was used to investigate the effects of immune function changes after splenectomy on fracture healing in a rat model.

Material and methods

Patients

All procedures were approved by the ethics committee of Shengjing Hospital of China Medical University (Shenyand, China). From May 2, 2010 to July 7, 2015, all hip fracture patients were reviewed at our hospital. Patients with the following diseases were excluded: osteomalacia, vitamin D deficiency and hyperthyroidism, as they can affect the normal development of bone. The hip fracture patients who also received splenectomy were selected as the splenectomy group (SFG), according to the International Statistical Classification of Diseases and Related Health Problems v. 9, Clinical Modification (ICD--9-CM).[9,10] Participants without splenectomy were randomly selected as the "hip-fracture-only group" (FG). The 2 groups were matched by sex, age and baseline characteristics (Table 1). A total of 84 patients (42 received splenectomy) who received a hip fracture operation in our hospital were selected.

Bone quality analysis

Bone quality at a non-fracture site was assessed according to the criteria offered by Singh.[11] Bone quality is classified by 6 grades: from grade 1 (poor bone quality) to grade 6 (normal bone quality).

Healing complications

Fracture healing complications were investigated after surgical repair and after up to 1 year. The complications included the rates of infection, delayed union and surgical re-intervention.

ELISA analysis of bone healing biomarkers in serum

The ELISA kits used to analyze the serum levels were the Human TNF-alpha ELISA Kit (ab46087), Human IL-6 ELISA Kit (ab46042) and Human BMP-2 ELISA Kit (ab119581). All the kits were purchased from Abcam Trading Company Ltd. (Shanghai, China).

Animals

To investigate the molecular mechanism for splenectomy delaying fracture healing, an animal model was established and the main molecules were examined. Healthy 12-week-old Sprague-Dawley (SD) rats (SPF grade, body weight: 300 ±35 g) were provided by Shengjing Hospital of China Medical University Experimental Animal Center. They were randomly divided into 5 groups (n = 8 for each group) by random number table: group A: femoral fractures + splenectomy; group B: femoral fractures only; group C: splenectomy only; group D: femoral fracture + sham splenectomy; group E: sham fracture. The rats were housed in the Experimental Animal Center, Shengjing Hospital of China Medical University. This study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (2013PS11K).

Surgical methods

Femoral fractures were made by a 0.8 mm diameter wire saw. After anesthesia, a lateral femoral incision of about 2.5 cm was made in the left leg of the rat. Through the skin incision and intermuscular space, the full-length femur was exposed, and then a transverse fracture was made at the midpoint of the femoral shaft using a 0.8 mm diameter wire saw, followed by fixation with a 2.0 intramedullary

Kirschner wire. After washing with povidone-iodine and saline, the muscle and skin were sutured. In the femur-fracture sham group, a suture was performed after just exposing and disinfecting the femur.

After anesthesia and skin preparation, with the rat in the right lateral position, a vertical incision was made at 1.5–2.0 cm under the left costal arch. Under sterile conditions, the stomach was put to the right to dissociate the spleen (a long strip of about 4 cm), and then the splenic arteries were clamped and cut off (about 4–6 arteries, arranged in parallel). Finally, the spleen was removed and the cut was sutured. The splenic arteries in the sham group were briefly exposed but not cut off, and the spleen was not removed.

Radiography

Digital radiography (DR) of the femur was performed to evaluate the femur status. All the images were analyzed as grayscale density by using Image-Pro-Plus 6.0 (Media Cybernetics Inc., Rockville, USA). The bone callus areas were analyzed in the DR images. Callus grayscale rates were calculated as ratios of the grayscale densities of the callus area to those of the normal bone areas. After surgery, the rats in groups A, B and D underwent digital radiography on day 7, 14, 28, 42, 56, 70, and 84.

Real-time polymerase chain reaction

A blood sample (1.0 mL) was taken via the orbital venous plexus, placed in a sterile EP tube with EDTA, and preserved under 4°C. The extraction of total RNA was performed with a TaKaRa extraction Kit (Takara Bio, Dalian, China) according to the manufacturer's instructions. The extracted RNA had an OD260/OD280 ratio of 1.7–2.0.

To 1.0 μL total RNA, 2.0 μL 5 × gDNA Eraser buffer, 1.0 μL gDNA Eraser, and RNase free dH_2O were added to a total volume of 10 μL, and then the solution was kept at 42°C for 2 min to remove genomic DNA. The treated solution was mixed with 4.0 μL 5 × PrimeScript® Buffer, 1.0 μL PrimeScript® RT Enzyme Mix I, 1.0 μL RT Primer Mix and 4 μL RNase free H_2O at 37°C for 15 min, and 85°C for 5 s for reverse transcription reaction. The resulting rDNA was finally stored at 4°C.

Every 10 μL of polymerase chain reaction (PCR) mixture contained 1 μL cDNA solution, 5 μL SYBR® Premix Ex Taq™ (2×), 1 μL PCR primers (5 μM), 3 μL dH_2O. A Roche LightCycler® 480II quantitative real-time PCR amplification system was used for the PCR. The mRNA levels of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and bone morphogenetic protein (BMP) were determined on day 0, 2, 7, 14, 21, 28, and 42.

Western blot

Protein was isolated using a protein isolation kit (Cat. No. ab65400, Abcam Trading Company Ltd.). Polyclonal rabbit anti-rat interferon alpha (ab191903), anti-rat IL-6 (ab7737), anti-rat BMP antibodies (ab118520) and goat anti-rabbit IgG H&L (ab6721) were purchased from Abcam Trading Company Ltd. (Shanghai, China). A rabbit anti-rat β-actin polyclonal antibody (Cat No. 4967, Cell Signaling Technology, Danvers, USA) was used as a loading control. All protein bands were visualized using an enhanced chemiluminescence substrate (Sangon Biotech Co. Ltd., Shanghai, China). The image intensity of the protein band was quantified by using NIH ImageJ software (Bethesda, USA).

Data analysis

All data was presented as number, mean values ±SD, and were analyzed using SPSS statistical software 20.0. Callus grayscale rates were calculated in group A, B and D. Fold induction was calculated to show the relative expression of the target gene in the experimental group.[12] The χ^2 test and Student's t-test were used to compare the 2 sets of data for determining whether there were significant differences between the 2 groups. There were statistically significant differences if $p < 0.05$.

Results

Baseline characteristics

After careful selection, there was no significant difference for healing index, bone quality and healing complications between SFG and FG groups ($p > 0.05$) (Table 1). Meanwhile, other parameters were also matched between the 2 groups ($p > 0.05$) (Table 1).

Outcome after one-year follow-up

After 1-year follow-up, the healing index and bone quality were higher in the fracture-operated-only group than in the splenectomy group ($p < 0.001$) (Table 2). In contrast, the rate of healing complications was lower in the fracture-operated-only group (FG) than in the splenectomy group. The biomarker analysis showed that the serum levels of TNF-α, IL-6 and BMP were higher in the fracture-operated-only group than in the splenectomy group ($p < 0.001$) (Table 2). Splenectomy was associated with worse calcification at the fracture site (fracture healing index ≤3) and worse bone quality (bone quality index >3) than in the FG group ($p < 0.001$) (Table 2). Moreover, patients receiving splenectomy had more complications following hip fracture fixation than those in the FG group ($p < 0.001$) (Table 2).

Table 1. Baseline characteristics of all participants

Variable		FG (n = 42)	SFG (n = 42)	χ^2 statistic/t-value	p-value
Gender [male/female]		27/15	25/17	0.2019	[a]0.653
Age [years]		39.8 ±11.2	40.6 ±10.6	1.34164	[b]0.114
BMI		24.8 ±5.6	25.2 ±4.8	0.738	[b]0.244
Fracture healing index	≤3	3	2	0.213	[a]0.645
	>3	39	40	0.213	[a]0.645
Bone quality index	>3	3	4	0.156	[a]0.693
	≤3	39	38	0.156	[a]0.693
Healing complications	yes	40	41	0.346	[a]0.557
	no	2	1	0.346	[a]0.557
Baseline comorbidities					
Cystic kidney disease		1	1	0	[a]1
Human immuno deficiency virus diagnosis		–	–	–	–
Diabetes mellitus		3	2	0.213	[a]0.645
Drug dependence		2	3	0.213	[a]0.645
Urolithiasis		2	1	0.346	[a]0.557
Urinary tract infection		1	1	0	[a]1
Biomarker analysis					
TNF-α [pg/mL]		19.4 ±4.8	18.7 ±4.4	0.862	[b]0.274
IL-6 [pg/mL]		11.5 ±3.0	12.1 ±3.6	2.178	[b]0.715
BMP [pg/mL]		61.9 ±17.2	65.3 ±16.5	0.623	[b]0.137

TNF-α – tumor necrosis factor alpha; IL-6 – interleukin 6; BMP – bone morphogenetic protein; [a] p –value was calculated by χ^2; [b] calculated with Student's t-test for independent samples. There were statistically significant differences if $p < 0.05$. FG – hip-fracture-only patients without splenectomy were randomly selected as the fracture group; SFG – the hip fracture patients who also received splenectomy were selected as the splenectomy group.

Table 2. Analysis of bone healing after 1-year follow-up

Variable		FG (n = 42)	SFG (n = 42)	χ^2 statistic/t-value	p-values
Fracture healing index	≤3 >3	38 4	23 19	13.4711	0.001
Bone quality index	>3 ≤3	40 2	25 17	15.303	0.001
Healing complications	yes no	4 38	28 14	29.077	0.001
Biomarker analysis					
TNF-α [pg/mL]		18.7 ±5.8	12.1 ±4.0	6.123	0.002
IL-6 [pg/mL]		12.6 ±3.2	8.7 ±2.9	8.637	0.001
BMP [pg/mL]		60.1 ±18.6	54.3 ±17.3	9.794	0.001

TNF-α – tumor necrosis factor alpha; IL-6 – interleukin 6; BMP – bone morphogenetic protein; [a] calculated with Student's t-test for independent samples; p-value was calculated by χ^2 test. There were statistically significant differences if $p < 0.05$. FG – hip-fracture-only patients without splenectomy were randomly selected as the fracture group; SFG – the hip fracture patients who also received splenectomy were selected as the splenectomy group.

Fracture status of rat models

After femur fracture, the formation of the callus was demonstrated by radiography. Figure 1A shows that the callus formation was delayed. Figures 1B and 1D show that the bone callus was formed. Figures 1C and 1E show normal femur bone structure.

The changes of the grayscale intensity ratio of the callus area to normal area are shown in Fig. 2. There was a significant difference between group A and group B or D, while the difference between group B and group D was not significant. No fracture was observed in group C and group E.

Relative mRNA levels of *TNF-α*, *IL-6* and *BMP* in the rat model

As shown in Fig. 3A, 3B and 3C, the mRNA levels of *TNF-α*, *IL-6* and *BMP* in the rats from group A and C reached a peak on day 7 and then decreased. For the rats without splenectomy (groups B, D and E), the mRNA levels of *TNF-α* (Fig. 3A), *IL-6* (Fig. 3B) and *BMP* (Fig. 3C) did

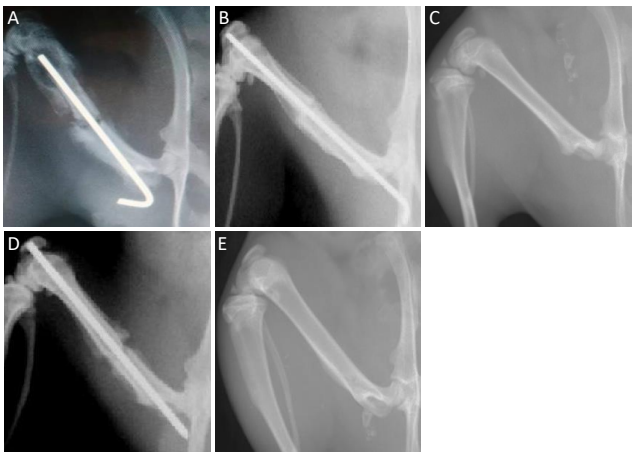

Fig. 1. X-rays show the femur of the rats 28 days after surgery

A: femoral fractures + splenectomy; B: femoral fractures only; C: splenectomy only; D: femoral fracture + sham splenectomy; E: sham fracture.

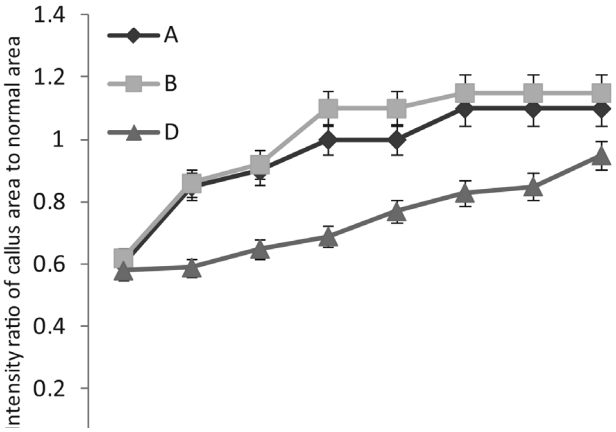

Fig. 2. Changes of the grayscale intensity ratio of the callus area to normal area in the X-ray images

A – femoral fractures + splenectomy; B – femoral fractures only; D – femoral fracture + sham splenectomy.

not show significant changes (Fig. 3C), even 42 days after surgery.

Protein level of TNF-α, IL-6 and BMP in the rat model

The protein levels of TNF-α, IL-6 and BMP were analyzed by western blot. As shown in Fig. 4, the protein level of TNF-α, IL-6 and BMP in the rats from group A and C reached a peak at day 7, and then decreased. For the rats without splenectomy (in groups B, D and E), the TNF-α, IL-6 and BMP did not show significant changes (Fig. 4).

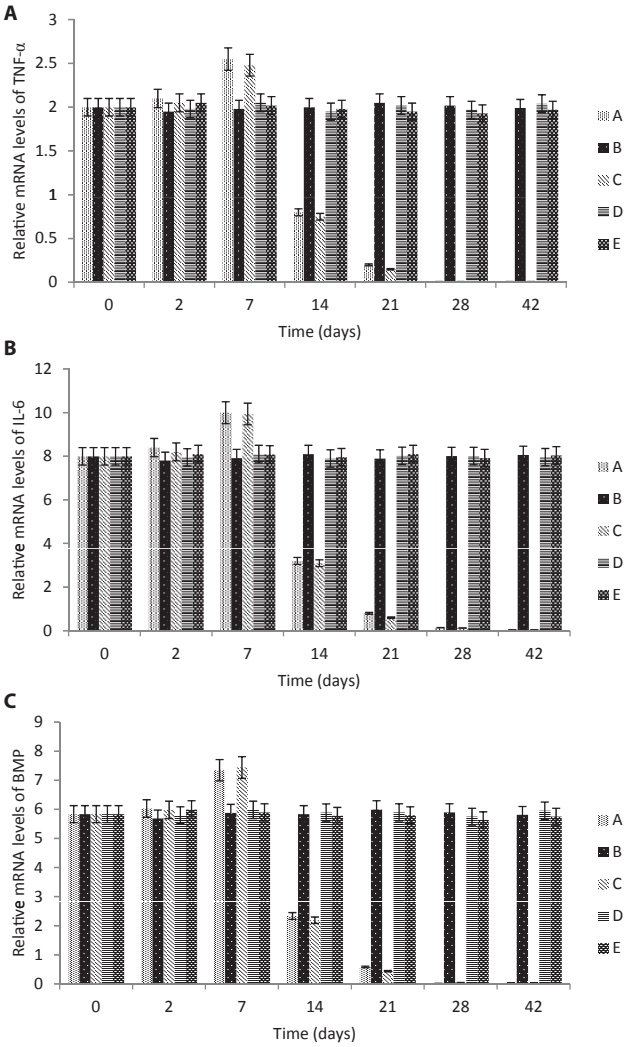

Fig. 3. Quantitative reverse transcriptase real-time PCR (qRT-PCR) analysis of the mRNA levels of *TNF-α*, *IL-6* and *BMP* in peripheral blood

A – mRNA level of *TNF-α*; B – mRNA level of IL-6; C – mRNA level of BMP. Group A – experimental group, femoral fractures + splenectomy; group B – femoral fractures only; group C – splenectomy only; group D – femoral fracture + sham splenectomy; group E – sham fracture.

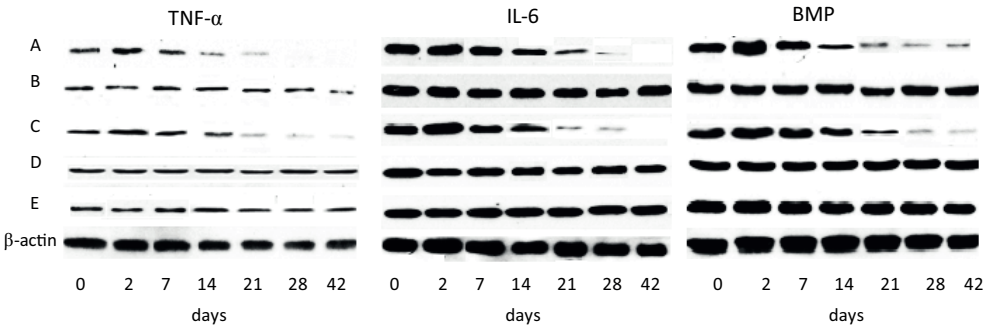

Fig. 4. Western blot analysis of the protein levels of TNF-α, IL-6 and BMP from different groups

Group A – experimental group, femoral fractures + splenectomy; group B – femoral fractures only; group C – splenectomy only; group D – femoral fracture + sham splenectomy; group E – sham fracture.

Discussion

Abdominal injuries combined with fractures caused by the accident trauma result in splenectomy in many patients. The splenectomy group was significantly different from the fracture-operated-only group (Table 2), which demonstrated that splenectomy delays the fracture healing process.

The spleen is an important organ of immunity, containing antigen-presenting T cells and B cells, which produce cytokines like TNF-α or IL-6. There are a lot of cytokines involved in the process of fracture healing. After splenectomy, T lymphocyte subsets in the peripheral blood change. The number of helper T lymphocytes reduces and the number of suppressor T lymphocytes increases. Previous studies have shown that splenectomy leads to a reduction of CD4+ T cells and a reduction of TNF-α and IL-6 produced by other organs.[13,14] The spleen is involved in endotoxin-mediated generation of IL-6. IL-6 levels in dog plasma was reduced by 75% after splenectomy. In this study, in the first 2 weeks after splenectomy, mRNA expression of IL-6 was significantly lower than normal.

TNF-α has been extensively studied in bone and cartilage metabolism, and it has been considered an important intermedia of osteoclasts for many years.[15–17] This study showed that the *TNF-α* mRNA levels after splenectomy increased in the first 7 days and reached a peak on day 7. High levels of TNF-α can promote osteoclast activity and inhibit osteoblast activity. Over time, the mRNA levels of *TNF-α* decreased significantly and became much lower than normal. According to a previous study, lack of the TNF-α signal caused a persistence of the cartilage healing tissue and delayed absorption of the mineralized cartilage tissue, which thereby delayed the original bone reconstruction. And it has been proven that BMP plays a pivotal role in the healing process.[18] Another study showed that TNF-α can induce production of BMP-2.[19] Our study found that splenectomy caused a decrease in the mRNA levels of BMP.

IL-6 is a multifunctional cytokine which is produced by both lymphoid and non-lymphoid tissue cells. IL-6 receptors are found in the cell membrane of various cells, such as activated B cells, quiescent T cells, macrophages, osteoblasts, and osteoclasts, which is the material base of IL-6 function.[20] Many studies have shown that IL-6 plays an important role in bone formation and repair. IL-6 can stimulate the production of the RANK ligand in osteoblasts and promote the transformation of peripheral blood mononuclear cells into bone cells.[21] IL-6 can also induce the differentiation of osteoblast precursors, inhibit the formation of bone medium bone nodules and induce production of cathepsin B.[22–24] In the process of distraction osteogenesis, the osteoblasts, hematopoietic cells and cartilage cell growth-related cells can produce IL-6.[25] A fracture healing rat model has shown that in the process of cartilage reconstruction and cartilage ossification, increased production of IL-6 is observed.[26,27] Thus, continued low mRNA expression of *IL-6* after splenectomy in some way affects the expression of BMP, and results in a delay of the secondary bone reconstruction process.

In short, the fracture healing process is complex and many cytokines are involved. In this pilot study, we studied the trends of *TNF-α*, *IL-6* and *BMP* mRNA levels after splenectomy. The underlying regulatory mechanism is unclear at present.

Conclusions

Changes in the immune function after splenectomy delayed the fracture healing process. The delay of the fracture healing after splenectomy was related to low-level expression of TNF-α, IL-6 and BMP.

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IL-4RA gene expression in PBMC with regard to place of living and atopy status

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

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Abstract

Background. IL-4 and IL-4RA are key factors in allergic inflammation. IL-4 stimulates both IgE production and Th2 lymphocyte differentiation. Increased levels of IL-4 and IL-4RA have been shown in allergic patients. Genetic analyses have confirmed that polymorphisms within the *IL-4RA* gene influence the risk of allergy and can change the expression of the protein. Due to gene-environment interactions, this process is also likely to be modified by environmental exposure.

Objectives. The aim of the study was to evaluate the *IL-4RA* gene expression in peripheral blood mononuclear cells (PBMC) from atopic and non-atopic subjects with regard to place of living (urban vs rural).

Material and methods. We enrolled 38 subjects into the study, 18 of whom were atopic. Atopy was estimated according to the results of a skin prick test. PBMC were isolated from whole blood, total RNA was extracted and reverse transcribed into cDNA. We performed real-time PCR to measure gene expression, the *ACTB* gene was chosen as a reference and the delta-delta Ct (ΔΔCT) method was applied for relative quantification. The Mann-Whitney U test was used for statistics.

Results. We did not observe any statistically significant differences in the gene expression profile between atopic and non-atopic subjects regardless of their place of living. However, a trend was observed for atopic rural inhabitants to have lower levels of *IL-4RA* gene expression than atopic subjects living in the town.

Conclusions. The regulation of *IL-4RA* gene expression is complex and probably influenced by both genetic and environmental factors, such as farming exposures, which could provide the counterbalance to atopy.

Key words: gene expression, asthma, atopy, *IL-4RA*

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Atopy, regarded as a susceptibility to react with IgE to common allergens, is a complex trait involving gene-environment interactions. Even though the genetic background seems to be obvious due to family inheritance of atopic disorders such as asthma or allergic rhinitis, recent genetic studies using gene-candidate and Genome Wide Association Study (GWAS) approaches have not provided a simple solution for disease susceptibility. We know much more about the pathogenesis owing to these studies, but there is still "a missing heritability", and the genetic effect associated with a single nucleotide polymorphism (SNP) or gene seems to be of minor importance. Environmental factors influence genes changing their expression pattern by silencing or activating specific pathways, and this process is crucial as a disease trigger.[1]

In recent years, there have been several studies in which the expression profile was estimated in relation to atopy or asthma. As an example, global gene expression studies in asthma have been performed in peripheral blood mononuclear cells (PBMC), bronchial biopsy specimens and epithelial cells from nasal lavage.[2–4]

This genetic profiling could be used as a new marker of atopic disorders or an indicator of disease severity. In that context, transcriptomic profiles of airway smooth muscle cells have been used for distinguishing atopic asthma subjects from atopic patients without asthma.[5] The cap analysis of gene expression (CAGE) method has been performed in children with asthma (study sample n = 37) to reveal that in subjects with severe asthma, 1305 transcription start sites and 816 known genes are differentially expressed.[6] Further, epithelial gene expression patterns have been pursued to differentiate atopic children from healthy controls (study sample n = 45).[7]

Both atopy per se and atopic disorders, are associated with increased Th2 cell activity. This is characterized by excessive production of IgE in response to different antigens. IL-4 is the key cytokine in IgE-dependent reactions, as it activates Th2 differentiation and directs the immunoglobulin production to generate IgE from IgM. Together with IL-13, IL-4 is also responsible for increasing mucus production and remodeling in asthma. IL-4 exerts its function by binding to IL-4RA, which is the common receptor for both IL-4 and IL-13. The signal is transmitted by activation of Janus kinase (JAK) and phosphorylation of STAT6.[8]

Increased expression of the *IL-4RA* gene has been revealed in only a few studies regarding different atopic phenotypes. One example is the recent research that showed that transfectants carrying SNP within the *FCER2* gene (rs2228137), which is associated with severe asthma, presented higher expression of the *IL-4RA* gene after stimulation with CD23. This phenomenon was related strictly to *IL-4RA*, and did not affect the gene expression of *IL-1A*, *IL-2*, *IL-4*, *TNFA* or *IL8*.[9] Two further studies were done in patients with asthma with the same result.[10,11]

The *IL-4RA* gene has long been considered a good candidate for the asthma and atopy susceptibility gene. In different types of studies – linkage analysis, gene-candidate studies and GWAS – its role has been proven.[12,13] The gene is highly polymorphic. The majority of SNPs are inherited in blocks. The most relevant are present in exon 12 (E375A, S411L, S478P, and Q551R), 1 identified in the promoter (C3223T), and 1 coding for amino acid changes in the extracellular portion of the receptor (I50V). SNPs within the *IL-4RA* gene have been described as associated with severe asthma exacerbations, lower lung function, and increased mast-cell-related tissue inflammation.[14] Cells presenting different forms of IL-4RA (I50 or V50) have been shown as presenting an altered phosphorylation pattern of STAT6 during IL-4 stimulation, thus affecting IL-4RA function.[8] Previously, we have demonstrated the effect of C3223T polymorphism within the promoter of the *IL-4RA* gene and I50 on atopy susceptibility.[15] These polymorphisms are also associated with a decreased level of soluble IL-4R.[16]

Here we report the difference in *IL-4RA* gene expression with regard to atopy status and place of living (urban vs rural).

Material and methods

In the study, we enrolled 38 subjects attending an allergy outpatient or inpatient clinic, 18 of whom were atopic. Atopy was estimated according to the results of a skin prick test to common allergens (dust mites – *Dermatophagoides pteronyssinus, Dermatophagoides farinae* – cat, dog, birch, mix of grasses) with at least 1 positive result of ≥3 mm. Control subjects had a negative skin prick test result. The procedure was performed according to the European Academy of Allergy and Clinical Immunology standards. Venous blood samples were taken into ethylenediaminetetraacetic acid (EDTA) probes (SARSTEDT AG & Co., Nümbrecht, Germany). PBMC were isolated from whole blood using the gradient method. We extracted total RNA using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, USA) according to the manufacturer's protocol. Reverse transcription to cDNA was carried out using the First Strand DNA Reverse Transcription Kit (Roche Ltd., Basel, Switzerland) according to the manufacturer's protocol. We performed real-time PCR for gene expression using LightCycler 1.5 with specific hybridization probes (Universal ProbeLibrary, Roche, Basel, Switzerland) and LightCycler TaqMan Master Mix (Roche) according to the manufacturer's protocol. We chose the *ACTB* gene as a reference gene. Delta Ct (ΔCT) and delta-delta Ct (ΔΔCT) values were determined (ΔCT = Ct GENE – Ct ACTB) for relative quantification and 2^–ΔΔCT for the related fold change estimation. The Mann-Whitney U test was used for statistics to compare ΔCT between the groups. The study was approved by the Ethics Committee of Wroclaw Medical University, Poland. All participants signed an informed consent.

Results

The characteristics of the study group are presented in Table 1. The group consisted of children and adults. The expression level of *IL-4RA* in children vs adults did not differ significantly. Four subjects skipped the skin prick tests, so the atopy status was not estimated.

We did not observe statistically significant differences in *IL-4RA* expression within the groups ($p > 0.05$). Atopic subjects had slightly higher *IL-4RA* gene expression levels than non-atopic subjects (Fig. 1). When comparing gene expression according to the place of living (urban vs rural), a trend was observed for rural dwellers to have lower gene expression levels of the *IL-4RA* than their urban counterparts. The urban inhabitancy was a similar stimulator for *IL-4R* expression and atopy (2^-ΔΔCT 2.07 vs 2.0139) (Fig. 2). This phenomenon was more pronounced in atopic subjects (urban vs rural 2^-ΔΔCT 2.75) (Fig. 3).

Table 1. Characteristics of study group

Parameter	Control (n = 16)	Atopic (n = 18)
Age	mean 14.84 ±12.1 min 1.5 max 47 median 10.5 (7.5–18.5)	mean 11.55 ±7.42 min 3 max 36 median 11.5 (6–14)
Sex	males 5 females 11	males 7 females 11
Place of living	village 4 town 12	village 5 town 13

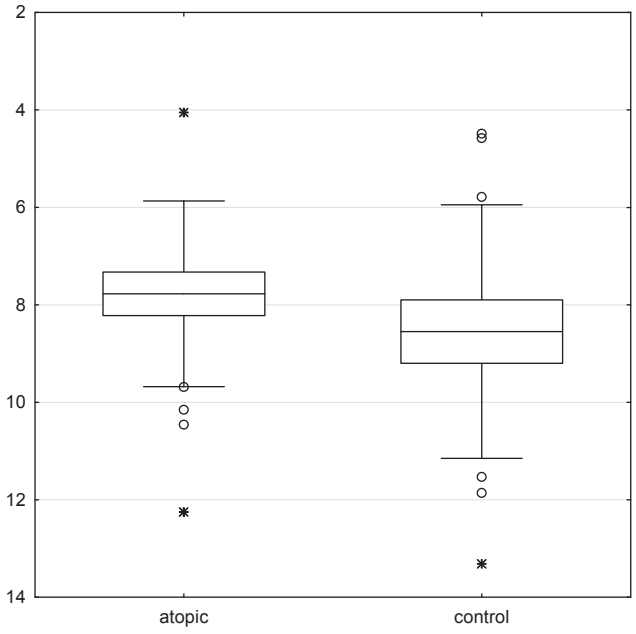

Fig. 1. *IL-4RA* gene expression in PBMC in atopic vs control subjects

Gene expression of the *IL-4RA* gene was expressed as ΔCT, where ΔCT = Ct *IL-4RA* – Ct *ACTB* for each sample; the lower the ΔCt values, the greater the amount of gene expression; the Y-axis has been reversed to better illustrate the effect; data is given as mean ±SE and ±SD; fold change 2^-ΔΔCT = 2.0139.

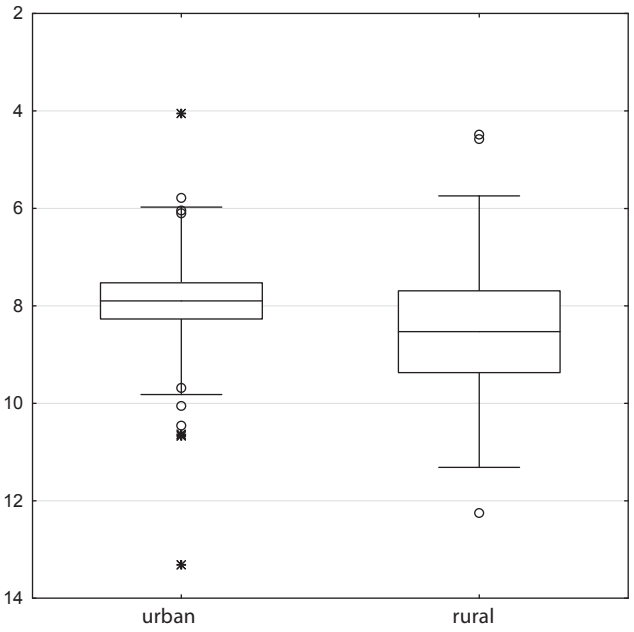

Fig. 2. *IL-4RA* gene expression in PBMC in urban vs rural subjects

Gene expression of the *IL-4RA* gene was expressed as ΔCT, where ΔCTC = Ct *IL-4RA* – Ct *ACTB* for each sample; the lower the ΔCt values, the greater the amount of gene expression; the Y-axis has been reversed to better illustrate the effect; data is given as mean ±SE and ±SD; fold change 2^-ΔΔCT = 2.07.

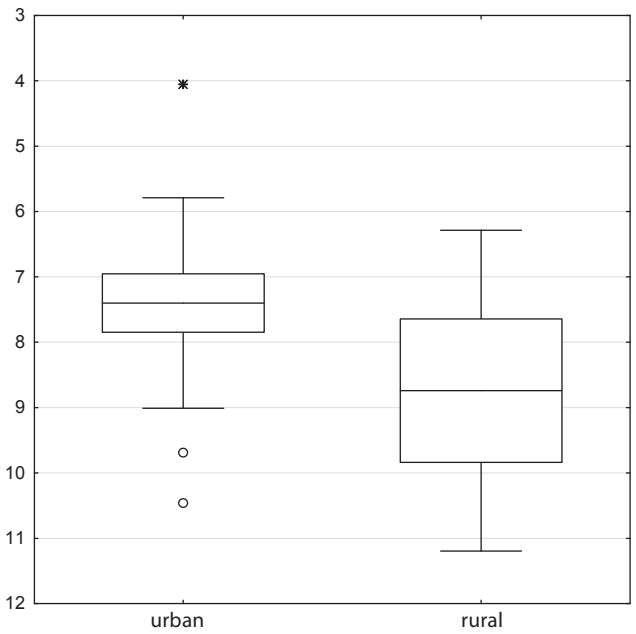

Fig. 3. *IL-4RA* gene expression expressed as ΔCT in atopic subjects living in urban vs rural environment

Gene expression of the *IL-4RA* was expressed as ΔCT, where ΔCT = Ct *IL-4RA* – Ct *ACTB* for each sample; the lower the ΔCt values, the greater the amount of gene expression; the Y-axis has been reversed to better illustrate the effect; data is given as mean ±SE and ±SD; fold change 2^-ΔΔCT = 2.75.

Discussion

In this study, we report that *IL-4RA* gene expression did not differ significantly in terms of atopy and place of living (urban vs rural). Only slight differences were observed for atopic and urban subjects to have higher *IL-4RA* expression. Most interestingly, atopic subjects living in rural settings tend to have lower *IL-4RA* gene expression in comparison to atopic subjects living in urban ones. In relation to that fact, it could be presumed that the expression of *IL-4RA* is regulated by both atopy and environmental factors. What is more, farming exposures associated with the rural environment seem to be a protective factor, corresponding with lower levels of *IL-4RA* expression.

IL-4RA gene expression has been studied in several genome-wide expression analyses as one of the components; however, there were only a few studies focused specifically on *IL-4RA* expression. In 2014, Pascual et al. performed genome-wide expression profiling of B lymphocytes and revealed the IL-4RA protein-coded transcript was one of the most differentially expressed transcriptomes of the B cells between the group of patients with allergic asthma and the controls.[10] These results were further validated within the relative expression analysis of *IL-4RA* in CD19+ B cells from 10 subjects and PBMC from 25 subjects. It has also been shown recently that decreased expression of *IL-4RA* in B cells is observed during anti-IgE biological treatment for severe asthma.[11] Some evidence of the environmental interaction with *IL-4RA* gene expression was provided by a study conducted in the Republic of Karelia in 2014, which showed that the maternal genetic variants in IL-4/IL-13 pathway genes, among them *IL-4*R Ile50Val, influenced IgE levels in school children, independently of the children's genetic effects. These effects differed in "Western or Eastern" environments (Finnish and Russian Karelian populations). These alternations could be explained by the epigenetic mechanism related to both the genetic background of the mother and environmental exposures in the prenatal period.[17]

Environment exposures associated with farming have been proposed as protective against atopy and this relation was proved in several studies. However, it has been shown that different farm exposures have different effects on the expression of genes related to innate immunity, i.e., toll-like receptors, possibly highly tied with early development of the atopic phenotype.[18] One of these exposures – raw milk consumption in the first year of life – was shown to change the expression of these genes in farm children.[19] Another study found that in farm children there was an increase in gene expression of *IRAK-4* and *RIPK1*, and innate regulatory modules, such as *SOCS4*, *IL10* and *TGFB*, as well as lower expression of *IFN-γ* and *IL-4* and higher of Th2-associated molecules, such as *GATA3*. However, the authors were not able to associate these differences with atopic conditions. These findings provide arguments that the molecular basis of allergic disorders possibly cannot be explained by the Th2 paradigm alone.[20] There are also studies in which farming is indicated as a risk factor for different conditions associated with the respiratory system, particularly on large farms.[21] Others conclude that the protective effects, seen as the altering gene expression of innate immunity, depend on the specificity of farm exposure.[20]

The IL-4 receptor has recently been proposed as a therapeutic target for asthma and atopic dermatitis.[22] Hamilton et al. observed in patients with atopic dermatitis that the transcriptome profile and disease severity changed during the treatment with dupilumab, which is human mAb directed at IL-4RA.[23] Specifically, in the skin biopsy specimens, the change before and after treatment was related to the suppression of immune and epidermal response, but no change in IL, IL13, IL5, and IL31 was observed. Dupilumab treatment was also used in uncontrolled persistent asthma as add-on therapy in a phase IIb clinical trial. It was effective for both FEV1 improvement and decrease of exacerbation rate.[24] Bechert et al. also described anti-IL-4RA effectiveness in the therapy of nasal polyps refractory to GKSs.[25]

The question arises about the exact role of IL-4. Is it simply an atopy cytokine? After all, IL-4 also regulates the response to parasites.[26] The cord blood CD34+ progenitor cell response to LPS, which is a component of the bacterial wall, is dependent on IL-4. IL-4 but not IL-13 reduces cord blood progenitor cell Eo/B differentiation in response to LPS and this process is dependent on IL-4R signaling.[27] Simple allergy triggering seems not the only action taken by IL-4. There are studies in cancer in which the expression of IL-4 pathway genes was shown to change depending on disease status.[28] IL-4 is also fundamental for cognition and brain function. In mice, the performance of learning and memory tasks is dependent on an increase of T cells in the meninges, producing cytokines such as IL-4. Mice lacking T cells exhibit cognitive impairment.[29] IL-4 can also counterbalance the aging process in the brain and could possibly play a role in Alzheimer's disease.[30]

The effect of IL-4 could depend on the specificity of the tissue and local environment. In a mouse model of asthma, IL-4 function depends on the cell type producing this cytokine. Both IL-4 and IL-13 are produced by T cells and a variety of cell types of the innate immune system. IL-4/IL-13 derived from the innate immune cells are responsible for the Th2 response in lungs and intestines, and protect against parasite *Nippostrongylus brasiliensis*. On the other hand, IL-4/IL-13 from T cells are critical for the induction of allergic inflammation and bronchial hyperresponsiveness, with IgG1 and IgE production, eosinophil and basophil recruitment to the lungs, goblet cell hyperplasia, expression of Muc5ac, Clca3, RELM-β, and the differentiation of alternatively activated macrophages.[31]

Conclusions

The regulation of *IL-4RA* gene expression is complex and probably influenced by both genetic – specific polymorphism – and environmental factors, such as farming exposures, which could provide a counterbalance to atopy.

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Analysis of prevalence of selected anamnestic factors among women with pelvic organ prolapse

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Abstract

Background. Pelvic organ prolapse is the most frequent medical condition in women in the postmenopausal age. The pathophysiology is multifactorial.

Objectives. The purpose of this paper was to analyze the prevalence of selected anamnestic factors in the population of women treated due to pelvic organ prolapse in the 2nd Department and Clinic of Obstetrics and Gynecology Wroclaw Medical University (Poland).

Material and methods. A total of 104 medical histories of women treated in the 2nd Department and Clinic of Obstetrics and Gynecology in the years 2012–2013 due to pelvic organ prolapse were analyzed.

Results. The most frequent type of defect was the complex defect concerning both cystocele and rectocele. Intensity of dysfunctions was determined by age, obstetric history (parity, newborn's body mass and process of labor), and woman's constitutional characteristic (her BMI and height). A comparison based on the type of defect revealed no differences between the groups except for BMI, which was the highest in the rectocele group (31.15 ± 5.84; $p = 0.0069$).

Conclusions. The multifactorial ethology and differential clinical presentation including several types of this defect make this disorder difficult to prevent and treat. The obtained results confirm that there exists a relation between the data from the medical history and the prevalence of pelvic organ prolapse. Anamnesis can be useful when predicting prevalence and, in the future, may even help to decrease the prevalence of this type of disorder.

Key words: risk factors, epidemiology, pelvic organ prolapse

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Introduction

Pelvic organ prolapse is one of the most frequent disorders connected with age that makes women visit their gynecologist. In the United States, this problem may affect even 24% of the women's population, whereby the percentage depends mainly on age. Among women between 20 and 39 years of age, it concerns 10% of the population, whereas it involves up to 50% of women in their eighties.[1]

With regard to the ageing process of the society, this problem will involve a higher rate of the total women's population. One estimates that in 2050 it will concern over 30% of women over 20 years old.[2] In the United States, 11.8% of women have undergone surgical procedures connected with one of the types of prolapse, which constitutes the most common indication for surgical procedure. In developed countries, approx. 20% of surgical procedures among women are carried out due to pelvic organ prolapse.[3–5] It is also worth mentioning that the problem is probably more frequent, because only 10% of the population struggling with pelvic organ prolapse in their everyday life seek help from a gynecologist, and the majority never ask for it.[6]

We can distinguish a few types of pelvic organ prolapse depending on the defect. Anterior prolapse occurs when the supportive tissue between a woman's bladder and vaginal wall weakens and stretches, allowing the bladder to bulge into the vagina (cystocele). Posterior prolapse occurs when the thin wall of fibrous tissue that separates the rectum from the vagina weakens, allowing the vaginal wall to bulge (rectocele). Small bowel prolapse occurs when the small intestine (small bowel) descends into the lower pelvic cavity and pushes at the top part of the vagina, creating a bulge (enterocoele). Uterus and vagina prolapse is with the uterus dislocation toward the vagina opening and finally its prolapse with complete eversion (descensus et prolapsus uteri). The consequences of the prevalence of the disorders are mainly connected with the discomfort they cause in such cases. Among the most frequent symptoms are the following: a sensation of a mass bulging into the vagina, a sensation of something coming or falling out of the vagina, urinary incontinence, fecal incontinence, having to push up on the perineum or digitate the vagina in order to urinate or defecate, discomfort during sexual intercourse.[3,7,8]

A properly functioning pelvic ligamentous and muscular system as well as the pelvic organ support system should sustain pelvic organs in anatomical position, ensuring a comfortable life for every woman regardless of her age. Damage to anatomical structures, connective tissue and nerves leads to different pelvic dysfunctions resulting in pelvic organ prolapse.[9–11] The reasons for pelvic organ prolapse are complex and arise from mechanical damage to the connective tissue of the true pelvis muscular system, weakening of the connective tissue fibers which follow the incompleteness of tissue structures, and vascularization dysfunctions or innervation of the anatomical structures responsible for sustaining pelvic organs in anatomical position. Among the most common risk factors are perinatal injuries, large body mass of a fetus during delivery, forceps delivery, strenuous physical work, family history of pelvic organ prolapse, obesity, chronic pulmonary disorders, diabetes, and constipation.[11–14]

The objective of this study was to analyze the prevalence of selected anamnestic factors in the population of women treated at the university clinic due to pelvic organ prolapse.

Material and methods

Medical histories received from 104 patients of the university clinic who were treated due to pelvic organ prolapse in the years 2012–2013 were analyzed, taking into consideration the following factors: frequency and type of defect; education and place of residence; age; smoking habits; constitutional characteristics; parity (number of labors, critical fetus body mass); additional disorders such as diabetes, pulmonary dysfunctions; history of subtotal hysterectomy and total hysterectomy; and history of hormonal replacement therapy (HRT).

The study was conducted in accordance with the Declaration of Helsinki after obtaining approval from the local Bioethics Committee.

Statistical analysis was carried out with STATISTICA software package v. 10 (StatSoft Inc., Tulsa, USA). The data was presented as means and percentages. Associations between anamnestic and demographic factors were assessed by Spearman's rank correlation coefficient within the formed groups according to diagnosis. Verification of the hypothesis of means equality for groups of the same diagnosis was performed by one-way analysis of variance (ANOVA test) and Pearson's χ^2 test. For post-hoc comparison, the method of least significant difference (LSD) was used. The criterion for statistical significance was set at $p < 0.05$.

Results

The data of 104 patients aged 64.43 years (SD = 9.66) was analyzed. The most frequent type of prolapse was the combined defect, which was diagnosed in 49 (47%) patients. The majority of the study group had increased body weight; only 30% of patients had their BMI within the normal range. Most of them lived in the city (65%), and the largest percentage had higher education (46%). The most frequent type of surgery performed in the patients studied was combined repair (39%); however, 25% of patients were treated non-invasively. Half of the study group reported 2 vaginal deliveries in the past. Among chronic diseases, diabetes was the most common and it was reported by 23% of patients. Most women had not suffered from any gynecological condition in the past, but some reported

total abdominal hysterectomy (14.29%), amputation of the body of the uterus (7.79%), and uterine myoma (9.09%). It is worth underlining that the standard procedure at our clinic is to suspend the vagina stump or the uterine cervix to round, cruciate and suspensory ligaments in each procedure of subtotal hysterectomy or total hysterectomy. The detailed characteristics of the demographic and clinical data as well as risk factors are presented in Table 1.

Analysis of variance was conducted for subgroups divided according to diagnosis. It did not reveal any differences of means in regard to variables such as age ($p = 0.7395$), age at the last delivery ($p = 0.7378$), the highest newborn birth weight ($p = 0.9429$), and patient's height ($p = 0.5134$). Significant differences were found in regard to patient's body weight ($p = 0.0371$) and BMI ($p = 0.0069$). The results of the post-hoc comparison for BMI are shown in Table 1.

Spearman's rank correlation coefficient did not reveal any clinically important correlations between coexisting diseases and the sociodemographic and clinical data from anamnesis.

Discussion

Our retrospective study on patients treated due to pelvic organ prolapse showed the existence of different risk factors connected with the type and stage of pelvic dysfunction. The most frequent pelvic disorder reported in this group of patients was the defect connected with both cystocele and rectocele. This may lead to the conclusion that cystocele is the most common type of dysfunction throughout the whole group of women with pelvic organ disorders. Similar results were obtained by Hendrix et al. on a large group where the most frequently observed disorder was also cystocele.[14]

Among the women who were examined, the largest percentage of them had higher education – 46%. This stands in contradiction to the data available in the literature, where the largest group included women with primary and vocational education, as in Cooper et al's. paper, in which 45% of women declared such educational background.[6] Neither was there a relation between education and the prevalence of pelvic disorders in Chiaffarino et al.'s study, who had examined a representative group of 108 women with moderate or high grade of pelvic organ prolapse.[11] This contradiction may be the effect of different groups examined in these papers – in the case of Cooper et al.'s, the cross-sectional study concerned a large group of patients with pelvic organ prolapse, while our analysis included patients who were admitted to hospital for pelvic organ prolapse surgery.

The age group and average age of patients who underwent the corrective procedures due to pelvic disorders were defined and compared with the above-mentioned studies. The feedback was similar – the highest proportion of patients belonged to the group of 60–69 years.[14] Both in Chiaffarino et al.'s study and Cooper et al.'s study, the age of the patients who underwent procedures due to pelvic organ prolapse in most cases did not exceed 56 years.[6,11]

In numerous reports, no association was found between smoking habits and a higher risk of pelvic disorders.[11,14–16] Hendrix et al. showed that current smoking is connected with a decrease in the prevalence of both cystocele and rectocele.[14] In the present study, the highest rate of smokers was noted among the patients with uterine prolapse – 9 patients; however, current smokers and women with smoking habits in their medical history were not diversified.

One of the best documented and frequently discussed prolapse risk factors in the literature is the constitutional status of patients evaluated on the basis of BMI index. Many authors indicate a strong relation between obesity and the prevalence of vaginal wall and uterine descent.[5,14,16,17] This is explained by increased intraabdominal pressure on the diaphragm along with increased BMI leading to pathological changes such as limitations in the support function and loosening of tissues supporting the pelvic organ in anatomical position. Other authors did not observe any correlation between obesity and the prevalence of pelvic organ prolapse.[11] Our findings showed significantly higher BMI in the rectocele group; however, our study group included patients of whom 70% had their BMI above the normal range, confirming the fact that being overweight or obese is associated with the development of prolapse. Due to these discrepancies, many authors attempt to build questionnaires which would make it possible to carry out a classified risk evaluation of the individual prevalence of pelvic organ prolapse based on constitutional data, family history and other data that could help estimate the future risk individually for each patient. An example of such a questionnaire may be the UR-CHOICE system. Here, the woman's height <160 cm is said to be the increased risk factor.[18,19] In our paper, the average height was 160.86 cm; however, it is worth emphasizing that 55.3% of patients were shorter than 160 cm.

There were not any nulliparae in the examined group, all patients in their medical history declared at least 1 spontaneous delivery, and most were after 2 or more deliveries, which may indicate a strong connection between spontaneous delivery and remote consequences occurring as pelvic organ prolapse. This is in line with the results obtained in earlier population studies.[1,11,14,16] During spontaneous delivery, support tissues may stretch, muscle fibers may be damaged, and the pelvis fundus structures responsible for sustaining the true pelvis may become partially denervated, which may lead to the dysfunction of the structures, pelvic disorders and urinary incontinence. However, the data from different sources concerning the pathomechanism and possible risks of denervation of the structures of the true pelvis fundus is contradictory.[9–11,20,21] Recently the role of perinatal damage to the levator ani muscle is being emphasized. The determinants may be age during

labor, head circumference of the fetus, duration of the 2nd phase of labor, use of forceps, or the fetu's body mass.[1]

In the present study, the average body mass of the biggest of the fetuses born was 3773 g. This can be compared with the result obtained in Erata et al.'s study, where the critical body mass increasing the risk of surgical intervention causing pelvic organ prolapse was 3800 g.[22] This study provided information on the relation between the critical body mass and the type of defect, confirming, at the same time, that the occurrence of higher than critical body mass is most frequent among women with more advanced disorders. Chiaffarino et al. did not find a negative influence of the newborn's body mass on subsequent risk of pelvic disorders.[11] At the same time, Chiaffarino et al.'s study, interestingly, concerned patients who had undergone surgical treatment due to pelvic organ prolapse, which is rather unclear. In our study, the newborn's birth weight was not significantly different among women with the 4 types of pelvic organ prolapse examined; however, the highest newborn's weight appeared in the cystocele and rectocele groups and reached 3850 g. Additionally, 78% of women with both cystocele and rectocele, and 79% of women with uterine prolapse had given birth to children of high body mass.

Some studies mention an interesting relation between the prevalence of pelvic organ prolapse and the pregnancy period itself and relaxin secretion among pregnant women. Relaxin is supposed to modulate support tissues and favors the subsequent development of pelvic organ prolapse, which would exclude elective cesarean section as a method of preventing pelvic disorders.[5,6,15] The above-mentioned conclusions could not be compared with the results obtained in our analysis, because in the examined group of women, none of the patients had had a cesarean section. The matter of the influence of anamnestic factors in this period of pregnancy needs to be analyzed more thoroughly.

Many authors point to pulmonary disorders with chronic cough and diabetes as independent factors increasing the risk of pelvic disorders.[5,12] In our study, no increased risk was stated concerning the prevalence of these illnesses among patients examined at the 2nd Department and Clinic of Obstetrics and Gynecology in Wrocław, neither was such relation found in Uustal Fornell et al.'s study.[22]

The literature mentions hysterectomy as a factor which has a negative influence on pelvic organs. The pathomechanism of the prolapse is multifactorial and applies to the disorders of few support points according to DeLancey, with the significant role of the tissues alongside the vagina (paracolpium) of the 1st level in situations where proper fixation of the vagina stump apex exists.[23,24] Our results did not confirm the negative influence of the former procedures, because only 10.6% of all examined patients had undergone prior abdominal hysterectomy, 5.7% reported amputation of the body of the uterus, and uterine myoma was observed among 6.7% of patients. It is worth emphasizing that at our clinic we suspend the vagina stump or uterine cervix to round, cruciate and ovarian suspensory ligaments as a standard procedure in every case when we perform total hysterectomy or subtotal hysterectomy. Most women had not suffered from gynecological disorders in the past. No relation was observed between hysterectomy and the subsequent lowering of the vagina walls and uterine cervix, unless additional suspension of the vagina/uterine cervix was applied during the hysterectomy or amputation of the body of the uterus. An 11-year analysis by Jurića et al. confirms the above-mentioned conclusions.[24] They applied the technique of suspending the vagina stump during abdominal hysterectomies performed in the group of 982 patients and did not observe posterior stump and vagina wall prolapse.

In our study, no relation was proved regarding the type of defect and applying hormonal substitutive therapy. Similar conclusions may be found in the literature – neither in Chiaffarino et al.'s study nor in Hendrix et al.'s study, is there evidence that using hormonal substitutive therapy is connected with the prevalence of pelvic organ prolapse.[11,14]

In this study, it was shown that the crucial factors relating to the prevalence of pelvic organ prolapse are: parity, method of delivery and critical body mass of the newborn as well as the woman's constitutional characteristics, her BMI and height. These anamnestic factors predominantly determine the prevalence of pelvic organ prolapse and may, in consequence, result in the necessity of applying surgical treatment. The results obtained by our team confirm the connection between the data from medical history and the risk of the prevalence of pelvic organ prolapse. In the future, this might help to predict and perhaps avoid the prevalence of this type of disorder.

This study has its limitations. One of them is the lack of possibility to evaluate more the common ways of delivery as risk factors in the population examined. The reason for this is that the patients who were examined had finished reproduction before the percentage of cesarian sections rose, and none of these women had a cesarian section in their medical history. Another limitation is the fact that the evaluation of prolapse could have been partly subjective, because it was performed based on the Valsava maneuver in speculum and bimanual examination.

Conclusions

The multifactorial etiology and differential clinical presentation, including several types of this defect, make this disorder difficult to prevent and treat. Our study confirms an association between the data from the medical history and the prevalence of pelvic organ prolapse. Factors such as mode of delivery, newborn's body mass, woman's BMI and height determined the occurrence of pelvic organ prolapse. Patients with different types of pelvic organ prolapse presented similar medical history except for BMI, which was significantly higher in the rectocele group. A therapeutic

Table 1. Clinical and demographic characteristics of the study group

Factor/type of defect, n (%)	Cystocele; 23 (22.1)	Rectocele; 6 (5.8)	Combined defect; 49 (47)	Prolapse uteri; 26 (25.1)	Total	p-value
Age [years], mean (SD)	62.43 (9.84)	64.67 (6.74)	65.10 (9.33)	64.88 (10.88)	64.43 (9.66)	0.740
Patients heigh, tmean (SD)	161.44 (4.89)	161.33 (3.08)	161.32 (5.89)	159.29 (5.40)	160.87 (5.43)	0.513
BMI, [kg/m^2], mean (SD)	27.15 (3.35)[a]	31.15 (5.84)[a,b]	26.09 (4.10)[b,c]	29.00 (4.35)[c]	27.32 (4.35)	0.007*
Body weight [kg] (n, %) normal overweight obese pathological obesity	 5 (5.32) 9 (9.57) 5 (5.32) 1 (1.06)	 1 (1.06) 1 (1.06) 2 (2.13) 2 (2.13)	 19 (20.21) 16 (17.02) 10 (10.64) 1 (1.06)	 3 (3.19) 12 (12.77) 5 (5.32) 2 (2.13)	 28 (29.79) 38 (40.43) 22 (23.40) 6 (6.38)	0.075
Body weight [kg], mean (SD)	71.11 (8.83)[d]	81.17 (15.63)[d,e]	68.05 (12.07)[e,f]	73.62 (10.48)[f]	70.87 (11.75)	0.037*
Age at last delivery [year], mean (SD)	30.81 (4.51)	29.00 (4.97)	29.74 (5.37)	31.23 (6.46)	30.38 (5.46)	0.738
The highest newborn weight [g], mean (SD)	3850.00 (495.70)	3850.00 (494.97)	3750.00 (551.89)	3721.111 (352.51)	3773.12 (465.05)	0.943
Place of residence, n (%) city rural area	 11 (10.58) 12 (11.54)	 4 (3.85) 2 (1.92)	 36 (34.62) 13 (12.50)	 17 (16.35) 9 (8.65)	 68 (65.38) 36 (34.62)	0.208
Education, n (%) higher secondary vocational primary	 4 (7.41) 1 (1.85) 1 (1.85) 6(11.11)	 2 (3.70) 0 (0.00) 2 (3.70) 1 (1.85)	 14 (25.93) 4 (7.41) 2 (3.70) 4 (7.41)	 5 (9.26) 4 (7.41) 1 (1.85) 3 (5.56)	 25 (46.30) 9 (16.67) 6 (11.11) 14 (25.93)	0.213
HRT, n (%)	2 (1.92)	3 (2.88)	6 (5.77)	3 (2.88)	14 (13.46)	0.058
Smoking, n (%)	6 (5.77)	0 (0.00)	7 (6.73)	9 (8.65)	22 (21.15)	0.104
Type of treatment, n (%) no surgery anterior repair posterior repair combined repair vaginal hysterectomy	 8 (7.69) 15 (14.42) 0 (0.00) 0 (0.00) 0 (0.00)	 1 (0.96) 0 (0.00) 5 (4.81) 0 (0.00) 0 (0.00)	 9 (8.65) 1 (0.96) 1 (0.96) 38 (36.54) 0 (0.00)	 9 (8.65) 1 (0.96) 0 (0.00) 3 (2.88) 13 (12.50)	 27 (25.96) 17 (16.35) 6 (5.77) 41 (39.42) 13 (12.30)	0.000
Vaginal deliveries, n (%) 1 2 3 4 5 6 8	 1 (1.01) 9 (9.09) 8 (8.08) 4 (4.04) 0 (0.00) 0 (0.00) 0 (0.00)	 2 (2.02) 1 (1.01) 2 (2.02) 1 (1.01) 0 (0.00) 0 (0.00) 0 (0.00)	 5 (5.05) 30 (30.30) 7 (7.07) 2 (2.02) 1 (1.01) 0 (0.00) 1 (1.01)	 7 (7.07) 10 (10.10) 6 (6.06) 1 (1.01) 0 (0.00) 1 (1.01) 0 (0.00)	 15 (15.15) 50 (50.51) 23 (23.23) 8 (8.08) 1 (1.01) 1 (1.01) 1 (1.01)	0.169
Comorbidities, n (%)[1] diabetes pulmonary disorders uterine myoma post-abdominal hysterectomy intestinal disorders post-uterus amputation post-repair	 2 (2.60) 1 (1.30) 1 (1.30) 1 (1.30) 1 (1.30) 1 (1.30) 0 (0.00)	 0 (0.00) 0 (0.00) 1 (1.30) 1 (1.30) 0 (0.00) 1 (1.30) 0 (0.00)	 6 (7.79) 0 (0.00) 1 (1.30) 1 (1.30) 0 (0.00) 1 (1.30) 2 (2.60)	 10 (12.99) 3 (3.90) 4 (5.19) 8 (10.39) 0 (0.00) 3 (3.90) 2 (2.60)	 18 (23.38) 4 (5.19) 7 (9.09) 11 (14.29) 1 (1.30) 6 (7.79) 4 (5.19)	0.441

[1] presence of the particular disease does not exclude other comorbidities; BMI – body mass index; normal – BMI 18.5–24.9; overweight – 25–29.9; obese – 30.00–34.00; pathological obesity – 35; n – number of subjects; SD – standard deviation; HRT – hormone replacement therapy; [a] cystocele vs rectocele, p = 0.040; [b] rectocele vs combined defect, p = 0.006; [c] combined defect vs prolapse uteri, p = 0.008; [d] cystocele vs Rectocele, p = 0.064; [e] rectocele vs combined defect, p = 0.010; [f] combined defect vs prolapse uteri, p = 0.068.

strategy based on the type of pelvic organ prolapse along with the data from anamnesis can be useful in predicting prevalence and, in the future, may even help to decrease the prevalence of this type of disorder.

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Oxidative stress markers predict early left ventricular systolic dysfunction after acute myocardial infarction treated with primary percutaneous coronary intervention

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Abstract

Background. Despite successful primary percutaneous coronary intervention (PCI) after ST-segment elevation myocardial infarction (STEMI), some patients develop left ventricular systolic dysfunction (LVSD) and acute heart failure (HF). Identifying patients with an increased risk of developing LVSD by means of biomarkers may help select patients requiring more aggressive therapy.

Objectives. The aim of this study was to evaluate the relationship between the levels of oxidative stress markers and development of LVSD and acute HF early after STEMI.

Material and methods. The study enrolled 148 patients with the first STEMI, who were treated by primary PCI < 12 h from the onset of symptoms. We assessed the impact of different biomarkers for developing LVSD and acute HF (Killip ≥ 2) including: markers of necrosis – peak creatine kinase (CK), markers of myocardial stretch – B-type natriuretic peptide (BNP), inflammatory markers – C-reactive protein (CRP), leucocyte and neutrophil count, as well as oxidative stress markers – total thiol groups, catalase, superoxide dismutase (SOD) and glutathione reductase (GR).

Results. In multivariate analysis, thiol groups, peak CK, anterior wall infarction, and age were predictors of LVEF ≤ 40%. Out of 16 variables significantly associated with the Killip ≥ 2 in univariate logistic regression analysis, 5 appeared to be independently associated with acute HF in multivariate analysis: catalase, BNP, leucocytes, neutrophil count, and size of left atrium.

Conclusions. In this study, we have shown for the first time that thiol groups and catalase are independent predictors of STEMI complication – LVSD and acute HF, respectively. Beside routine used biomarkers of necrosis and myocardial stretch, thiol groups and catalase may provide additional information regarding the risk stratification.

Key words: oxidative stress, heart failure, acute myocardial infarction, percutaneous coronary intervention, left ventricular systolic dysfunction

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Introduction

Primary percutaneous coronary intervention (PCI) is a first choice reperfusion therapy for ST-segment elevation myocardial infarction (STEMI). Primary PCI, in most cases, provides early and complete restoration of blood flow in the infarct related artery (IRA) and improves myocardial viability, ventricular function and patient survival.[1] Despite primary PCI, some patients develop left ventricular systolic dysfunction (LVSD) and acute heart failure (HF), which have an adverse impact on short and long-term outcomes.[2] The sudden reperfusion of occluded artery leads to injuries mainly induced by oxidative and inflammatory mechanisms. Restored oxygen supply increases free radical production in the electron transport chain on the inner mitochondrial membrane, while inflammatory cells, especially neutrophils, release free oxygen species and cytokines with negative inotropic effects and further aggravate myocardial dysfunction and contribute to HF.[3] Early identification of patients at an increased risk of developing left ventricular (LV) dysfunction after acute myocardial infarction (AMI) could help in the selection of patients requiring more aggressive therapy. The transthoracic echocardiographic examination remains the most often used method for non-invasive detection of LV dysfunction and risk stratification after AMI. Ejection fraction (EF) or other closely related parameters are strong predictors of the risk for future events.[4]

Besides a physical examination and an ultrasound, a laboratory evaluation has an essential role. Biomarkers are indispensable tools to present a diagnosis and prognosis in acute coronary events, and some of them are now introduced into the current guidelines for HF.[5]

Most data on biomarkers has been derived from patients with chronic HF, but risk stratification in patients with acute HF remains a challenge. In the development of LVSD and HF after AMI participate different pathophysiological processes, which are reflected by different biomarkers.[6] Apart from the commonly used B-type natriuretic peptide (BNP) and N-terminal pro-brain natriuretic peptide (NT-proBNP), risk stratification may be refined by the use of new biomarkers.[7] Markers of oxidative stress are also widely studied in chronic HF, but their role in the prediction of early systolic dysfunction after STEMI is largely unknown. We focused on oxidative stress parameters that are cheap, easy to perform and widely available. Thiol groups are found in many proteins and have an essential role for their function. Thiol/disulphide homeostasis is disrupted in AMI and could serve as an additional marker for diagnosis.[8] Catalase, another very often used marker of oxidative stress, was found to be increased in an end-stage failing heart, while higher catalase activity was observed in AMI as well in subjects with high cardiovascular risk.[9,10] Considering the important role of oxidative stress on myocardial injury during the reperfusion, we postulated that lower antioxidant status early after AMI will predict development of low ejection fraction.

In this paper, we described the role of markers of oxidative stress in the prediction of left ventricular systolic dysfunction and acute heart failure early after AMI.

Patients and methods

Patients

The study enrolled 148 patients aged 18 years or more, with the first STEMI, who were treated by primary PCI <12 h from the symptoms onset. Patients were admitted to the Coronary Care Unit of Clinical Centre of Serbia, from October 2013 to December 2014. All patients signed consent forms, whilst the study was approved by the Ethical Committee of Clinical Centre of Serbia. The primary endpoint of this study was to establish if oxidative stress markers were predictors of early LVSD in patients with STEMI. The role of oxidative stress markers in the prediction of acute HF was a secondary endpoint.

The STEMI was defined according to European Society of Cardiology guidelines.[11] A standard 12-lead electrocardiography (ECG) was performed on admission and 60 min. after revascularization with PCI. Reduction of ≥50% in ST-segment elevation after PCI was considered significant resolution of ST-segment elevation (STR).[12] The exclusion criteria were: the presence of any contraindication to dual antiplatelet therapy and contrast agents, inability or refusal to provide written informed consent, prior coronary revascularisation, prior history of heart failure, permanent atrial fibrillation, left bundle branch block, hemodynamically severe valvular heart disease, primary cardiomyopathies, chronic pulmonary disease, implantable pacemaker, acute or chronic inflammation, history of autoimmune disease, history of febrile disorders and malignancy.

Coronary angiography and primary PCI were performed using the standard technique. Patients were treated on admission and after PCI according to current guidelines.[11] The angiogram of all patients was graded according to the number of diseased coronary arteries. The coronary flow in the IRA was graded according to the classification used in the thrombolysis in myocardial infarction (TIMI) trial. Successful reperfusion was defined as the establishment of TIMI grade 3 flow in the IRA.[13] Time from symptoms onset to the treatment was defined as the time from the symptom onset to the first balloon inflation.

All patients underwent astandard transthoracic echocardiography on the 4th or 5th day of admission. The size of the heart chambers, left ventricular ejection fraction and wall motion score index (WMSI) were assessed. LVEF was calculated by modified biplane Simpson's method and defined as EF ≤ 40%.[5,14] According to the values of LVEF, patients were divided into groups with LVSD (the group with LVEF ≤ 40%) and without LVSD (the group

with LVEF > 40%). WMSI was calculated according to the 17-segment model division of the LV and assigned a score of 1, 2, 3, and 4 points for normokinesia, hypokinesia, akinesia, and dyskinesia, respectively.[14] Significant regional LVSD was considered WMSI ≥ 1.5. Patients who developed HF during hospitalization were categorized according to the Killip status of I–IV: class I – no rales and no third heart sound; class II – pulmonary congestion with rales up to 50% of the lung fields, sinus tachycardia or third heart sound; class III – pulmonary edema with rales over 50% of the lung fields; and class IV – included cardiogenic shock.[11] Killip of class ≥ 2 was considered as acute HF.

Blood sampling and biochemical analysis

Blood samples were collected after admission and before PCI. Plasma and serum were derived, aliquoted and immediately frozen at −80°C until analysis. Routine laboratory analyses were determined from separated samples obtained at the same time. Creatine kinase (CK), B-type natriuretic peptide (BNP), C-reactive protein (CRP), complete and differential blood count were determined according to standard laboratory procedures. Glutathione reductase (GR) was determined by using commercially available kits according to manufacturer instructions (Randox Laboratories Ltd., Crumlin, UK). Total thiol groups were determined by Ellman's reagent (5,5-dithiobis-(2-nitrobenzoic acid)-DTNB) as previously described.[15] Catalase was determined exactly as previously described.[16] Superoxide dismutase (SOD) was measured according to method based on autoxidation of adrenaline.[17]

Statistical analysis

Distribution of the continuous variables was assessed using the Kolmogorov-Smirnov test. Depending on the normality, continuous data was presented as mean ±SD or median (interquartile range – IQR). Categorical data is summarized as numbers (percentages). Comparison of continuous features of patients with and without HF was done using the Student's t-test or Mann-Whitney U test. Pearson's χ^2 test or the Fisher's exact test were used for comparing categorical characteristics between the 2 groups. Spearman's correlation were used to estimate the association of LVEF with biochemical and echocardiographic parameters. In order to estimate which of the biochemical, clinical and echocardiographic parameters from a baseline were independently associated with LVEF ≤ 40% and clinically apparent HF (Killip ≥ 2), univariate and multivariate logistic regression analyses were done. Variables that were significantly associated with HF in univariate logistic regression at the significance level < 0.1 were entered into the multivariate logistic regression model (Hosmer-Lemeshow method).[18] Odds ratios (OR) with 95% confidence intervals were computed and Pearson goodness of fit test was performed to assess the overall fitness of the models. All statistical tests were two-sided and were performed at the 5% significance level. The statistical analysis was performed using IBM SPSS Statistics v. 20.0 software (IBM Corp., Armonk, USA).

Results

The study included 148 patients (111 men and 37 women) with a mean age of 58.7 ±11.7 years. Baseline characteristics of patients are shown in Table 1. The patients with LVSD were more frequently male, with an anterior wall infarction, heart rhythm disorders, acute HF, and significantly lower STR. There were no significant differences between the 2 groups with regard to the risk factors and renal function.

Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD) and the left atrial (LA) size were significantly higher in the group with LVEF ≤ 40%. Additionally, WMSI ≥ 1.5 was more prevalent in this group of patients (61 (88.4%) vs 2 (2.5%); p < 0.001)). Angiographic data revealed that TIMI flow grade after the primary PCI, presence of multi-vessel coronary disease and the number of the implanted stents did not differ between the 2 groups; however, there was a significantly higher prevalence of left anterior descending (LAD) artery as the culprit artery in group with LVEF ≤ 40%. During hospitalization, 4 patients with LVSD died, while none of the patients died in the group without LVSD (p < 0.001).

Thiol groups, as a marker of oxidative stress, were significantly lower in the group with LVEF ≤ 40% (p = 0.034), while the peak CK, a marker of myocardial necrosis, was significantly higher in the group with reduced LVEF than in patients with LVEF > 40% (Table 2). The inflammatory parameters, CRP, leukocytes and neutrophils were significantly higher in the group with LVEF ≤ 40% compared with patients who had LVEF > 40% (p = 0.006), (p < 0.001) and (p = 0.011), respectively. The same results were observed for markers of myocardial stretch. Plasma levels of BNP were higher in the group of patients with LVSD than patients without LVSD (p < 0.001).

Regarding the acute HF during hospitalization, patients who developed Killip ≥ 2 had significantly lower concentration of thiol groups but higher concentrations of peak CK, CRP, leukocyte and neutrophil count compared to patients with Killip < 2 (Table 3).

Spearman's correlation showed significant association between LVEF and thiol groups (r = 0.187; p = 0.023), and negative correlation with peak CK (r = −0.505; p < 0.001), plasma BNP levels (r = −0.326; p < 0.001), leukocytes (r = −0.384; p < 0.001), neutrophils (r = −0.223; p = 0.006) and CRP (r = −0.261; p = 0.001). Considering echocardiographic features, LVEF showed negative correlation with WMSI (r = −0.924; p < 0.001), LVEDD (r = −0.480; p < 0.001) and LVESD (r = −0.480; p < 0.001).

Table 1. Baseline characteristics of patients with and without left ventricular systolic dysfunction

Variable	LVEF ≤ 40% (n = 69)	LVEF > 40% (n = 79)	p-value
Baseline characteristics			
Age [years]†	58.88 ±12.43	58.51 ±11.42	0.848
Male gender, n (%)	59 (85.5)	52 (65.8)	0.006
BMI [kg/m^2]†	28.04 ±4.06	26.83 ±4.14	0.075
Smokers, n (%)	45 (65.2)	48 (60.8)	0.576
Hypertension, n (%)	38 (55.1)	55 (69.6)	0.068
Diabetes, n (%)	11 (15.9)	20 (25.3)	0.162
Hyperlipidemia, n (%)	44 (63.8)	49 (62.0)	0.827
Family history, n (%)	21 (30.4)	30 (38.0)	0.336
eGFR < 60 mL/min, n (%)	9 (13.0)	5 (6.3)	0.164
Clinical features			
Time from onset of chest pain to PCI [min]¥	300 (345.0)	240 (240)	0.057
Anterior wall infarction, n (%)	62 (89.9)	25 (31.6)	<0.001
Killip class ≥ 2, n (%)	13 (18.8)	3 (3.8)	<0.001
Echocardiography			
LVESD [cm]†	4.37 ±0.58	3.44 ±0.41	<0.001
LVEDD [cm]†	5.76 ±0.62	5.24 ±0.45	<0.001
LA [cm]†	3.89 ±0.54	3.68 ±0.38	0.009
WMSI ≥ 1.5, n (%)	61 (88.4)	2 (2.5)	<0.001
Electrocardiography			
STR, n (%)	55 (79.7)	77 (97.5)	0.001
Ventricular arrhythmias, n (%)	11 (15.9)	3 (3.8)	0.001
Angiographic findings			
Multi-vessel disease, n (%)	34 (49.3)	41 (51.9)	0.750
IRA, n (%)			
LM	1 (1.4)	1 (1.3)	<0.001
LAD	61 (88.4)	21 (26.6)	
LCx	4 (5.8)	11 (13.9)	
RCA	3 (4.3)	46 (58.2)	
Number of stents implanted¥	1 (0)	1 (1)	0.795
Final TIMI flow, n (%)			
TIMI flow 0	0	0	0.598
TIMI flow 1	0 (0.0)	0 (0.0)	
TIMI flow 2	2 (2.9)	1 (1.3)	
TIMI flow 3	67 (97.1)	78 (98.7)	
Therapy, n (%)			
ACE inhibitors	56 (81.2)	77 (97.4)	0.51
Beta blockers	62 (89.9)	70 (88.6)	0.114
Diuretics	25 (36.2)	4 (5.1)	<0.001
Digitalis	8 (11.6)	5 (6.3)	0.068
Antiarrhythmics	17 (24.6)	3 (3.8)	<0.001
In-hospital mortality, n (%)	4 (5.8)	0 (0)	<0.001

Table 2. Biomarkers of patients with and without left ventricular systolic dysfunction

Variable	LVEF ≤ 40% (n = 69)	LVEF > 40% (n = 79)	p-value
Oxidative stress			
Thiol groups [μM]¥	39.4 (14.5)	44.6 (22.0)	0.034
Catalase [U/mg Prot]¥	98.20 (7.20)	96.1 (10.70)	0.161
GR [U/mL]†	8.94 ±8.88	8.00 ±7.57	0.536
SOD [U/mL]†	2.91 ±1.19	3.16 ±0.89	0.141
Necrosis			
Peak CK [U/L]¥	3153 (3867.5)	1162 (1432)	<0.001
Strech			
BNP [pg/mL]¥	49 (156.8)	14.9 (53.8)	<0.001
Inflammation			
Sedimentation [mm/h]¥	18.0 (22.0)	19 (19.75)	0.886
C-reactive protein [mg/L]¥	12.1 (33.0)	8.4 (8.4)	0.006
Leukocyte count [× 10^9/L]†	12.58 ±4.03	10.23 ±2.45	<0.001
Neutrophil count [× 10^9/L]¥	83.3 (10.9)	79 (11.3)	0.011
Fibrinogen [g/L]¥	2.0 (1.36)	2 (0.0)	0.078

† values are mean ±SD; ¥ values are median (IQR); GR – glutathione reductase; SOD – superoxide dismutase; CK – creatine kinase; BNP – B-type natriuretic peptide.

Table 3. Biomarkers of patients with and without acute heart failure

Variable	Killip class ≥ 2 (n = 16)	Killip class 1 (n = 132)	p-value
Oxidative stress			
Thiol groups [μM]¥	35.6 (21.3)	44.1 (21.1)	0.046
Catalase [U/mg Prot]†	102.5 ±8.5	93.7 ±21.3	0.106
GR [U/mL]†	8.24 ±6.32	7.90 ±4.21	0.536
SOD [U/mL]†	3.0 ±0.9	3.1 ±1.1	0.705
Necrosis			
Peak CK [U/L]¥	4228.5 (3185.3)	1731.5 (2494.0)	<0.001
Stretch			
BNP [pg/mL]¥	82.4 (128.6)	30.0 (81.4)	<0.001
Inflammation			
Sedimentation [mm/h]¥	21 (32.8)	18.0 (18.0)	0.451
C-reactive protein [mg/L]¥	30.9 (45.2)	9.4 (10.5)	0.002
Leukocyte count [× 10^9/L]†	14.7 ±4.4	11.1 ±3.1	<0.001
Neutrophil count [× 10^9/L]†	85.8 ±3.9	78.3 ±9.2	<0.001
Fibrinogen [g/L]¥	2.0 (0.0)	2.0 (1.0)	0.815

† values are mean ±SD; ¥ values are median (IQR); GR – glutathione reductase; SOD – superoxide dismutase; CK – creatine kinase; BNP – B-type natriuretic peptide.

† values are mean ±SD; ¥ values are median (interquartile range – IQR); BMI – body mass index; eGFR – estimated glomerular filtration rate; LVESD – left ventricular end-systolic diameter; LVEDD – left ventricular end-diastolic diameter; LA – left atrium; WMSI – wall motion score index; STR – resolution of ST-segment elevation; IRA – infarct related artery; LM – left main coronary artery; LAD – left anterior descending artery; LCx – left circumflex artery; RCA – right coronary artery; TIMI – thrombolysis after myicardial infarction; ACE – angiotensin-converting enzyme.

Independent predictors of left ventricular systolic dysfunction and acute heart failure

After including all significant variables from the univariate logistic regression into multivariate logistic regression model, 4 variables remained significant predictors of LVEF ≤ 40%: thiol groups (OR 0.82, 95% CI 0.698–0.966; p = 0.017), peak CK (OR 1.001, 95% CI 1.000–1.001; p = 0.003), anterior wall infarction (OR 22.21, 95% CI 6.81–72.48; p < 0.001), and age (OR 1.06, 95% CI 1.01–1.11; p = 0.021) (Table 4).

Table 5 shows the results of multiple logistic regression analysis employed to identify independent predictors of HF during hospitalization (Killip ≥ 2). Out of 16 variables significantly associated with the Killip ≥ 2 in univariate logistic regression analysis, 5 appeared to be independently associated with acute HF in multivariable analysis: catalase (OR 1.11, 95% CI 1.02–1.21; p = 0.020), BNP (OR 1.01, 95% CI 1.001–1.012; p = 0.028), leucocytes (OR 1.32, 95% CI 1.04–1.67; p = 0.025), neutrophil count (OR 1.20, 95% CI 1.04–1.39; p = 0.014), and size of LA (OR 5.76, 95% CI 1.31–25.37; p = 0.021).

Table 4. Independent predictors of left ventricular systolic dysfunction (LVEF ≤ 40%)

Variable*	OR	95% CI	p-value
Thiol groups	0.821	0.698–0.966	0.017
Peak CK	1.001	1.000–1.001	0.003
Anterior wall infarction	22.212	6.807–72.475	<0.001
Age	1.056	1.008–1.106	0.021

* Multivariate logistic model was started with 16 variables. The following variables were excluded from the model: BNP, leukocyte count, neutrophil count, C-reactive protein, fibrinogen, STR, LA, RCA, ventricular arrhythmias, LVEDD, male gender and BMI. Odds ratios correspond to 1 unit increase in predictor variable.
CK – creatine kinase; BNP – B-type natriuretic peptide; STR – resolution of ST-segment elevation; LA – left atrium; RCA – right coronary artery; LVEDD – left ventricular end-diastolic diameter; BMI – body mass index.

Table 5. Independent predictors of acute heart failure (Killip class ≥ 2)

Variable*	OR	95% CI	p-value
Catalase	1.110	1.017–1.211	0.020
BNP	1.006	1.001–1.012	0.028
LA	5.763	1.309–25.371	0.021
Leukocyte count	1.315	1.036–1.669	0.025
Neutrophil count	1.199	1.037–1.386	0.014

* Multivariate logistic model was started with 16 variables. The following variables were excluded from the model: thiol groups, CK, C-reactive protein, STR, anterior wall infarction, ventricular arrhythmias, WMSI ≥ 1.5, LVESD, LVEDD, male gender, and BMI. Odds ratios correspond to 1 unit increase in predictor variable.
BNP – B-type natriuretic peptide; LA – left atrium; CK – creatine kinase; STR – resolution of ST-segment elevation; WMSI – wall motion score index; LVEDD – left ventricular end-diastolic diameter; LVESD – left ventricular end-systolic diameter; BMI – body mass index.

Discussion

Appearance of left ventricle dysfunction after AMI has a great adverse impact on patient outcomes. Although several biomarkers of HF are well studied and widely accepted as reliable in routine clinical practice, their significance in prediction of LVSD early after AMI remains uncertain.[19] In this study, we assessed the impact of different biomarkers for developing LVSD and acute HF during hospitalization measured in the early phase of acute myocardial infarction including: markers of necrosis (peak CK), markers of myocardial stretch (BNP), inflammatory markers (CRP, leucocyte and neutrophil count), as well as oxidative stress markers (total thiol groups, catalase, SOD and GR). Peak CK, anterior wall infarction, age, and total thiol groups appeared as independent predictors of LVSD. Interestingly, BNP, although being a sensitive marker of HF, was not an independent predictor for future development of LVEF ≤ 40%. However, BNP levels were significantly higher in patients with systolic dysfunction.

Peak CK correlated with the extent of myocardial necrosis, a major factor contributing to the development of LVSD and HF after admission.[11] Similarly, Hamdan et al. found that peak CK in patients after STEMI undergoing primary PCI was one of the most powerful predictors of LV function.[1] Anterior localization of myocardial infarction is generally associated with extensive myocardial necrosis and worse prognosis.[4] Age as a predictor of adverse outcomes after STEMI is also well known and described in many studies.[20]

Thiol groups showed a direct correlation with EF and were an independent predictor for LVSD in our study group. To the best of our knowledge, the predictive role of thiol groups for LVSD development after AMI has not been described, albeit a decrease of serum thiol group was observed in coronary artery disease patients and AMI.[8,21] Moreover, the disulphide/total thiol ratio was independently related to AMI.[8] Different thiol compounds interact with circulating nitric oxide and produce S-nitrosothiols, a potent endogenous vasodilators.[22] Protein S-nitrosylation has an essential role in inflammatory response, apoptosis, nitric oxide synthase activity, myocardial contractility, and response to hypoxia and may be involved in pathogenesis of atherosclerotic heart disease, pulmonary hypertension and cardiac arrhythmias.[23] Considering that detection of total thiol groups is a cheap, simple one-step colorimetric method, that can easily be automated, their determination may be in the future of great importance in AMI.

Not all patients with LVSD develop acute HF, and not all patients who develop HF have LVSD. In our study, independent predictors of acute HF (Killip ≥ 2) were BNP, leucocytes, neutrophils, catalase, and LA size. Although patients with acute HF had lower concentrations of thiol groups, they were not an independent predictor of HF, while they were an independent predictor of LVSD probably because of the relatively small number of patients who developed HF and skewed distribution of thiol groups (box

plot shown in supplementary material). The evaluation of BNP concentration is a useful tool for detecting LVSD and a prognostic factor for an adverse clinical outcome after STEMI. BNP correlates with myocardial damage, LVSD and acute HF.[24,25] Similarly, in our patients, BNP levels were significantly higher when LVSD was present, and displayed a significant negative correlation with LVEF. Interestingly, some authors found that the levels of NT-proBNP early after AMI do not predict the development of LVSD.[26] Higher BNP levels in patients with LVSD were not an independent predictor of low EF. However, BNP was a predictor of acute HF during hospitalization. This could be explained by the fact that BNP and NT-proBNP are stretch dependent peptide derivatives whose synthesis requires some time to complete.[19]

Leucocytes and neutrophils were also independent predictors of acute HF in our group of patients. Neutrophil and leucocyte counts were described as predictors of severe microvascular injury and LVSD.[27] Neutrophils release variety of inflammatory cytokines that are directly involved in endothelial injury. After cholesterol crystals ingestions, neutrophils produce neutrophil extracellular traps (NET), structures composed of extracellular neutrophil DNA and proteins that are highly thrombogenic.[28] Moreover, NETs induce macrophages to produce IL-1β, a major cytokine responsible for endothelial inflammation. In animal models NETs formed in situ after reperfusion are responsible for myocardial microvasculature obstruction and consequent appearance of HF.[29]

Catalase has an important role in hydrogen peroxide degradation. Despite its relatively low toxicity, hydrogen peroxide may mediate synthesis of highly reactive hydroxyl radical. Patients with coronary artery disease have lower catalase levels and increased oxidative stress than aged matched controls.[30] Data related to catalase status in AMI is rather conflicting. Several papers reported lower as well as higher catalase activity in AMI in comparison to healthy controls.[9] In the end-stage, failing heart catalase gene as well as catalase tissue activity were clearly elevated, probably as compensatory mechanism in response to pronounced oxidative stress.[10] In our group of patients, catalase at admission was an independent predictor for development of acute HF.

Conclusions

In this study, we have shown for the first time that thiol groups and catalase are independent predictors of AMI complications – LVSD and acute HF, respectively. Apart from the routinely used biomarkers of necrosis and myocardial stretch, thiol groups and catalase may provide additional information regarding the risk for LVSD development. Considering the simplicity of laboratory procedures necessary to determine catalase and thiol group and their usefulness, these 2 biomarkers could be important and novel tools for risk stratification in AMI.

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Temporomandibular disorders in adolescents with headache

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Abstract

Background. Headache is a common complaint in all age groups and is a frequent cause of medical consultations and hospitalization.

Objectives. The aim of this study was to evaluate the prevalence of bite and non-bite parafunctions as well as the signs and symptoms of temporomandibular disorder (TMD) in adolescents presenting with primary headaches.

Material and methods. Parents of adolescents presented with headaches to the Department of Developmental Neurology within a 12-month period were asked to complete a questionnaire developed by the authors of this study. Of the 1000 patients evaluated, 19 females and 21 males, aged 13 to 17 years, met the inclusion criterion – a confirmed clinical diagnosis of migraine or a tension headache according to the International Classification of Headache Disorders, 2nd edition. The diagnostic algorithm of the study group consisted of a full medical history, an assessment of the occurrence of bite habits and a physical examination based on the Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD).

Results. Bite and non-bite parafunctions were found in 36 of the study group patients. A significant difference ($p = 0.0003$) between the number of bite parafunctions and non-bite parafunctions was found in females but not in males. However, bite parafunctions were more frequent in boys compared to girls ($p = 0.01$).

Conclusions. Our findings suggest that it may be useful for pediatricians and neurologists to include TMD dysfunctions as a part of a standard examination of adolescents presenting with persistent headaches.

Key words: temporomandibular disorders, headache, adolescents

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Introduction

Headaches are one of the most common complaints in all age groups and a frequent cause of medical consultation and hospitalization.[1] They also have a significant impact on quality of life, with a detrimental effect on cognitive, emotional and social behavior.[2] Chronic headaches, with an estimated prevalence of 10.0% in the pediatric population, contribute to school absence, decreased participation in extracurricular activities and poor school performance.[3–7] Headaches can be broadly classified as primary or secondary.[8] In the case of primary headaches, the problem lies in the pain itself – mainly migraine and tension-type pain. The diagnosis of primary headache is by means of the generally accepted clinical criteria developed by the International Headache Society, 2nd edition (IHS-II).[9] According to IHS-II, a headache can be attributed to temporomandibular disorders (TMD) when the coexistence of pain, mobility and acoustic symptoms of the temporomandibular joint are present. In the case of both headaches and TMD, pain can occur in the frontal, temporal, parietal and occipital regions.[9] Population-based studies have previously examined the associations between TMD and psychological variables, pain conditions, and oral parafunctional habits.[10,11] The international core tool used for TMD diagnosis is the Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) developed by Dworkin and LeResche and based on a biopsychosocial model.[12] The RDC/TMD is derived from a consensus of leading researchers and clinicians and utilizes operationalized diagnostic criteria.[12] The tool standardizes the evaluation and classification of patients, including young people with TMD, and assesses both physical disorder factors and psychosocial illness impact factors, axis I and axis II.[13]Axis I is a set of diagnostic clinical procedures that provides data necessary for a physical diagnosis. Axis II assesses behavioral, psychosocial and quality-of-life aspects related to TMD pain diagnosis and treatment.[12–15]

In adolescents, a variety of TMD symptoms, such as pain in the pre-auricular area, tenderness during mandibular movements, joint crepitus or restricted mandibular movements, may be detected, although less frequently than in adult populations.[16] The etiology of TMD is multifactorial in adolescents and may result from trauma, repetitive microtrauma from oral parafunctions (both bite and non-bite) and occlusal, systemic and psychological factors.[10,11,17–20]

It has been suggested that parafunctional activities may overload the dentition and masticatory system and may play an etiological role in the development of TMD.[21] The detrimental effects of oral parafunctions will depend on their frequency, intensity and duration, as well as habits. Both masticatory and facial muscles respond to emotional states.[17,22] The prevalence of TMD increases with age due to the escalating exposure of children and adolescents to stress at school, during exams and while adapting to adult life.[21,23] Among the different oral parafunctional habits, teeth clenching and grinding, nail biting and gum chewing are most prevalent.[24,25]

Based on the results of Karibe et al., other pain conditions and parafunctional habits may exist that are related to TMD symptoms, even in a young population, and these factors should receive urgent attention.[26] Oral bite and non-bite parafunctional habits involving repeated or sustained occlusal contact can be harmful to teeth and other components of the masticatory system and can affect the muscles and/or temporomandibular joint (TMJ). Hence, we investigated the prevalence of TMD symptoms in adolescents (12–17 years) with headaches, and assessed the occurrence of parafunctional habits and symptoms of TMD. In this study, we tested the hypothesis that TMD symptoms are associated with parafunctions in adolescents suffering from headaches.

Material and methods

Parents of all adolescents presenting with headaches in the Department of Developmental Neurology at Poznan University of Medical Sciences in Poland, from March 2013 to March 2014, were asked to complete a questionnaire survey developed by the authors of the study. Out of the 1000 patients evaluated in the Department, 40 children and adolescents aged 13–17 years met the inclusion criterion of the study – a confirmed clinical diagnosis of migraine or tension type headache according to the International Classification of Headache Disorders, 2nd edition (ICHD-2).[9] The mean age of the patients was 14.9 ±1.7 years. During hospitalization, all the subjects in this group were examined by a certificated child neurologist and a full medical history was provided. Data from the questionnaire survey was also used to confirm the ICHD-2 criteria for migraine and tension type headache. The questionnaire study was approved by the Ethics Committee of the Poznan University of Medical Sciences, Poland.

Questionnaire survey

The diagnoses of all subjects in the investigated group were confirmed both by questionnaire and clinical assessment. The diagnostic algorithm used for the study group included a medical history, the occurrence of bite and non-bite parafunctions and a physical examination based on the RDC/TMD.[12–15] The questionnaire survey focused on the characteristics of the headache, its frequency and the presence of additional symptoms. It also included questions about the history of head and neck injuries, mouth breathing, incorrect ingestion, and parafunctional habits. Parafunctional habits were categorized according to the following classification: bite habits (that involve the contact of opposing teeth), such as teeth grinding or/and habitual teeth clenching; non-bite habits (which do not involve the contact of opposing teeth) such as tongue, lip/cheek biting, biting or chewing on objects and/or nail biting.

Examination of TMD – RDC/TMD questionnaire (axis II) and examination (axis I)

All functional examinations of the stomatognathic system were performed by the same dentist as well as the evaluation of the occurrence of clinical TMD signs or symptoms. The clinical examination required about 10–15 min, whereas completing the questionnaire required up to 30–45 min.

The axis II component of the RDC/TMD is a measure of depression and non-specific physical symptoms, typical pain intensity (characteristic pain intensity) and pain-related disability classification which are reliable and valid indicators with respect to the selected reference standards and for application to this patient population. Questions regarding sexual behavior were excluded. If a patient has experienced facial pain, or pain in the stomatognathic system or temples for the last month, the second part of the form was completed – the Chronic Pain Grade Protocol.[12,13] This included pain insensitivity, disability due to pain, depression, fainting accidents, dizziness, heartache, and stomach or throat discomfort.

The physical examination was performed according to axis I of the RDC/TMD. The classification included 3 major groups: 1. muscle disorders; 2. disc displacements; 3. arthralgia, osteoarthritis, and osteoarthrosis of the TMJ. In order to diagnose a muscle-related TMD, pain must be present in the jaw, temples, face, pre-auricular area or inner ear at rest or during activities; tenderness/palpation must be present in at least 3 out of 20 palpation sites, with at least 1 tender site situated ipsilaterally to the complaint pain. The following areas were palpated bilaterally (with tenderness ranges of no pain, mild pain, moderate pain, and severe pain): the posterior/middle/anterior temporal muscle, superior/middle/inferior masseter muscle, posterior mandibular region, submandibular region, lateral/posterior pole of the temporomandibular joint, lateral pterygoid area and tendon of temporal muscle. Using the observed signs and symptoms, the RDC/TMD specifies diagnostic algorithms along with a classification system dividing TMD diagnoses into 3 main groups and 8 subgroups (Table 1). This classification system only applies to the most common TMD diagnoses. The examination included: measurement of the range of mandibular movements, assessment of pain in joints and muscles in motion, and palpation of clicks or crepitus during mandibular movements.

The presence of bite and non-bite parafunctions, occurrence of etiological factors of TMD, prevalence of TMD according to RDC/TMD, prevalence of headache in each TMD– elicited from patient history, and clinical examinations are presented in the tables as counts (in absolute numbers) while frequencies are expressed in percentages. A test for differences between 2 proportions was used to detect the differences between the occurrence of parafunctions and gender. To check the dependency between gender and TMD type, the χ^2 test was used. The data obtained from the study was then analyzed using STATISTICA v. 10.0 (StatSoft Inc., Tulsa, USA). The level of significance was set at $p \leq 0.05$.

Table 1. Categories of clinical conditions present in TMD, according to RDC/TMD axis I

Main groups	Diagnoses
I – myofascial pain	Ia – myofascial pain Ib – myofascial pain with limited opening
II – disc displacements	IIa – disc displacement with reduction IIb – disc displacement without reduction, with limited opening IIc – disc displacement without reduction, without limited opening
III – arthralgia, osteoarthritis, osteoarthrosis	IIIa – arthralgia IIIb – osteoarthritis of the temporo-mandibular joint IIIc – osteoarthrosis of the temporomandibular joint

Results

The study group consisted of 19 female (47.5%) and 21 male (52.5%) patients, aged 13 to 17 years. All participants had experienced episodes of headache for at least 3 months and this was the main reason for their admission to the department. A total of 12 participants (30.0%) from the study group manifested migraine headaches according to ICHD-2 diagnostic criteria, while 28 participants (70.0%) fulfilled the diagnostic criteria for tension type headaches.

In the examined group of 40 adolescents presenting with primary headaches, bite and non-bite parafunctions were found in 36 (90.0%) patients. The study found no correlation between gender and the presence of parafunctional habits in general. There was a significant difference ($p = 0.0003$) between the number of bite (31.6%) and non-bite parafunctions (89.5%) in female patients, which was not observed in male patients ($p = 0.73$). Boys (71.4%) presented more frequently with bite parafunctions compared to girls (31.6.0%; $p = 0.01$) (Table 2). In patients with a history of head trauma, malocclusion, abnormal swallowing or mouth breathing, the number of bite and non-bite parafunctional habits was high, ranging from 81.8% to 100% (Table 3).

RDC/TMD examination (axis I)

Using the axis I RDC/TMD diagnostic criteria, it was found that among 40 adolescents presenting with headaches, 16 patients (40.0%) suffered from muscle disorders (Ia), 13 patients (32.5%) had disc displacement with reduction (IIa) and 11 patients (27.5%) showed no dysfunctions. Bite and non-bite parafunctions were present in 100% of cases with muscle disorders (Ia), 100% of cases with disc displacement with reduction (IIa) and in 63.6% of patients with no symptoms of TMD (Table 4). Analysis of the 16 patients with muscle disorder (Ia) showed that the

Table 2. Presence of bite and non-bite parafunctions in the group of adolescents presenting with headache

Study group n (%)	Parafunctions				p-value
	bite parafunctions n (%)		non-bite parafunctions n (%)		
Girls 19 (47.5)	6 (31.6)	p = 0.01	17 (89.5)	ns	p = 0.0003
Boys 21 (52.5)	15 (71.4)		16 (76.2)		ns
Total 40 (100)	21 (52.5)		33 (82.5)		–

There was a significant difference (p = 0.0003) between the number of bite (31.6%) and non-bite parafunctions (89.5%) in girls, which was not observed in the case of boys (p = 0.72). The study revealed no statistically significant differences (ns) between girls and boys in the case of non-bite parafunctions (p = 0.3). However, bite parafunctions (p = 0.01) presented more frequently in boys.

Table 3. Occurrence of parafunctions depending on TMD etiological factors in adolescents

Etiological factors of TMD	n = 40 (100%)	Bite and non-bite parafunctions n (%)
Malocclusion	18 (45.0)	18 (100)
Head injuries	11 (27.5)	9 (81.8)
Breathing through the mouth	11 (27.5)	10 (90.9)
Incorrect ingestion	10 (25.0)	9 (90.0)

pain was mainly located in the frontal (50.0%) and temporal region (43.8%). Among the 13 patients manifesting disc displacement with reduction (IIa), 38.5% experienced pain in the temporal, parietal and occipital region and 30.8% in the frontal region. Among the 11 patients showing no symptoms from TMJ, 90.9% of cases experienced headache in the frontal region (Table 5).

Chronic Pain Grade classification and depression RDC/TMD questionnaire survey (axis II)

The prevalence of chronic pain located in the facial area (Chronic Pain Grade) according to RDC/TMD axis II is presented in Fig. 1. To facilitate the interpretation of data, the 4 grade scale of chronic pain used in RDC/TMD questionnaire was simplified to: 0 – absence of pain and 1 – presence of pain. In 16 subjects presenting with muscle disorder (Ia) chronic pain was diagnosed in 62.5%. Among 13 patients presenting with disc displacement with reduction (IIa) chronic pain was present in 69.2%. Among

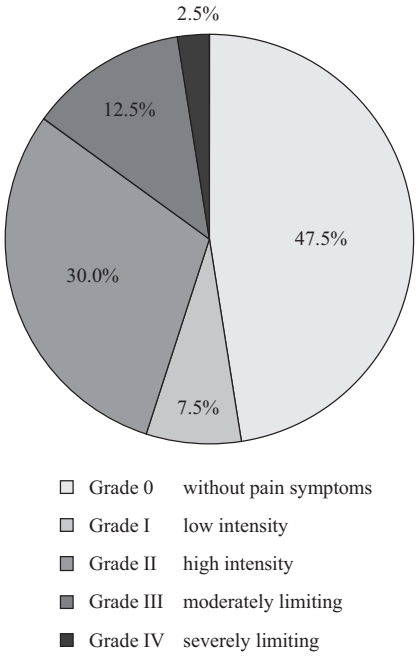

Fig. 1. Classification of the severity of chronic pain according to RDC/TMD axis II (headache groups)

Table 4. Prevalence of TMD in the group of adolescents suffering from headache according to RDC

TMD diagnoses (axis I)	Total n = 40 (%)	Girls n = 19 (%)	Boys n = 21 (%)	Bite and non-bite parafunctions of every TMD n = 40 (%)
Ia – myofascial pain	16 (40.0)	8 (42.1)	8 (38.1)	16 (100)
IIa – disc displacement with reduction	13 (32.5)	7 (36.8)	6 (28.6)	13 (100)
0 – no disorder	11 (27.5)	4 (21.1)	7 (33.3)	7 (63.6)
Total	40 (100)	19 (100)	21 (100)	36 (90.0)

Table 5. Prevalence of headache in each TMJ disorder according to RDC/TMD axis I diagnostic criteria

TMD diagnoses (axis I)	Frontal region n (%)	Parietal region n (%)	Temporal region n (%)	Occipital region n (%)	Frontal + temporal + parietal + occipital region n (%)
Ia – myofascial pain (n = 16)	8 (50.0)	1 (6.3)	7 (43.8)	1 (6.3)	2 (12.5)
IIa – disc displacement with reduction (n = 13)	4 (30.8)	5 (38.5)	5 (38.5)	5 (38.5)	1 (7.7)
0 – no disorder (n = 11)	10 (90.9)	0	0	1 (9.1)	0

Table 6. Prevalence (%) of depression and non-specific physical symptoms with a pain component and non-specific physical symptoms without a pain component in RDC/TMD diagnoses, axis I (headache groups)

TMD diagnoses (axis I)	Depression n (%)	Non-specific physical symptoms, with pain items n (%)	Non-specific physical symptoms, without pain items n (%)
Ia – myofascial pain (n = 16)	1 (6.3)	3 (18.8)	1 (6.3)
IIa – disc displacement with reduction (n = 13)	1 (7.7)	4 (30.8)	1 (7.7)

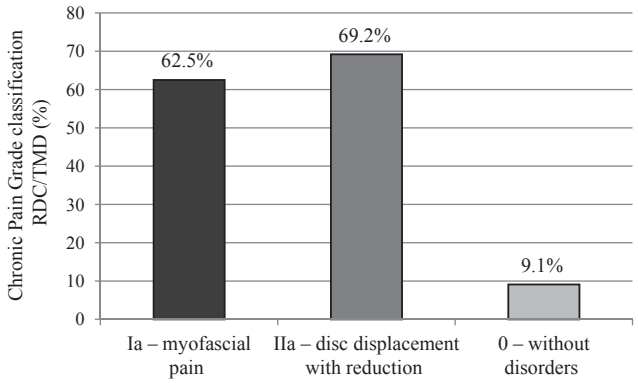

Fig. 2. Prevalence of chronic pain (according to Chronic Pain Grade) RDC/TMD axis II in TMD diagnoses (axis I) (headache groups)

patients showing no symptoms from TMJ chronic pain was experienced by only 9.1% (Fig. 2).

The depression and somatization symptoms grade scale, according to the RDC/TMD diagnostic criteria, was also simplified as: 0 – absence of pain and 1 – presence of pain. Depression was diagnosed in 1 patient in the Ia group and 1 patient in the IIa group. Non-specific physical symptoms were present in 3 cases in the Ia and 4 cases in the IIa group (Table 6). Subjects without TMD did not show any depression or non-specific physical symptoms.

Discussion

Recent studies of young (preschool to maturity) outpatients and inpatients have revealed that the number of headache cases has increased over recent years.[10,27,28] An accurate analysis of headache characteristics is crucial for a proper differential diagnosis.[29,30] Long term stress provokes emotional anxiety and increases muscle hyperactivity and tension.[9,10,31] Chronic muscle tension can lead to headaches located in the temporal, frontal, parietal or occipital region. Liljeström et al. observed a correlation between TMD and headache in a group of adolescents with primary headache and concluded that TMD should always be considered when headaches are associated with ear pain, difficulty in opening the mouth and fatigue or stiffness of the jaw.[32] Bertoli et al. evaluated the signs and symptoms of TMD in 50 children, aged 4–18 years, who had headaches.[18] They found a higher prevalence of signs and symptoms of TMD in patients with headaches compared to the control group.

Adolescents with TMD have reported significantly higher levels of stress and psychosocial problems.[33] Other researchers have demonstrated a correlation between headache and stress level, parafunctional habits, pops and clicks in the TMJ, and pain during mandibular movement.[21,26,34] Subjects may be unaware of diurnal clenching and nocturnal tooth grinding while sleeping.[11,35] Considering the high frequency of parafunctions and TMD in children and adolescents, epidemiological research into the morphological and functional causes would be of great benefit.[11,16,27,36,37]

In our study, in the group of adolescents presenting with primary headache, 36 (90.0%) of the subjects were found to have bite and non-bite parafunctions. Bite parafunctional habits were present in 52.5% of cases while 82.5% of subjects revealed non-bite habits. It was demonstrated that occlusal parafunctions appeared more frequently in male patients. There was also a significant difference between the number of bite parafunctions (31.6%) and non-bite parafunctions (89.5%) in girls, but not in boys. No significant difference was noted in non-bite parafunctional habits between female and male patients.

All patients with a diagnosed muscle disorder or disc displacement with reduction displayed bite and non-bite parafunctional habits. The results are consistent with that of Glaros et al.[38] Farsi et al. in their population studies of school children did not find any differences in the number of TMD symptoms between genders, but revealed a more frequent occurrence of non-bite parafunctions compared to bite parafunctions.[25] Nail biting was the most common non-bite oral parafunction (27.7%) while bruxism proved to be the least common parafunction (8.4%).

Among the etiological factors, traumas, including head traumas, often occur in children and adolescents, and may be a source of headaches as well as TMJ disorders.[39] Katzberg et al. showed that traumas are the cause of 26.0% of TMD in children and adolescents.[40] TMJ injury can occur due to impact (car accidents, contact sports), biting on hard objects or opening the mouth too wide. In the group of 40 subjects, 27.5% of participants had a history of head trauma with 81.8% exhibiting bite and non-bite parafunctions. Other etiological factors of these dysfunctions included malocclusion, abnormal swallowing or mouth breathing. Such activities in combination with

parafunctional habits may accelerate the development of TMJ disorders.

Carlsson et al. reported malocclusion in 35.0–95.0% of randomly selected 7-, 11- and 15-year-old subjects.[41] Our results demonstrate the presence of malocclusion in 45% of cases, mouth breathing in 27.5% of cases, abnormal swallowing in 25% of cases, and parafunctions in 81.8–100% of cases. Over the past several decades, the age of patients presenting with TMD has been gradually decreasing.[41] In individuals from 7 to 11 years old, the number of disorders increased rapidly from 30.0% to 60.0%; while the age group 12 to 14 showed no increase until the age of 19, when the disorders increased to 80.0%. Studies of adult subjects have revealed that TMD symptoms are twice as frequent in females than in males.[42,43] However, there was no significant difference in TMD incidence between males and females in the age group studied.

Analysis of the location of pain was also performed in 16 patients with muscle disorders, where (Ia) pain affected the forehead (50.0%) and temples (43.8%). Of the 13 subjects presenting with disc displacement with reduction (IIa), the pain was located in the temporal, parietal and occipital region (38.5%), and frontal region (30.8%). Among the 11 patients without TMD, 90.9% of cases involved the frontal region. Ballegaard et al. examined adult patients presenting with headaches and discovered a high (83.0%) percentage of disabilities caused by chronic pain of the face and TMJ.[44] In contrast, our findings demonstrate that chronic pain (Chronic Pain Grade) in children occurs to a lesser extent (52.5%). Among the subjects presenting with muscle disorders (Ia), 62.5% of patients suffered from pain. However, 69.2% of patients presenting with disc displacement with reduction (IIa) experienced pain. Some children with TMD symptoms did not develop any facial pain.

Currently, the significance of psychophysical factors as a cause of diseases and TMJ disorders is being emphasized. List et al. revealed that, in adolescents with TMD, psychosocial factors such as increased levels of stress, somatic complaints and emotional problems seem to play a prominent role.[33] The chief factors include: stress, nervousness, depression, and anxiety. The depression scale SCL-90 elaborated by Derogatis et al. has been included in the RDC/TMD diagnostic criteria by Dworkin et al.[12,45] Mental state assessment by Ballegaarda et al. revealed that 54.5% of patients with headache were affected by moderate to severe depression.[44] Our findings revealed that children and adolescents suffered from depression and nonspecific physical syndromes to a lesser degree: depression in 6.3–7.7% of cases and nonspecific physical syndromes in 18.8–30.8% of cases. Patients without any TMD symptoms showed no depression or nonspecific physical syndromes.

Conclusions

1. On the basis of patient history and clinical examination of adolescents presenting with primary headache, a high incidence of TMD symptoms, bite and non-bite parafunctional habits was found.

2. This paper underscores the need for a multilevel diagnostic and therapeutic approach, the importance of a rigorous examination of the stomatognathic system and the identification of a destructive influence of parafunctional habits in adolescents suffering from headache.

3. RDC/TMD classification is a helpful tool for diagnosing TMD in children and youth presenting with primary headache.

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Morphometric study of the triangle of Petit in human fetuses

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Abstract

Background. The inferior lumbar triangle of Petit is bounded by the iliac crest, lateral border of the latissimus dorsi and the medial border of the external oblique.

Objectives. In the present study, we aimed to quantitatively examine the base, sides, area, and interior angles of the inferior lumbar triangle in the human fetus so as to provide their growth dynamics.

Material and methods. Using anatomical dissection, digital image analysis (NIS-Elements AR 3.0), and statistics (Student's t-test, regression analysis), we measured the base, 2 sides, area and interior angles of Petit's triangle in 35 fetuses of both sexes (16 male, 19 female) aged 14–24 weeks.

Results. Neither sex nor laterality differences were found. All the parameters studied increased commensurately with age. The linear functions were computed as follows: $y = -0.427 + 0.302 \times$ age for base, $y = 1.386 + 0.278 \times$ age for medial side, $y = 0.871 + 0.323 \times$ age for lateral side, and $y = -13.230 + 1.590 \times$ age for area of the Petit triangle.

Conclusions. In terms of geometry, Petit triangle reveals neither male–female nor right–left differences. An increase in both lengths and area of the inferior lumbar triangle follows proportionately. The Petit triangle is an acute one in the human fetus.

Key words: digital-image analysis, Petit triangle, inferior lumbar triangle, side, area

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Introduction

The inferior lumbar triangle of Petit is a topographical element of the lower back with an area of minor resistance of the posterior abdominal wall. The base of that triangle is limited by the iliac crest, with the opposite apex directed toward the inferior angle of the scapula, the medial side is constituted by the lateral border of the latissimus dorsi, and the lateral side is composed of the medial border of the external oblique muscle. The floor of the Petit triangle is part of the internal oblique muscle covered with the superficial fascia and subcutaneous tissue.[1–7]

Precise data on the quantitative anatomy of the inferior lumbar triangle may be useful in anesthesiology, especially in anesthesia of the transversus abdominis plane (TAP), and in fetal surgery. TAP is located in the anterior abdominal wall between the transversus abdominis and the internal oblique muscle, and includes the lower intercostal nerves with concomitant blood vessels destined for the anterolateral abdominal wall.[6–9] To date, autopsy material of adult individuals only has been used for the geometrical analysis of Petit's triangle.[6,7,10] Therefore, this is the first report in the professional literature to present a numerical analysis of Petit's triangle in human fetuses.

The objectives of the present study were:

- morphometric analysis of the inferior lumbar triangle in human fetuses with respect to its linear and planar parameters, and interior angles in order to determine their normative values at varying gestational ages;
- establishing the possible sexual and bilateral differences regarding the analyzed parameters;
- establishing developmental dynamics for the analyzed parameters, including mathematical growth models best matched for fetal age.

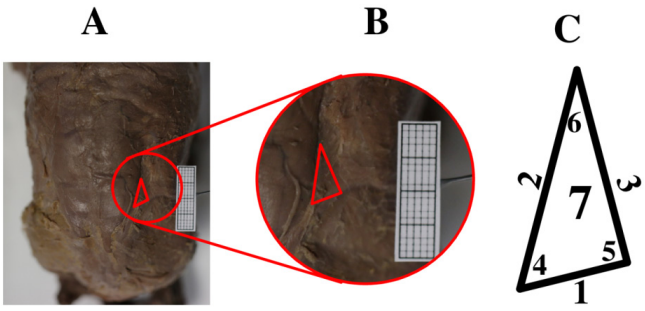

Fig. 1. Petit's triangle (A, B) in a female fetus at 24 weeks showing the measured parameters (C)

Material and methods

The study material consisted of 35 fetuses of both sexes (16 males and 19 females) aged 14–24 weeks of fetal life, originating from spontaneous abortions and stillbirths. The material was acquired before the year 2000 and remains part of the specimen collection of the Department of Normal Anatomy of Nicolaus Copernicus University in Toruń. This experiment was sanctioned by the Bioethics Committee of the Nicolaus Copernicus University in Toruń (approval No. KB 186/2016). The fetal age was determined on the crown-rump length. Table 1 lists the characteristics of the study group, including age, number and sex of the fetuses.

Using anatomical dissection, the inferior lumbar triangle was visualized on both sides, then recorded using a Sony α330 digital camera and subjected to morphometric analysis using digital image-analysis (NIS-Elements AR 3.0 software, Nikon, Minato, Japan). In each Petit's triangle, the following 7 parameters were measured (Fig. 1):

Table 1. Characteristics (age, number and sex) of the fetuses studied

Gestational age weeks (Hbd-life)	Crown-rump length [mm]				Number of fetuses	Sex	
	mean	SD	min	max		♂	♀
14	88.00		88.0	88.0	1	0	1
15	94.67	2.31	92.0	96.0	3	2	1
16	114.75	1.71	113.0	117.0	4	1	3
17	123.00	5.00	118.0	128.0	3	2	1
18	139.33	3.21	137.0	143.0	3	1	2
19	152.00	4.12	145.0	155.0	5	2	3
20	164.00	2.65	162.0	167.0	3	1	2
21	175.00	2.94	171.0	178.0	4	1	3
22	188.67	2.31	186.0	190.0	3	2	1
23	197.00	4.18	192.0	201.0	5	4	1
24	206.00		206.0	206.0	1	0	1
Total					35	16	19

♂ – male; ♀ – female.

1. base – length of the iliac crest (mm);
2. medial side – length of the lateral border of the latissimus dorsi (mm);
3. lateral side – length of the medial border of the external oblique muscle (mm);
4. basomedial angle – between the iliac crest and the medial border of the external oblique muscle;
5. basolateral angle – between the iliac crest and the lateral border of the latissimus dorsi;
6. apical angle – between the medial border of the external oblique and the lateral border of the latissimus dorsi;
7. area – calculated semiautomatically after outlining the triangle (mm^2).

The obtained numerical data was analyzed statistically using STATISTICA v. 12.5 software (StatSoft Inc., Tulsa, USA). The results are expressed as arithmetic means with standard deviations (SD). To compare the means, Student's t-test for dependent (left–right) and independent (male–female) variables, and one-way analysis of variance were used. The characterization of the developmental dynamics of the analyzed parameters was based on linear and curvilinear regression analysis. The match between the estimated curves and numerical data was evaluated due to coefficient of determination (R^2). Differences were considered significant at $p < 0.05$.

Results

The entire fetal material unveiled the inferior lumbar triangle on both sides within its typical boundaries: a base at the iliac crest, a medial side constituted by the lateral border of the latissimus dorsi, and a lateral side constituted by the medial border of the external oblique muscle.

The statistical analysis revealed neither sexual nor bilateral differences concerning all the analyzed parameters. Therefore, we investigated the developmental dynamics of the 7 established parameters without taking sex or age into account. The numerical data of the inferior lumbar triangle has been presented in tables, as follows: its base and 2 sides in Table 2, its interior angles in Table 3, and its area in Table 4.

The developmental dynamics of the base, sides and area of the inferior lumbar triangle followed linear functions, as displayed in Table 5.

Discussion

The inferior lumbar triangle is an area of minor resistance of the posterior abdominal wall and a gateway to lower lumbar hernias.[1–7] As reported, the most tenuous point of the lumbar triangle is the Hartmann fissure located at its apex.[11–13] Lower lumbar hernias may be both congenital and acquired, constituting 20% and 80% of cases, respectively.[4,5,10,11,14] Congenital defects of the posterior abdominal wall are the most common reasons for the lower lumbar hernias in children.[15,16] In turn, acquired lower lumbar hernias are divisible into primary and secondary types, the former resulting from an excessive tonus of abdominal musculature, e.g., in obese elderly people, and the latter being a consequence of damage to the abdominal muscles and resulting in scar formation.[16–18] In adults, lower lumbar hernias affect men 3 times more often than women, especially those aged 40–60 years, and with a greater tendency to occur on the right side.[15,19–22] The main symptom reported by patients is a pain or discomfort in the lumbar region, usually when tightening the abdominal musculature.[11,15,20–22] However, lower lumbar hernias may be asymptomatic as well.[14,20,21]

In anesthesia, the inferior lumbar triangle is a natural and safe anatomical gateway to transversus abdominis plane punctures, as there are no neurovascular structures.[7,10] The transversus abdominis plane block was developed in 2001 by Rafi for an easy and effective anesthesia within the hypogastrium, especially in cesarean sections, inguinal hernia surgery and laparoscopic procedures.[6,23] Hebbard et al. described a method of TAP anesthesia through a block of the lower intercostal nerves that are readily accessible via the inferior lumbar triangle.[24] The Petit triangle is also conducive in urology, neurosurgery and surgery as an approach to the retroperitoneal space.[25–28]

In this study, the inferior lumbar triangle was bilaterally present in all human fetuses considered. In an autopsy study conducted in adults, Loukas et al. observed Petit's triangle in 82.5% of cases, and Starczewski in 89% of cases, somewhat more often on the left side.[10,28] In cases with absent lumbar triangles, the latissimus dorsi was overlapped by the external oblique muscle.

As presented in the current study, in terms of quantity, the Petit triangle did not demonstrate any sexual or bilateral differences. Between weeks 14 and 24 of gestation, the dimensions of the inferior lumbar triangle increased as follows: its base from 3.63 to 6.91 mm, its medial side from 5.24 to 6.84 mm, and its lateral side from 5.40 to 8.82 mm. These parameters were deliberated in adults by Loukas et al., Jankovic et al. and Starczewski et al., who also did not note any sexual differences in this aspect.[6,10,28] As reported by Loukas et al., in the inferior lumbar triangles on the right and left sides, the mean base measured at the iliac crest was 2.57 cm and 3.1 cm, respectively, the mean medial side measured at the latissimus dorsi was 3.44 cm and 4.57 cm, respectively, and the mean lateral side measured at the external oblique muscle was 4.53 cm and 3.25 cm, respectively.[10] In the same study, symmetrical triangles were observed most often (25%) with type I, less often (17.5%) with type II, and least often (3.7%) with type III, according to the classification by Loukas et al. detailed below.[10] In addition, the inferior triangles located on the left side were larger. Jankovic et al., in 26 individuals aged 72–102 years with the mean height of 161.8 cm ±9.9 cm, demonstrated that the triangle base

Table 2. Base, medial and lateral sides of the Petit's triangle

Gestational age (weeks)	Number of fetuses	Base					Medial side					Lateral side				
		right		left		p-value	right		left		p-value	right		left		p-value
		mean	SD	mean	SD		mean	SD	mean	SD		mean	SD	mean	SD	
14	1	3.72		3.54			5.28		5.21			5.42		5.37		
15	3	4.06	0.17	4.01	0.11	<0.05	4.84	0.40	4.82	0.35	<0.05	5.04	0.72	4.92	0.75	<0.05
16	4	4.57	0.69	4.50	0.69	<0.05	6.15	0.45	6.13	0.40	<0.05	6.36	0.46	6.24	0.55	<0.05
17	3	4.65	0.80	4.68	0.73	<0.05	6.23	0.63	6.25	0.63	<0.05	6.93	0.69	6.94	0.75	<0.05
18	3	5.46	0.37	5.49	0.42	<0.05	7.05	0.54	7.07	0.60	<0.05	7.37	0.43	7.38	0.40	<0.05
19	5	5.42	0.18	5.38	0.18	<0.05	6.87	0.21	6.85	0.18	<0.05	6.93	0.25	6.92	0.22	<0.05
20	3	5.63	0.06	5.66	0.05	<0.05	7.03	0.18	7.03	0.14	<0.05	7.32	0.19	7.37	0.10	<0.05
21	4	5.77	0.33	5.78	0.30	<0.05	7.13	0.49	7.15	0.52	<0.05	7.89	0.08	7.95	0.12	<0.05
22	3	6.37	0.04	6.43	0.07	<0.05	7.37	0.45	7.35	0.42	<0.05	7.51	0.54	7.53	0.62	<0.05
23	5	6.39	0.24	6.39	0.22	<0.05	7.79	0.46	7.80	0.47	<0.05	8.13	0.38	8.13	0.38	<0.05
24	1	6.83		6.98			6.92		6.76			8.81		8.83		

Table 3. Angles of the Petit's triangle

Gestational age (weeks)	Number of fetuses	Basomedial angle (α)					Basolateral angle (β)					Apical angle (γ)				
		right		left		p-value	right		left		p-value	right		left		p-value
		mean	SD	mean	SD		mean	SD	mean	SD		mean	SD	mean	SD	
14	1	47.85		48.13			72.44		72.03			59.71		59.84		
15	3	49.99	3.49	49.93	3.62	<0.05	69.69	3.09	69.88	3.32	< 0.05	60.31	0.74	60.18	0.33	<0.05
16	4	49.53	2.13	49.32	1.90	<0.05	73.40	5.20	72.66	5.65	< 0.05	57.06	3.13	58.01	3.76	<0.05
17	3	47.23	1.40	47.16	2.35	<0.05	77.57	4.73	77.19	5.32	<0.05	55.19	3.34	55.63	3.19	<0.05
18	3	47.05	1.05	46.52	0.71	<0.05	75.53	0.83	75.76	0.32	< 0.05	57.42	1.74	57.71	0.99	<0.05
19	5	47.18	1.56	47.22	1.60	<0.05	78.00	3.66	77.76	4.06	<0.05	54.81	2.47	55.01	2.58	<0.05
20	3	46.59	0.33	46.71	0.81	<0.05	77.33	2.64	77.86	1.95	<0.05	56.08	2.32	55.43	1.15	<0.05
21	4	46.77	0.55	46.79	0.41	<0.05	78.57	2.30	78.49	2.20	<0.05	54.65	2.46	54.71	2.03	<0.05
22	3	46.54	1.05	46.83	1.37	<0.05	78.12	2.65	78.41	2.74	<0.05	55.33	1.69	54.75	1.49	<0.05
23	5	47.76	3.69	47.50	3.50	<0.05	78.95	4.31	78.68	4.35	<0.05	53.28	1.58	53.81	2.21	<0.05
24	1	54.95		55.72			77.93		78.69			47.12		45.59		

Table 4. Area of the Petit's triangle

Gestational age (weeks)	Number of fetuses	Area (mm²)									Sex	
		mean		SD		p-value	min		max		♂	♀
		right	left	right	left		right	left	right	left		
14	1	8.92	9.20				8.92	9.20	8.92	9.20	0	1
15	3	9.15	8.87	1.53	1.37	<0.05	7.51	7.44	10.56	10.18	2	1
16	4	13.36	13.04	2.76	2.84	<0.05	10.43	10.01	17.09	16.87	1	3
17	3	14.26	14.38	3.55	3.38	<0.05	10.16	10.51	16.51	16.70	2	1
18	3	18.20	18.34	2.02	2.33	<0.05	16.08	15.86	20.11	20.50	1	2
19	5	17.19	17.05	0.74	0.80	<0.05	16.35	16.28	18.30	18.21	2	3
20	3	18.54	18.69	0.49	0.26	<0.05	18.16	18.53	19.10	18.99	1	2
21	4	19.77	19.90	1.31	1.46	<0.05	18.10	17.88	21.32	21.41	1	3
22	3	21.39	21.57	1.66	1.86	<0.05	19.47	19.43	22.42	22.82	2	1
23	5	23.22	23.25	1.52	1.50	<0.05	20.80	21.01	24.80	25.14	4	1
24	1	23.25	23.23				23.25	23.23	23.25	23.23	0	1
Total											16	19

♂ – male; ♀ – female.

Table 5. The growth dynamics of the base, sides and area of the Petit triangle

Parameter	Regression equation	R^2	F	p-value
Base [mm]	y = –0.427 + 0.302 × age	0.843	364.83	0.000
Medial side [mm]	y = 1.386 + 0.278 × age	0.702	160.15	0.000
Lateral side [mm]	y = 0.871 + 0.323 × age	0.739	192.88	0.000
Area [mm²]	y = –13.230 + 1.590 × age	0.858	412.27	0.000

was 2.3 cm ±1.03 cm, the medial side was 2.2 cm ±1.38 cm, and the lateral side was 3.3 cm ±1.36 cm.[6] Numerical data by Starczewski indicated that for the right and left sides, the mean triangle base was 24.8 mm ±11.8 mm and 25.3 mm ±9.0 mm, respectively, the mean medial side was 24.3 mm ±9.9 mm and 25.1 mm ±8.7 mm, respectively, and the mean lateral side was 33.0 mm ±10.1 mm and 32.2 mm ±11.0 mm, respectively.[28]

Of note, the present study has been the first report in the literature to explicitly display mathematical growth models of the inferior lumbar triangle as a function of fetal age. Its morphometric parameters increased proportionally, following the functions: y = –0.427 + 0.302 × age for base, y = 1.386 + 0.278 × age for medial side, and y = 0.871 + 0.323 × age for lateral side of the inferior lumbar triangle.

In our study, the basomedial angle of the lumbar triangle was between 47.85° and 54.95° on the right side, and between 48.13° and 55.72° on the left side. The basolateral angle was between 72.44° and 77.93° on the right side, and between 72.03° and 78.69° on the left side. Obviously, the basal angles determined the apical angle, the value of which was between 59.71° and 47.12° on the right, and between 59.84° and 45.59° on the left. Furthermore, all observed interior angles were smaller than 90°, and thus all inferior lumbar triangles observed in this study were acute ones. As reported by Starczewski et al., the basomedial angle was 47° ±15° on the right and 49° ±17° on the left.[28] In turn, the basolateral angle was 84° ±29° on the right side and 81° ±26° on the left side. These authors reported 2 types of the Petit triangle: acute (59%) and obtuse (41%).

In our study, at the fetal age of 14–24 weeks, the inferior lumbar triangle area increased from 8.92 mm² to 23.25 mm² on the right, and from 9.20 mm² to 23.23 mm² on the left side. This increase in area of Petit's triangle followed the linear function: y = –13.230 + 1.590 × age. Based on its area value, Loukas et al. described 4 types of the inferior lumbar triangle: type I (43.7%) – small with the area of up to 8 cm²; type II (26.2%) –intermediate with the area between 8 and 12 cm², and type III (12.5%) – large, with the area exceeding 12 cm²; type IV (17.5%) was defined as a lack of the Petit triangle.[10] Starczewski introduced a 3-degree classification of the Petit triangle, according to its area.[28] Type I or small (20%) involved triangles with their area not exceeding 3 cm²; type II or intermediate (44%) included triangles with their area of 3–6 cm²; type III or large (36%) referred to triangles with their area above 6 cm². The mean Petit triangle area was 3.6 cm ±2.2 cm². Similarly, in the study by Jankovic et al., the mean Petit triangle area was 3.63 cm ±1.93 cm².[6] Furthermore, the authors demonstrated that the inferior lumbar triangle was enormously variable in its size and shape, and was located more medial than it had been previously expected. The orthocenter of the Petit triangle was, on average, 6.9 cm more posterior

with relation to the mid-axillary line, while the measurements taken superficially were somewhat smaller (9.3 cm). Due to the observed differences in size and shape of Petit's triangles, the authors concluded that the presumptive location of the inferior lumbar triangle may prevaricate the physician when administering TAP anesthesia.

Conclusions

In terms of geometry, Petit's triangle reveals neither male–female nor right–left differences. An increase in both lengths and area of the inferior lumbar triangle follows proportionately. The Petit triangle is acute in the human fetus.

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The effect of high intensity physical exercise and hypoxia on glycemia, angiogenic biomarkers and cardiorespiratory function in patients with type 1 diabetes

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Abstract

Background. An integral part of the treatment of diabetes is physical activity. Scientific reports have shown the beneficial effects of hypoxia and exercise on cardiovascular and metabolic variables in patients with diabetes.

Objectives. The aim of the study was to assess the effect of normobaric hypoxia and exercise on the serum concentrations of proangiogenic factors and glycemia in patients with type 1 diabetes.

Material and methods. A total of 28 adults (aged 30.4 years ±9.7 years), suffering from diabetes for 12.1 years ±6.0 years and healthy individuals, participated in the following trials: normoxic (Nor) and hypoxic (Hy) rest and Nor and Hy incremental exercise test (Ex) ($FIO_2 = 15.2\%$). The Altitude Trainer Hypoxico System (HYP-123 Hypoxic Generator, LOWOXYGEN Technology GmbH, Berlin, Germany) corresponding to a height of about 2500 m above sea level was used in the study. Exercise tests were performed on a cycle ergometer Excalibur Sport (Lode B.V., Groningen, The Netherlands). Cardiorespiratory variables, glycemia, angiogenic and hematological indices were measured at rest and in response to both exercise protocols.

Results. The present data confirmed that the patients with type 1 diabetes demonstrated a good level of aerobic capacity and fitness. NorEx and HyEx resulted in a significant decrease in serum glucose concentration ($p < 0.05$ vs $p < 0.01$). Patients with diabetes had higher baseline hypoxia induced factor-1alpha levels compared to healthy adults ($p < 0.05$), which increased after exposure to hypoxia and hypoxia with exercise ($p < 0.001$). Hypoxia significantly decreased baseline transforming growth factor-β (TGF-β) ($p < 0.05$) and had a significant effect on tumor necrosis factor-α level (TNF-α) ($F = 4.9$; $p < 0.05$).

Conclusions. Our study demonstrated that hypoxia combined with exercise reduces glycemia and may induce significant benefits in the prevention of diabetes cardiovascular complications.

Key words: angiogenesis, type 1 diabetes mellitus, exercise tolerance, hypoxia-inducible factor-1

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Introduction

Type 1 diabetes (T1DM) is an autoimmune disease resulting in the targeted destruction of pancreatic β-cells and permanent loss of insulin production. Currently, there is no clear evidence of the benefits of regular physical activity on glucose control in type 1 diabetes. However, regular exercise decreases the risk of diabetes related complications and ameliorates quality of life.[1–3] It is widely accepted that individuals who have been using intensive insulin therapy can practice almost all types of physical activity.[3,4] Recent studies suggest that an increasing number of individuals with type 1 diabetes are also practicing extreme sports (e.g., mountain climbing), even in high altitude conditions.[5,6]

Regular exercise increases glucose metabolism through an insulin-independent pathway, leads to increasing muscle oxidative capacity and constitutes the most effective stimulus for improvement in cardiovascular control.[7–10] However, in people with diabetes, it may also be responsible for the occurrence of some adverse reactions, such as hypoglycemia, hyperglycemia, ketosis and diabetes related complications. The metabolic and cardiovascular side effects of exercise may depend on the starting levels of glycemia, type and/or intensity of exercise, and use of exogenous insulin. During intense exercise (> 85% of maximal oxygen consumption – VO_{2max}), the epinephrine response can augment hepatic glucose output and may lead to post exercise hyperglycemia. These can be mitigated by administrating short-acting insulin analogues before high intensity exercise. Prolonged exercise at moderate intensity (40–60% of VO_{2max}) activates lipolysis, glycogenolysis and gluconeogenesis to allow an adequate supply of substrates for oxidative phosphorylation in contracting muscles, which exposes the patient to the risk of post-exercise hypoglycemia.[2–4]

Scientific reports have shown that hyperglycemia is mainly responsible for the development of micro- and/or macroangiopathy.[11,12] Chronically elevated blood glucose concentration induces endothelium dysfunction, manifested mainly by impaired vascular dilation. Impaired vascular dilation, as well as hyperglycemia-induced disorders of platelet function, cause greater monocyte chemotactic activity and inflammation initiating the development of vascular complications.[13] Diabetes related angiopathy can be aggravated by angiogenesis, which is mainly stimulated by hypoxia and more specifically by hypoxia-inducible factor-1 alpha (HIF-1α). Hypoxia increases the level of HIF-1α, which activates a number of genes related to hypoxia and stimulates angiogenic molecules such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), transforming growth factor alpha (TGF-α), and platelet-derived growth factor (PDGF).[13,14]

In diabetes, the chronic exposure of blood cells to hyperglycemia modifies the function of neutrophils which lose diapedesis ability. They plug the lumen of the vessel, leading to local hypoxia and potentially to angiogenesis intensification.[14]

Contrary to these observations, Mackenzie et al. support the findings that acute hypoxia and physical exercise appear to improve some aspects of glycemic control and might be a valuable therapeutic method in the treatment of people with type 2 diabetes.[15] Exposure to hypoxia and muscle contractions improved short-term glycemic control, and decreased pro-inflammatory cytokines and insulin resistance.[16] It has been hypothesized that the effects of hypoxia may induce inflammatory responses indispensable for the ischemia-related neovascularization process in physically active patients with diabetes.[14]

Type 1 diabetes is associated with reduced skeletal muscle capillarization and deregulation of complex angiogenesis pathways.[11,17,18] The severity of microvascular complications is associated with an increase in VEGF concentrations, which is the main angiogenic factor in poorly controlled patients. Chronic hyperglycemia may be responsible for the intensification of angiogenesis in the arterial walls, which can lead to atherosclerosis.[19,20] The significance of other factors stimulating the synthesis of serum VEGF and VEGF-A mRNA expression, such as cytokines, insulin-like growth factor-1 (IGF-1), nitric oxide, glycation end products, and reactive oxygen species, has also been reported.[12,16,20]

The physiological responses to lowered inspired oxygen pressure, either mediated by barometric pressure reduction (hypobaric hypoxia) or by lowering the oxygen fraction (normobaric hypoxia), in diabetes mellitus are, however, controversial.[10,16,21,22] Hence, we aimed to evaluate the effect of normobaric hypoxia with and without physical exercise on the serum concentrations of proangiogenic factors, glycemic control, and cardiorespiratory adaptation in patients with type 1 diabetes mellitus.

Material and methods

Subjects

Fourteen patients suffering from type 1 diabetes (T1DM) were randomly assigned to the study. All subjects were recruited at the Diabetes Clinic of the Silesian Center in Poland. Mean duration of diabetes was 12.1 (SD 6.0) years, and glycated hemoglobin (HbA_{1c}) was 55.2 (7.8) mmol/mol (Table 1). All individuals with T1DM adhered to intensive insulin therapy (NovoRapid – Novo Nordisk, Denmark; Lantus – Lantus SoloStar, Sanofi-Aventis GmbH, Frankfurt, Germany, or Humalog – Humalog Eli Lilly, Houten, Nederland) and performed self-monitoring of blood glucose on glycemic control. Half of the patients were using continuous subcutaneous insulin infusion while the other half used multiple insulin injections. Patients were advised to reduce their basal insulin infusion by 50% prior to each physical test.

Only patients free of diabetic complications were enrolled. The other criteria for inclusion were no personal history of other cardiovascular or metabolic diseases, no simultaneous participation in another trial, being free of any acute infections up to 1 week prior to the study, nonsmokers and with good exercise tolerance confirmed by the direct measurement of VO_{2max}, and HbA_{1c} < 8.0%. The medical history and information about diabetes etiology of the study participants were prepared by medical personnel of the Silesian Center. The control group consisted of 14 healthy nonsmoking individuals (CG) of a similar age and aerobic efficacy.

A few days before the examination, the subjects were asked to abstain from exercise, and also from the consumption of alcohol and caffeinated drinks. For the entire duration of experiment, their diet, fasting glycemia and insulin dosage were monitored. Composition of the diet was calculated with a dedicated software (Dietus, B.U.I. InFit, Warszawa, Poland).

The study protocol was approved by the Local Ethics Committee and conformed to the standards set by the Declaration of Helsinki.

Testing protocol

Throughout the experiment, the patients and healthy control individuals participated in the following trials: normoxic (NorRest: FIO_2 = 20.9%; P = 990 hPa) and hypoxic rest (HyRest: FIO_2 = 15.2%; P = 990 hPa) and normoxic (NorEx: FIO_2 = 20.9%; P = 990 hPa) and hypoxic (HyEx: FIO_2 = 15.2%; P = 990 hPa) graded exercise test. The protocol was based on 3 laboratory studies (ambient conditions: 21°C, 60% relative humidity) after an overnight fast and all studies were separated by at least 7 days. The participants' body mass and composition were determined by means of Bioelectrical Impedance Analysis (BIA) (InBody 220 Data Management System, Biospace, Seoul, Korea).

The Altitude Trainer Hypoxico System (HYP-123 Hypoxic Generator, LOWOXYGEN Technology GmbH, Berlin, Germany). corresponding to a height of about 2500 m above sea level was used in the study.

Normoxic and hypoxic rest

In the first part of the experiment, the patients and healthy control individuals rested for 40 min under Nor and Hy conditions. Physiological variables and biochemical variables were measured immediately before the test (PreEx) and after 40 min of rest (NorRest and HyRest).

Normoxic and hypoxic exercise test

All subjects participated in 2 graded exercise tests performed on a cycle ergometer Excalibur Sport (Lode B.V., Groningen, The Netherlands) in normoxic (NorEx) and hypoxic (HyEx) conditions. The tests started with a 3-min warm-up; the intensity was then increased by 30 W every 3 min up to maximal exercise intensity. Pulmonary ventilation (VE), oxygen uptake (VO_2), and carbon dioxide output (CO_2) were measured continuously from the 6th min prior to exercising and throughout each stage of the exercise load under both Nor and Hy conditions (Ergospirometr Metalyzer 3B-2R; Cortex Biophysik GmbH, Leipzig, Germany).

Blood response measures and biochemical variables were measured immediately before (PreEx) and at maximal exercise intensity (NorEx; HyEx). Blood glucose concentrations, insulin dosages, and hypo- or hyperglycemia events were controlled up to 24 h after exercise tests. Hypoglycemia was diagnosed with a BG concentration < 70 mg/dL, hyperglycemia with fasting BG > 130 mg/dL.[4] Prior to and after the hypoxic exposure, as well as during both exercise protocols, pulse oxygen saturation ($SatO_2$, pulse oximeter), heart rate (HR, PE-3000 Sport-Tester; Polar Inc., Kempele, Finland) and systolic and diastolic blood pressure were measured (HEM-907XL; Omron Corporation, Kyoto, Japan).

Biochemical analyses

Blood samples were taken from the cubital vein in the morning at rest for the determination of HbA_{1c} (Ames DCA-2000^{TM} Immunoassay Analyzer – normal range: 4.2–6.5%), BG (Glucose 201+, HemoCue) and cytokine/hormone concentrations. The levels of HIF-1α, VEGF, and TGF-β were measured by enzyme-linked immunosorbent assay ELISA kit (BlueGene Bitech Co., Ltd., Shanghai, China). Serum insulin (INS) and tumor necrosis factor alpha (TNF-α) were measured by immunoassays (DIAsource ImmunoAssays, Ottignies-Louvain-la-Neuve, Belgium). Serum IGF-1 was determined using an immunoradiometric assay IRMA kit (IGF-1-RIACT Cisibo, Gif-sur-Yvette, France). Blood red blood cells (RBC), white blood cells (WBC), lymphocytes (LYM), monocytes (MON), absolute neutrophil count (ANC), and hemoglobin (HGB) level were measured (ABX MICROS 60, HORIBA, Montpellier, France). Blood lactate concentrations (LA) were determined using the Biosen C-line method (EKF Diagnostic GmbH, Barleben, Germany); blood gases and acid-base balance were also analyzed (RapidLab 348; Bayer Diagnostics, Leverkusen, Germany). The D-max method was used to predict the anaerobic threshold based (LAT) on the recorded blood lactate levels. Data estimated in plasma after exercise was corrected for the after-exercise hematocrit change value. The obtained serum was aliquoted and frozen at –80°C until assay.

Statistical analysis

All results are presented as mean ±SD. The data was analyzed by a two-way ANOVA followed by the Student-Newman-Keuls test, when appropriate. The significance of the differences between groups was also assessed with the post

hoc Bonferroni correction. All analyses were performed using STATISTICA v. 10 software package (StatSoft, Tulsa, USA). Statistical significance was set at $p < 0.05$.

Results

Blood glucose, insulin dosage and diet

Subjects with T1DM and CG were matched for age and they did not differ in body weight and composition (Table 1). Average calorie supply with diet, mean daily fat and protein intake on successive days of the experiment did not differ significantly between the CG and T1DM groups. The T1DM group demonstrated lower carbohydrate consumption compared to CG ($p < 0.05$) (Table 2). The statistical analyses did not reveal any significant differences in fasting glycemia, nor in insulin dosage on the 1st and 2nd day in Nor and Hy conditions (Table 3).

Table 1. Subject characteristics; mean (SD)

Variable	CG (n = 14)	T1DM (n = 14)
Age [years]	24.0 (5.2)	30.4 (9.7)
Body height [cm]	174.9 (7.9)	177.6 (9.6)
Body weight [kg]	70.6 (9.4)	76.0 (11.1)
BMI [kg/m^2]	23.2 (2.4)	24.1 (3.1)
BFM [%]	17.1 (5.9)	18.3 (8.2)
FFM [kg]	58.5 (8.4)	61.6 (10.6)
TBW [kg]	43.0 (6.2)	45.3 (7.8)
WHR [cm]	82.7 (5.2)	87.2 (4.9)
HbA_{1c} [mmol/mol]	n.a.	55.2 (7.8)
Duration of T1DM [years]	n.a.	12.1 (6.0)

BMI – body mass index; PBF – percentage body fat; BFM – body fat mass; FFM – fat-free body mass; TBW – total body water; WHR – waist-to-hip ratio; HbA_{1c} – glycated hemoglobin; n.a. – not analyzed.

Table 2. Mean calorie supply with diet, mean daily fat, carbohydrate and protein intake on successive days of the experiment in CG and T1DM; mean (SD)

Variable	CG (n = 14)	T1DM (n = 14)
Calorie supply with diet [kcal/kg/day]	35.7 (9.4)	31.1 (11.9)
Fat intake [g/kg/day]	1.4 (0.4)	1.2 (0.7)
Carbohydrate intake [g/kg/day]	4.4 (1.7)	3.6 (1.5)#
Protein intake [g/kg/day]	1.7 (0.5)	1.5 (0.8)

# $p < 0.05$ significant differences between CG and T1DM.

Table 3. Fasting glycemia and insulin dosage on day 1 and day 2 of experiment in T1DM

Variable	Nor	Hy
Fasting glycemia day 1 [mg/dL]	123.5 (19.1)	132.0 (21.2)
Fasting glycemia day 2 [mg/dL]	115.5 (44.5)	116.0 (17.0)
Insulin day 1 [units/day]	34.7 (11.6)	33.9 (12.6)
Insulin day 2 [units/day]	35.9 (13.6)	35.9 (11.6)

Fasting glycemia – blood glucose concentration after an overnight fast; insulin – insulin dosage; day 1 – the day of the Nor and Hy exercise; day 2 – the day following the Nor and Hy exercise tests.

Normoxic and hypoxic rest

Significantly lower $SatO_2$ was observed after 40 min exposure to HyRest compared to NorRest (91.0 vs 96.0%, respectively; $p < 0.05$). Blood glucose (BG) concentrations were lower in CG compared to patients with T1DM at baseline and in response to resting hypoxia (Table 4). Forty-minute hypoxia did not induce significant changes in BG in T1DM. No significant effect of hypoxia was observed regarding WBC, RBC, HCT, and HBG concentrations (Table 5). The resting hypoxic intervention significantly altered HIF-1α ($F = 5.1$; $p < 0.05$). Significant increases of rest HIF-1α level were observed in the T1DM group ($p < 0.001$) and CG ($p < 0.05$). Diabetes has a significant impact on resting HIF-1α level ($p < 0.001$) in comparison to healthy controls. Significantly lower TGF-β_{rest} level ($p < 0.05$) in Hy compared to Nor conditions was observed in T1DM subjects (Table 6).

Normoxic and hypoxic exercise test

Peak oxygen consumption (VO_2), the criterion measure for aerobic capacity, showed good exercise tolerance in patients with T1DM (Table 4). A tendency toward increased VO_{2max} and significant higher minute pulmonary ventilation was observed in both groups in response to exercise and hypoxia. ANOVA showed significant interaction effects of the test conditions (NorEx vs HyEx) and group (T1DM vs CG) on VE_{max} ($F = 21.1$, $p < 0.001$), HR_{max} ($F = 4.1$; $p < 0.05$), and LA ($F = 6.4$, $p < 0.01$). Post-hoc analysis confirmed higher values of VE_{max} in HyEx than NorEx for both T1DM and CG ($p < 0.01$). Pre- and post-exercise HR as well as SBP/DBP did not differ between both protocols ($p > 0.05$).

No statistically significant effect of hypoxia on lactate threshold (LAT) was observed. However, the participants in both groups exhibited a tendency toward lower LAT in HyEx compared to NorEx. Moreover, significantly greater 15 min post-exercise LA level in HyEx compared to NorEx (8.8 ±1.0 vs 10.3 ±0.8 mmol/L, respectively; $p < 0.05$) was observed in T1DM subjects. Significantly lower $SatO_2$ was observed after both exercise protocols ($p < 0.001$) and post-exercise pO_{2max} ($p < 0.01$) and pCO_{2max} ($p < 0.01$) (Table 4).

Two-way ANOVA revealed a significant effect of hypoxia and physical exercise on blood glucose concentrations ($F = 6.1$; $p < 0.01$). In the T1DM group, lower glucose levels were observed in normobaric hypoxia compared to baseline and post-exercise levels in normoxia ($p < 0.05$). Hypoxia and exercise (HyEx) had a significant impact on HyEx ($F = 5.8$; $p < 0.01$). Compared to baseline, NorEx and HyEx resulted in a significant decrease in BG ($p < 0.05$ vs $p < 0.001$). Significantly lower BG levels were observed at

Table 4. Cardiopulmonary indicators in hypoxia (Hy) and normoxia (Nor) for T1DM and CG; mean (SD)

Variable	T1DM		CG	
	Hy	Nor	Hy	Nor
VO_{2max} [mL•kg^{-1}•min^{-1}]	43.9 (7.8)	40.3 (7.3)	46.4 (7.7)	45.4 (9.7)
P_{max} [W]	207.7 (9.2)	216.9 (12.0)	218.0 (8.6)	230.0 (11.0)
LA_{max} [mmol/L]	9.4 (1.2)	9.2 (1.5)	9.3 (1.8)	9.4 (1.2)
LAT [W]	145.9 (35.0)	153.7 (45.0)	140.3 (23.7)	156.0 (31.5)
VE_{max} [L/min]	121.0 (26.0)	101.6 (24.0)**	121.9 (21.3)	102.3 (24.0)**
HR_{pre} [L/min]	81.0 (14.0)	88.0 (15.0)	82.0 (15.0)	86.0 (15.0)
HR_{max} [L/min]	177.0 (10.0)	178 (12.0)	181.0 (12.0)	183.0 (11.0)
SBP_{pre} [mm Hg]	119.0 (18.0)	118.0 (12.0)	116.0 (12.0)	112.0 (8.0)
SBP_{max} [mm Hg]	150.0 (9.0)	163.0 (13.0)	149.0 (12.0)	145.0 (13.0)
DBP_{pre} [mm Hg]	77.0 (7.0)	72.0 (6.0)	72.0 (7.0)	70.0 (8.0)
DBP_{max} [mm Hg]	76.0 (8.0)	72.0 (6,0)	72.0 (7.0)	74.0 (7.0)
$SatO_{2max}$ [%]	90.8 (4.1)	97.0 (3.1)***	92.0 (0.8)	96.8 (1.0)***
pO_{2max} [mm Hg]	62.8 (1.8)	86.5 (1.6)**	60.2 (2.0)	87.3 (1.5)**
pCO_{2max} [mm Hg]	29.0 (1.0)	33.4(1.2)**	29.1 (0.9)	33.1 (1.0)**
BG_{pre} [mg/dL]	182 (10.8)	196.4 (12.8)	92.5 (12.0)###	92.9 (16.6)###
BG_{max} [mg/dL]	141.3 (9.2)	170.4 (11.3)**	92.4 (10.6)###	91.1 (15.7)###

VO_2 – oxygen consumption; P – power; LA – blood lactate concentration; LAT – lactate threshold; VE – lung minute ventilation; HR – heart rate; SBP – systolic blood pressure; DBP – diastolic blood pressure; $SatO_2$ – oxyhemoglobin saturation; pO_2 – partial pressure of oxygen in blood; pCO_2 – partial pressure of carbon dioxide in blood; BG – blood glucose; $_{pre}$ – measured at rest before exercise; $_{max}$ – measured in the last minute of the exercise test; * $p < 0.05$ and ** $p < 0.001$ indicate statistically significant differences between Nor and Hy conditions; ### $p < 0.001$ indicates statistically significant differences between CG and T1DM.

Table 5. Blood response measures following hypoxic (Hy) and normoxic (Nor) conditions; mean (SD)

Variable		TIDM			CG		
		Rest	PreEx	Ex_{max}	Rest	PreEx	Ex_{max}
LYM [10^9/L]	Hy	2.1 (0.7)	2.2 (0.8)	4.2 (0.3)**	2.1 (0.4)	2.0 (0.6)	4.1 (0.5)***
	Nor	2.1 (0.6)	2.1 (0.6)	3.2 (0.3)*	2.2 (0.5)	1.9 (0.4)	3.4 (0.3)***
ANC [10^9/L]	Hy	2.1 (1.6)	2.3 (1.0)	3.2 (0.5)	3.2 (1.2)	3.4 (1.3)	4.8 (0.5)**#
	Nor	2.4 (0.8)	2.0 (0.5)	2.6 (0.9)	3.1 (1.1)	3.8 (1.1)	5.2 (0.8)**#
MON [10^9/L]	Hy	0.7 (0.3)	0.7 (0.3)	0.7 (0.1)	0.5 (0.3)	0.4 (0.2)	0.9 (0.1)***
	Nor	0.6 (0.2)	0.5 (0.2)	0.6 (0.1)	0.3 (0.1)	0.3 (0.2)	0.5 (0.1)*
WBC [10^9/L]	Hyp	5.8 (2.3)	4.9 (1.9)	8.7 (0.9)**	5.0 (1.1)	5.8 (1.6)	9.8 (1.2)***
	Nor	5.0 (1.9)	4.7 (1.6)	7.1 (1.1)	4.7 (1.2)	6.0 (2.0)	9.0 (1.1)***
HGB [g/L]	Hy	14.8 (0.9)	15.1 (0.9)	15.9 (0.5)	15.5 (0.6)	15.9 (0.9)	16.1 (0.8)
	Nor	15.1 (0.9)	15.1 (0.9)	16.0 (0.8)	16.1 (0.9)#	15.3 (1.1)	15.6 (0.6)
HCT [L/L]	Hy	43.7 (3.8)	44.2 (2.0)	46.8 (1.7)	47.2 (1.9)	46.0 (1.9)	47.6 (2.2)
	Nor	44.2 (2.0)	43.7 (3.8)	48.7 (2.9)	45.9 (2.1)	45.2 (2.0)	47.9 (2.2)
RBC [10^{12}/L]	Hy	4.9 (0.2)	4.9 (0.5)	5.3 (0.2)	5.4 (0.2)#	5.3 (0.4)	5.5 (0.2)
	Nor	4.9 (0.4)	4.9 (0.2)	5.2(0.3)	5.5 (0.3)#	5.5 (0.2)#	5.5(0.3)

LYM – lymphocytes; ANC – absolute neutrophil count; MON – monocytes; WBC – white blood cells; HGB – hemoglobin; HCT – hematocrit; RBC – red blood cells; rest – measured at rest after 40 min of Nor and Hy; preEx – measured at rest before exercise; Ex_{max} – measured in the last minute of the exercise tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences between max and preEx value; # $p < 0.05$ indicates statistically significant differences between CG and T1DM group.

maximal exercise intensity ($p < 0.01$) and in response to a 15-min and 24-h recovery period after Hy Ex ($p < 0.05$) compared to NorEx (Fig. 1). A tendency toward lower fasting glycemia on day 2 after graded exercise compared to day 1 was observed (Table 3). The analysis of the incidence of hyper- or hypoglycemia indicated that hyperglycemia episodes were more frequent in patients after NorEx compared to HyEx (30% and 20%, respectively).

Significant increases in WBC ($p < 0.01$) and LYM count ($p < 0.01$) were observed in response to HyEx compared

Table 6. Angiogenic factors in hypoxia (Hy) and normoxia (Nor) for T1DM and CG; mean (SD)

Variable	T1DM		CG	
	Hy	Nor	Hy	Nor
HIF-1α_{rest} [ng/mL]	217.5 (18.0)	36.1 (22.0)***	32.2 (5.2)###	20.1 (10.7)*#
HIF-1α_{max} [ng/mL]	163.0 (48.6)	48.7 (21.6)**	38.0 (16.4)###	23.6 (8.9)*#
$VEGF_{rest}$ [pg/mL]	15.0 (8.5)	10.0 (8.7)	11.4 (6.5)	22.6 (15.8)
$VEGF_{max}$ [pg/mL]	10.3 (8.1)	23.7 (12.3)*	14.4 (8.1)	17.4 (11.3)
TGF-β_{rest} [pg/mL]	113.0 (38.8)	141.0 (28.7)*	121.5 (45.5)	131.0 (18.8)
TGF-β_{max} [pg/mL]	144.7 (34.2)	152.0 (32.1)	158.0 (34.8)	143.0 (42.0)
TNF-α_{rest} [pg/mL]	61.6 (23.5)	35.6 (16.9)	50.2 (30.0)	35.1 (10.4)
TNF-α_{max} [pg/mL]	43.6 (14.0)	39.4 (14.4)	43.2 (14.3)	34.3 (7.7)
IGF-1_{rest} [ng/mL]	212.0 (80.0)	214.0 (86.0)	486.2 (75.0)##	474.0 (60.0)##
IGF-1_{max} [ng/mL]	233.0 (83.0)	215.0 (98.0)	573.3 (58.3)##	550.0 (99.0)#
INS_{rest} [ng/mL]	7.4 (4.0)	5.1 (2.0)	14.2 (5.3)	14.2 (5.3)
INS_{max} [ng/mL]	7.1 (2.9)	4.4 (0.6)	21.7 (12.0)	15.0 (8.2)

* $p < 0.05$ and ** $p < 0.001$ indicate statistically significant differences between normoxic and hypoxic conditions; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ indicate statistically significant differences between CG and T1DM.

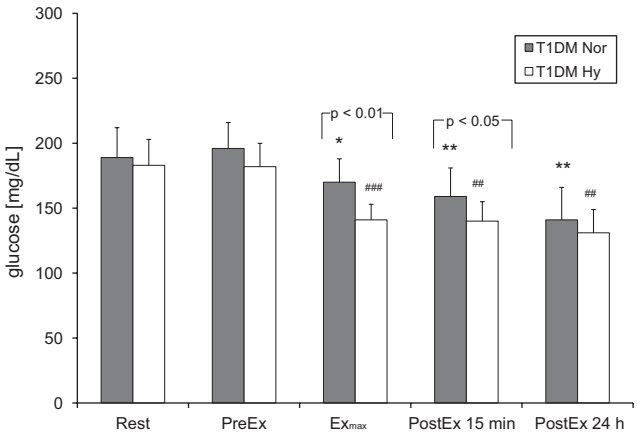

Fig. 1. Blood glucose concentrations at rest, pre-exercise (PreEx), at maximal exercise intensity (Ex_{max}), and in response to 15-min (PostEx 15 min) and 24-h recovery (PostEx 24 h) in normoxia (Nor) and hypoxia (Hy) in patients with diabetes (T1DM)

* $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$ indicate statistically significant differences between preEx and post exercise value in Nor; ## $p < 0.01$, ### $p < 0.001$ indicate statistically significant differences between preEx and post exercise value in Hy.

to preEx count in T1DM. Exercise under Hy and Nor conditions significantly increased LYM ($p < 0.001$), ANC ($p < 0.01$), MON ($p < 0.001$) and WBC counts ($p < 0.001$) in CG. ANC was significantly higher at maximal exercise intensity (Hy and Nor) in CG compared to T1DM ($p < 0.05$). T1DM participants exhibited a significant reduction in RBC ($p < 0.05$) (Table 5).

The HyEx had a significant effect on serum HIF-1α concentrations ($F = 5.1$; $p < 0.05$). Significant increases of post-exercise HIF-1α (HyEx; $p < 0.01$) were observed compared to Nor in T1DM and CG ($p < 0.05$). Diabetes has a significant impact on post-exercise HIF-1α level ($p < 0.001$). A tendency toward higher VEGF level was observed after NorEx compared to HyEx ($p = 0.05$) and a non-significant decrease was seen in response to HyEx compared to HyRest in T1DM. Hypoxia significantly decreased baseline TGF-β ($p < 0.05$) and had a significant effect on TNF-α level ($F = 4.9$; $p < 0.05$). No significant effects of hypoxia were observed regarding pre- and post-exercise INS and IGF-1 levels. In both conditions, significantly lower serum IGF-1 concentrations were observed in T1DM ($p < 0.01$). There was a tendency toward higher IGF-1 levels after exercise in HyEx compared to NorEx in both groups (Table 6).

Discussion

Effects of high intensity exercise in normoxia and normobaric hypoxia

According to scientific reports, the primary aim of diabetes treatment is to achieve as stable glycemic control as possible to prevent the long term complications.[1–4] The present data confirmed that hypoxia combined with physical activity have been shown to induce beneficial effects on glycemic control, the cardiovascular system and exercise tolerance. Glucose control was improved after low- and moderate-intensity exercise, but also in response to high-intensity exercise combined with hypoxia.[3,8,10,15,16,23]

Mackenzie et al. (in 2011 and 2012) have recently demonstrated that combining physical exercise with hypoxia has an additive effect on glucose utilization in people with T2DM.[15,23] The authors also noted that aerobic as well as anaerobic exercise improve glycemia control, however, to a different extent.

The major findings of our study are that a single bout of graded exercise reduces glycemia in T1DM. The greatest

and a safe decline in blood glucose concentration was stated after exercise combined with exposure to hypoxia. In this study, intense aerobic exercise in hypoxic conditions decreases the hyperglycemic episodes compared to NorEx and allows more effective control of post-exercise glucose homeostasis. In patients with T1DM in normoxic conditions, a high intensity exercise-induced increase of BG concentration is followed by hypoglycemia hours after completing the exercise. Indeed, in the present study, episodes of hyperglycemia were mainly observed in response to NorEx. Blood glucose concentration in the post-exercise period could have been influenced by patients' diet and insulin administration. However, on day 2, patients in fact applied the same doses of insulin compared to day 1. Therefore, the differences of hyperglycemia mainly depended on the exercise test conditions. Hypoxia and muscle contractions reduce elevated blood glucose levels more effectively, improve short-term glycemic control and may be important in preventing post-exercise hyperglycemic events.

It is well established that constant administration of various insulin preparations, proper diet and regular physical activity are essential to optimize glycemia control and minimize potential diabetes complications.[4,8] Recent investigations also indicate that paracrine mechanisms, observed during hypoxia exposure in isolated insulin-resistant muscle tissue and in humans, may exert alternative or parallel actions.[21,24] Mackenzie et al. have shown lower BG concentrations during hypoxia exposure associated with an increase in INS sensitivity in T2DM.[15,23] In this study, the markedly lower BG level in T1DM patients after exercising in hypoxic conditions may depend on higher absorption of insulin in hypoxia due to vasodilatation with increased endothelium-dependent dilator capacity, higher production of endothelial nitric oxide (NO), and eNOS expression. The possible mechanism might consist of up-regulation of the body's glycolytic energy pathways to compensate for hypoxia-induced reduction in mitochondrial respiration. In our study, we could only assume that a significant increase in post-exercise LA level and lower LAT in HyEx might suggest higher stimulation of anaerobic glycolytic pathways under hypoxic conditions. These observations seem to confirm the abovementioned significant role of anaerobic metabolism in adapting to hypoxia and the possible use of glycolytic processes in patients with T1DM.

Data concerning the effects of exercise and exposure to hypoxic conditions on aerobic performance and health condition is still sparse. Most studies report beneficial effects of this type of exercise compared to exercise in normoxia in healthy individuals.[25,26] However, there is not enough data on the effects of hypoxia with and without exercise on the condition of the cardiovascular and immune systems of people with diabetes.[5,6,10]

Angiogenic factors in response to normobaric hypoxia

It has been previously documented that diabetes is associated with angiopathy, the latter being possibly aggravated by angiogenesis.[11,13] An important result presented in this study is the significant effect of diabetes on the level of proangiogenic factors. Patients with diabetes had significantly higher baseline HIF-1α levels and lower serum IGF-1 concentrations compared to healthy control. There were no differences in baseline VEGF and TNF-α between T1DM and CG. The higher HIF-1α level and the lower serum IGF-1 concentrations observed in T1DM compared to healthy subjects might suggest that these factors may modify angiogenesis processes.

The major finding of the study is that 40 min exposure to hypoxia significantly decreased TGF-β concentrations in patients with T1DM. This factor has anti-inflammatory action, so if hypoxia in T1DM lowers TGF-β levels, it could suggest that hypoxia lowers the anti-inflammatory potential of these patients. However, the tendency toward higher TGF-β and lower HIF-1α, VEGF and TNF-α observed in response to exercise after hypoxia compared to HyRest and lower VEGF after HyEx compared to NorEx may suggest the beneficial effects of exercise in hypoxia on the regulation of pro-angiogenic pathways and possible stabilization of angiogenesis in physically active patients.

According to Sanchez-Elsner et al., HIF-1α stimulates the secretion of TGF-β.[27] Interestingly, TGF-β stimulates angiogenesis by inducing apoptosis of endothelial cells. It has been shown that elevated glycemia activates inflammatory cells to the production of this factor. Excessive secretion and activity of TGF-β was confirmed in diabetic nephropathy and retinopathy, and also in other pathological conditions.[28] In the present study, baseline levels of TGF-β were lower in Hy conditions in the T1DM group. Since evaluation of TGF-β is a valuable tool for the diagnosis of diabetic complications, the study findings could suggest a beneficial effect of hypoxia (FIO_2 = 15.2%) and exercise in the prevention of these disorders. No significant differences in post-exercise TGF-β concentrations in the T1DM group compared to healthy subjects allows us to assume that this factor could not contribute to cardiovascular complications in response to high intensity exercise.

In our study, we also analyzed blood markers for immune function in people with diabetes and healthy individuals. A more pronounced increase in circulating monocytes and neutrophils as well as higher LYM and WBC counts immediately after exercise in response to HyEx and NorEx in CG seemed to suggest greater immune activation – probably in response to higher sympathetic activity compared to T1DM subjects. Type 1 diabetes mellitus is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency.[1] Under physiological conditions, there is a balance between pathogenic T cells that mediate disease and regulatory cells

that control autoimmunity. However, an imbalance in the activity between the pathogenic actions of auto-reactive effector T cells (Teffs) and a second T cell subtype, known as regulatory T cells (Tregs), may be crucial in the breakdown of peripheral tolerance, leading to the development of T1DM.[29]

According to our study, hypoxia and exercise did not increase cytokine concentrations. Moreover, the results of leucocyte measurements indicated that exercise under hypoxic conditions may induce lower immune response in T1DM compared to CG and allows a hypothesis that exposure to HyEx (FIO_2 = 15.2%) is not a factor leading to an increased number of diabetes complications. It is worth pointing out that our participants with T1DM had baseline HbA_{1C} levels slightly over the reference range, suggesting proper long-term glycemia control and the effects of diabetes on the immune system might not be so spectacular.

Since multiple signaling pathways may be responsible for preventing T1DM complications, we also analyzed exercise-induced serum IGF-1 responses. The previous data demonstrated that administration of IGF-1 and/or a complex of IGF-1/IGFBP-3 effectively enhanced β-cell resistance to cytokine-induced cytotoxicity and might prove an efficacious therapy for T1DM complication prevention.[30] On the other hand, the results of in vitro and in vivo studies have demonstrated that IGF-1 activates the phosphatidyl inositol (PI), 3-kinase/Akt and mitogen-activated protein kinase (MAPK) signaling pathways and induces the growth of myocytes. The pathways play a significant role in the induction of muscle hypertrophy and an increase in IGF-1 secretion in response to hypoxic conditions accompanied by reduced glycemia. This might constitute an important mechanism in the prevention of skeletal muscle hypotonia and cardiomyocyte dysfunction in T1DM patients. Our results allow us to assume that HyEx might constitute an important factor leading to IGF-1 elevation. As we did not assess differences in the levels of other hormones, we could only hypothesize that the exercise- and hypoxia-induced increase in IGF-1 availability in tissues might act as an important protective factor against the aforementioned diabetes complications.

Another important result presented in our study is the significant effect of Hy on the levels of proangiogenic factors. Diabetes has a significant impact on both resting and post-exercise HIF-1α levels, but not on the main angiogenic factor, i.e., VEGF. In this study, lower VEGF levels were observed after HyEx compared to NorEx. Importantly, lower serum concentrations of TGF-β and a tendency toward higher IGF-1 were seen in response to exercise in hypoxia compared to normoxia in T1DM. However, no significant effect of exogenous insulin dose was observed in serum INS concentrations in Hy compared to NorRest and NorEx in both T1DM groups.

Diabetes mellitus is a generally accepted risk factor for vascular dysfunction, contributing to the development of serious complications.[11,13,20] The coexistence of enhanced angiogenesis, such as in proliferative retinopathy or atherosclerotic plaque angiogenesis, and impaired neovascularization in diabetes is defined as "the angiogenic paradox".[31] Diabetes related vascular complications can be caused by micro- and macroangiopathy depending on the increase in glycemia, inflammatory and oxidative stress, and is associated with deregulation of cellular and tissue response to ischemia. VEGF is a potent angiogenic factor. It stimulates the proliferation and differentiation of endothelial cells, prevents apoptosis of muscle and endothelial cells, and regulates vasodilatation. VEGF expression has been evidenced to increase under hypoxic conditions largely due to HIF-1α.[19,20] Hence, HIF-1α mediates VEGF expression and stimulates inflammatory cells to secrete a direct stimulator of angiogenesis (TGF-β, TNF-α), which has a pro- and antiangiogenic effect. The results of several studies on HIF-1α expression in diabetes show both its inhibition and enhancement. Xiao et al. emphasized the stimulating effect of high BG on HIF-1α synthesis and transcriptional activity in human epithelial cells.[32] Thangarajah et al. reached the opposite conclusions, suggesting an inhibiting effect of hyperglycemia on HIF-1α secretion in diabetes.[19] The results of the present study revealed significantly higher baseline levels of HIF-1α in T1DM subjects associated with hyperglycemia. Hypoxia increased the concentration of HIF-1α probably in response to the effects of inhibition of its degradation. It could be suggested that hypoxia is much more challenging to patients with diabetes than to healthy subjects. Higher baseline HIF-1α concentration during a hypoxic state could lead to the conclusion that hypoxia is not as beneficial for T1DM patients as for healthy individuals. However, it might be hypothesized that reduced hyperglycemia in response to hypoxia with exercise contributes to lower local stimulation of endothelial cells to express HIF-1α and VEGF. Poor glycemic control causes higher serum HIF-1α concentration and could be associated with marked serum VEGF increase. Hence, the improvement of glycemic control with the significant reduction in proangiogenic factors observed in our study in response to hypoxia and exercise helps decrease the risk of severe microvascular complications in T1DM. A revision of the recent findings published in the literature regarding the angiogenic paradox will be performed. Apparently, endothelial dysfunction, as well as molecules such as VEGF and HIF-1α, play a major role in vascular complications. In addition, the monocytes/macrophages are important in endothelium activation for arteriogenesis and its arteriogenic response is reduced, leading to impaired collateral artery growth. Moreover, the molecular mechanisms involved will be addressed, including abnormalities in growth factor, cytokines and metabolic derangements.[33]

Conclusions

In summary, our results show that short-time hypoxia combined with graded exercise increases cardiorespiratory adaptation to exercise and allows more effective control of glucose homeostasis in patients with type 1 diabetes. The pattern of observed changes in pro- to anti-angiogenic factors suggests that hypoxia may provide benefits in the prevention and management of diabetes complications, even in patients participating in high intensity physical training. Whether this therapy might result in a long-term clinical benefit needs further investigation.

Limitations

Intensive insulin therapy impedes the interpretation of the effects of different study protocols on glycemic control in the group of patients. In addition, the concentrations of angiogenic and inflammatory factors in blood serum were assessed only immediately after exercise, which limits the ability to draw conclusions about the long-term therapeutic effects.

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Nutrient intake assessed with Diet History Questionnaire II, in relation to long-term calcium-phosphate control in hemodialysis patients with end-stage renal failure

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Abstract

Background. Diet is a key factor that determines proper alignment of calcium-phosphate and nutritional status among hemodialysis (HD) patients.

Objectives. To assess the nutrient intake in relation to long-term calcium-phosphate control in HD patients with end-stage renal failure.

Material and methods. The study included 107 patients (66 men, 41 women) from 10 dialysis centers in the Upper Silesia region of Poland. To analyze the diet composition during the previous year, a portion-sized version of the Diet History Questionnaire II (DHQ-II) from National Institutes of Health was used. The nutrient intake was assessed in accordance with the most complex recommendations on HD patients' nutrition – K/DOQI Clinical Practice Guidelines for nutrition in chronic renal failure. Poor long-term alignment of calcium-phosphate homeostasis was defined as the presence of over 50% monthly phosphorus concentrations exceeding 5 mg/dL, and for calcium 10.2 mg/dL, during the last 6-month period.

Results. Lower than recommended protein intake was found in 63% of HD patients (average consumption: 0.9 ±0.5 g/kg/day). Most of the patients consumed too much fat (33.5 ±6.7% of daily energy intake) and sodium (2912 ±1542 mg/day). In 42% of patients, dietary phosphorus intake was consistent with the recommendations (13.3 ±7.5 mg/kg/day). Protein intake over 1.2 g/kg/day resulted in an increased consumption of phosphorous, but did not increase the risk of misalignment of phosphorus concentrations (OR = 1.15 [0.40–3.27]); p = 0.8). Poor control of serum phosphorus concentrations was observed in 69% of patients (they were on average 8 years younger). The average intake of protein and phosphate in the groups with good or not satisfactory serum phosphorus alignment did not differ significantly.

Conclusions. Adequate control of protein intake is not sufficient to obtain phosphorus alignment, especially in younger HD patients.

Key words: diet, hemodialysis, food frequency questionnaire, calcium-phosphate control, nutrient intake

Introduction

Cardiovascular disease is the most common cause of death in patients with end-stage kidney disease (ESKD) on renal replacement therapy. Epidemiological studies have shown that one of the major causes of increased morbidity and mortality in this group of patients are the disturbances of the calcium-phosphate metabolism.[1] Diet is a key factor that determines both calcium-phosphate balance and nutritional status in hemodialysis (HD) patients, and is an essential component of the therapeutic approach in this population.[2]

The primary role of diet in the non-dialysis stage of chronic kidney disease (CKD) is to reduce uremic toxemia and to diminish the risk of developing renal osteodystrophy and hyperkalemia. This includes a restriction of daily protein intake up to 0.6–0.75 g/kg of body mass, and food rich in phosphorus additives. Nutritional recommendations change considerably after the initiation of dialysis. Due to an increased risk of developing protein-energy malnutrition, higher daily protein intake is recommended (1.2 g/kg body mass/day), while maintaining the dietary phosphate restriction (Table 1).[3,4]

A low-phosphate diet is difficult to follow for a long period of time, as most nourishment with high protein content (products of animal origin, e.g., meat and dairy products) are also a rich source of phosphorus. This often complicates the choice of food, leading to monotony of the diet, and consequently a lack of acceptance of the recommendations by numerous patients.[5]

The basic criterion for good calcium-phosphate control, in accordance with the recommendations of the Working Group of the Polish Society of Nephrology on the quality of treatment in hemodialysis ESKD patients, is obtaining proper values of serum concentrations of calcium (8.4–10.2 mg/dL) and phosphorus (2.5–5.0 mg/dL). The concentration of native (intact) parathyroid hormone (PTH) should be in the range of 2–9 times the upper limit of the reference kit (~130–600 pg/mL).[6] It should be emphasized that there are some discrepancies between Polish recommendations and the latest (2009) Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group clinical practice guidelines for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease – mineral and bone disorder, and older (2003) National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF K/DOQI) guidelines on bone metabolism and disease in chronic kidney disease.[7,8] KDIGO guidelines do not give specific reference ranges of calcium, phosphorus, and intact PTH levels. They emphasize the role of trends in laboratory values on therapeutic decision-making, and recommend that clinical laboratories should inform clinicians on the actual method used and report any change in the assays. According to K/DOQI recommendations, serum levels of phosphorus should be maintained between 3.5 mg/dL and 5.5 mg/dL, serum levels of corrected total calcium within the normal range for the laboratory, preferably toward the lower limit (8.4–9.5 mg/dL), and the target range of plasma levels of intact PTH in dialysis patients should be of 150–300 pg/mL.[8]

The percentage of patients with hyperphosphatemia in dialysis units, according to the Working Group of the Polish Society of Nephrology, should be less than 45%.[6] Serum calcium should be definitely below 10.2 mg/dL, as greater values have been related to increased mortality in HD patients.[7,9]

Table 1. Recommended energy, macro- and micronutrient intake in HD patients (in accordance with K/DOQI recommendations (*) or EBPG on nutrition (†))[11,12]

Macronutrient intake	Recommended intake
Protein intake[a]	1.2 g/kg body mass/day (≥1.2–1.3 g/kg body mass/day for patients who are actually ill or have more severe protein-energy wasting)* at least 1.1 g/kg ideal body mass/day in clinically stable chronic HD patients†
Energy intake[b]	35 kcal/kg body mass/day for those who are <60 years of age and 30–35 kcal/kg body mass/day for individuals of 60 years or older* 30–40 kcal/kg ideal body mass/day, adjusted to age, gender, and to the best estimate of physical activity level†
Fat intake	30% of daily energy intake*
Saturated fat	up to 10% of daily energy intake*
Polyunsaturated fatty acids	up to 10% of daily energy intake*
Monounsaturated fatty acids	up to 20% of daily energy intake*
Carbohydrates[c]	the rest of non-protein calories*
Total fiber	20–25 g/day*
Sodium	750–2000 mg/day* ≤2000–2300 mg of sodium or 5–6 g (75 mg/kg body mass)/day of sodium chloride†
Potassium	up to 70–80 mEq/day* 50–70 mmol (1950–2730 mg) or 1 mmol/kg ideal body mass in patients with pre-dialysis serum potassium >6 mmol/L†
Phosphorus	10–17 mg/kg body mass/day* 800–1000 mg†
Calcium[d]	≤1000 mg/day* ≤2000 mg/day†
Magnesium	200–300 mg/day*
Iron	requirements vary according to the dose of administered erythropoietin* 8 mg/day for men, and 15 mg/day for women is recommended†
Zinc	15 mg/day* 8–12 mg/day of elemental zinc for women, and 10–15 mg/day for men†

EBPG – European Best Practice Guidelines; [a] minimum of 50% of total protein intake should be derived from high-quality protein; [b] in overweight or obese patients, limitation of total energy intake is recommended in order to reduce weight; [c] preferably complex carbohydrates; [d] both dietary calcium intake and oral calcium-based phosphate binders.

There is a lack of data regarding the percentage of HD patients that follow the recommended levels of macro- and micronutrients in their diet. Therefore, the aim of the study was to assess nutrient intake in relation to long-term calcium-phosphate control in hemodialysis ESKD patients.

Material and methods

Study population and data collection

The study population consisted of 107 patients (66 males, 41 females) from 10 dialysis centers in the Upper Silesia Region of Poland undergoing hemodialysis 3 times per week in morning sessions, who had been on HD therapy for at least 6 months and who gave written consent for participation in this study. Among the exclusion criteria were gastrointestinal tract diseases and current hospitalization. The study protocol was approved by the Bioethical Committee of the Medical University of Silesia in Katowice (KNW 22/KB1/185/I/11/12). Figure 1 shows a flow chart of the study.

Clinical and laboratory data was retrieved from the medical records of the dialysis centers. Residual renal function was assessed on the basis of residual diuresis reported by the patients.

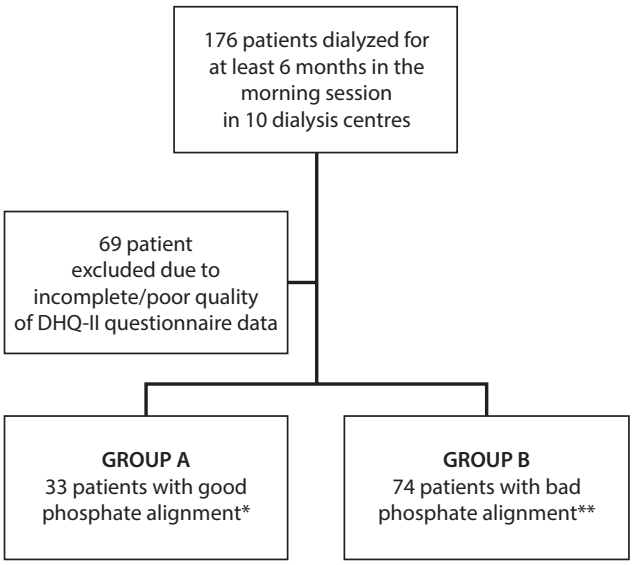

Fig. 1. Flow chart of the study

* <50% monthly phosphorus levels ≥5 mg/dL; ** ≥50% monthly phosphorus levels ≥5 mg/dL.

Diet assessment

To analyze the composition of patient diet during the previous year, a portion-size version of the food frequency questionnaire by the National Institutes of Health – Diet History Questionnaire II (DHQ-II) was used.[10] Patients were instructed on how to complete the questionnaire, and written instructions were provided. The analysis of the survey was performed using the computer program Diet Calc (National Cancer Institute, Bethesda, USA), and adopted as the standard value in accordance with the most complex recommendations on HD patients' nutrition – K/DOQI Clinical Practice Guidelines for nutrition in chronic renal failure (Table 1).[11] Consumption of energy, protein and phosphorus was expressed per kilogram of current body mass.[7] Other nutrient intake (carbohydrates and fats) was presented as total daily intake and percentage of daily energy. Nutritional status was assessed on the basis of anthropometric measurements (body mass and height) used to calculate body mass index (BMI; kg/m^2), stratified in line with WHO recommendations for Caucasians.

Analysis of calcium-phosphate parameter alignment

To evaluate the parameters of the calcium-phosphate metabolism, the results of monthly routine assessments of phosphorus, calcium, and iPTH concentrations were used. These included results from the last 6 months preceding the analysis of the diet. Recommended daily doses of oral phosphate binding drugs (calcium carbonate and sevelamer hydrochloride) were incorporated. The criterion of bad calcium-phosphate alignment was the occurrence of phosphorus concentrations exceeding 5 mg/dL, and calcium over 10.2 mg/dL in ≥50% of monthly assessments (according to the recommendations of the working group of the Polish Society of Nephrology concerning the quality criteria of dialysis in ESKD patients).[6]

Statistical analysis

For the statistical analysis, Statistica software v. 11.0 (StatSoft Inc., Tulsa, USA) was used. Data was presented as mean values ±standard deviation. The distribution of the examined variables was checked by Shapiro-Wilk test. Categorical variables were compared using χ^2 tests, while quantitative variables with ANOVA or Mann-Whitney U test, as appropriate. Logistic regression was used to calculate odds ratios (OR). The threshold for statistical significance was set at $\alpha = 0.05$. The statistical review of the study was performed by a biomedical statistician.

Results

Overall characteristics of patients

Detailed patient characteristics, including anthropometric data, causes of CKD, concomitant diseases, dialysis parameters, pharmacotherapy, and the degree of calcium-phosphate alignment is shown in Table 2.

Table 2. Patients' characteristics, mean ±SD (group A – patients with <50% monthly phosphorus levels ≥5 mg/dL; group B – patients with ≥50% monthly phosphorus levels ≥5 mg/dL)

Variables	All patients (n = 107)	Group A (n = 33)	Group B (n = 74)	p-value
Gender (male/female)	66/41	19/14	47/27	ns
Age [years]	61.9 ±14.8	67.4 ±12.5	59.4 ±15.2	p < 0.01
Body mass [kg]	73.0 ±15.0	74.8 ±14.1	72.5 ±15.4	ns
Height [m]	1.68 ±0.08	1.69 ±0.07	1.67 ±0.09	ns
BMI [kg/m^2]	26.1 ±4.8	26.3 ±4.4	26.0 ±5.0	
underweight [n (%)]	2 (1.9)	0	2 (2.7)	
normal weight [n (%)]	46 (43.0)	14 (42.4)	32 (43.2)	
overweight [n (%)]	38 (35.5)	12 (36.4)	26 (35.1)	ns
obesity [n (%)]	21 (19.6)	7 (21.1)	14 (18.8)	
I grade obesity [n (%)]	15 (14.0)	6 (18.2)	9 (12.2)	
II grade obesity [n (%)]	6 (5.6)	1 (3.0)	5 (6.8)	
Time on dialysis [months]	53 ±52	35 ±32	61 ±58	p < 0.01
Kidney transplantation [n]	9	0	9	
Renal failure cause				
diabetes [n]	31	10	21	
hypertension [n]	12	6	6	
nephrolithiasis [n]	6	4	2	
glomerulonephritis [n]	15	2	14	
interstitial nephritis [n]	6	0	4	
ADPKD [n]	9	2	7	
vasculitis [n]	3	1	2	
ischemia [n]	2	1	1	
other or unknown [n]	23	7	16	
Co-morbidities				
hypertension [n (%)]	99 (92.5)	33 (100)	66 (89.2)	ns
ischemic heart disease [n (%)]	57 (53.3)	24 (72.7)	33 (44.6)	p < 0.01
myocardial infarction [n (%)]	20 (18.7)	8 (24.2)	12 (16.2)	ns
stroke [n (%)]	6 (5.6)	3 (9.1)	3 (4.1)	ns
diabetes [n (%)]	40 (37.4)	14 (42.4)	26 (35.1)	ns
hypercholesterolemia [n (%)]	25 (23.4)	10 (30.3)	15 (20.3)	ns
parathyroidectomy [n (%)]	6 (5.6)	2 (6.1)	4 (5.4)	ns
cancer [n (%)]	17 (15.9)	6 (18.2)	11 (14.9)	ns
PCI [n (%)]	9 (8.4)	1 (3.0)	8 (10.8)	ns
CABG [n (%)]	7 (6.5)	4 (12.1)	3 (4.1)	ns
Dialysis parameters				
vascular access				
arterio-venous fistula [n (%)]	75 (70.0)	22 (66.7)	53 (71.6)	ns
central venous catheter [n (%)]	32 (29.9)	11 (33.3)	21 (28.4)	ns
dialysis session duration [h]	3.8 ±0.4	3.8 ±0.5	3.8 ±0.4	ns
ultrafiltration [L]	2.5 ±0.9	2.2 ±1.0	2.6 ±0.8	p < 0.05
residual diuresis [mL]	492 ±534	597 ±535	446 ±531	ns
residual diuresis >500 mL/day [n (%)]	31 (29.0)	12 (36.4)	19 (25.7)	ns
Pharmacotherapy				
iron [mg/week]	33 ±40	35.6 ±41.5	32.1 ±39.6	ns
calcium carbonate [g/day]	3.3 ±2.7	3.5 ±2.5	3.3 ±2.8	ns
alfadiol [n (%)]	36 (33.6)	9 (27.3)	27 (36.5)	ns
cinakalcet [n (%)]	15 (14.0)	1 (3.0)	12 (16.2)	ns
sevelamer [n (%)]	3 (2.8)	0	3 (4.1)	ns
Biochemical parameters				
phosphorus [mg/dL]	5.8 ±1.5	4.3 ±0.5	6.5 ±1.3	p < 0.001
calcium [mg/dL]	8.6 ±0.9	8.5 ±1.0	8.6 ±0.8	ns
poor serum calcium alignment [n (%)]	2 (1.9)	0	2 (2.7)	ns
iPTH [pg/mL]	466 ±441	451 ±501	472 ±414	ns

BMI – body mass index; ADPKD – autosomal dominant polycystic kidney disease; PCI – percutaneous coronary intervention; CABG – coronary bypass grafting; iPTH – intact parathyroid hormone.

Assessment of calcium-phosphate alignment

The mean 6-month serum calcium and phosphorus levels in the study group were 8.6 ±0.9 mg/dL and 5.8 ±1.5 mg/dL, respectively. Nearly 70 % of patients (n = 74) had bad

phosphate alignment (≥50% monthly phosphorus levels ≥5 mg/dL; group B). Patients with good phosphate alignment (group A) were on average 8 years older than those with poorly controlled phosphate metabolism ($p < 0.05$), and they were also characterized by a nearly 2-fold shorter dialysis vintage and lower ultrafiltration ($p < 0.005$ and $p < 0.05$, respectively). Both groups did not differ in the other assessed factors except bad calcium alignment, which occurred only in group B (Table 2).

Macro- and micronutrient intake of HD patients in relation to the recommendations

The intake of energy, and macro- and micronutrients in relation to the recommended values in HD patients is shown in Table 3. A high percentage of patients (80%) had lower than recommended energy (22.7 ±12.4 kcal/kg/day) and fiber (14.2 ±7.1 g/day) intake. Almost two thirds of patients consumed less protein than is recommended (0.9 ±0.5 g/kg/day). The majority of patients consumed more fat, sodium and calcium (33.5 ±6.7%; 2912 ±1542 mg/day; and 583 ±364 mg/day, respectively) compared to recommendations. The average intake of phosphorus was 13.3 ±7.5 mg/kg body mass/day. Only in 40% of patients was the intake within the recommended range.

The degree of phosphorus alignment in relation to the intake of energy, macro- and micronutrients is shown in Table 4. The mean intake of protein and phosphorus in patients with good and bad phosphorus alignment did not differ significantly. High intake of protein (>1.2 g/kg body mass/day) resulted in a significant increase in phosphate intake ($p < 0.05$) (Fig. 2). However, protein intake >1.2 g/kg body mass/day did not increase the risk of phosphorus misalignment (OR = 1.15 [0.40–3.27]; p = 0.8).

Discussion

Currently, there are 2 specific renal nutrition guidelines available for daily practice – the Clinical Practice Guidelines for nutrition in chronic renal failure, released in 2000 by NKF K/DOQI, and the more recent (2007) European

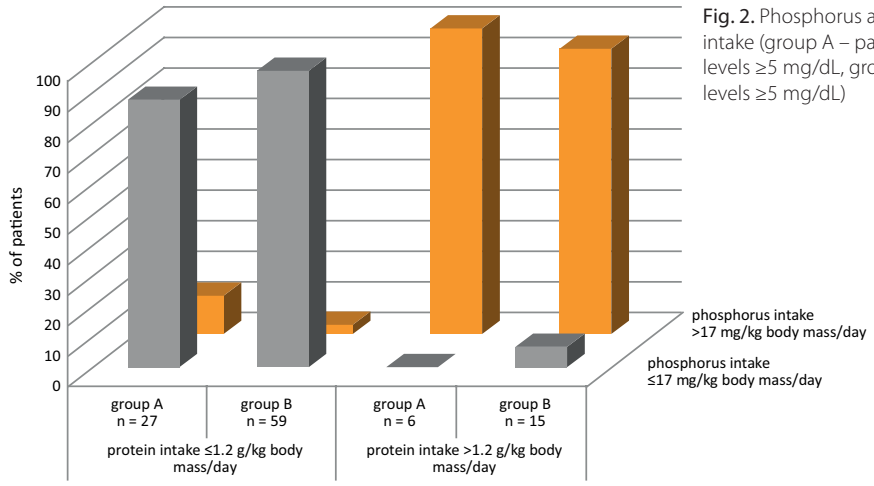

Fig. 2. Phosphorus alignment according to protein and phosphorus intake (group A – patients with less than 50% monthly phosphorus levels ≥5 mg/dL, group B – patients with ≥50% monthly phosphorus levels ≥5 mg/dL)

Table 3. Energy, macro- and micro-nutrient intake in 107 hemodialysis patient diets in comparison to K/DOQI recommendations

Variable	% of patients		
	below recommended level	according to recommendation	over recommended level
Protein intake[a]	62.6	17.8	19.6
Energy intake	82.8	9.5	7.6
Fat intake (% of daily energy)[b]	10.3	16.8	72.9
Carbohydrate intake (% of daily energy)[c]	24.3	74.7	0.9
Fiber intake	81.3	13.0	5.6
Sodium	3.7	26.1	70.0
Potassium	65.4	10.3	24.3
Phosphorus	34.6	42.0	23.4
Calcium	0	18.6	81.4
Magnesium	46.7	35.5	17.7

[a] refers to 1.0–1.2 g/kg body mass/day; [b] refers to 25–30% of daily energy intake; [c] refers to 45–75% of daily energy intake.

Table 4. Energy, macro- and micronutrient intake in HD patients with good and bad phosphorus alignment

Variable	Group A (n = 33)	Group B (n = 74)	p-value
Protein intake [g/kg body mass/day]	0.9 ±0.4	0.9 ±0.6	ns
Energy intake [kcal/kg body mass/day]	23.8 ±12.4	22.2 ±12.5	ns
Fat intake [% of daily energy]	34.6 ±6.0	33.1 ±7.0	ns
Carbohydrate intake [% of daily energy]	50.6 ±7.2	51.4 ±9.2	ns
Dietary fiber intake [g/day]	16.5 ±8.9	13.1 ±6.0	p = 0.06
Sodium intake [mg/day]	3189 ±1619	2789 ±1501	ns
Potassium intake [mEq/day]	68.5 ±34.6	59.6 ±33.4	ns
Phosphorus intake [mg/kg body mass/day]	13.8 ±7.0	13.1 ±7.7	ns
Calcium intake [mg/day]	4102 ±2454	3809 ±2850	ns
Magnesium intake [mg/day]	247 ±129	212 ±106	ns

Best Practice Guideline (EBPG) on Nutrition in Chronic Kidney Disease.[11,12] It should be emphasized that there are several differences between those 2 documents (Table 1), and consensus has not been published yet.

There is limited data in the literature concerning nutrition knowledge and its adequacy to the nutritional recommendations in CKD patients in Poland, and they refer mostly to the non-dialysis population.[13,14] One potential reason that could explain this fact is the difficulty in assessing the nutritional habits and diet of HD patients, mainly due to the need for precise recording of the size and type of food portions when using quantitative methods of diet assessment (a 24-hour diet interview or a 3-day food diary). Qualitative methods of diet assessment such as the DHQ-II questionnaire used in this study may be an alternative in the population of HD patients.

The unbalanced diet of dialysis patients may lead either to the shortage or the excess of specific nutrients.[15] Proper energy intake in HD patients (30 kcal/kg body mass/day for patients ≤60 years of age, and 30–35 kcal/kg body mass/day for those over 60 years according to K/DOQI) is one of the determinants of normal nutritional status, and protects against malnutrition. In the present study, energy intake that was too low was the most common disorder seen in HD patients; it affected more than 80% of respondents. Similarly, energy intake that was too low was observed in 2 other studies using a 24-h diet interview in 92 and 38 Polish HD patients.[16,17] Also in these studies, inadequate protein and fiber consumption was frequently observed. Recently, a paper by Luis et al. reported similar results for the Spanish population. Performing an analysis of 3-day dietary records of 91 HD patients, they observed that only 11% and 15% of patients (based on K/DOQI guidelines and EBPG, respectively) fulfilled the recommended energy intake. This study also showed that a relatively low percentage of patients consumed enough protein and fiber (41% and 22%, respectively).[18] It is more difficult to increase the intake of fiber than of protein. The main sources of dietary fiber are fruits, vegetables, whole grains, and seeds. These sources are often rich also in potassium and phosphorus. However, a limited number of selected fruits (e.g., black currants, red currants, raspberries, white currants, and blackberries) and vegetables (e.g., green peas, broad beans, Brussels sprouts, and celeriac) should be more recommended than others to increase fiber intake (Table 5).

It should be emphasized that a significant association between the state of education concerning kidney diseases and its treatment (including the nutritional aspect), patient compliance and the number of complications (e.g., hyperphosphatemia) was observed.[2,19] In the present study, we did not assess the level of patients' knowledge on diet recommendations specific for the HD population, nor the degree of compliance with the prescribed doses of phosphate-binding drugs, which is one of the limitations of the study.

Despite education regarding the need to limit dietary sources of phosphorus, and common use of phosphate-binding drugs, the percentage of HD patients diagnosed with hyperphosphatemia remains high. In a previously published study, we observed serum phosphorus levels exceeding 5.5 mg/dL in 56% of HD patients.[20] In a recent epidemiological study that assessed the rank of calcium and phosphorus alignment among HD patients in Poland in the years 2003–2009, phosphorus concentrations higher than 6 mg/dL were observed in 51% of patients.[21] With more stringent recommendations, consistent with current NKF K/DOQI criteria, the percentage of people characterized by poorly controlled serum phosphorus levels in the study group was significantly higher, as high as 69%.[21]

It is worth noting that the group with poorly controlled serum phosphorus concentration was significantly younger (on average by 8 years) compared to patients with good alignment, as in the previous study.[20] Both groups had similar doses of oral phosphate binders prescribed, and phosphorus intake from protein. In addition, almost all patients with increased protein intake (>1.2 g/kg body mass/day) consumed excessive amounts of phosphorus. Therefore, the reason for poor serum phosphorus alignment is rather related to the fact that inorganic phosphorus intake (i.e., beverages and highly processed foods) is greater in the younger population.

Table 5. Amount of protein, phosphorous and potassium in fiber-rich fruits, vegetables and seeds (according to Polish tables of composition and nutritional value of food)[23]

Variables	Dietary fiber [g/100 g]	Protein [g/100 g]	Potassium [mg/100 g]	Phosphorus [mg/100 g]
Fruits				
Dried figs	12.9	3.6	938	122
Dried dates	8.7	2.0	688	58
Black currants	7.9	1.3	336	58
Red currants	7.7	1.1	259	33
Raspberries	6.7	1.3	203	33
Dried bananas	6.4	3.8	1493	76
White currants	6.4	1.0	275	23
Avocado	3.3	2.0	600	41
Blackberries	3.2	0.8	62	14
Gooseberries	3.0	0.8	230	26
Vegetables				
Soy (dry seeds)	15.7	34.3	2132	743
White beans (dry seeds)	15.7	21.4	1188	437
Peas (dry seeds)	15.0	23.8	937	388
Red lentils (dry seeds)	8.9	25.4	874	301
Horseradish	7.3	4.5	740	120
Green peas	6.0	6.7	353	122
Broad beans	5.8	7.1	261	57
Brussels sprouts	5.4	4.7	416	33
Parsley root	4.9	2.6	399	77
Celeriac	4.9	1.6	320	80
Seeds				
Poppy seeds	20.5	20.1	963	1022
Sesame seeds	7.9	23.2	387	775
Sunflower seeds	6.0	24.4	793	784
Pumpkin seeds	5.3	24.5	810	1170
Flax seeds	3.9	24.5	762	722

It should be emphasized that the questionnaire used in the study did not take into account the intake of inorganic phosphates, which are often present in large amounts in highly processed foods, and account for a significant percentage of added preservatives (e.g., in meats or soft drinks). These products may be a source of the so-called 'hidden phosphorus', easily absorbed by the gastrointestinal tract.[22] Therefore, in CKD patients the DHQ-II questionnaire cannot be the only tool used to assess phosphorus intake.

Conclusions

Adequate control of protein intake is not sufficient to obtain phosphorus alignment, especially in younger HD patients.

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New predictor of acute necrotizing pancreatitis: Red cell distribution width

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Abstract

Background. Acute pancreatitis (AP) is inflammation of the pancreas of various severity ranging from mild abdominal pain to mortality. AP may be classified as acute interstitial edematous pancreatitis (AEP) or acute necrotizing pancreatitis (ANP), according to the revised Atlanta criteria. Most of the patients with AP are AEP (75–85% of patients), while 15–25% of patients have ANP. The mortality rate is 3% in AEP and 15% in ANP. Thus, it is important to predict the severity of AP to decrease the morbidity and mortality.

Objectives. The aim of the study was to evaluate the relationship between red cell distribution width (RDW) and the severity of AP on admission to hospital.

Material and methods. Patients admitted to Adana Numune Research and Educational Hospital with a diagnosis of AP through the time frame of January 2014–May 2016 were included in our study. Diagnosis of AP was made according to the revised Atlanta classification. Patients' age, sex, etiology of AP, and RDW values were recorded on admission to the hospital.

Results. A total of 180 patients were included in the study. Eighty patients (44%) were male and 100 patients were female. Mean age was 56.25 ±18.3 years (52.66 ±14.4 in males; 59.84 ±20.2 in females). There was no statistically significant difference between patients' age. The most frequently observed etiologic factor was gallstone disease followed by alcohol intake and the use of pharmaceuticals. Drug-related AP was associated with azathioprine, furosemide, and thiazide diuretics. One hundred forty-four (80%) patients had AEP and 36 (20%) patients had ANP. RDW values showed a statistically significant difference between patients with AEP and ANP ($p = 0.011$). The cut-off value of RDW was 16.4 and the area under curve (AUC) value was 0.591 ($p = 0.0227$) with a sensitivity of 29.2% and specificity of 89.83%.

Conclusions. Red cell distribution width could be used to evaluate the prognosis of acute pancreatitis.

Key words: acute pancreatitis, acute necrotizing pancreatitis, red cell distribution width

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Introduction

Acute pancreatitis (AP) is a disease with inflammation of the pancreas of various severity ranging from mild abdominal pain to mortality.[1] AP is characterized by 3 phases. In the 1st phase, enzyme activation and cellular damage cause early symptoms. In the 2nd phase, systemic inflammatory response and intrapancreatic inflammatory reaction occur by the release of proinflammatory and anti-inflammatory mediators. In the 3rd phase, complications of AP occur.[2] AP is classified as acute interstitial edematous pancreatitis (AEP) or acute necrotizing pancreatitis (ANP) according to the revised Atlanta classification.[3] AEP constitutes 75–85% of patients with AP, while ANP consists of 15–25% of patients with AP. The mortality rate is 3% in AEP and 15% in ANP.[4] Since mortality is 5 times higher in ANP than in AEP, it is important to discriminate ANP from AEP to predict morbidity and mortality. Several scoring systems have been developed to detect the severity of AP. These scoring systems include the Ranson criteria, Acute Physiology and Chronic Health Evaluation II (APACHE II), systemic inflammatory response syndrome criteria, bedside index of severity in acute pancreatitis, harmless acute pancreatitis score, and Balthazar score.[5] Recent studies have shown newer markers to detect AP severity. These include pancreatic protease activation peptides, interleukin 6 and interleukin 8, polymorphonuclear elastase, procalcitonin, mean platelet volume (MPV), and proteinuria.[6,7] Red cell distribution width (RDW) is calculated by dividing the standard deviation of red blood cell volume by mean corpuscular volume (MCV) and multiplying it by 100 to express the results as percentages.RDW reflects the variability of the size of the circulating erythrocytes.[8] Several studies have shown that RDW is significantly associated with inflammatory markers such as C-reactive protein and fibrinogen.[9,10] In this study, we aimed to evaluate the RDW levels in patients with AEP and ANP.

Material and methods

The study included 180 patients who were admitted to the gastroenterology department of Adana Numune Research and Educational Hospital (Turkey) with a diagnosis of AP through the time frame of January 2014–May 2016. AP was diagnosed using the revised Atlanta classification.[3] Patients' age, sex, etiology of AP, and RDW were recorded.

White cell count (WCC), red blood cell (RBC) count, platelet (PLT) count, RDW, hemoglobin (HGB) level, MCV, and mean platelet volume (MPV) were determined using the XE-2100 automated hematology analyser (Sysmex, Kobe, Japan) with Sysmex reagents (Sysmex). The normal reference range for RDW in the laboratory of our hospital is 11.6–15%.

Exclusion criteria comprised history of chronic pancreatitis or pancreas carcinoma, heart failure, hyperlipidemia, peripheral vascular disease, hematologic disorders, accompanying acute or chronic inflammatory diseases, any other accompanying carcinomas and chronic liver diseases.

Statistical analysis

Descriptive statistics were used to define the continuous variables and are expressed as mean ±standard deviation. Student's t-test was used for independent and normally distributed variables. Mann-Whitney U test was used for independent and not normally distributed variables. Receiver operating characteristic (ROC) curve analysis was performed for the variables found to be significant in univariate analysis to determine the cut-off point. Statistical significance level was determined as 0.05. The analysis was made using MedCalc Statistical Software v. 12.7.7 (MedCalc Software BVBA, Ostend, Belgium).

Results

A total of 180 patients were included in the study. Eighty patients (44%) were male and 100 patients (56%) were female. Mean age was 56.25 ±18.3 years (52.66 ±14.4 years for males; and 59.84 ±20.2 years for females). There was no statistically significant difference between the age of male and female patients. Patients were grouped into AEP and ANP. Patients' age was 53.75 ±13.3 years and 55.65 ±15.6 years in AEP and ANP groups, respectively. There was no statistically significant difference between patients' age.

Patient characteristics are shown in Table 1. The drug-induced AP among the patients was caused by azathioprine, furosemide, and thiazide type diuretics. Imaging studies (ultrasonography, computed tomography and magnetic resonance cholangiopancreatography) and laboratory values revealed no other etiologic factors in the idiopathic pancreatitis group.

One hundred forty-four patients (80%) were included in the AEP group, and 36 patients (20%) were included in the ANP group. There was a statistically significant difference in the RDW values between the groups (p = 0.011). There

Table 1. Characteristics of patients with acute pancreatitis

Variable	AEP n = 144 (80%)	ANP n = 36 (20%)	p-value
Age [years]	53.75 ±13.3	55.65 ±15.6	ns
Sex (male/female)	60/50	40/30	ns
RDW (%)	14.5 ±2.1	17.3 ±2.3	0.011
Etiology			
biliary	116 (80.5%)	27 (75%)	ns
alcohol	15 (10.4%)	4 (11.1%)	
drugs	3 (2.1%)	1 (2.8%)	
idiopathic	10 (7%)	4 (11.1%)	

AEP – acute interstitial edematous pancreatitis; ANP – acute necrotizing pancreatitis; RDW – red cell distribution width; ns – non-significant.

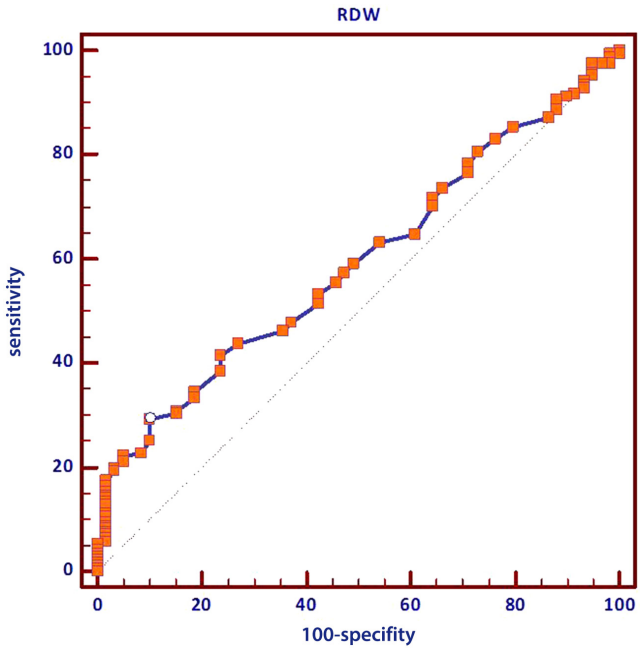

Fig. 1. The receiver operating characteristic (ROC) curve of red cell distribution width (RDW) values for predicting acute necrotizing pancreatitis in patients with acute pancreatitis

was no statistically significant difference between age, sex and etiologic factors in the patient groups. (Table 1).

Receiver operating characteristic (ROC) curve analysis was used to evaluate the values of RDW to diagnose necrotizing AP. The area under curve (AUC) and cut-off values were calculated. The AUC for the RDW value was 0.591 (p = 0.0227). The cut-off value to diagnose necrotizing AP patients was 16.4 (sensitivity: 29.2%, 95% CI 22.5–36.7; specificity: 89.83%, 95% CI 79.2–96.2) (Fig. 1).

Discussion

There has been research to evaluate the relationship between RDW and mortality in patients with AP before. In our study, we found a relationship between the disease severity and the RDW values in patients with AP. Earlier studies included a small number of patients, while our study had the largest patient group.

RDW is calculated as part of complete blood count. It is used to distinguish the etiology of anemia and it shows heterogeneity. Normal values for RDW are 11.5–14.5%. There is no case of RDW levels below normal limits. Thus, RDW is expressed as normal or high. Increased RDW shows a higher variation of erythrocyte size than normal. RDW is increased in hematologic and inflammatory diseases. The RDW value shows no sensitivity and specificity for a specific disease, and also does not exclude a diagnosis. RDW values are increased in hemolytic anemia, in the case of erythrocyte transfusion, and deficiency of vitamin B_{12}, folic acid and iron.[9,11,12]

Many studies have shown a correlation between higher RDW values and prognosis, but these studies were about cardiovascular and cerebrovascular diseases, chronic renal failure, liver disease, pancreatitis and venous thromboembolism.[13–17] Kurt et al. showed a correlation of nonvalvular atrial fibrillation and higher RDW values in 320 patients.[13] Demir et al. conducted a study of 37 patients with cerebral sinus thrombosis and 101 patients with primary headache with a total of 138 patients. In that study, increased RDW values were correlated with the existence of vertebral sinus thrombosis. They showed that increased RDW values might be a predictor of cerebral sinus thrombosis in patients with primary headache.[14] In a study of 367 patients with grade I–V chronic renal failure, Solak et al. reported a relationship between increased RDW values and flow mediated dilatation, which shows endothelial dysfunction independent of anemia, diabetes mellitus and inflammation.[15] Kim et al. showed the correlation between increased RDW values and advanced fibrosis in 24,547 patients with nonalcoholic liver disease.[16]

The inflammation status of the disease may change the RDW values. The association of RDW with mortality may be explained by inflammation. Inflammation promotes the death of RBCs or inhibits the maturation of RBCs, thus decreasing RBC lifespan. RDW values may reflect the inflammation status of acute pancreatitis and may be used to predict severe acute pancreatitis. Bone marrow function and iron metabolism may be influenced by inflammation, and inflammatory mediators suppress erythrocyte maturation and cause larger and younger reticulocytes to enter the circulation, thus increasing RDW. Inflammation also increases oxidative stress leading to elevated RDW by reducing RBC survival and increasing the release of large, premature RBCs into the circulation. Inflammation itself also alters RBC membrane structure, contributing to changes in RBC morphology.[17]

Acute pancreatitis may cause local inflammation and also systemic effects. Mortality is higher in ANP than in AEP, so early diagnosis and treatment of ANP may improve the prognosis.[17] At present, there is no single marker showing ANP. Biochemical tests, imaging studies and scoring systems are used in all clinics to detect the severity of AP, but 20–30% of severe AP may not be diagnosed with these tests.[18] An ideal marker – objective, simple, cheap, reproducible, sensitive and specific to AP, should be available in all clinics.[17] RDW is used to show the severity and prognosis of various diseases, and has been found to have the above-mentioned qualities.[13–17] Wang et al. showed higher mortality rates with RDW values > 13.4%.[17] In our study, we found sensitivity of 88.2% and specificity of 91.8%, with a cut-off value of 14.35% in ROC analysis. In a study including 103 patients, Şenol et al. observed that high RDW values on admission were associated with mortality.[19] They found a cut-off value of 14.8% and predicted mortality in 77% of cases. They reported a sensitivity of 47.6% and a specificity of 96.3%.[19] Yao and Lv also reported a cut-off value of 14.2%, and found higher RDW values in terminal

patients with a sensitivity of 75% and specificity of 89.8% in 106 patients.[20] Our study included 180 patients with AP. 144 patients had AEP, and 36 patients had ANP. We found that high RDW values on admission to the hospital were associated with severe disease. We found 16.4 as the cut-off value of RDW. Sensitivity was 29.2% and specificity was 89.83% for this value.

In conclusion, RDW can effectively distinguish ANP from AEP, and be used to evaluate the prognosis of AP. It is simple, cheap and reproducible.

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Social and medical determinants of quality of life and life satisfaction in women with Turner syndrome

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Abstract

Background. Turner syndrome (TS) appears in women as a result of the lack of part or the whole of one of the X chromosomes. It is characterized by the occurrence of low height, hypogonadism, numerous developmental defects, and is often accompanied by psychological disturbances.

Objectives. Although the phenotype characteristics of women with TS are quite well documented, the knowledge of the impact of Turner syndrome on the satisfaction with life is still insufficient. The aim of our study was to assess the impact of TS on selected variables of quality of life, and hence also life satisfaction in women with this syndrome.

Material and methods. The research was carried out in a group of 176 women with TS starting March 1995. The patients underwent anthropological and medical examinations, and their medical histories were taken using a questionnaire that included demographic and psychosocial items as well as issues related to selected variables of quality of life. In our research model, general life satisfaction was a dependent variable. The statistical analysis was conducted using the eta and Cramer's V correlation coefficients as well as a multidimensional logistic regression model.

Results. The main determinants of dissatisfaction with life in women with TS were short stature and feelings of loneliness and being handicapped.

Conclusions. The determinants of life satisfaction in women with Turner syndrome were closely related to the private life of the study participants, in particular self-perception and feelings concerning their health status.

Key words: education, life satisfaction, health-related quality of life, Turner syndrome, sexual sphere

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Introduction

Turner syndrome (TS) is named for Dr. Henry Turner, who described the disorder in 1938. TS occurs in women and is characterized by abnormalities in the quantity or structure of sex chromosomes. The syndrome is most frequently characterized by gonadal dysgenesis and somatic disorders, the most important of which is short stature.[1,2] Hormonal studies typically reveal low circulating concentrations of ovarian steroids and high concentrations of gonadotropins in women aged 14 and above. In very few women with TS, the ovaries show cyclic hormonal activity manifested by the menstrual cycle and even fertility.[3] TS affects one in every 2,500 women. Hence, it can be speculated there are approx. 8000 women with Turner syndrome in Poland, of whom 5000 are above 18 years of age.[4] Although the characteristics of the TS phenotype have been well documented, the knowledge on the impact of the disorder on quality of life, including body image, feelings, social functioning and interaction, and general life satisfaction, remains limited. Social stigmatization makes women with TS feel different compared to healthy women. The disease significantly hinders their psychosocial functioning, in particular at those life stages when the sense of attractiveness associated with appearance is developing; women with Turner syndrome are concerned about short stature and femininity disorders.

Entering the world of independent social relations partly depends on how women with Turner syndrome accept their life situation. However, during the period of primary socialization, it also depends on their readiness to perform different social roles equal to those of healthy women, and not to condemn themselves for social isolation. The studies concerning different aspects of psychosocial functioning of women with TS have not yielded clear results. Some studies indicated there were certain difficulties and limitations in the social functioning of women with TS, while other reports confirmed the chance of normal functioning despite the limitations caused by Turner syndrome.[5–8]

Quality of life assessment has been a subject of theoretical considerations and empirical studies. At present, most researchers agree regarding the multidimensionality and subjective nature of quality of life (QoL) assessments. Some degree of simplification allows the identification of 2 QoL levels: 1) quality of life as a general subjective assessment of an individual's life progress; life satisfaction is one of the measures of quality of life; 2) QoL is limited to a subjective assessment of the disease that an individual suffers from, and the impact of treatment.

Turner syndrome affects development and is characterized by a variety of medical implications; it also has an impact on several aspects of patients' lives. In our research model, general life satisfaction was a dependent variable. Deeper insight into health-related quality of life based on subjective assessments of women with TS might increase the clinicians' awareness regarding these women's needs, and thus allow better tailoring of the treatment process.

Objectives

The aim of this study was to assess the impact of Turner syndrome and its biological and psychosocial effects on quality of life, determined on the basis of life satisfaction. We employed the following strategies to accomplish this goal: 1) we described variables that influence quality of life; 2) we searched for life satisfaction parameters which could help assess general quality of life.

Material and methods

The research was carried out on a group of 176 Polish women with TS who responded to recruitment materials published in newspapers and broadcast in radio and television advertisements. Patients were invited to participate in the examinations starting March 1995. All participants gave their informed written consent to participate in the study. The study procedures were approved by the Bioethics Committee of the Medical University of Silesia. The women's medical histories were taken using a standardized questionnaire, and all medical records were checked by the same group of investigators.

The aim of the questionnaire was not only to collect demographic data but also to gather information on quality of life including social functioning, feelings and sexual activity of women with Turner syndrome. Sex drive and sexual initiation (i.e., having sexual experience) were analyzed as factors linked to the sexual sphere. The effects of TS on psychosocial functioning were analyzed based on the attitudes and behaviors of patients with TS, but also attitudes and behaviors toward these women. The respondents were asked about the causes of TS and how they felt about their short statures. They were also requested to present their assessments regarding the impact of TS on their educational level, occupational status, social life, and attractiveness to men. The women were asked whether their parents' knowledge of the occurrence of Turner syndrome in the family had affected the parents' attitudes toward them. We also asked if Turner syndrome had caused the women's parents to view them differently from their siblings. The participants were then requested to assess the attitudes of other people toward them, and whether they had experienced positive or negative consequences of these attitudes. The psychosocial aspects of life satisfaction were also investigated, including having friends, a sense of loneliness, of being perceived as a worthy human being, self-esteem, assessment of own intellectual abilities, capacity to perform work, self-assessment of own health condition, and feeling handicapped in different areas of life.

The study participants underwent anthropological measurements and medical examinations to identify stature characteristics, body build abnormalities such as webbed necks, turned-in elbows, and genu varum (bow legs), foot and hand anomalies, abnormal appearance of fingers, thin/hypoplastic toenails and thin/hypoplastic/hyperconvex fingernails,

excessive hair growth, low hairline, pigmented nevi, auricular septal defects, sagging eyelids (ptosis), epicanthal folds, rich eye frames, strabismus, daltonism, and infertility.

Age, educational level, marital status, and habitation were among the variables that were taken into account during the analyses. Age and stature were measured on an interval scale, whereas the other variables were measured on a nominal scale. They were then transformed to binary variables, which reflected the occurrence or non-occurrence of a given attribute or attitude.

In order to determine QoL, the women with TS were asked questions concerning life satisfaction. The strength of the relationship between the interval variables and life satisfaction was estimated using the eta correlation coefficient; the strength of the relationship between the nominal variables and life satisfaction was estimated using the Cramer's V correlation coefficient. The hypotheses that the correlation coefficient and the obtained logistic regression coefficients differed from 0 were rejected at the significance level of $p = 0.05$.

Quality of life variables were analyzed using a multidimensional logistic regression model. The statistical analysis was performed using SPSS 12 PL for Windows.

Results

Variables that influenced quality of life and the characteristics of life satisfaction measurement

Social and demographic characteristics

The examined women were at the age of 18–53 years, mean age 25 ±7.6 years. Regarding the skewness of age distribution, other measures of central tendency were considered: the median age was 22 years; the 1st quartile age was 19 years, and the 3rd quartile age was 29 years. The demographic characteristics of our study participants are shown in Table 1.

It should be emphasized though that over 50% of women had secondary or university education. The majority of the respondents were single, but approx. a quarter of them were married or had a partner. Most women lived with their parents; 17% lived with a husband or a partner. The majority were inhabitants of urban areas (81.3%).

The data in Table 2 shows that approx. 1/3 of the study participants negatively assessed the attitudes of other people; 2/3 confirmed they had actually experienced negative attitudes. Approximately 15% of women believed that Turner syndrome affected their family's attitude toward them, while about 8% thought that their family position was different from that of their siblings. Almost all had a sense of being perceived as a worthy human being.

The mean height of the examined women was 144.7 ±7.2 cm; the median height was 144 cm, and the 1st and 3rd quartiles were 140 cm and 149 cm, respectively. Dissatisfaction with short stature was expressed by approx. 60% of women; approx. 40% believed that their height was the reason for their low physical attractiveness to men. Only approx. 11% of the examined women thought there was a relationship between their short stature and educational level, and 1/4 believed that their short stature had affected their professional activity. Genu varum (bow legs) was found in 24% of women. Only 16.5% admitted having no sex drive (Table 3).

Table 1. Social and demographic characteristics of study participants

Social and demographic variables		n	%
Education	elementary	38	21.8
	basic technical/trade	45	25.9
	secondary	80	45.9
	university	11	6.4
Marital status	single	126	71.6
	married	32	18.2
	has a partner	9	5.1
	had a partner	9	5.1
Habitation	with parents only	131	74.4
	with a partner	30	17
	alone	8	4.5
	other	7	4
Area	town	143	81.3
	country	33	18.7

Table 2. Assessment of other people's attitudes

Variable	Answers	n	%
Negative assessment of other people's attitudes	no	122	69.3
	yes	54	30.7
Experienced negative attitudes of other people	no	59	33.5
	yes	117	66.5
Impact of Turner syndrome on parents' attitude	no	148	84.4
	yes	27	15.6
Incorrect attitude of their parents	no	135	76.7
	yes	41	23.3
Different position in the family compared to brothers and sisters	no	162	92
	yes	14	8
Sense of being perceived as a worthy human being	selectively	17	9.8
	yes	157	90.2
Loneliness	no	124	70.9
	yes	51	29.1

Feeling of being handicapped

The authors developed a scale to measure the feeling of being handicapped in various spheres of life. Variables related to this feeling exhibited strong correlations. The following binary variables were used as components

Table 3. Health and body image assessment

Variable	Answers	n	%
Unhappiness about short stature	no	70	40.9
	yes	101	59.1
Negative impact of short stature on attractiveness to men	no	104	60.8
	yes	67	39.2
Negative impact of short stature on education	no	156	88.6
	yes	19	11.4
Negative impact of short stature on occupational status	no	141	76.1
	yes	34	23.9
Genu varum (bow egs)	no	133	75.6
	yes	43	24.4
No sex drive	no	147	83.5
	yes	29	16.5

of the scale (the answers in parentheses are those for which the respondents were given a point):

– Do you feel handicapped? (yes)

– Do you perceive your capacity to perform work as limited? (yes)

– Do you believe your health is impaired? (yes)

– Do you perceive your intellectual abilities as impaired? (yes)

– How do you compare with other people (self-esteem)? (worse)

The scale was characterized by Cronbach's alpha of 0.77 (Table 4).

The distribution of results regarding the degree of feeling handicapped was as follows: score 0 (no feeling of being handicapped) – 100 women (57.5%); score 1 – 18 (10.3%); score 2 – 25 (14.4%); score 3 – 17 (9.8%); score 4 – 9 (5.2%); score 5 (the strongest feeling of being handicapped) – 5 (2.9%). Almost 67.8% of the examined women did not feel handicapped, and only 8.1% admitted having a feeling of being heavily handicapped.

Satisfaction with life

Life satisfaction of the study participants was assessed with the question: "Are you satisfied with your life?". The responses were: "yes" (subgroup 1); "no" or "do not know" (subgroup 2). Response distribution was as follows: 129 "yes" (73.3%); 19 "no" (10.8%); and 28 "do not know" (15.9%) options. In further analysis, the "no" and "do not know" responses were combined into 1 category. The majority of women with Turner syndrome (73.3%) confirmed that they were satisfied with life.

Determinants of general life satisfaction with respect to demographic, medical and psychosocial factors

Determinants of life satisfaction were sought among the demographic variables, each woman's subjective feelings and opinions about herself, the women's subjective assessments of the views and attitudes of other people, and their actual life situation.

Among the demographic variables, age and social environment at the place of residence were found to be essentially linked to life satisfaction; the magnitude of the

Table 4. Factor analysis (without rotation) and analysis of the internal consistency of feeling handicapped

Variable	Correlation with the principal component		"Question–scale" correlation	Alpha after disregarding the question
	1	2		
Feeling handicapped	0.9	–0.3	0.78	0.62
Work capacity assessed as "rather limited"	0.74	0.37	0.55	0.72
Health assessed as "rather impaired"	0.72	–0.6	0.54	0.72
Intellectual abilities assessed as "rather impaired"	0.7	0.2	0.5	0.74
Self-esteem compared to patient's perception of other people "worse or unstable"	0.52	0.55	0.35	0.79

Table 5. Demographic variables and life satisfaction in women with Turner syndrome

Variable		Life satisfaction				Cramer's V	p-value
		yes		no/do not know			
		n	%	n	%		
Habitation	with parents only	100	76.3	31	23.7	0.24	<0.05
	with partner	22	73.3	8	26.7		
	alone	2	25	6	75		
	other	5	71.4	2	28.6		
Area of residence	town	100	69.9	43	30.1	0.16	<0.05
	country	29	87.9	4	12.1		

correlations was approximately the same. Women who lived alone and in a city reported life dissatisfaction more frequently (Table 5).

The study participants' self-assessment regarding stature, attractiveness and worthiness were most strongly associated with life satisfaction. Life dissatisfaction was more frequently reported by women who were unhappy about their short stature and those who negatively evaluated the impact of short stature on attractiveness to men. A nearly equally strong relationship was found between a sense of being perceived as a worthy human being and life satisfaction. The feeling of loneliness had the strongest impact on life satisfaction, while the negative assessment of the impact of short stature on educational and professional status had a weaker influence on life satisfaction (Table 6).

The relationship between body height and life satisfaction was the strongest, with an eta of 0.48. That is, taller women were more satisfied with life.

Other variables that had a substantial impact on quality of life of women with TS were genu varum (bow legs) and actual experience of negative attitudes of other people. However, these correlations were slightly weaker than those for the demographic variables.

Considering the attitudes of other people, the recognition that parents' attitudes were incorrect and that the disease affected families' and friends' attitudes toward them had the strongest impact on life satisfaction of women with TS. The feelings that their family position was different from that of their siblings, and that other people had negative attitudes toward them were less strongly correlated with life satisfaction. The degree of feeling handicapped was also a significant predictor of life satisfaction, with an eta of 0.43 (Table 7).

Although this study was correlational rather than experimental, an attempt was made to assess the potential influence of some factors on general quality of life; the assessment was based on life satisfaction. In the logistic regression model, life satisfaction was the main dependent variable, while the other variables were predictors.

The results of multidimensional analysis (the logistic regression model) showed that unhappiness about short stature, loneliness and feeling handicapped were independent

Table 6. Self-assessment of own limitations vs satisfaction with life in women with Turner syndrome

Variable	Answers	Satisfaction with life				Cramer's V	p-value
		yes		no/do not know			
		n	%	n	%		
Dissatisfaction with short stature	no	62	88.6	8	11.4	0.29	<0.001
	yes	63	62.4	38	37.6		
Negative impact of short stature on education	no	115	76.2	36	23.8	0.17	<0.05
	yes	10	52.6	9	47.4		
Negative impact of short stature on professional situation	no	106	77.9	30	22.1	0.23	<0.005
	yes	18	52.9	16	47.1		
Negative impact of short stature on attractiveness to men	no	86	82.7	18	17.3	0.27	<0.001
	yes	39	58.2	28	41.8		
Sense of being perceived as a worthy human being	yes	121	77.1	36	22.9	0.28	<0.001
	selectively	6	35.3	11	64.7		
Loneliness	no	105	84.7	19	15.3	0.41	<0.001
	yes	23	45.1	28	54.9		

Table 7. Assessment of other people's attitudes as a determinant of life satisfaction in women with Turner syndrome

Variable	Answers	Satisfaction with life				Cramer's V	p-value
		yes		no/do not know			
		n	%	n	%		
Incorrect attitude of parents	no	109	80.7	26	19.3	0.31	<0.001
	yes	20	48.8	21	51.2		
Impact of TS on parents' attitude	no	117	79.1	31	20.9	0.31	<0.001
	yes	11	40.7	16	59.3		
Different position in the family compared to siblings	no	119	77.3	35	22.7	0.26	<0.001
	yes	5	35.7	9	64.3		
Negative assessment of other people's attitudes	no	98	80.3	24	19.7	0.24	<0.005
	yes	31	57.4	23	42.6		

Table 8. Logistic regression: determinants of life satisfaction: "no"/"do not know" vs "yes"

Variables		OR	95% CI		p-value
Age over 32 years		0.96	0.23	3.98	ns
Education	elementary	1	–	–	–
	basic technical/trade	3.93	0.84	18.3	ns
	secondary and university	2.31	0.59	9.13	ns
Habitation	with parents only	1	–	–	–
	with partner	1.8	0.4	8.06	ns
	alone	8.01	0.77	83.32	ns
	other	3.16	0.25	40.6	ns
Height		0.98	0.90	1.06	ns
No sex drive		2.69	0.73	9.91	ns
Dissatisfaction with short stature		4.11	1.28	13.19	<0.05
Negative impact of short stature on attractiveness to men		2.02	0.67	6.07	ns
Incorrect attitude of parents		2.85	0.91	8.94	ns
Sense of being perceived as a worthy human being		3.07	0.60	15.76	<0.05
Loneliness		3.28	1.20	8.94	<0.05
Degree of feeling handicapped		1.53	1.08	2.16	ns

95% CI – 95% confidence interval; OR – odds ratio; ns – statistically non-significant.

predictors of life satisfaction, and substantially increased the risk of life dissatisfaction in women with Turner syndrome (Table 8).

Although variables such as living alone, education higher than elementary, and incorrect parents' attitudes were not statistically significant (p = 0.05), they also clearly affected satisfaction with life as determined by multidimensional logistic regression.

Discussion

The study was performed on a relatively large group of women with TS and we believe the results obtained considerably expand the knowledge on quality of life in women with TS. The drawback of the study design was the method of patient recruitment (by response to adverts), which introduced some significant bias. It should be expected that patients with lower quality of life were less ready to answer such adverts. Our study group consisted of well-educated women whose life situation, including the limitations resulting from TS, could be better, although other studies indicate the opposite. Women who participated in the examinations did so voluntarily, indicating higher awareness and better adaptation to life. The psychosocial dimensions of quality of life in women with Turner syndrome are quite diverse; nevertheless, our findings are quite consistent with other studies carried out in this group of women.

The previous studies focused primarily on the results of hormonal treatment, particularly growth hormone (GH), administered to improve quality of life. Carel et al. studied quality of life in young women (aged 22.6 ±2.6 years) with TS who had already undergone growth hormone treatment for 5 years, and concluded that quality of life was normal and unaffected by height in young adults with Turner syndrome treated with GH.[9] The beneficial effects of growth hormone therapy on the patient's quality of life were also reported by Bannink et al.[10] Ross et al. observed a positive effect of estrogen on the psychological well-being of girls with TS, and emphasized the need to initiate estrogen replacement therapy by the age of 12–14 years in this population.[11]

There are also papers that document the positive influence of GH administered in childhood on quality of life and psychological well-being, although not as explicitly as that of Ross et al.[11]

Amundson et al. compared quality of life in TS women treated with GH and/or oxandrolone to promote growth with quality of life in a healthy population; they concluded that social isolation was more commonly reported in the whole TS cohort than in the general population. Except for less pain, no significant impact on quality of life attributable to GH treatment could be found, despite the mean +5.1 cm final height.[12] Taback and Van Vliet used the Short Form (36) Health Survey (SF-36) and found no benefit to or adverse effect on health-related quality of life (HRQoL), either from receiving or not receiving GH injections. Young adult women with TS had normal HRQoL, suggesting that they adjusted well to life challenges.[13]

Women with TS have reduced levels of androgens due to ovarian failure; androgen insufficiency plays a role in TS-impaired body composition, neurocognition and quality of life. Oral methyl testosterone given for 1 year improved attention, reaction time and verbal memory, and had no effect on executive functions or spatial cognition. Patients reported improved quality of life, including general health,

coping with stress and sexual desire.[14] These results indicate that the lower quality of life in women with TS can be improved with pharmacological treatment.

After analyzing the changes in quality of life after childhood GH treatment, Zenaty concluded that, although the women's quality of life appeared to be similar to that of the healthy population, the presence of cardiovascular and otological diseases, and delayed feminization did impair quality of life. Hence, early diagnosis and regular screening from childhood to adulthood are essential to reduce morbidity and improve self-esteem.[15] Turtle et al. share this opinion.[16] In the research by Azurah et al. and Robbins et al., it was hypogonadism and amenorrhea that affected life satisfaction, especially regarding sexual function.[17,18] A great number of factors are related to a patient's QoL. Special and individual protocols should also be assessed in patients with TS.[19]

The results of our research indicate that life satisfaction can be affected by both health-related and demographic variables. Our study revealed lower quality of life in those women with Turner syndrome who were troubled by their short statures, loneliness, feeling of being handicapped, and negative attitudes of other people.

Educational and professional background are essential for QoL, including women with TS. Verlinde et al. assessed the health status, education, occupation, and life situation of 102 women, of whom 40% reported health problems (most frequently hypertension) and 25% had undergone spontaneous pubertal development.[5] Compared to the general population, more women with TS had higher education; 45% were employed, 70% lived with their parents, and 17% were married or lived with partners.

Konradsen and Nielsen examined 69 women with TS as well as their sisters, and found that the disease had no impact on educational level or profession.[20] It is essential to emphasize that psychosocial functioning in women with TS changes over time and that psychosociological resources are more complex and involved in younger women with TS.[21] As mentioned in the introduction, our study group consisted of well-educated women, whose life situation could be better if they did not suffer from TS.

The sexual sphere is one of the most important areas of a woman's life,[6] but Job et al. found that 64% of their study participants with TS did not show interest in the opposite sex.[22] Hettmer et al. found that all women identified as feminine and heterosexual.[23] Women who were put on hormone replacement therapy too late showed lack of mental and social well-being as well as disturbances in sexual function. In our previous paper, we found that the percentage of sexually active women with TS was much smaller than that in the general population, and that these women initiated sex later. Eighty percent of women with TS reported being sexually attracted to men, but only 29% initiated sexual activity. Women with TS differ from healthy women in the general Polish population in that they show less interest in men, less frequent sexual activity, later initiation of sexual activity, and they have orgasms less frequently. The most frequent reason for diminished sexual activity is a lack of a regular partner. We concluded that the quality of sexual life of women with TS differs from that of women in the general population.[6] Women with TS also differ from healthy women in lifestyle.[24]

Sexual activity as a component of QoL was also considered in a variety of aspects, including medical (hypogonadism, amenorrhea) and pharmacological ones (GH, oxandrolone, methyltestosterone).[17,18,25] Only in the latter study did the patients report improved QoL. Other authors found no differences in QoL between sexually active and inactive women with TS. Nevertheless, the sexually active patients did have poorer arousal outcomes compared to the general population. No differences between sexually active and inactive women with TS were found in their SF-36 scores or clinical and anthropomorphic characteristics.[18] Data collected from women with TS and their families indicated that the women, their families, health care workers, and society did not have sufficient information about Turner syndrome and did not know how to help patients with TS.[26]

To sum up, research results confirm that Turner syndrome has a substantial impact on women's satisfaction with life. TS-related stigmatization (short stature and feelings of being handicapped and lonely) significantly determines the women's general assessments of their quality of life.

Conclusions

Life satisfaction was the lowest in those women with Turner syndrome who were unhappy about their short stature. Loneliness diminished satisfaction with life in women with Turner syndrome. The feeling of being handicapped in different areas decreased life satisfaction in women with Turner syndrome. The determinants of life satisfaction in women with Turner syndrome were closely related to the private life of the study participants, and their feelings concerning health status.

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Real-life use of thromboprophylaxis in patients hospitalized for pulmonary disorders: A single-center retrospective study

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Abstract

Background. Underuse of thromboprophylaxis in hospitalized medical patients is still common worldwide. Little is known about the use of thromboprophylaxis in patients with pulmonary diseases in everyday hospital practice.

Objectives. The aim of this study was to assess the use of pharmacological prophylaxis of venous thromboembolism (VTE) in real-life patients with pulmonary diseases.

Material and methods. In this retrospective study, 2 validated scoring systems, i.e., the Padua prediction score and Caprini VTE risk assessment, were used to assess the VTE risk in 2011 patients (1133 men and 878 women), aged 18 years or more, hospitalized for pulmonary diseases (median 6 days) in a single tertiary pulmonary medical center from January to December 2014.

Results. Using the Padua prediction score, we identified 428 (21.28%) patients at a high risk for VTE, including 167 (39.01%) who received thromboprophylaxis with low-molecular-weight heparin, and 261 (60.98%) individuals at a high risk without thromboprophylaxis ($p < 0.001$). A total of 888 (44.16%) patients who scored 5 points or more using the Caprini VTE risk assessment were identified as subjects at a high risk for VTE, including 34.79% of patients receiving thromboprophylaxis. From among patients at a high risk for VTE, 579 (65.20%) did not receive appropriate thromboprophylaxis ($p < 0.001$). Underuse of thromboprophylaxis was observed more commonly among patients hospitalized for lung cancer or pneumonia (50.60% and 24.87% of patients at a high risk for VTE without prophylaxis, respectively).

Conclusions. Thromboprophylaxis is underutilized in hospitalized patients with pulmonary diseases regardless of the scoring system used. Implementation of thromboprophylaxis should be markedly improved in this patient group.

Key words: venous thromboembolism, thromboprophylaxis, pulmonary diseases, Padua prediction score, Caprini VTE risk assessment

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Introduction

Hospitalization is associated with an increased risk of developing venous thromboembolism (VTE) and leads to 10–20% of the VTE episodes in the general population.[1–3] Importantly, 10% of in-hospital deaths are related to VTE.[4–6] It has been estimated that 42% of hospitalized patients are at an intermediate or high risk for VTE.[1] An increased risk of VTE is also observed during the 30-day post-discharge period.[1,7]

Appropriate pharmacologic prophylaxis to prevent VTE with its consequences has been recognized as a key medical intervention among patients admitted to hospital, as it directly increases their safety, reduces the occurrence of VTE, and reduces the cost of medical treatment. Appropriate thromboprophylaxis reduces the risk of VTE by half.[8,9] Surgical patients benefit more than medical patients. The 2012 and 2016 guidelines of the American College of Chest Physicians (ACCP) strongly recommend pharmacologic prophylaxis among hospitalized patients at a high risk for VTE (grade 1B), or mechanical thromboprophylaxis in patients at a high risk for VTE as well as for bleeding, or those who bleed actively (grade 2C).[9,10] Identification of high-risk VTE patients who should receive thromboprophylaxis during the hospital stay can be done by means of validated scoring systems, which usually divide patients into high and low risk groups. The former group should receive thromboprophylaxis. The ACCP recommends the Padua prediction score,[11] a validated risk assessment model identifying patients at a high risk for VTE (4 points or more) and those at a low risk for VTE (fewer than 4 points). There is an alternative to the Padua prediction score, the Caprini VTE risk assessment, in which a high risk for VTE is defined as 5 points or more. It is important to consistently use 1 system in everyday hospital practice. The recommended thromboprophylaxis during hospitalization is heparin, most common worldwide, low-molecular-weight heparin (LMWH) s.c., or, infrequently, unfractionated heparin (UFH), or, very rarely, fondaparinux.[9,10] Neither the use of aspirin nor the use of antiplatelet agents is recommended as a prophylaxis of VTE.[9,10] The effectiveness of thromboprophylaxis was assessed in 3 large clinical trials, namely MEDENOX (the Medical Patients with Enoxaparin Trial), in which enoxaparin was used once daily;[12] PREVENT (the Prospective Evaluation of Dalteparin Efficacy for Prevention of VTE in Immobilized Patients Trial), in which dalteparin was used once daily;[13] and ARTEMIS (the Affordability and Real-World Antiplatelet Treatment Effectiveness After Myocardial Infarction Study), in which fondaparinux was used once daily.[14] Some newer oral anticoagulants, e.g., apixaban, dabigatran, and rivaroxaban, can be used as thromboprophylaxis after hospitalization due to surgical indications solely in orthopedic patients.[9,10] The optimal duration of VTE prophylaxis in medical patients is unknown. The current approach is to use it during the whole hospitalization period, and under some circumstances it might be continued for 28 days after discharge.[9,10]

Physicians who refer medical patients to hospital and then take care of them may still fail to put them on thromboprophylaxis in accordance with the ACCP guidelines. They commonly perceive some patients as low-risk individuals, especially if the planned hospital stay is short. On the other hand, there is a subset of patients with a low or moderate risk for VTE who receive pharmacologic thromboprophylaxis during hospital stay, which increases the risk of bleeding, and cost.

A particular group of medically-treated patients who often require thromboprophylaxis during hospitalization are patients with pulmonary disease. For example, the risk of VTE in chronic obstructive pulmonary disease (COPD) exacerbation ranges from 5% to even 29%.[15–18] Postmortem examination of patients who died due to COPD exacerbation have demonstrated pulmonary embolism (PE) in 28–51% of cases.[19,20] Lung cancer is a well- established potent risk factor for developing VTE.[21–25] Respiratory diseases of low prevalence are reported to be associated with an elevated VTE risk. The risk of VTE in patients with pulmonary fibrosis has been estimated as 34% higher than in the background population, and 44% and 54% greater than among patients with COPD and lung cancer, respectively.[26] Asthma is also increasingly perceived as a disease related to an increased VTE risk.[27]

The Epidemiologic International Day for the Evaluation of Patients at Risk for Venous Thromboembolism in the Acute Hospital Care Setting (ENDORSE) study was also performed in Poland and its results were published in 2007.[28,29] It was found that as few as 51.8% of high-risk patients received the thromboprophylaxis recommended by the ACCP (54.7% of surgical patients and 32.5% of nonsurgical patients). In this study, pulmonary patients constituted 26.8% of all evaluated subjects. The main cause of hospitalization was pneumonia (17.6%) and acute respiratory infections (9.2%).[29] Recently, the proportion of hospitalized, medically-treated patients with pulmonary disorders has been increasing in the aging populations; however, PE still remains the main preventable cause of death in this population.[4–6]

Objectives

To our knowledge, there have been no published reports on VTE risk in real-life patients hospitalized in pulmonology wards in recent years. Therefore, the aim of our study was to evaluate the risk of VTE among hospitalized patients and the use of thromboprophylaxis in a ward in which patients with respiratory disorders are treated. We sought to assess the current trends in thromboprophylaxis in patients hospitalized for pulmonary diseases in tertiary specialist hospitals.

Material and methods

In this retrospective study, we enrolled all the patients aged 18 years or more, hospitalized from January 1, 2014 to December 31, 2014 in the Pulmonology Ward of the John Paul II Hospital in Krakow, Poland. Solely patients who stayed in the hospital for more than 24 h were eligible. The hospitalized patients did not need intensive care. No exclusion criteria were used for patients admitted to the ward. The study was carried out in accordance with local legal regulations.

All patients were classified into one of 6 core groups based on the main cause of hospitalization identified at discharge.

Medical data, i.e., demographics, basic and concomitant diseases, duration of hospitalization, and medications, was collected on the basis of hospital records. Patients' physical activity when hospitalized, reduced mobility making a patient stay in bed for more than 30 min during 3 consecutive days as well as any non-pharmacologic thromboprophylaxis were determined on the basis of nursing care records.

We selected 2 validated scoring systems, the Padua prediction score and Caprini VTE risk assessment, and evaluated the VTE risk in all patients.[9,11] 2014 was the last year prior to the implementation of 1 recommended VTE risk assessment tool for the patients hospitalized in the ward.

The Padua prediction score was one model used to identify patients at a high risk for VTE when hospitalized. The group included patients whose score was 4 or higher. Those whose score was lower than 4 were identified as at a low risk for VTE when hospitalized. The Caprini VTE risk assessment was the other model used to identify patients at a high risk for VTE when hospitalized. Those whose score was 4 or lower were identified as not at a high risk for VTE when hospitalized. The group included those whose score was 5 or higher.

Administration of enoxaparin (40 mg once daily from the first to the last day of hospitalization) was used as thromboprophylaxis for all patients at a high risk for VTE. Mechanical thromboprophylaxis was not used.

Not using thromboprophylaxis in patients at a high risk for VTE was recognized as the underuse of prophylaxis, while the overuse of prophylaxis was recognized as using any thromboprophylaxis in patients identified as at a low risk for VTE.

Statistical analysis

The variables were presented as number and percentage. Categorical variables in the subgroups were compared by the Pearson's χ^2 test or the Fisher's exact test for 2 × 2 tables. All p-values presented were two-sided and were considered as statistically significant if below 0.05. All calculations were done with JMP® v. 12.2.0 (SAS Institute Inc., Cary, USA). The Caprini VTE risk assessment and Padua prediction score were expressed as median and interquartile range (IQR), and were compared with the Mann-Whitney U test and the Kruskal-Wallis test.

Results

A total of 2011 individuals aged between 18 and 94 (median: 66) years, including 1133 (56.4%) men and 878 (45.6%) women, were analyzed (Table 1). The mean duration of hospitalization was 6 days. The most common causes of hospitalization were pneumonia (n = 780; 38.8%) and lung cancer (n = 551; 27.4%). Eighty-seven (4.3%) patients were on anticoagulation with vitamin K antagonists (VKAs) on admission. During hospitalization, 368 (18.3%) patients received thromboprophylaxis with enoxaparin.

Padua prediction score

Using the Padua prediction score, we identified 428 (21.28%) patients at a high risk for VTE, including 167 (39.01%) who received thromboprophylaxis. As many as 60.98% of high-risk patients did not receive thromboprophylaxis (Table 2). Out of the 1583 (78.7%) patients with a low risk of VTE, 201 (9.99%) received thromboprophylaxis ($p < 0.001$) (Table 2).

Caprini VTE risk assessment

A total of 888 (44.16%) patients scored 5 points or more using the Caprini VTE risk assessment, and were identified as subjects at a high risk for VTE, including 309 (34.79%) who received thromboprophylaxis. On the other hand, 579 (65.20%) individuals who were at a high risk for VTE did not receive appropriate thromboprophylaxis (Table 3). The number of patients identified as at a low risk for VTE was 1123 (55.88%). Fifty-nine of them received thromboprophylaxis despite having no indications, which makes up 5.25% of the cohort ($p < 0.001$) (Table 3).

The main cause of hospitalization

The main causes of hospitalization among patients requiring thromboprophylaxis were diseases of airways, lung cancer, interstitial lung disease, pneumonia, and respiratory failure. A total of 138 (15.54%) patients with diseases of airways scored 5 points or more using the Caprini VTE risk assessment, and were identified as at a high risk of VTE. Ninety (15.54%) individuals were identified as underusing thromboprophylaxis (Table 3). Using the Padua prediction score with this group, 12 (4.60%) patients were demonstrated to underuse thromboprophylaxis (Table 2).

Among lung cancer patients, 293 (50.60%) individuals were identified as underusing thromboprophylaxis according to the Caprini VTE risk assessment (Table 3). Using the Padua prediction score in this group, 218 (83.52%) patients were demonstrated to underuse thromboprophylaxis (Table 2).

According to the Caprini VTE risk assessment, 38 (6.56%) of the interstitial lung disease patients were also underusing thromboprophylaxis (Table 3). Using the Padua prediction score with these patients, 3 (1.15%) individuals

Table 1. Characteristics of the study cohort

Characteristics	The whole population n = 2011	Patients who received thromboprophylaxis n = 368 (18.3%)	Patients who did not receive thromboprophylaxis n = 1643 (81.7%)	p-value
Age > 70 years	632 (31.4)	177 (8.8)	455 (22.6)	<0.001
Men	1133 (56.4)	213 (10.6)	920 (45.7)	0.52
BMI > 30 kg/m^2	349 (17.3)	77 (3.8)	272 (13.5)	0.05
Acute patients	124 (6.2)	13 (0.6)	111 (5.5)	0.02
Elective patients	1887 (93.8)	355 (17.6)	1532 (76.2)	0.02
Cause of hospitalization				
Airways diseases[1]	332 (16.5)	65 (3.2)	267 (13.3)	<0.001
Interstitial lung disease[2]	268 (13.3)	11 (0.5)	257 (12.8)	<0.001
Lung cancer	551 (27.4)	160 (7.9)	391 (19.4)	<0.001
Pneumonia	780 (38.8)	77 (10.5)	703 (34.9)	<0.001
Pulmonary embolism	52 (2.6)	46 (2.3)	6 (0.3)	<0.001
Respiratory failure	28 (1.4)	9 (0.4)	19 (0.9)	<0.001
Comorbidities				
Arrhythmia	79 (3.9)	63 (3.1)	16 (0.8)	<0.001
Diabetes	127 (6.3)	27 (1.3)	100 (5.0)	0.41
Arterial hypertension	859 (42.7)	215 (10.7)	644 (32.0)	<0.001
Heart failure	14 (0.7)	10 (0.5)	4 (0.2)	<0.001
Thyroid disorders	35 (1.7)	3 (0.1)	32 (1.6)	0.18
Previous venous thromboembolism	13 (0.6)	9 (0.4)	4 (0.1)	<0.001
Varicose veins	164 (8.2)	68 (3.4)	96 (4.8)	<0.001
Other diseases	44 (2.2)	20 (1.0)	24 (1.2)	<0.001
Medications on admission				
Oral corticosteroids	27 (1.3)	12 (0.6)	15 (0.7)	0.01
VKA	87 (4.3)	67 (3.3)	20 (1.0)	<0.001
Risk scores				
Padua prediction score low risk (<4 points)	1583 (78.7)	201 (10.0)	1382 (68.7)	<0.001
Padua prediction score high risk (≥4 points)	428 (21.3)	167 (8.3)	261 (13.0)	<0.001
Caprini VTE risk assessment score low risk (1–2 points)	322 (16.3)	7 (0.3)	315 (16.0)	<0.001
Caprini VTE risk assessment score medium risk (3–4 points)	795 (39.5)	52 (2.6)	743 (36.9)	<0.001
Caprini VTE risk assessment score high risk (≥5 points)	888 (44.2)	299 (15.4)	589 (28.8)	<0.001
Death	7 (0.3)	2 (0.1)	5 (0.2)	0.62

Data was shown as number (percentage). VKA – oral anticoagulant therapy; BMI – body mass index; [1] asthma, chronic obstructive pulmonary disease and bronchiectasis; [2] idiopathic pulmonary fibrosis, sarcoidosis, nonspecific interstitial pneumonia, and hypersensitivity pneumonitis.

were in the same group of patients underusing thromboprophylaxis (Table 2).

Underuse of thromboprophylaxis in hospitalized patients was also observed in patients with pneumonia. As many as 203 (22.86%) of them scored 5 points or more using the Caprini VTE risk assessment, and were identified as at a high risk for VTE. A total of 144 (24.87%) individuals were identified to underuse thromboprophylaxis (Table 3). Using the Padua prediction score, 27 (10.34%) patients were demonstrated to underuse thromboprophylaxis (Table 2).

Among respiratory failure patients, as few as 10 (1.73%) individuals were identified to underuse thromboprophylaxis according to the Caprini VTE risk assessment (Table 3). Using the Padua prediction score, none of them was demonstrated to underuse thromboprophylaxis (Table 2).

Using the Padua prediction score, the overuse of thromboprophylaxis was common in the following groups based

Table 2. Use of thromboprophylaxis according to Padua prediction score

Variable	Patients who should receive thromboprophylaxis according to Padua prediction score ≥ 4 points	Patients who should not receive thromboprophylaxis according to Padua prediction score < 4 points	Patients who received thrombo-prophylaxis	Patients who did not receive thrombo-prophylaxis despite indication	Patients who received thrombo-prophylaxis without indication	p-value
Padua prediction score ≥ 4 points	428 (21.28)	1583 (78.7)	167 (39.01)	261 (60.98)	201 (9.99)	<0.001
Main cause of hospitalization						
Airways diseases[1]	14 (3.27)	318 (20.09)	2 (13.17)	12 (4.60)	63 (31.34)	<0.001
Lung cancer	359 (83.88)	192 (50.03)	141 (84.43)	218 (83.52)	19 (9.45)	<0.001
Interstitial lung disease	3 (0.70)	265 (16.74)	0 (0.00)	3 (1.15)	11 (5.47)	0.0006
Respiratory failure	0 (0.00)	28 (1.77)	0 (0.00)	0 (0.00)	9 (4.48)	<0.001
Pneumonia	41 (9.58)	739 (46.68)	14 (8.38)	27 (10.34)	63 (31.34)	<0.001
Pulmonary embolism	11 (2.57)	41 (2.59)	10 (5.99)	1 (0.38)	36 (17.91)	<0.001
Components of Padua prediction score						
Active cancer	419 (97.89)	68 (4.29)	160 (95.81)	259 (99.23)	7 (3.48)	<0.001
Previous VTE	9 (2.10)	4 (0.25)	7 (4.19)	2 (0.77)	1 (0.50)	0.0071
Advanced age (>70 years)	173 (40.42)	459 (29.00)	67 (40.12)	106 (40.61)	110 (54.73)	0.0035
Heart or respiratory failure	5 (1.17)	9 (0.57)	5 (2.99)	0 (0.0)	5 (2.49)	0.0253
Acute infection and/or rheumatic disease	367 (85.75)	599 (37.84)	149 (89.22)	218 (83.52)	140 (69.65)	<0.001
BMI > 30 kg/m^2	75 (17.52)	274 (17.31)	36 (21.56)	39 (14.94)	41 (20.40)	0.1562
Hormonal treatment	17 (3.97)	10 (0.63)	10 (5.99)	7 (2.68)	2 (0.99)	0.0189
Thrombophilia	1 (0.23)	1 (0.06)	1 (0.60)	0 (0.00)	1 (0.05)	0.4837

[1] Asthma, chronic obstructive pulmonary disease and bronchiectasis.

on the main causes of hospitalization: diseases of airways (n = 63; 31.34%); pneumonia (n = 63; 31.34%); and pulmonary embolism (n = 36; 17.91%) (Table 2). Using the Caprini VTE risk assessment, the overuse of thromboprophylaxis was common in patients with diseases of airways (n = 17; 28.81%), pulmonary embolism (n = 15; 25.42%), and pneumonia (n = 18; 30.51%) (Table 3).

Underuse of thromboprophylaxis was identified in some components of the Padua prediction score: active cancer (99.23%); acute infection or rheumatic disease (83.52%); and advanced age (40.61%) (Table 2). Taking into account the components of the Caprini VTE risk assessment and underuse of thromboprophylaxis, we observed among the patient groups: abnormal pulmonary function (99.65%); serious lung diseases (75.24%); and BMI > 25 kg/m^2 (63.73%) (Table 3).

Discussion

To our knowledge, this study is the largest analysis of the current everyday practice in thromboprophylaxis in patients hospitalized for pulmonary diseases. Given the rising prevalence of several pulmonary diseases in the general population, e.g., COPD, the significant risk for VTE during a hospital stay should also be acknowledged in this subset of medical patients. The current guidelines make it possible to choose 1 of a few validated scoring system to evaluate the VTE risk; however, using 2 of the tested scores, namely the Padua prediction score and Caprini VTE risk assessment, the proportion of patients with pulmonary disease who are deprived of benefits from the prophylactic use of LMWH is substantial. This observation indicates that every patient hospitalized for medical reasons should be assessed as a potential candidate for thromboprophylaxis.

There was a large subset of patients with pulmonary diseases hospitalized, i.e., 60.98% according to the Padua prediction score and 65.20% according to the Caprini VTE risk assessment, who did not receive proper prophylaxis of VTE. A much lower proportion of patients hospitalized for pulmonary disorders, i.e., 9.99% based on the Padua prediction score and 5.25% based on the Caprini VTE risk assessment, received prophylaxis of VTE, but did not need it according to the current recommendations. Compared to the 2007 ENDORSE study, the proportion of pulmonary patients without thromboprophylaxis during a hospital stay is comparable to the data obtained in non-surgical wards.[29] Our findings highlight the need for widespread use of thromboprophylaxis in medical patients, including those from pulmonary wards. The proportion of patients on thromboprophylaxis during a hospital stay is still suboptimal, and without significant improvement after about 10 years.

We identified some subsets of patients with pulmonary disorders who are more likely not to receive

Table 3. Use of thromboprophylaxis according to Caprini VTE risk assessment

Variable	Patients who should receive thrombo-prophylaxis according to Caprini VTE risk assessment ≥ 5 points	Patients who should not receive thrombo-prophylaxis according to Caprini VTE risk assessment < 5 points	Patients who received thrombo-prophylaxis	Patients who did not receive thrombo-prophylaxis despite indication	Patients who received thrombo-prophylaxis without indication	p-value
Caprini VTE risk assessment ≥ 5 points	888 (44.16)	1123 (55.88)	309 (34.79)	579 (65.20)	59 (5.25)	<0.001
Main cause of hospitalization						
Airways diseases[1]	138 (15.54)	194 (17.27)	48 (15.53)	90 (15.54)	17 (28.81)	0.028
Lung cancer	449 (50.56)	102 (9.08)	156 (50.48)	293 (50.60)	4 (6.78)	<0.001
Interstitial lung disease	47 (5.29)	221 (19.68)	9 (2.91)	38 (6.56)	2 (3.39)	0.052
Respiratory failure	16 (1.80)	12 (1.07)	6 (1.94)	10 (1.73)	3 (5.08)	0.214
Pneumonia	203 (22.86)	577 (51.38)	59 (19.09)	144 (24.87)	18 (30.51)	0.061
Pulmonary embolism	35 (39.77)	17 (1.51)	31 (10.03)	4 (0.69)	15 (25.42)	<0.001
Components of Caprini VTE risk assessment						
Age 41–60 years	93 (10.47)	396 (35.26)	28 (9.06)	65 (11.23)	18 (30.51)	<0.001
Age 61–75 years	431 (48.53)	434 (38.65)	146 (47.25)	285 (49.22)	27 (45.76)	0.7848
Age > 75 years	357 (40.20)	50 (4.45)	127 (41.10)	230 (39.72)	2 (3.39)	<0.001
History of VTE	10 (1.13)	1 (0.09)	8 (2.59)	2 (0.35)	0 (0.00)	0.0056
Varicose veins	127 (14.30)	37 (3.29)	61 (19.74)	66 (11.40)	7 (11.86)	0.0027
Congestive heart failure	12 (1.35)	2 (0.18)	10 (3.24)	2 (0.35)	0 (0.00)	0.0008
Swollen legs	76 (8.56)	17 (1.51)	64 (20.71)	12 (2.07)	7 (11.86)	<0.001
Serious lung diseases	682 (76.80)	299 (26.62)	262 (84.79)	420 (72.54)	32 (54.24)	<0.001
Abnormal pulmonary function	885 (99.66)	1078 (95.99)	308 (99.68)	577 (99.65)	59 (100.00)	0.9035
Cancer	475 (51.46)	12 (1.07)	166 (53.72)	309 (53.37)	1 (1.69)	<0.001
Thrombophilia	2 (0.22)	0 (0.00)	2 (0.65)	0 (0.00)	0 (0.00)	0.1263
BMI > 25 kg/m^2	587 (66.10)	478 (42.56)	218 (70.55)	369 (63.73)	20 (33.90)	<0.001

[1] Asthma, chronic obstructive pulmonary disease and bronchiectasis.

thromboprophylaxis during a hospital stay. It is disturbing to demonstrate that more than 50% of lung cancer patients did not receive thromboprophylaxis despite clear indications. Approximately 3% of lung cancer patients develop VTE within 2 years of diagnosis and this complication is associated with a 50% higher risk of death within 2 years.[23,24] These findings strongly support the need for much more common use of thromboprophylaxis in cancer patients.

The issue of prophylaxis in pulmonary embolism (PE) patients deserves a comment. Most of patients with PE, including incidental pulmonary embolism, received thromboprophylaxis in spite of being at a low risk for VTE (36 patients at a low risk for VTE according to the Padua prediction score, and 15 individuals according to the Caprini VTE risk assessment). It seems that this observation results from better implementation of the recommendations for PE treatment in the pulmonary ward. Obviously, patients with confirmed PE were treated, as recommended, mostly with therapeutic doses of LMWH during the hospital stay.

This study has several limitations. The study is retrospective, which implies some problems with data acquisition and their precision. We did not have data from other years to compare the trends in thromboprophylaxis in our hospital. In some patients, the diagnosis could have been not convincingly established and, for example, we could not address the issue as to whether asthma was associated with a comparable risk of VTE vs COPD. We did not assess the impact of certain comorbidities and high-risk VTE factors, e.g., recent myocardial infraction, stroke, injury or surgery, since none of the enrolled patients experienced such disease states. An analysis of the clinical outcomes of under- or overuse of thromboprophylaxis during a hospital stay and follow-up was beyond the scope of the current study.

From the practical point of view, it is important to put more focus on a proper assessment of patients at a high risk for VTE, and consistently use 1 assessment model to identify patients at a high risk for VTE. We believe that since at the John Paul II Hospital the Caprini VTE risk assessment was implemented as a preferred tool for assessing the patients' risk of VTE during hospitalization, the proportion of patients who can benefit from LMWH has been improving, also leading to better clinical outcomes among patients treated for pulmonary diseases.

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Review

Selenium – a fascinating antioxidant of protective properties

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Abstract

Selenium is a trace element which fulfils important functions in the organism. Its deficit may cause acute disorders, but an overdose can also lead to severe consequences. The functions of selenium in the organism are mainly connected with its antioxidant properties, as it is an essential part of important antioxidant enzymes. Disturbances of oxidant balance have been found to be involved in the activity of numerous harmful factors as well as in the pathogenesis of diverse illnesses. Selenium administration has proved to be effective against the toxicity of many agents and the side effects of drugs. However, the narrow range between therapeutic and toxic doses of selenium, as well as the dependence of its effect on the applied form, dose and method of treatment, makes the choice of the most effective supplement a very complex issue. Divergent forms of selenium are still being studied, including both inorganic and organic compounds as well as Se-enriched natural products. The newest research has also involved selenium nanoparticles. The aim of this review is to present the great potential of selenium for protecting the organism against a wide variety of environmental pollutants, drugs and physical factors.

Key words: oxidative stress, selenium, drug-related side effects, protective agents

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Introduction

Selenium belongs to trace elements essential for humans. In the body it is involved in numerous processes, among other things immune functions and antioxidant defense. Its deficit may result in cardiac, muscular, osseous, and immune disturbances.[1,2] The biological functions of selenium result from the occurrence of the selenocysteine amino acid in proteins. The research has revealed that about a hundred selenoproteins can be found in mammal organisms.[2] The most important of them are the antioxidant enzymes – glutathione peroxidase and thioredoxin reductase, as well as selenoprotein P, responsible for the storage and transport of selenium.[3–6] Selenium supplementation has been proved to be protective against a very wide range of harmful factors, both chemical, such as drugs exerting severe side effects, heavy metals, carcinogens, mycotoxins, or pesticides, and physical, such as heat stress or magnetic fields. However, the narrow range between therapeutic and toxic doses of selenium, as well as the dependence of its effect on the applied form, dose and method of treatment, make the choice of the most effective supplement a very complex issue.[5,7–10]

The average lethal dose established in animal models for sodium selenite (7 mg Se/kg b.w.) is almost 20 times smaller than that obtained for selenium sulfides, and more than 900 times smaller than for elemental selenium.[11] According to the US National Academy of Sciences, for adults, 55 μg is the recommended daily selenium intake, whereas 400 μg is the threshold which should not be exceeded.[7] The toxic dose for adults was established as more than 700 μg/day.[12] The symptoms of selenium toxicity include fatigue, disturbances in connective tissue as well as in cardiovascular, gastrointestinal, nervous, and respiratory systems.[11,13] As the interest in selenium and its effects on human health is still growing, diverse compounds of selenium are still being studied, both inorganic and organic, Se-enriched natural products like probiotics, yeast and green tea, as well as selenium nanoparticles.[3,8,10,14–18] Organic compounds have been widely studied recently due to the similarity of the activity of some of them (e.g., ebselen or diphenyl diselenide) to that shown by glutathione peroxidase.[19] Diphenyl diselenide has also been proved to possess many beneficial pharmacological properties: anti-hyperglycemic, anti-hyperlipidemic, hepatoprotective, antiulcer, and antidepressant.[19,20]

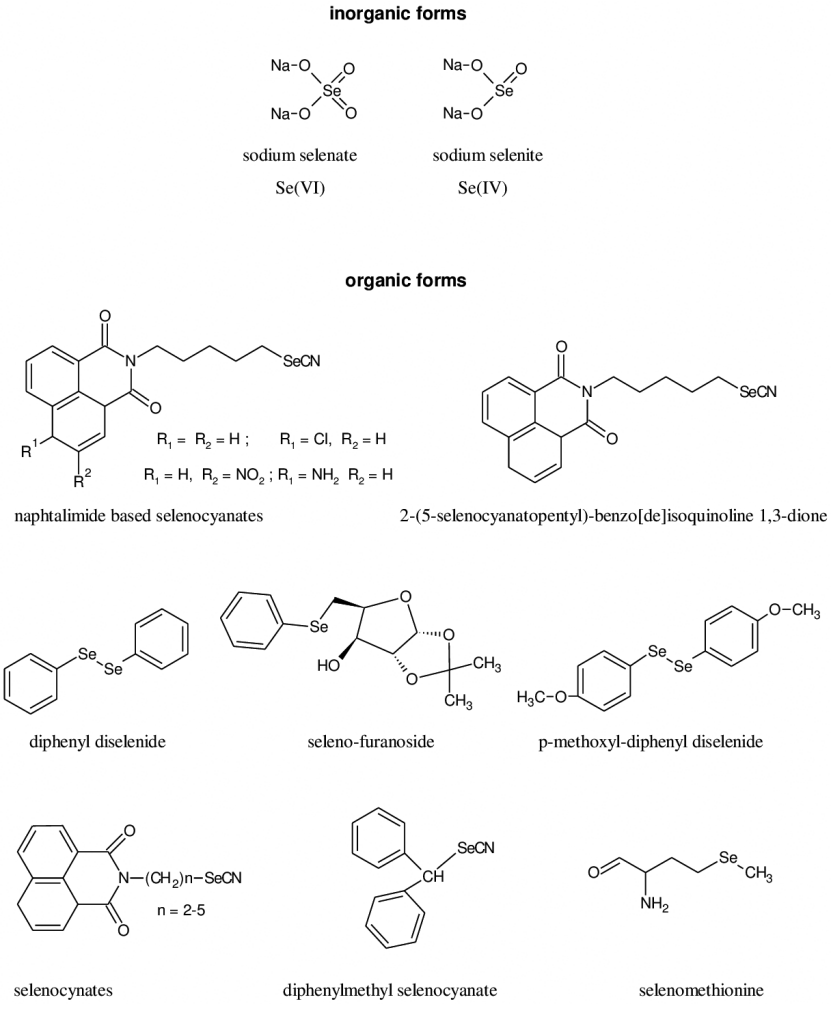

Fig. 1. The different forms of selenium used in scientific research

The aim of this review is to present the great potential of selenium for protecting the organism against the damage caused by environmental pollutants, drugs and physical factors, as well as the dependence of the selenium impact on the form and model of administration.

Comparison of the effect of different selenium forms on organisms

The effect of selenium on organisms shows strong dependence on its form. A distinct difference between organic and inorganic compounds has been found. The forms of selenium used are presented in Fig. 1.

Sodium selenite remains the most often studied inorganic form of selenium, containing Se(IV), but other forms are also commonly studied. A comparison of selenite and selenate, containing Se(VI), was performed on the mycelia of *Pleurotus ostreatus* exposed to cadmium and silver (1.25 mg/L, 2.5 mg/L and 5 mg/L). Selenium (2.5 mg/L or 5 mg/L) prevented the malonyldialdehyde (MDA) increase caused by the metals, but while Se(IV) showed full effectiveness, Se(VI) was either less effective or even intensified the pro-oxidant processes.[21]

The research more and more often includes selenoorganic compounds – either naturally occurring forms or newly synthesized substances, e.g., selenomethionine, naphthalimide-based selenocyanates, 2-(5-selenocyanatopentyl)-benzo[de]isoquinoline-1,3-dione, diphenyl diselenide, selenofuranoside, p-methoxyldiphenyl diselenide, selenocyanates, and diphenylmethyl selenocyanate.[4–6,9,16,17,19,20,22–26] The results regarding the comparison of inorganic and organic compounds are not fully consistent, although the newest studies usually reveal

Table 1. Protective effect of selenium against cisplatin toxicity

Authors, citation number	Animals, way of administration, dose, time of exposure	Harmful effects of cisplatin	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Chakraborty et al. 2011[9]	mice intraperitoneally 5 mg/kg b.w. 5 days	kidney GST, GPx, CAT, SOD, GSH ↓; kidney TBARS, serum creatinine, blood urea nitrogen ↑	synthetic organic diphenylmethyl selenocyanate, 3 mg/kg b.w., oral gavage, 2 models: concomitant treatment (Se from day 1 to day 9, and cisplatin from day 1 to day 5); pretreatment (Se 7 days before cisplatin, and then from day 1 to day 9, cisplatin from day 1 to day 5)	kidney GPx, CAT, SOD, serum creatinine, blood urea nitrogen (±); kidney GSH, GST, TBARS (+); pretreatment model more effective	none
Yazici et al. 2014[30]	rats intraperitoneally 16 mg/kg 3 days	drug-caused edema and subsequently retinal thickness increase	Na_2SeO_3 1.5 mg of Se/kg, oral gavage twice daily, for 5 days before drug and for 3 days concomitantly	selenium reduced the effects of cisplatin and showed the antiapoptotic influence	not studied
Rezvanfar et al. 2013[31]	rats intraperitoneally, single injection 7 mg/kg before Se	serum testosterone ↓; sperm abnormality ↑; blood and testis lipid peroxidation ↑; blood and testis peroxynitrite ↑; blood and testis SOD, CAT, GPx ↓	selenium nanoparticles 2 mg/kg/day, orally 10 days	serum testosterone (±); sperm abnormality (±); blood and testis lipid peroxidation (+); blood and testis peroxynitrite (+); blood and testis SOD, CAT, GPx (+)	none
Wilhelm et al. 2012[25]	mice intraperitoneally, single injection 10 mg/kg on day 3	plasma urea and plasma creatinine ↑; kidney vitamin C, GSH, GST, GPx, GR, CAT, δ-ALA-D ↓	p-methoxyl-diphenyl diselenide 50 mg/kg or 100 mg/kg, orally 6 days	lower dose: plasma urea (±); plasma creatinine (0); kidney GSH, GST, GPx, GR, δ-ALA-D (±); kidney vitamin C, CAT (+) higher dose: plasma urea and plasma creatinine (±), kidney GSH, GST, GPx, GR, vitamin C, CAT (+) kidney δ-ALA-D (±)	none

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection; GST – glutathione S-transferase; GPx – glutathione peroxidase; CAT – catalase; SOD – superoxide dismutase; GSH – reduced glutathione; TBARS – thiobarbituric acid-reactive substances; GR – glutathione reductase; δ-ALA-D – δ-aminolevulinic dehydratase.

Table 2. Protective effect of selenium against toxicity of different drugs

Authors, citation number	Animals, way of administration, dose, time of exposure	Harmful effects of drug	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Gürgen et al. 2013[32]	rats, cyclophosphamide intraperitoneally 75 mg/kg b.w. once a week, for 3 weeks	degeneration of the ovarian tissue; serum GSH ↓	40 ppm/day/rat, oral gavage 3 weeks	reducing the degeneration of ovarian tissue; serum GSH (±)	not studied
Ghosh et al. 2015[5]	mice, cyclophosphamide intraperitoneally 25 mg/kg b.w. 10 days or 25 days	pulmonary ROS, NO, lipid peroxidation ↑; GSH, GST, GPx, SOD, CAT ↓	a synthetic organic compound 2-(5-selenocyanatopentyl)-benzo[de]isoquinoline-1,3-dione 3 mg/kg b.w. (non-toxic dose), oral gavage, 2 models: 10 days or 25 days; 2 models: concomitant co-administration of selenium (10 days); Se-pretreatment (15 days before drug-exposure and subsequent co-administration)	oxidant parameters considerably improved by selenium; the pretreatment model was more effective, which was confirmed by histopathological studies of the lung: in co-administered animals moderate changes were observed, in the pretreatment model no distinct alterations were displayed compared with control	none
Danesi et al. 2006[33]	rats adriamycin, single intraperitoneal dose 10 mg/kg b.w.	plasma reactive oxygen metabolites ↑; plasma total antioxidant activity ↓; reactive oxygen metabolites in heart ↑	pretreatment with dietary Na_2SeO_3 or Se-enriched potato obtained by foliar Se-supplementation during growth 0.1 mg/kg 60 days	plasma reactive oxygen metabolites and total antioxidant activity (0); heart reactive oxygen metabolites: selenite (±), Se-enriched potato (+)	not studied
Saied and Hamza 2014[34]	rats isoretinoin (a retinoid used in dermatology) gastric tube 7.5 mg/kg b.w. 28 days	ALT, AST, ALP, total cholesterol and triglycerides, TBARS ↑; HDL, GSH, SOD, CAT ↓	Na_2SeO_3 500 µg/kg/day, gastric tube, 28 days	ALT, ALP, TBARS, total cholesterol, triglycerides (±); SOD, CAT (0); AST, HDL, GSH (+)	SOD, CAT, HDL ↑
Mossa et al. 2014[35]	rats aspirin 22.5 mg/kg b.w. 28 days	erythrocytes SOD, CAT, GPx ↓; erythrocytes MDA ↑	Na_2SeO_3 200 µg/kg b.w./day, orally 28 days	erythrocytes SOD, CAT, GPx, MDA (+)	none

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection. GSH – reduced glutathione; ROS – reactive oxygen species; NO – nitrogen oxide; GST – glutathione S-transferase; GPx – glutathione peroxidase; SOD – superoxide dismutase; CAT – catalase; ALP – alkaline phosphatase; AST – aspartate aminotransferase; ALT – alanine aminotransferase; HDL – high-density lipoproteins.

that the organic forms are more beneficial and less likely to induce their own toxic effects. In mice treated with either sodium selenite or naphthalimide-based synthetic organoselenocyanates (oral gavage; 1.2 mg/kg b.w. and 3 mg/kg b.w., respectively), selenite significantly decreased hemoglobin as well as increased aspartate aminotransferase (AST), alanine aminotransferase (ALT), and hepatic lipid peroxidation. Organic selenium depressed AST and ALT as well as blood urea nitrogen and creatinine, indicating the potential hepatoprotective and nephroprotective effects of this form. Histopathological studies have confirmed these outcomes. The advantage of the organic form was also observed in the case of liver antioxidant parameters.[24]

In one of the newest studies, sodium selenite, selenomethionine and selenium yeast were compared in vitro (1 µg/mL in cell medium). In HepG2 cells exposed to lead nitrate (40 µg of Pb/mL or 80 µg of Pb/mL), DNA damage was reduced by organic selenium, whereas selenite not only was ineffective as a protective agent, but also intensified DNA injury.[6]

Four dietary selenium forms (selenite, lactate-protein complex, Se-proteinate, and Se-yeast), given to goats before (0.3 mg/day/goat) and after parturition (0.9 mg/day/goat), were studied as potential Se-supplements. Tissue selenium content in different organs of the younglings were the highest in the Se-yeast group, but the other organic forms were also more effective as supplements than selenite.[27]

Sodium selenite was also compared with dextrin stabilized selenium nanoparticles (both forms used at doses 10–100 µg/mL). An in vitro investigation revealed that the viability of the nanoparticle-treated cells was much higher than in the case of those treated by selenite. In an in vivo

Table 3. Protective effect of selenium against cadmium toxicity

Authors, citation number	Animals, way of administration, dose, time of exposure	Cadmium-induced harmful effects	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Li et al. 2013[14]	chickens $CdCl_2$ 150 mg/kg of diet 60 days	liver lipid peroxidation, NO level and nitric oxide synthase activity ↑; liver SOD, GPx ↓; the number of apoptotic cells ↑	dietary Na_2SeO_3 10 mg/kg, 60 days	liver lipid peroxidation, NO level, nitric oxide synthase activity, SOD, GPx (±); the number of apoptotic cells (±)	GPx ↑
El-Boshy et al. 2015[37]	rats cadmium 40 mg/L in drinking water as $CdCl_2$ 30 days	serum tumor necrosis factor α, interleukins IL-6, IL-10, IL-β, MDA ↑; serum interferon γ, GSH, GPx, CAT, SOD ↓; ALT and AST, urea and creatinine ↑	Na_2SeO_3 0.1 mg/kg b.w., orally	serum tumor necrosis factor α, IL-6, IL-10, interferon γ, MDA, GSH, GPx, CAT, SOD, ALT, AST, urea and creatinine (+); IL-β (0)	serum interferon γ (IFN-γ), GSH, GPx, CAT ↑; IL-10 ↓
Sk and Bhattacharya 2006[26]	mice $CdCl_2$ intraperitoneally 1 or 2 mg/kg b.w. 20 days	serum ALT and AST, hepatic microsomal lipid peroxidation ↑; liver cytosol GST, SOD, CAT, GSH ↓	synthetic selenocyanates 3 mg/kg b.w., by gavage 2 models: concomitant; pretreatment (selenium given 7 days before cadmium, and then throughout the experimental period of 20 days)	serum ALT and AST, hepatic microsomal lipid peroxidation, liver cytosol GST, SOD, CAT, GSH (±); pretreatment model more effective	not studied
Vargas et al. 2013[17]	mice $CdCl_2$, single intraperitoneal dose	ovary δ-aminolevulinate dehydratase activity ↓	synthetic seleno-furanoside 100 µmol (32.9 mg)/kg subcutaneously	in $CdCl_2$ 2.5 mg/kg group (±); in $CdCl_2$ 5 mg/kg group (+)	none

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection. NO – nitrogen oxide; SOD – superoxide dismutase; GPx – glutathione peroxidase; MDA – malonyldialdehyde; GSH – reduced glutathione; CAT – catalase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GST – glutathione S-transferase.

study, rats with arthritis showed depletion of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the liver, kidney and spleen as well as an increase in serum C-reactive protein. These disturbances were reversed by oral administration of nanoparticles (100 µg/kg b.w., 250 µg/kg b.w., 500 µg/kg b.w., or 750 µg/kg b.w.).[28] On the other hand, treatment with selenium nanoparticles (5–25 µg/mL) was reported to cause dose-dependent malformations in zebrafish embryos, whereas the same doses of sodium selenite did not show any harmful effects.[29]

A comparison of Se-enriched probiotics and sodium selenite was performed on rats exposed to carbon tetrachloride. CCl_4 significantly increased serum ALT and AST, and disturbed liver oxidant balance. Both selenium forms alleviated the harmful effects, but the Se-enriched probiotics were more effective.[3]

Protecting effect of selenium against toxicity of different substances

Protective properties of selenium against toxicity of diverse drugs: Animal model research

Administration of drugs can be connected with side effects causing the impairment of organism functions. Selenium was found to protect against the toxicity of different drugs, including the chemotherapeutic agents – cisplatin and cyclophosphamide, antibiotics and dermatological medications as well as aspirin. The research also showed the pretreatment application to be more effective. The details of the studies performed and their results are presented in Tables 1 and 2.

Table 4. Protective effect of organic selenium against toxicity of aluminum, mercury and arsenic

Authors, citation number	Animals, way of administartion, dose, time of exposure	Induced harmful effects	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Viezeliene et al. 2013[38]	mice $AlCl_3$, intraperitoneally 25 mg Al^{3+}/kg b.w. 16 h	serum interleukin IL-6 ↑; liver total GSH ↓	Na_2SeO_3, 1.25 mg of Se^{4+}/kg b.w., intraperitoneally 16 h	IL-6 (±); liver total GSH (+)	liver total GSH ↑; serum IL-6 ↑
Lakshmi et al. 2015[39]	rats $AlCl_3$ 100 mg/kg, orally	brain CAT, GSH, GR, AChE ↓; MDA ↑	selenium 1 mg/kg, orally	brain CAT, AChE (±); GSH, GR, MDA (+)	brain GSH, GR ↑; MDA ↓
Agarwal and Behari 2007[1]	rats mercuric chloride, intraperitoneally 0.4 mg/kg/day 20 days	brain, liver and kidney lipid peroxidation ↑; brain, liver and kidney GSH ↓; brain, liver and kidney Hg concentration ↑	Na_2SeO_3 0.2 mg/kg/day, intraperitoneally 4 h before mercury, 20 days	brain, liver and kidney lipid peroxidation and GSH, brain Hg (0); liver and kidney Hg concentration (–)	liver MDA ↑; kidney GSH ↓
Glaser et al. 2013[19]	mice methylmercury (MeHg), orally in drinking water 40 mg/L 21 days	GPx, respiratory chain enzymes in cortical mitochondrial preparations ↓; GR, TBARS in cortical mitochondrial preparations ↑; cerebral cortex Hg deposition ↑	diphenyl diselenide 5 µmol/kg, subcutaneously 21 days	TBARS, respiratory chain enzymes in cortical mitochondrial preparations (+); GPx, GR in cortical mitochondrial preparations (0); cerebral cortex Hg deposition (±)	GR ↑; TBARS ↓; cerebral cortex Hg deposition ↑
Prasad and Selvaraj 2014[40]	human lymphocytes sodium arsenite $NaAsO_2$ 10 µM 1 h	increased cell death; DNA damage	selenium nanoparticles 0.01 µg/µL 1 h	protecting from DNA damage and cell death	not studied
Xu et al. 2013[41]	rats sodium arsenite $NaAsO_2$, in drinking water 13 mg/L 20 weeks	liver MDA ↑; liver CAT, GPx, SOD ↓; serum ALT and AST ↑	Na_2SeO_3 17.0 mg/L, in drinking water 20 weeks	liver MDA (+); liver CAT, GPx, SOD (±); serum ALT and AST (+)	liver CAT ↓

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection; (–) – intensification of harmful effects. GSH – reduced glutathione; CAT – catalase; GR – glutathione reductase; AChE – acetylcholinesterase; MDA – malonyldialdehyde; GPx – glutathione peroxidase; TBARS – thiobarbituric acid-reactive substances; SOD – superoxide dismutase; ALT – alanine aminotransferase; AST – aspartate aminotransferase.

Protective properties of selenium against environmental contaminants and physical factors: Animal model research

Selenium has been studied in regard to its possible protective effect against numerous environmental pollutants, and the results obtained seem to be very promising. The harmful effects of toxic elements (cadmium, aluminum, mercury, arsenic, lead, chromium) and compounds, e.g., carbon tetrachloride, carcinogens (acrylamide, hydrocarbons), mycotoxins (patulin, aflatoxin), and pesticides (beta-cyfluthrin), diazinon, acephate) were found to be reversed or alleviated by selenium treatment. Sodium selenite was most often used, but organic forms were also studied. Some researchers applied very interesting, new natural forms like polysaccharides isolated from selenium-enriched Ziyang tea, or meat from lambs receiving Se-nanoparticles in drinking water.[7,10] Synthetic organoselenium compounds also revealed their advantage over inorganic forms. Diphenyl diselenide was proved effective against the toxicity of mercury in rodents, while selenite showed no efficacy.[1,19] It was also found that the influence of selenium was dependent on its dose.[10,36] The detailed information is collected in Tables 3–8.

In vitro studies of protective properties of selenium

The beneficial impact of different forms of selenium against the toxicity of diverse factors has also been confirmed by in vitro studies.

In neuronal cells exposed to the addictive drug methamphetamine, a decrease in GPx isoforms (GPx 1 and GPx 4), and the depletion of GPx activity and intracellular reduced glutathione (GSH) was observed. However, in cells cultured in Se-containing media (10 nM or 100 nM as sodium selenite), before and during methamphetamine exposure, these effects were alleviated, although in the case of GPx

Table 5. Protective effect of selenium against toxicity of lead and chromium

Authors, citation number	Animals, way of administration, dose, time of exposure	Induced harmful effects	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Han et al. 2014[8]	weaned rats $PbCl_2$, in drinking water 2 mmol/L 3 weeks	deteriorations of synaptic structural plasticity as well as impaired spatial learning and memory	Se-enriched yeast 6 µg/100 g b.w., by gavage 3 weeks after Pb exposure	Pb-induced effects were improved by the subsequent treatment with selenium	not studied
Baş et al. 2015[42]	rats lead nitrate, by gavage 22.5 mg/kg b.w. (1/100 LD_{50}) 28 days	MDA in erythrocytes and leucocytes ↑; SOD, CAT, GPx, GST in erythrocytes and leucocytes ↓; plasma antioxidant capacity ↓	Na_2SeO_3 1 mg/kg b.w., by gavage 28 days	MDA, SOD, CAT, GPx, GST in erythrocytes and leucocytes (±); plasma antioxidant capacity(±)	none
Soudani et al. 2011[43]	rats $K_2Cr_2O_7$, in drinking water 700 ppm 3 weeks	heart SOD, CAT, GPx, MDA ↑; heart GSH, vitamin C, non-protein thiols, LDH activity ↓; plasma LDL, ALT, AST, bilirubin, total cholesterol, triglycerides, LDL-cholesterol ↑; plasma HDL-cholesterol ↓	dietary Na_2SeO_3 0.5 mg/kg 3 weeks	heart SOD, CAT, GPx, MDA, vitamin C, non-protein thiols, LDH (±); heart GSH, plasma ALT, AST, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides (+); plasma bilirubin (±)	heart non-protein thiols ↑; heart SOD ↓
Soudani et al. 2012[44]	rats $K_2Cr_2O_7$, in drinking water 700 ppm 3 weeks	cerebrum and cerebellum MDA, SOD, GPx, CAT, plasma LDH ↑; cerebrum and cerebellum GSH, non-protein thiols, vitamin C, AChE, LDH ↓	dietary Na_2SeO_3 0.5 mg/kg 3 weeks	cerebrum and cerebellum MDA, SOD, GSH, non-protein thiols, vitamin C, GPx, LDH, AChE, cerebrum CAT (±); cerebellum CAT (0); plasma LDH (+)	cerebrum non-protein thiols ↑
Hassanin et al. 2013[18]	rats $K_2Cr_2O_7$, single intraperitoneal dose 60 µg/kg b.w. in Cr + Se animals, Cr was given on day 3	serum thyroid hormones (free triiodothyronine and thyroxine), GSH ↓; serum CAT, SOD and MDA ↑; in thyroid: hyperplasia of intrafollicular epithelium, follicular segregation, increased interfollicular spaces, increased collagen deposition	selenium nanoparticles, intraperitoneally 0.5 mg/kg b.w. 5 days	serum thyroid hormones (free triiodothyronine and thyroxine) (±); serum GSH, CAT, SOD and MDA (+); alleviated histopathological changes	none

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection; MDA – malonyldialdehyde; SOD – superoxide dismutase; CAT – catalase; GPx – glutathione peroxidase; GST – glutathione S-transferase; GSH – reduced glutathione; LDH – lactate dehydrogenase; LDL – low-density lipoproteins; ALT – alanine aminotransferase; AST – aspartate aminotransferase; AChE – acetylcholinesterase.

Table 6. Protective effect of organic selenium against toxicity of carbon tetrachloride and other carcinogens

Authors, citation number	Animals, way of administration, dose, time of exposure	Induced harmful effects	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Ding et al. 2010[45]	mice CCl_4, intraperitoneally 5 µL of a 20% solution in olive oil biweekly for 4 weeks	liver GPx ↓; liver MDA, serum ALT, liver fibrosis, liver α_1 (I) collagen mRNA ↑	Na_2SeO_3 4 mg/L, in drinking water started 2 days before CCl_4	liver GPx and MDA (+); liver fibrosis (±); serum ALT, liver α_1 (I) collagen mRNA (0)	liver GPx ↑; liver MDA ↓
Wang et al. 2014[10]	mice CCl_4, intraperitoneally 0.3 µL of a 0.8% solution in peanut oil once	serum ALT, AST and LDH ↑; liver MDA ↑; liver SOD and GPx ↓	polysaccharides isolated from selenium-enriched Ziyang tea 100, 200 and 400 mg/kg b.w. intragastrically daily for 14 days before CCl_4	serum ALT, AST and LDH (±); liver MDA, SOD: low dose (0), middle and high doses (±); liver GPx: all doses (±)	not studied
Ali et al. 2014[46]	rats acrylamide, gastric intubation 15 mg/kg b.w./day 28 days	erythrocytes, leucocytes and hematocrit, serum Zn and ALP ↓; MDA, Na, Ca in retinas ↑; retinas GPx and K ↓	0.1 mg/kg b.w./day, gastric intubation for 28 days	all studied parameters (+)	none
Ungvári et al. 2014[7]	mice hydrocarbon DMBA, 7,12-dimethyl-bez(a) antracene, single intraperitoneal dose 200 mg/kg b.w.	blood total antioxidant capacity, total white blood cells counts ↓; impaired granulopoiesis	meat from lambs receiving selenium nanoparticles in drinking water (0.1%)	blood total antioxidant capacity (+); total white blood cells counts (±); granulopoiesis (±)	blood total antioxidant capacity ↑; intensified granulo-poiesis

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection; DMBA – 7,12-dimethylbenz(a)anthracene; GPx – glutathione peroxidase; MDA – malonyldialdehyde; ALT – alanine aminotransferase; AST – aspartate aminotransferase; LDH – lactate dehydrogenase; SOD – superoxide dismutase; ALP – alkaline phosphatase.

Table 7. Protective effect of organic selenium against toxicity of mycotoxins and substances used in agriculture

Authors, citation number	Animals, way of administration, dose, time of exposure	Induced harmful effects	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Song et al. 2014[22]	mice patulin intraperitoneally 1 mg/kg once a week for 8 weeks	brain thiol group, GPx, GR, TrxR mRNA expressions ↓; brain oxidized glutathione, ROS generation, TBARS, protein carbonyls ↑	dietary Na_2SeO_3 or selenomethionine 0.2 mg Se/kg	all studied parameters (+)	not studied
Liao et al. 2014[47]	ducklings aflatoxin B_1, intragastrically 0.1 mg/kg b.w. 14, 21, and 28 days	serum ALT and AST↑; liver Bax and caspase-3 ↑; liver Bcl-2 ↓	Na_2SeO_3 1 mg of Se/kg b.w., intragastically, 5 min after aflatoxin for 14, 21, and 28 days	serum ALT and AST(+); liver caspase-3 (+); liver Bcl-2 and Bax (±)	not studied
Chen et al. 2013[36]	broilers aflatoxin B_1, in diet 0.3 mg/kg 7, 14, and 21 days	peripheral blood T-cell subsets, serum interleukin IL-2 and interferon-γ (IFN-γ) ↓	dietary Na_2SeO_3 0.6, 0.8 and 1.0 mg Se/kg	peripheral blood T-cell subsets: low and middle doses (±), high dose (–); IL-2: low dose (±), middle dose (+), high dose (±); IFN-γ: low dose (±), middle dose (+), high dose (0)	not studied
Jebur et al. 2014[48]	rats β-cyfluthrin, oral gavage 15 mg/kg b.w. (1/25 LD_{50}) 30 days	liver TBARS, LDH ↑; liver GSH, GPx, GR, SOD, CAT, GST, AST, ALT, ALP ↓	Na_2SeO_3, oral gavage 200 µg Se/kg b.w. 30 days	TBARS, GSH, GPx, GR, SOD, CAT, LDH (+); AST, ALT, ALP (±); GST (0)	serum ALT, TBARS ↓; serum GSH, GPx, GR, CAT, GST ↑
El-Demerdash and Nasr 2014[15]	rats diazinon, oral gavage 10 mg/kg b.w. 30 days	serum TBARS, ALT, AST, ALP, LDH ↑; serum GSH, SOD, CAT, GST, GPx, GR, AChE, HDL-cholesterol ↓; serum total lipids, total cholesterol, triglycerides, LDL-cholesterol ↑	Na_2SeO_3, oral gavage 200 µg Se/kg b.w. 30 days	all studied parameters (±)	TBARS ↓; GSH, CAT, GST, GPx, GR ↑
Acker and Nogueira 2014[20]	rats acephate, oral gavage 140 mg/kg once	plasma glucose, corticosterone, triglycerides ↑; liver tyrosine aminotransferase ↑; cerebral AChE ↓	synthetic organic diphenyl diselenide, oral gavage 10 or 30 mg/kg 1 h before acephate, once	plasma glucose (±); plasma corticosterone (0); plasma triglycerides (+); cerebral AChE (0); liver tyrosine aminotransferase: lower dose (0), higher dose (±)	none

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection; (–) – intensification of harmful effects; GPx – glutathione peroxidase; GR – glutathione reductase; TrxR – thioredoxin reductase; TBARS – thiobarbituric acid-reactive substances; ALT – alanine aminotransferase; AST – aspartate aminotransferase; LDH – lactate dehydrogenase; GSH – reduced glutathione; ALP – alkaline phosphatase; SOD – superoxide dismutase; CAT – catalase; AChE – acetylcholinesterase.

activity only the low dose, and in the case of GSH only the high one, were found to be effective.[51]

In vitro experiments have also shown the efficacy of selenium nanoparticles. This form was proved to prevent DNA damage and cell death in lymphocytes exposed to UVB radiation.[52] Another study performed on cardiomyoblast H9c2 cells revealed the occurrence of changes in the antioxidant level as well as in mitochondrial functions during ischemia and reperfusion which were prevented by selenium in the form of selenium incorporated guar gum nanoparticles.[53]

Natural selenium forms have also been found to show a protective effect under in vitro conditions. Two selenium-enriched medicines of herbal origin (IMOD and Angipars) (Rose Pharmed Biotechnology Co., Iran) were studied in an experiment performed on human lymphocytes. Both substances prevented the toxicity of an organophosphorus pesticide – chlorpyrifos. The beneficial influence included the amelioration of the chlorpyrifos-induced increase in TNF-α and reduction in cell apoptosis and necrosis.[54]

Table 8. Protective effect of selenium against negative effects caused by pathological condition

Authors, citation number	Animals pathological condition	Harmful effects	Selenium form, dose, method and time of administration	Effects of selenium co-administration	Effects of Se itself
Akil et al. 2011[49]	rats acute swimming exercise after the end of experiment period, once for 30 min	plasma MDA and lactate, erythrocyte GSH, serum SOD and GPx ↑	Na_2SeO_3 200 µg/day, intraperitoneally 4 weeks	plasma MDA and lactate (±); erythrocyte GSH, serum SOD and GPx further ↑	none
Prigol et al. 2009[16]	mice acute swimming exercise after Se-treatment, for 20 min; euthanized 1 h or 24 h after exercise	euthanized after 1 h: skeletal muscle MDA and vitamin C, lung MDA and CAT ↑; euthanized after 24 h: skeletal muscle MDA and vitamin C ↑	synthetic organic diphenyl diselenide pretreatment 5 mg/kg, orally 7 days	euthanized after 1 h: skeletal muscle and lung MDA (+); skeletal muscle vitamin C, lung CAT (±); euthanized after 24 h: skeletal muscle MDA (+); skeletal muscle vitamin C (±)	after 1 h: muscle MDA ↓ after 24 h: muscle MDA and vitamin C ↓; lung vitamin C ↑
Ghazi Harsini et al. 2012[4]	chicken heat stress 4 weeks	serum glucose, uric acid and copper, skeletal muscle MDA and SOD ↑; serum iron and zinc ↓	dietary selenomethionine 0.5 or 1 mg of Se/kg	lower dose: skeletal muscle MDA, serum glucose (±); serum uric acid, copper, iron and zinc (0); skeletal muscle SOD (–) higher dose: skeletal muscle MDA, serum glucose, copper and uric acid (±); serum iron and zinc (0); skeletal muscle SOD (–)	none
Ghodbane et al. 2011[50]	rats static magnetic field 128 mT/1 h/day for the last 5 consecutive days of Se-administration	kidney and muscle GPx ↓; kidney, muscle and brain Se level ↓	Na_2SeO_3 0.2 mg/L, in drinking water for 4 weeks	kidney and muscle GPx (+); kidney, muscle and brain Se level (+)	liver and muscle GPx ↑

↓ – decrease; ↑ – increase; (+) – full protection; (±) – partial protection; (0) – lack of protection; (–) – intensification of harmful effects; MDA – malonyldialdehyde; CAT – catalase; SOD – superoxide dismutase; GPx – glutathione peroxidase; GSH – reduced glutathione.

Protective properties of selenium: Human model research

Diverse human studies have also revealed the beneficial effects of selenium supplementation.

In young children (4.4–5.4 years) in rural Bangladesh, an inverse relationship between the exposure to cadmium (evaluated by the assay of its urinary level) and glomerular filtration rate was found, particularly in girls. This effect was alleviated in subjects with higher urinary selenium, which led the authors to suggest that higher Se-status seemed to exert a protective influence against cadmium nephrotoxicity.[55]

An interesting study on 933 mother-newborn pairs, performed in China, revealed that umbilical cord serum manganese was related to the risk of lower Neonatal Behavioral Neurological Assessment rank. However, this effect was alleviated in the cases where umbilical cord serum selenium was higher. The authors suggested that selenium supplementation might be considered in pregnant women, particularly in regions of low environmental selenium level.[56]

Based on a study involving cancerous patients subjected to cisplatin therapy, Ghorbani et al. suggested that selenium might prevent the renal toxicity of the drug. They found that acute kidney failure occurred in 11.5% of patients treated with cisplatin, whereas in those pretreated with a single selenium tablet (400 mcg), no such cases were observed.[57] Similarly, Mix et al. observed some protective influence of selenium treatment (selenomethionine, 1 week before as well as during therapy) in patients with inoperable, stage III non-small cell lung cancer undergoing concurrent chemoradiation (radiation, paclitaxel and cisplatin).[23]

Conclusions

In conclusion, the presented studies make it possible to suggest that selenium seems to be one of the most appealing agents to be examined in relation to its protective role against the toxic effects induced by different harmful factors, both chemical and physical. But it must be

emphasized that its effect depends on many factors, such as its chemical form as well as the applied dose and experimental model, so supplementation must be performed taking proper precautions to obtain the best results and avoid the toxicity of selenium itself.[5,10,24,36]

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Non-suicidal self-injury (NSSI) and suicidal: Criteria differentiation

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Abstract

There are 2 types of basic self-destructive behavior: suicide and non-suicidal self-injury (NSSI). Currently, more and more researchers point out significant disorders which are NSSI behavior. This phenomenon is not new; NSSI seemingly has always been present in society, and certainly in approx. 10% of the population worldwide in recent times. Despite the enormous scale of the phenomenon, so far it has been overlooked and marginalized. They were considered transient behavior, typical of adolescence, a part of youthful rebellion. Current research indicates that the disorder affects the adult population in almost equal measure. It is only in the latest diagnostic classification – Diagnostic and Statistical Manual of Mental Disorders, Fifth edition (DSM-5) by American Psychiatric Association – that has considered NSSI a separate class of behavior. Up to now, it was classified as a prelude to suicide or an element of personality disorders. NSSI is more commonly associated with disturbing behavior and suicide attempts.

Key words: non-suicidal self-injury, suicide, suicide attempt

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Introduction

There are 2 types of basic auto-destructive behavior: suicide and non-suicidal self-injury (NSSI). There are a number of distinguishing criteria, but the main one is the intention of death.

According to World Health Organization (WHO), suicide is a multidimensional phenomenon, resulting from the interaction between biological, psychological, genetic, and environmental factors.[1] According to the definition, suicide is the deliberate termination of life, and legal definition is extended with the statement that the death is a result of the direct or indirect action or negligence of the victim, who fully realizes the effect of their actions.[2]

NSSI has been defined by the International Society for the Study of NSSI as the deliberate, self-inflicted destruction of body tissue without suicidal intent and for purposes not socially sanctioned. NSSI can be divided due to its features and forms. The features are positive reinforcement, addition of desired stimulus, or negative reinforcement or subtraction of unwanted stimulus. NSSI is expressed in various forms from relatively mild, such as scratching, plucking hair or interfering with wound healing, to relatively severe forms, such as cutting, burning or hitting.[3]

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), published in the year 2013,[4] qualifies NSSI as a separate entity, among the disorders requiring further research. The proposed criteria for DSM-5 include the following:

- intentional self-inflicted injury performed with the expectation of physical harm, but without suicidal intent, on 5 or more days in the past year;
- the behavior is performed for at least 1 of the following reasons:
  - to relieve negative thoughts or feelings;
  - to resolve an interpersonal problem;
  - to cause a positive feeling or emotion;
- the behavior is associated with at least 1 of the following:
  - negative thoughts or feelings, or interpersonal problems that occur immediately prior to engaging in NSSI;
  - preoccupation with NSSI that is difficult to resist;
  - frequent urge to engage in NSSI;
- the behavior is not socially sanctioned and is more significant than nail biting or picking at a scab;
- the behavior causes clinically significant distress or impairment;
- the behavior does not occur exclusively in the context of another disorder and cannot be accounted for by another mental or medical disorder.

Objectives

The aim of the study was to differentiate between the self-destructive behaviors NSSI and suicide.

Methods

Using the Scopus, PubMed, EBSCO, Google Scholar, and Web of Science databases, medical literature on the topics of suicide and NSSI were searched and 12 differentiation criteria were created between the self-destructive behaviors suicide and NSSI.

Results

NSSI is commonly although incorrectly called suicide attempts. This misuse of the term occurs just as frequently in clinical practice and entails improper treatment of the patient. Therefore, it is important to tell these phenomena apart. So far in Polish literature, there have been no articles on the distinction between these behaviors. Below there are the 12 determinants by means of which NSSI can be distinguished from suicide attempts.

Table 1 explains the differences between a suicide attempt and NSSI (author's own work, after Walsh, 2006).

The fundamental criterion for distinguishing between non-suicidal self-destructive behaviors and suicide is the intention of death.[5] According to Shneidman, suicide involves not so much the desire to kill the body, but the wish to end one's own consciousness.[6] NSSI, in turn, has 2 functions. One of them is negative reinforcement and removing unwanted stimuli, i.e., dismissing unpleasant emotional states.[5,6] The most common categories of unpleasant emotional states declared by NSSI are fear, sadness, shame or guilt, tension, anxiety or panic, frustration, and contempt. The share and severity of these emotions varies individually.[6] The other function of NSSI is positive reinforcement – to boost a desired stimulus, i.e., to provide stimulation by experiencing strong emotions and get rid of the feeling of emptiness, which takes place in the vast minority of cases.[7]

Babikier and Armond suggest a different division of functions of NSSI: functions related with managing and surviving, functions related with ego and proper experience, and functions connected with punishing oneself and being a victim.[8] NSSI is a way of coping and it is a means of surviving, as it reduces tension and fear, helps to manage anger and it is applied to avoid or focus on pain in order to control it. An individual copes with unbearable feelings to distract oneself from fear and tension, and to pay attention to other, more attainable behaviors, such as self-harm. The functions of NSSI related with ego include reinforcement of a sense of control, intensification of a sense of reality and breaking the states of dissociation. The pain that accompanies NSSI is often an important element of the process of regaining the sense of self-control and integration of an individual. Another function of NSSI is creating opportunities to take care of oneself. In some individuals, the period following NSSI is the only moment when they allow themselves to feel relieved and experience physical care. On the other hand, coping with one's

Table 1. Criteria for differentiation of direct self-destructive behaviors

Criterion	Suicide	NSSI
Primary intention	intention to die	preservation of life, destruction of the body
General intention	escape from mental pain and consciousness	escape from mental pain and transformation into physical pain
Functions	reduction of tension, a sense of relief	managing and surviving (e.g., reduction of tension and fear), functions connected with ego, functions connected with proper experience, punishing oneself, providing stimulation
Potential death	high degree of mortality	low degree of mortality
Number of methods used	usually one	usually many (the number increases over time)
Chronicity	rarely	often
Mental pain	persistent, unbearable	discontinuous
Cognitive narrowing	high, suicide seen as the only solution	low or none
Hopelessness and helplessness	constant, occupies a central place	variable, does not dominate
Consequences in terms of the recognition of discomfort	discomfort intensifies after suicide attempt	discomfort decreases after NSSI
Associated disorders	psychotic disorders (schizophrenia), affective disorders, alcohol abuse, anxiety disorders	personality disorders, addictions, eating disorders, posttraumatic stress disorder
Main issue	depression, mental pain unbearable	negative image of ego

experience is expressed by experiencing again the feelings and traumas which had been denied previously, as well as by manifesting toward oneself their own experiences. Punishing oneself is also a significant function of NSSI, most frequently related with a negative image of oneself created in the period of childhood or under the influence of a traumatic experience.[8]

NSSI and suicide attempts also differ by their mortality rate, which results from the nature of the forms of self-destruction. The most common methods of suicide in Poland are hanging oneself – 75.04%, jumping from a height – 7.11%, poisoning – 4.49%, and jumping in front of a vehicle – 2.27%.[9] Suicide attempts are fatal in 10–20% of all cases, while other self-destructive behavior ends in death in 0.6% of cases and are most often caused by cutting an artery.[5,10,11] However, according to Whitlock et al., NSSI usually takes the form of: scraping the skin – 51.6%; beating – 37.6%; cutting – 33.7%; bruising – 24.5%; cutting symbols in the skin – 14.9%; scratching wounds – 13.5%; piercing with sharp objects – 12%; and plucking hair – 11%.[12] A general review of the literature shows that the most common methods of NSSI include cutting the skin, hitting oneself, scratching wounds, bruising, and biting.[6,8] Thus, suicide and NSSI involve different methods.

Another point of distinction between self-harm behavior is the number of methods used. Those making suicide attempts use the same method when making another suicide attempt.[13] Most people repeatedly attempt suicide by drug overdose.[6] In contrast, 78% of people who practice non-lethal self-injurious behavior use more than 1 method.[14] The use of more methods is related mainly to preferences. Many people using NSSI say they use a variety of methods depending on their mood. For example, some people engaged in NSSI claim that they cut their skin when they are emotionally wrecked, and hit themselves when experiencing nervousness. Others cut their skin when experiencing anxiety and burn themselves under nervousness. The scope of relations between the form of self-harm and the type of emotion is nearly infinite.[15]

Another issue that tells these types of behavior apart is the frequency of their occurrence. NSSI episodes in one person take place much more often than suicide attempts. Most people attempting suicide do it neither frequently nor repeatedly. Suicide attempts are usually made once or twice in a lifetime, in one's most stressful period of life.[15] However, the number of NSSI episodes is approx. 20–100 times over the course of several years.[11] The frequency of NSSI among teenagers may reach up to 20–30 episodes per year.[5]

The differences between the types of aggressive behavior are also seen in the level of psychological pain, which, like cognitive narrowing, is higher for suicidal behavior than NSSI.[5] According to Ringel's concept, around 80% of suicides are related to the narrowing of consciousness, which, apart from anger inhibition and directing it at oneself and suicidal thoughts, is an element of pre-suicidal syndrome. The narrowing of consciousness occurs when an individual does not perceive alternative forms of solving a problem (tunnel vision). Suicide attempts are preceded by dichotomous thinking, where the alternative is death. Hopelessness and helplessness in the face of mental pain are more often experienced by future suicides than NSSI, who do not declare their lack of control over pain; on the contrary, NSSI helps them maintain a sense of control.[16] NSSI is not characterized by dichotomous thinking. People engaged in NSSI are usually rather disorganized than limited in terms of their way of thinking. They do not limit their life to all-or-nothing attitudes. They consider

themselves to be capable to make life choices. One of those choices is the decision to harm themselves.

Research on suicide has long identified a sense of both hopelessness and helplessness as essential component of depression and suicidal behavior.[17–19] The feeling of helplessness, which involves the lack of hope, refers to the loss of control.[18] People who feel helpless believe that they have no real influence or real control over their situation. They are convinced that there is nothing they could do to change or improve their lives. Such cognitive pessimism is very characteristic of "surrender", which is part of suicide. The feeling of helplessness of people committing suicide is well illustrated by Beck's triad of depression.[17] In this perspective, people with suicidal tendencies think: "I am angry, the world is a hostile place and the future is unchangeable."

On the other hand, helplessness and hopelessness are not characteristic of people hurting themselves. Such people usually do not have a sense of lack of control over their physiological pain, which de facto conditions this sense of control. Control resulting from NSSI is the opposite of hopelessness. The future is not seen as a great suffering, because NSSI reduces emotional tension. An individual's psychological discomfort and crippling pessimism are temporary. Their suffering lacks a sense of permanence, which is typical of a suicidal crisis.

Muehlenkamp and Gutierrez made a comparative study of teenagers engaged in NSSI.[20] Their research showed that people engaged in self-injury without suicidal intentions were characterized by a lower rate of hopelessness, stronger future-orientation, more developed motivation to live as compared to people undertaking a suicide attempt. These results confirm the hypothesis that the level of emotional pain varies depending on the type of self-destructive behavior.

The difference is also observed in terms of psychological consequences. After a failed suicide attempt, a person usually experiences a worsening of mood, the feeling of failure associated with the fact they failed to commit suicide successfully. NSSI, on the other hand, is done to reduce tension, and as a result, a person usually experiences relief and an improved frame of mind.[5]

Research indicates that some disorders accompany suicide attempts, while others are associated with NSSI. Approximately 90% percent of suicides are associated with at least 1 psychiatric disorder.[21] The risk of committing suicide over a lifetime is 30.2% for the general population of the mentally ill.[2,21] These are mainly depressive disorders, addictions, cluster B personality disorders, and schizophrenia.[24] Schizophrenia is associated with the highest elevated risk of suicide. About 40–50% of schizophrenia patients have suicidal thoughts at some point in life, and 4–13% commit suicide, making it a leading cause of premature death among patients.[23] In addition, the risk of a suicide attempt is increased by 30% in those patients suffering from schizophrenia who have symptoms of depression.[24] Depressive disorders and alcohol abuse are placed second and third, respectively.[1] Suicidal behaviors are also largely related to anxiety disorders. The risk of suicide is further increased if anxiety and personality disorders are observed collectively.[25] Other important disorders associated with suicide are affective disorders. The risk of suicide in these patients is around 30–40%.[26] Throughout the course of life, suicidal behavior affects an average of 4% of people with mood disorders and 8% of those with bipolar disorder.[1]

By contrast, NSSI are often linked to destructive mental disorders, such as alcohol and drug abuse, eating disorders and personality disorders, and are observed in patients suffering from posttraumatic stress disorder (PTSD).[27,28] Up to 79% of those suffering have experienced violence or rejection in childhood.[28] Up to 50% of cases of self-destructive behaviors are associated with alcohol abuse.[1] In the context of substance abuse and addictions related to self-destructive behaviors, in its therapy it is necessary in the first place to deal with the substance abuse, which is the base for other self-destructive behaviors. The next step is to deal with the self-harm which derives from the addictions and substance abuse. Amongst the aforementioned behaviors, addictions and substance abuse are the easiest to overcome, which is caused by the fact that for a self-harming individual it is hard to function without self-harm, and at the same time self-harm seems the mildest means of self-destruction that enables the individual's existence.[10]

The source of the problems of people with suicidal tendencies fluctuates around depression, sadness and rage due to the primary source of pain. Maltsberger showed that suicides are caused not only by sadness, isolation and loneliness, but also include an element of "murderous hatred".[29] This hatred is directed both inward and outward. Help for those making suicide attempts is to rely on finding and reducing the original source of pain. Shneidman emphasizes that the task of the therapist is to add a third component to the dichotomous thinking in people with suicidal tendencies, one that reduces the risk of a suicide attempt.[6] Finding the source of unbearable suffering should be the first element in working with people at risk of suicide. The more accurate the clarification of the source, the more efficient the therapeutic work.

In contrast, research shows that the source of the problem in NSSI is a distorted body image.[6] The feeling of being cut off from the body or hatred toward the body leads to NSSI. The key question in the treatment of the source of self-injury should be: "What are the sources of such a relationship with one's body?" and "Why do you keep trying to inflict harm on your body?"

Summary and discussion

As it has been shown, suicides and NSSI differ in many respects. These differences are primarily qualitative. These behaviors are caused by other factors, they have a different intent and serve different functions. NSSI and those making a suicide attempt also suffer different consequences and psychological after-effects.[5] One should also pay attention to the coexistence of these self-destructive behaviors; NSSI often precedes a suicide attempt, as the individual embraces the notion of self-destruction, to start later using more and more destructive methods. NSSI youths are 3 times more likely to experience suicidal thoughts and attempt suicide.[11] Long-term NSSI often precedes suicide, even though the individual showed no intention of death at first.[30]

Recent years have brought a lot to the understanding of NSSI. Both NSSI and suicide attempts are phenomena of enormous magnitude. NSSI involves 7–14% of the population worldwide[6] and applies to 15–28% of young people,[27,30] and on average begins between 12 and 14 years of age.[31,32] However, according to WHO data, suicide is one of the 20 most common causes of death among the total population and represents one of the most common causes of death among young people.[33] Further research on the various types of self-destructive behavior is needed for a full understanding of the problem and to determine the appropriate directions of therapeutic work. Incorrect diagnosis of self-destructive behavior can cause inefficient therapeutic work, and even intensify the severity of the disorder occurrence.

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Review

Role of miR-181a in the process of apoptosis of multiple malignant tumors: A literature review

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Abstract

It has been recognized that miR-181a expression is dysregulated and intimately associated with clinical prognosis in a variety of human cancers. However, the direct role of miR-181a in tumor progression has been elusive. Moreover, mounting evidence has demonstrated that cellular apoptosis, a physiological process of programmed cell death, is disrupted in various categories of human malignancies. Multiple apoptosis-related genes have been proven to act as the target genes of miR-181a. In this study, we hypothesize that miR-181a probably plays a potential role in modulating the procession and apoptosis of cancer cells. We performed a literature review and elucidated how miR-181a modulated cellular apoptosis, especially the malignant neoplasm cells. We also unraveled the potential role of miR-181a in the diagnosis, treatment and clinical prognosis of multiple human malignancies – miR-181a plays a pivotal role in the development, treatment and prognosis of patients suffering from malignant tumors. It also participates in the development of cancer partially by modulating cellular apoptosis.

Key words: miR-181a, apoptosis, Bcl-2 family, P53, PRKCD

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Introduction

MicroRNAs (miRNAs) are defined as a class of highly-conserved, small non-coding RNA (22 nucleotides on average), which function to modulate gene expression at the posttranscriptional level by binding to the 3'-untranslated region (3'-UTR) of target genes. Previous investigations have demonstrated that the regulatory role of miRNAs is involved in multiple cellular processes, including cellular metabolism, proliferation, differentiation, development, and apoptosis. Aberrant expression of miRNAs contributes to the incidence and progression of malignant tumors, whereas the precise role of miRNAs in the development of malignant neoplasms remains elusive.[1] As one of multiple conserved miRNAs among vertebrates, miR-181a has been confirmed to be differentially expressed in a variety of diseases, especially various cancers, and plays a critical role in the occurrence and development of malignant neoplasms.

Apoptosis is a physiological process of programmed cell death that is essential for normal tissue development and homeostasis, through which damaged, unattached, mutant, and aged cells are eliminated. Mounting evidence has demonstrated that miR-181a is capable of modulating cellular apoptosis by targeting several apoptosis-related genes, probably related to the underlying mechanism of the role of miR-181a involved in tumor progression. Aberrations in the signaling pathway can lead to degenerative and autoimmune disorders, and even cancer.[2] This paper reviews the mechanism underlying the role of miR181-a involved in cellular apoptosis and investigates the correlation between miR-181a and varying human malignancies.

Apoptosis

Since apoptosis was first described in 1842 by Carl Vogt, accumulated studies have been performed to deepen the understanding of cellular apoptosis. As one of 2 major types of cell death, apoptosis is a highly-regulated process with specific and well-described morphological changes, and is critical for many physiological processes, including cell development, proliferation, differentiation, immune regulation, and eliminating defective and harmful cells. Dysfunction of apoptosis is central to multiple pathological states. For instance, enhanced apoptosis has been described in neurodegenerative diseases, e.g., acquired immunodeficiency syndrome (AIDS), transplant rejection and heart failure, whereas diminished apoptosis has been observed in autoimmune diseases, such as viral infections and even cancers.[3–7]

Currently, cancer is one of the major causes of a poor quality of life and human death. Common anticancer therapies, such as chemo- and radiotherapy, mainly induce the apoptosis of cancer cells by administration of cytotoxic agents. In 2014, Gong et al. demonstrated that Nexrutine® treatment could inhibit the growth of pancreatic cancer cells through induction of apoptosis.[8] Targeting components of the apoptotic pathway has been regarded as a therapeutic approach for cancers, because aberrant apoptosis is central to the growth of malignant tumors and the resistance to anticancer therapies. Failures in normal apoptotic pathways contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations. In turn, tumor cells employ a variety of molecular mechanisms to suppress apoptosis, thus establishing a tumor/cancer-promoting loop.[9]

With deepening the understanding of the relationship between apoptosis and cancer, researchers have recently found that aberrant expression of miRNAs in tumor cells results in the dysfunction of cellular apoptosis. For instance, miR-106a overexpression significantly aggravated cellular apoptosis induced by cisplatin in ovarian cancer A2780 cells through downregulating the expression of antiapoptotic protein Mcl-1.[10] Additionally, Ribeiro et al. summarized the function of human miRNAs in carcinogenesis and target genes.[11] The let-7 family acts as a tumor suppressor by targeting c-Myc. It has been proven that the miR-15-16 cluster is an oncogene by interacting with Bcl-2. Interestingly, miR-125b plays a dual role, serving as an oncogene by regulating p53 and Bak-1 expression, whereas acting as a tumor suppressor during interaction with Bcl-2, Mcl-1, Bcl-w, etc. All these miRNA targets, including Bcl-2, Bcl-w, Bak-1, and Mcl-1, are capable of encoding the proteins associated with cellular apoptosis. Consequently, apoptosis dysfunction is implicated in the progression of malignant tumors. Especially in cancer cells, miRNAs are potentially capable of regulating the process of cellular apoptosis.

miR-181a and cancer

As a member of the miR-181 family, miRNA-181a has been intensively studied; miRNA-181a is located on the chromosome 1 (37.p5) and miR-181a2 is situated on the chromosome 9 (37.p5). Similar to other miRNAs, miR-181a plays a pivotal role in many cellular processes, such as cell fate determination and cellular invasion.[12] It has been discovered to be abnormally expressed in solid malignant tumors and hematological malignancies. A significant downregulation of miR-181a level has been detected in squamous lung cell carcinoma, oral squamous cell carcinoma, salivary adenoid cystic carcinoma, acute myeloid leukemia, and non-small cell lung cancer, whereas evident overexpression of miR-181a level has been found in MCF-7 breast cancers, multiple myeloma, pancreatic and gastric cancer, and hepatocellular carcinoma.[13–22] All these investigations have demonstrated that aberrant expression of miR-181a contributes to the development and metastasis of malignant tumors. In patients diagnosed with

colorectal cancer, miR-181a overexpression promotes CRC cell growth, invasion and liver metastasis by targeting the 3'-UTR of tumor suppressor gene *WIF1*.[23] Additionally, upregulated expression of miRNA-181a can induce carcinogenesis by targeting E2F5 in hepatocellular carcinoma evolved from chronic hepatitis B.[24]

Additionally, it has been revealed that high expression of miR-181a is associated with short recurrence time and poor outcome in patients with epithelial ovarian cancer.[25] In a meta-analysis investigation, Lin et al. suggested that low expression of miR-181a/b is significantly associated with poor survival in patients with hematological malignancies.[26]

Decades of research have indicated that miR-181a plays a critical role in tumorigenesis and cancer progression, whereas the mechanism underling this function remains to be unraveled. Some scholars have indicated that, as a regulator of tumor phenotype, miR-181a is involved in tumorigenesis and downstream malignant processes through the ability to modulate the expression of critical genes and signaling networks which are mainly involved with regulating cell apoptosis.[25] Hence, the influence on apoptosis is believed to be a vital mechanism for miR-181a to participate in tumor progression and development. In this review, the mechanisms underlying the role of miR-181a in cancer cellular apoptosis were retrospectively analyzed, and the correlation between miR-181a and the diagnosis, treatment and prognosis of malignant tumors was also briefly discussed.

miR-181a modulates apoptosis by targeting apoptosis-related genes

To mediate cellular apoptosis, 2 signaling pathways have been accepted: extrinsic and intrinsic.[27] The extrinsic pathway of apoptosis is mediated by ligands activating death receptors, such as APO2L/TRAIL, FasL(Fasl/APOI/CD95), TNF(TNFR1), etc. The intrinsic pathway of apoptosis is initiated in the mitochondria, and tightly regulated by the balance between pro-apoptotic (e.g., Bcl-1, Bax, Bim, and P53) and anti-apoptotic genes (e.g., Bcl-1 and Mcl-1). Caspases, such as cysteine aspartic acid specific protease, serve as the central regulatory proteins in both extrinsic and intrinsic signaling pathways. Aberrant expression of regulatory genes results in deregulation of these pathways and subsequently leads to the incidence of multiple diseases. Recent research has verified that miR-181a could modulate cell apoptosis by targeting the apoptosis-related genes, including Bcl-2 family, *P53*, *ATM*, *PRKCD*, *PBX3*, and *RALA* (Table 1).

Table 1. Target genes and effect of miR-181a upon cellular apoptosis

Target	Relationship	Tissues	Activity
Bcl-2	negative	breast cancer cell[30]	enhance
		malignant glioma cell[32]	
		CLL cell[34]	
		AML cell[35]	
		prostate cancer cell[37]	
	positive	osteosarcoma cell[31]	inhibit
Mcl-1	negative	CLL cell[40]	enhance
Bax	positive	NSCLC cell[41]	enhance
Bim	negative	lymphoma cell[42]	inhibit
P53	positive	myeloma cell[19]	enhance
		CLL cell[40]	
ATM	negative	gastric cancer cell[44]	inhibit
PRKCD	negative	squamous cervical carcinoma cell[48,49]	inhibit
PBX3	negative	acute myelogenous leukemia[51]	enhance
RalA	negative	chronic myelogenous leukemia[55]	enhance

Bcl-2 gene family

The Bcl-2 gene family plays a pivotal role in the regulation of apoptosis via the mitochondrial pathway. The proteins of the Bcl-2 family are encoded, and consist of approx. 20 pro- and anti-apoptotic proteins falling into 3 groups. Anti-apoptotic multidomain members, such as Bcl-2, Mcl-1 and Bcl-w, contain BH1-4 domains, whereas pro-apoptotic multidomain proteins, including the Bax subfamily, contain BH1-3 domains. The BH3-only group, such as Bim, is proven to be pro-apoptotic. These proteins contribute to cellular apoptosis by regulating mitochondrial permeability, fission and fusion. Recent investigations have demonstrated that these proteins play a role in cellular homeostasis with respect to metabolism, calcium signaling, endoplasmic reticulum function, and autophagy.[28,29]

B-cell lymphoma 2

B-cell lymphoma 2 (Bcl-2), encoded by the *Bcl2* gene in humans, is the founding member of the Bcl-2 family. It has been recognized that miR-181a can suppress Bcl-2 expression by targeting the 3'-UTR of the *Bcl-2* gene.[30] In spite of the negative relationship, western blotting has shown that the upregulation of Bcl-2 in MG63 cells is associated with the overexpression of miR-181a.[31] In conclusion, miR-181a contributes to inducin gand suppressing apoptosis via interaction with the *Bcl-2* gene.

Chen et al. suggested that miR181a sensitizes human malignant glioma cells to radiation by targeting Bcl-2.[32] MTT assay detected that cell growth rate was significantly reduced in miR-181a overexpressed cells after 18.8 Gy irradiation. Furthermore, they proved that the upregulation of miR-181a (exogenous miR-181a expression) resulted in a decrease in the expression of Bcl-2 protein. Khanna et al.proposed that overexpression of miR-34a, miR-30e and miR-181a could

increase the rate of apoptosis, accompanied by a decline in Bcl-2 expression in miRNA-transfected mouse models.[33] Li et al. constructed a luciferase reporter vector and identified a target site of miR-181a in the *BCL*-2 3′ UTR.[34] K562 cells transfected with an miR-181a inhibitor had significantly higher survival compared to normal K562 cells, and K562/A02 cells transfected with a miR-181a mimic had a significantly lower survival than non-transfected K562/A02 cells, implying that miR181a can decrease the survival rate of the CLL cells treated by daunorubicin via targeting Bcl-2, regardless of whether the CLL cells are sensitive or resistant to daunorubicin. Bcl-2 was confirmed as a direct miR-181a target by immunoblot analysis and reporter gene assays. Bai et al. found that the Bcl-2 3′UTR contains a highly-conserved 8mer site complementary to the seed region of the miR-181a.[35] Moreover, they demonstrated that miR-181a can intensify caspase-dependent cell death through Bcl-2 in AML cells. Ouyang et al. established a mouse model and found that, compared to control cells, elevated expression of miR-181a reduced cell survival by 31%, whereas knockdown of endogenous miR-181a levels increased survival by 27%.[36] Moreover, the downregulated levels of miR-181a are associated with reduced cell death and oxidative stress, and preserved mitochondrial function in astrocytes via upregulating Bcl-2 protein level. Zhai et al. observed that the expression levels of miR-181a in PC-3 prostate cancer cells were upregulated after bufalin treatment, indicating that inhibition of miR-181a activity could attenuate bufalin-induced apoptosis in PC-3 cells.[37] Furthermore, miR-181a inhibitor can reverse the reduction of bufalin-induced Bcl-2, whereas miR-181a transfection was shown to downregulate the expression of Bcl-2 significantly, suggesting that induced miR-181a mediates downstream bufalin-induced apoptosis by repressing Bcl-2 protein in PC-3 cells. Moon et al. confirmed that N2a cells subjected to serum deprivation and oxidative stress yielded less cell death when the expression of miR-181a was downregulated, whereas it aggravated cell death at the upregulation of miR-181a levels.[38]

Myeloid cell leukemia-1

Myeloid cell leukemia-1 (Mcl-1) is the second member of the Bcl-2 family, which directly interacts with the BH3 alpha-helical domain of pro-apoptotic proteins, such as Bax, Bak, Bad, and Bim, and inhibits their functions.[39] Ouyang et al. and Zhu et al. revealed that Bcl-1 is not only a direct target of miR-181a, but also miR-181a could enhance the cell apoptosis via negative interaction with Mcl-1.[36,40]

Bax

As an apoptosis regulator, Bax, also known as Bcl-2-like protein 4, is a human protein encoded by the *Bax* gene. *Bax* is a member of the Bcl-2 gene family and promotes apoptosis by binding to and antagonizing the Bcl-2 protein.Galluzzi et al. suggested that miR-181a is capable of sensitizing NSCLC A549 cells to the lethal action of CDDP, carboplatin and oxaliplatin by stimulating Bax oligomerization, and the activation of pro-apoptotic caspases.[41] Khanna et al. observed that the miR-34a, miR-30e and miR-181a not only modulate Bcl-2 expression, but downregulate the expression of pro-apoptosis genes, such as *Bax*, and cleavage of caspases, and then gain neuronal survival in the brain of calorie-restricted mice.[33] In brief, the positive relationship between miR-181a and Bax validates that miR-181a plays a pro-apoptotic role in both human and mouse cells.

Bim

Bim, as a member of the BH3-only family and Bcl-a protein family, contains only 1 single BH-domain. Bim plays a key role in promoting apoptosis. Lwin et al. demonstrated that adhesion of mantle cell lymphoma and other non-Hodgkin lymphoma cells to follicular dendritic cells reduced cell apoptosis and was associated with downregulated levels of Bim.[42] Moreover, cell adhesion is capable of upregulating the expression of miR-181a; miR-181a overexpression decreased, whereas miR-181a inhibition increased Bim levels by directly targeting Bim. These results imply that miR-181a acts as a negative effector of the Bim-apoptosis signaling pathway.

Tumor protein p53

Tumor protein p53 (p53) is a transcription factor that activates or represses the expression of multiple genes. Numerous studies have established that p53 promotes apoptosis by transcriptionally activating or repressing the expression of a panel of pro- and anti-apoptotic proteins. Additionally, activation of p53-dependent apoptosis leads to mitochondrial apoptotic changes via both intrinsic and extrinsic pathways, triggering cell death notably by the release of cytochrome c and activation of caspase cascade.[43]

Pichiorri et al. demonstrated that the miR-181a targets p300-CBP-associated factor and through p53 indirectly controls p53 activity in myeloma, and functions as a positive regulator of p53.[1x9] Furthermore, Zhu et al. revealed that chronic lymphocytic leukemia cells transfected with miR-181a from p53 wild-type patients led to a significant increase in apoptosis, compared to miRNA controls.[40] However, enforced expression of miR-181a exerted no effect on B-CLL cells from p53-attenuated patients, implying that miR-181a can enhance cellular apoptosis by targeting p53.

Ataxia telangiectasia mutated

Ataxia telangiectasia mutated (ATM) is a serine/threonineprotein kinase that is recruited and activated by DNA double-strand breaks. ATM phosphorylates several

key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. Several of these targets, including p53, CHK2 and H2AX, are tumor suppressors. Zhang et al. demonstrated that ATM is a direct target of miR-181a, miR-181a mimics transfection downregulating the expression of ATM at both mRNA and protein levels.[44] Additionally, compared to negative control and blank groups, transfection of miR-181a inhibitor is capable of inhibiting proliferation, invasion and migration, while promoting the apoptosis of SGC7901 cells. This data collectively indicates that overexpression of miR-181a promotes the proliferation, while suppressing the apoptosis of gastric cancer cells through directly targeting ATM.

Protein kinase C delta type

Protein kinase C delta type (PKC-δ), an enzyme encoded by the *PRKCD* gene, acts as a substrate for caspase-3. A series of studies have shown that *PRKCD* activity is required for apoptosis induced by DNA damaging agents, such as cisplatin, etoposide, cytosine arabinoside, mitomycin C, and doxorubicin. The regulating effect of PRKCD upon cellular apoptosis is highly complex. PKC-δ participates in oxLDL-induced endoplasmic reticulum stress-dependent apoptotic signaling through the IRE1α/JNK pathway.[45] Moreover, PKC-δ plays a crucial role in the propagation of TNFα-induced endoplasmic reticulum stress-mediated JNK activation and CHOP/GADD53 induction.[46] Recent research has revealed that the expression of *PRKCD* is modulated by miR-181a. Bergman et al. have demonstrated that the *PRKCD* gene is a direct target of miR-181a in a rat model of multiple sclerosis.[47] Ke et al. and Chen et al. found that miR-181a could inhibit the irradiation- and cisplatin-induced apoptosis of human squamous cervical carcinoma cells via downregulating the expression levels of *PRKCD*.[48, 49]

PBX3

PBX3, a HOXA cofactor gene, can encode pre-B-cell leukemia transcription factor 3, which is capable of regulating the transcription of downstream targets by forming stable heterocomplexes with HOX and MEIS proteins. In human leukemic cells carrying *MLL* rearrangements, ectopic expression of miR-495 significantly inhibits cell viability and increases cell apoptosis via directly targeting the *PBX3* gene, implying that PBX3 could regulate cellular apoptosis, but the underling mechanism remains elusive.[50] Li et al. demonstrated that miR-181a possesses the same function as miR-495.[51] Upregulated expression of miR-181a significantly promotes cellular apoptosis, inhibits the viability and proliferation of leukemic cells, and delays leukemogenesis by downregulating the endogenous expression of *PBX3* at both the RNA and protein levels. The effect of miR-181a upon the growth and apoptosis of leukemic cells depends on the PBX3 signaling pathway.

RalA

RalA, as an important effector of Ras, is proven to be involved in tumorigenesis and cancer invasion, and overactivated in multiple human cancers, such as malignant peripheral nerve sheath tumor, non-small cell lung cancer and chronic myeloid leukemia.[52] Male et al. demonstrated that the proliferation and invasiveness of A549 cells were reduced upon silencing RalA, whereas apoptosis and necrosis were enhanced in such conditions in non-small cell lung cancer cell lines.[53] Zhu et al. investigated that siRNA RalA, used to reduce RalA protein level in K562 and KCL-22 cells, effectively inhibited cell viability by significantly increasing caspase 3 activity, and CML cells transfected with siRNA RalA acquired typical features of apoptosis, including nuclear pyknosis, fragmentation and apoptotic body at 48-h post-transfection.[54] This evidence validated that downregulation of RalA can induce cell apoptosis. Fei et al. demonstrated that miR-181a could downregulate the expression of RalA.[55] The dual-luciferase reporter and western blot assays confirmed that RalA contains a miR-181a binding site at its 3'-UTR and is directly regulated by miR-181a. Additionally, immunoblot and RT-PCR revealed that overexpression of miR-181a significantly downregulates the expression level of RalA mRNA, subsequently suppresses cell growth, and eventually induces G2-phase arrest and apoptosis in leukemia K562 cells.

Programmed cell death 4

The programmed cell death 4 (PDCD4) gene functions to encode a protein localized within the nucleus in proliferating cells. The product of PDCD4 is thought to play a role in apoptosis, but the specific role has not been determined. Previous investigations have indicated that PDCD4 protein was downregulated in HCC tissues, and Huh7 cells transfected with PDCD4 resulted in upregulated expression of cytosolic cytochrome complex (cyt *c*) and mitochondrial Bax accompanied by downregulated levels of mitochondrial cyt *c* and cytosolic Bax. Furthermore, a slight reduction of procaspase-8, and a significant reduction of procaspase-9 and procaspase-3 were observed after PDCD4 transfection. These results indicate that PDCD4 might induce apoptosis via mitochondria events and caspase cascade. Additionally, the *PDCD4* gene is originally identified as a tumor-related gene in humans and acts as a tumor suppressor in mouse epidermal carcinoma cells, prompting that the suppressing effect of *PDCD4* upon malignant tumors could be regulated by miR-21 and miR-106a.[56–58] Daisuke et al. demonstrated that *PDCD4* is a target gene of miR-181a, and the increased miR-181a levels were

significantly associated with shortened disease-free survival and overall survival of breast cancer patients, whereas low expression of *PDCD4* was significantly correlated with poorer disease-free survival.[59] However, no statistical significance was observed between *PDCD4* gene expression and accumulation of miR-181a. The regulating effect upon cellular apoptosis is complex, and deeper understanding of the relationship between miR-181a and *PDCD4* and other apoptosis-related genes is urgently required.

Conclusions

The mechanism underlying the role of miRNAs involved in the pathogenesis of malignant neoplasm remains an emphasis. Malignant neoplasm represents a broad group of diseases involving unregulated cell growth, implying that abnormal cellular apoptosis plays a pivotal role in the development of malignancies.

In this paper, we conducted a literature review to elucidate how miR-181a modulates cellular apoptosis. Specifically, miR-181a modulates apoptosis by targeting the apoptosis-related genes. The enhancing/inhibiting apoptosis balance is probably explained by the direct interaction between miR-181a and alternative apoptosis-related genes. It has been recognized that miR-181a is capable of enhancing apoptosis of cells, particularly in malignant tumor cells, by targeting p53, Bax, Bcl-2, PBX3, and RalA, while suppressing apoptosis by interacting with PRKCD, ATM, Bim, and Bcl-2.

Depending on the different functions involved in the apoptotic process and aberrant expression in cancer cells, miR-181a could dually act as oncogene and tumor suppressors, as illustrated in Table 2. Despite the fact that miR-181a is involved in cancer development, overexpression of miR-181a sensitizes cancer cells to drugs and radiation via targeting apoptosis-related genes.[29,31,36,42] Additionally, miR-181a contributes to the bufalin-induced apoptosis and cisplatin-induced apoptosis of cancer cells.[35,38] Based on the role of miR-181a in chemotherapy or radiotherapy, miR-181a might be a potential target for the treatment of varying cancers. The predictive role miR-181a plays in epithelial ovarian cancer and hematological malignancies verifies that miR-181a levels could be used as a potential prognostic biomarker predicting the clinical prognosis of cancer patients.

Taken together, miR-181a plays a pivotal role in the development, treatment and prognosis of patients suffering from malignant tumors. It participates in the development of cancer partially by modulating cellular apoptosis. Nevertheless, the exact role of miR-181a in different malignancies remains to be elucidated. If miR-181a could serve as a clinical parameter, prognostic biomarkers in different types of cancer should be subsequently validated by clinical investigations.

Table 2. Relationship between miR-181a and development and apoptosis of malignant tumors

Expression of miR-181a	Tissues	Regulation on apoptosis process	Activity
Reduced	malignant glioma cell[32]	enhance	oncogene
	CLL cell[34,40]		
	AML cell[16,52,51]		
	CML cell[55]		
	prostate cancer cell[37,60]		
	NSCLC cell[15,41]		
Increased	osteosarcoma cell[31]	inhibit	oncogene
	lymphoma cell[42]		
	gastric cancer cell[21,44]		
	squamous cervical carcinoma cell[48,49]		
	breast cancer cell [8,30]	enhance	tumor suppressor
	myeloma cell[19]		

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The role of hypoxia-inducible factors in leukemias

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Abstract

Hypoxia, understood as low partial oxygen pressure, has become one of the most explored fields in recent years. Cellular response to hypoxia is mediated by hypoxia-inducible factors (HIFs) – potent transcription regulators, and their downstream pathways. In general, HIFs modify energy metabolism, inflammation and immune response, enhance cancer invasion, metastasis, resistance to treatment, and relapse. The influence of HIFs on the progression of leukemia is still under investigation in various studies, but in mice and some human models HIFs have been recognized as leukemia immortalizers by promoting leukemic stem cell quiescence and inhibiting their cell cycle. This makes leukemic stem cells resistant to most known treatment approaches. The role of HIFs in solid tumors and leukemia makes them almost ideal targets for an anticancer treatment. Although the first attempts with new molecules are encouraging, there is a need to investigate the ambiguous role of HIFs to develop a modern antileukemic treatment.

Key words: leukemia, hypoxia, hypoxia-inducible factor-1

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Leukemias remain an issue for contemporary medicine. The huge success in chronic myeloid leukemia (CML) treatment has not yet been matched by treatments for acute leukemias. Even tyrosine kinase inhibitors (TKI) – the main players in CML treatment – are not able to eradicate leukemic stem cells (LSCs). LSCs are a small subset of quiescent, self-renewable cells capable of re-establishing the whole tumor after chemotherapy. LSCs share marrow niches with hematopoietic stem cells (HSCs), but the niches are also inhabited by mesenchymal stem cells, bone cells, immune cells, and other cell types. The marrow microenvironment contributes considerably to the protection and development of leukemic cells. The bone marrow hematopoietic niche is characterized by low partial oxygen pressure, and this is essential to retaining HSCs, LSCs and other progenitor cells for long periods. Hypoxia is a very strong stimulus for adaptive cell changes mediated by the hypoxia-inducible factor (HIF) family. It has been shown that through HIFs, hypoxia optimizes solid tumor growth via metabolic modifications, stimulation of angiogenesis, cancer stem cell (CSC) maintenance and differentiation, and modification of the function of inflammatory cells. However, in leukemias, the role of hypoxia remains unclear, and the influence of HIFs on leukemogenesis is still controversial. On the other hand, some research has established the influence of HIFs on leukemic cell proliferation and resistance to chemotherapy. This paper attempts to summarize current knowledge about the role of hypoxia and HIFs in leukemias.

Hypoxia

Hypoxia is defined as a state of reduced or inadequate oxygen availability. Although different tissues and cells have varying degrees of susceptibility to hypoxia, at the cellular level, hypoxia and hypoxic responses generally occur at $PO_2 \leq \sim 1$ kPa ($\leq$~7–10 mm Hg or ~1% O_2).[1] There are multiple physiologic and pathological contexts in which cells experience conditions of insufficient oxygen availability.[2]

Hypoxia has been reported to contibute in a variety of pathological states, including solid tumors with incomplete neoplasmatic vascularization; ischemic injury, such as myocardial infarction or transplants; and obesity with impaired adipose tissue metabolism.[2] Other conditions that can be added to this list include vascular diseases (e.g., sickle cell disease), obstructive sleep apnea and chronic infection (e.g., granulomas).

Tissue oxygen concentration is much lower than in arterial blood enriched with oxygen from the air in the lungs. Tumor cells are not only prepared to survive total anoxia, but are skilled at taking advantage of the state to ensure uninhibited and uncontrolled growth through completely altered (but impaired) metabolism.[3] Local oxygen concentration plays a leading role in tumor progression due to impaired and incomplete vessel networks, resulting in reduced cell nutrition in hypoxic zones. Low oxygen levels cause the activation of mTOR kinase 1 (mTORC1) and modify tumor metabolism through the HIF signaling pathway, leading to tumor growth.[4]

The role of hypoxia-inducible factors

As Semenza wrote: "[HIF] transcription factors are master regulators of the cellular response to hypoxia and coordinate a transcriptional program that ensures optimal functional, metabolic, and vascular adaptation to O_2 shortages".[5] HIF-1α is also known to be responsible for cancer angiogenesis, growth and survival, glucose metabolism, invasion and metastasis, and immune regulation via the control of Th17/Treg balance.[6–10] HIF-2α is also expressed in a variety of cells, including endothelial cells and immune cells such as tumor-associated macrophages, and is reported to play an opposite role to HIF-1α in the regulation of angiogenesis, but mainly in iron metabolism, erythrocytosis control, and somatic stem cell self-renewal.[11–16]

HIF is a heterodimeric complex consisting of 2 subunits: an oxygen-sensitive HIF-α and an unchangeable oxygen-stable HIF-β (aryl hydrocarbon receptor nuclear transporter – ARNT).[17] Both subunits are members of the basic helix-loop-helix (bHLH) PAS family of transcription factors.[17] Three HIF-α homologs have been discovered: HIF-1α, HIF-2α and less known HIF-3α.[18–20] HIF-1α and HIF-2α heterodimerize to HIF-1β and translocate to the nucleus, where the complex is bound to hypoxia response elements (HREs) in the promoters of target genes. When oxygen is available, prolyl-hydroxylases (PHDs) are active and HIF1-α is degraded by the PHD-mediated oxygen-hydroxylation of proline (Pro-403 or Pro-564).[21] The hydroxylated HIF1-α is recognized by the von Hippel-Lindau protein (pVHL), which is the recognition component of an E3 ubiquitin-protein ligase. This leads to ubiquitination and consequent degradation by proteasome.[21] In hypoxic conditions, PHDs are inactivated, which leads to HIF-α accumulation. However, cells are doubly protected against HIF activation effects, and the shield is factor-inhibiting HIF (FIH) hydroxylating asparagine residues in HIF1-α and HIF-2α, which prevents HIFs from building active transcriptional complexes with cofactors.[22] Both PHDs and FIH require a-ketoglutarate (2-oxoglutarate) as a co-substrate. On the other hand, hypoxia diminishes PHD- and FIH-dependent HIF-α hydroxylation, resulting in full activation of the HIF-α pathway.[23]

HIF1-α expression is also regulated by growth factors, cytokines and other signaling pathways, such as the phosphatidylinositide 3-kinase (PI3K) pathway and mitogen-activated protein kinase (MAPK) pathway. HIF1-α plays a role in cancer progression by activating the transcriptional programs to maintain the ability to self-renew and the multipotency of cancer stem cells in a hypoxic

environment.[24] This has been described for renal cell cancer, hepatocellular cancer, colorectal cancer and other cancers.[25–27]

In the O_2-independent mechanism of HIF stabilization, bacterial products are recognized by toll-like receptors (TLRs) expressed on myeloid cells, signaling through the nuclear factor-light-chain-enhancer of activated B cells (NF-κB) to increase HIF1-α transcription.[28] Similarly, T cell receptor (TCR) ligation upon antigen presentation on T lymphocytes results in increased HIF1-α transcription and HIF-1α protein accumulation, even in the presence of oxygen. The mechanism of HIF-1α mRNA expression has not yet been identified, but activation of PI3K and mTOR seems to be involved in TCR-related activation of HIF-1α.[29] On the other hand, HIF-1α controls the Th17/Treg balance by promoting transcription of RORγt – a key regulator of Th17 differentiation – and proteosomal degradation of forkhead box P3 (FOXP3) – a key regulator of Treg differentiation. This leads to a sustained proinflammatory and autoaggressive reaction.[10]

HIF transcriptional targets also include glycolysis and angiogenesis regulating genes, e.g., glucose transporter 1 (*GLUT1*), phosphoglycerate kinase 1 (*PGK1*) and vascular endothelial growth factor (*VEGF*).[30–32] As McNamee et al. wrote: "While some HIF-1α targets are conserved across multiple cell types, HIF-1α is also clearly capable of mediating cell type-specific transcriptional responses. HIF-1α is regulated at multiple stages, including transcriptional, translational, and post-translational levels."[2]

Hematopoietic stem cells (HSCs) are able to enter quiescence and are characterized by self-renewal capability, and HIF-1α protein is reported to enhance these features.[33] Conditional deletion of the HIF-1α gene causes HSC proliferation and reduces self-renewal potential in serial transplantations.[34] On the other hand, HSCs cultured in hypoxic conditions have increased quiescence in vitro and in vivo when the HIF-1 pathway is activated.[35]

HIF-2α is not necessary for the function of adult HSCs in vivo.[35,36] Human bone marrow CD34+ cells hardly express HIF-2α, but HIF-2α itself has been identified as a STAT5 target gene in HSCs. STAT5 plays crucial roles in self-renewal in mouse and human HSCs, and its persistent activation leads to leukemic transformation.[37] As Forristal et al. wrote: "It is clear that normal adult mouse and human [bone marrow] hematopoietic cells in steady state have low levels of HIF-2α".[35] HIF-2α is also expressed by some hematological neoplastic cells, such as acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) cells.[35,38–40]

HIF-2α is also an important transcriptional regulator of the cellular hypoxia response, including hypoxic regulation of erythropoietin (EPO) synthesis and macrophage function.[12,41] The interaction between HIF-1α and HIF-2α in hypoxic gene regulation remains unclear. However, these transcriptional regulators can have competitive or even antagonistic functions.[2,42]

In summary, the HIF complex can cause transactivation of the target genes, leading to cell adaptation to hypoxic conditions, and plays a crucial role in many processes including enhanced cell proliferation (renal and colorectal cancer), cancer vascularization via VEGF, metastases, glycolysis regulation multidrug resistance (MDR), HSC quiescence and self-renewal, and many other processes. All these pathways have been widely described for solid tumors, but the roles of HIFs in leukemia seem to be inconsistent and remain unclear.

Leukemias, hypoxia and HIFs

Leukemia is the uncontrolled and uninhibited proliferation of hematopoietic cells. Acute leukemias are defined by proliferation of immature hematopoietic cells that fail to differentiate and accumulate in bone marrow and other organs, inhibiting the growth of normal hematopoietic cells. Acute myeloid leukemia (AML) has a high risk of relapse, despite therapeutic advances. Most known treatments target cycling cells, so the concept of relapse deriving from a quiescent surviving population of cells has arisen. LSCs derive from HSCs homing to the most hypoxic bone marrow areas.[43–45] As mentioned above, tissue oxygen concentration is much lower than in arterial blood enriched in O_2 from the lungs. In bone marrow, the oxygen concentration is even lower than in other tissues, ranging from < 6% oxygen near blood vessels to anoxia in not vascularized regions. The components of the hematopoietic niche vary in oxygen level.[45,46] The boosting role of hypoxia in rapid growth, proliferation, metabolism, metastases and mortality of solid tumors has been described.[47] The role of hypoxia in leukemias is not as well established, but the hypoxic BM niche increases poor response to treatment.[45]

It is accepted that gradients of O_2 from below 1% in the hypoxic niche to 6% in the sinusoidal cavity exist in human bone marrow. Hypoxia is essential for long-term HSC survival and function.[48] Molecular regulation of the influence of hypoxia on HSCs has not been established yet, but some studies have shed new light on the crucial role of HIF1 in mediating the effect of hypoxia on HSCs.[34]

Resistance to standard treatment modalities leading to leukemia relapse may be related to increased HIF expression.[49] Poor outcomes of antileukemic treatment have been linked with overexpression of HIF-1α in some studies, including an impact on survival.[50–52] In other studies, overexpression of HIF-1α has simply been reported – in AML, acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML).[34,35] Similarly, HIF-2α overexpression has been described in both AML and ALL, but has not yet been correlated with outcomes.[35]

There is a variety of mechanisms and molecular pathways that allow both HIF-1α and HIF-2α to contribute to leukemia survival, including metabolism, promoting cells in quiescence and immune dysregulation. HIF-1α

also plays the opposite role, inhibiting the expression of tumor expression genes.[34,53,54] Most of the widely used antileukemic drugs target cycling cells, so AML cells quiesced by HIFs become resistant to standard cytosine treatment. Another HIF-1α-mediated mechanism promoting leukemia resistance is LSC support, as in CML, T cell acute lymphoblastic leukemia (T-ALL) and AML.[44] This is a vicious circle: on one hand, LSCs sheltered in naturally hypoxic bone marrow niches stabilize HIF-1α, on the other hand, that same HIF-1α keeps them in quiescence and lets them survive any treatment, as in CML.[55] Moreover, in AML cells, HIF-1α is stabilized under normoxic conditions as well.[56] HIF-1α (in cooperation with the Notch pathway) is able to arm LSCs with a powerful tool: self-renewal, which lets them survive all known treatment modalities.[57]

Perspectives and conclusions

Taking into account the data cited above, it has been assumed lately that HIF inhibitors or PHD stimulators/enhancers could be a potent weapon in the antileukemic war. Some new agents have been tested; one of the first was echinomycin, which was known to inhibit HIF-1α DNA binding activity. This antibiotic targets AML cells through apoptosis. Echinomycin has no impact on self-renewal and differentiation of HSCs, which makes it a perfect drug to eradicate leukemia.[58] Another well-known drug, L-ascorbic acid in high concentrations, has also been shown to inhibit the expression of HIF-1α in CML cells. It is particularly important in CML treatment to find a molecule capable of impacting LSCs that are completely resistant to TKI-based modalities. EZN-2208 (pegylated SN38) has also been shown to inhibit the expression and transcriptional activity of HIF-1α in APL.[59] None of these molecules target HSCs. Another molecule, TH-302, is a hypoxia-activated prodrug that has been reported to preferentially decrease proliferation, reduce HIF-1α expression and induce cell-cycle arrest in AML cells.[60] This is an example of an alternative strategy "using" hypoxia to activate prodrugs in the bone marrow niche and target LSCs in their homeland.

On the other hand, some research has shown evidence that PHD inhibition can inhibit tumor growth and invasiveness.[57] This data derives from solid tumor investigation; evidence is still needed in relation to leukemia, but those trials definitely demonstrated the complex and ambiguous role of HIFs in cancer.

This data corroborates the view that hypoxia and HIF-mediated signaling play a crucial role in leukemia. As noted above, there are some confusing and even contrary results, but most mouse trials have unequivocally confirmed the proleukemic role of HIFs. Therefore, it can be assumed that HIFs inhibitors may potentially be successful in treating human leukemia.

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Telemedicine and eHealth in Poland from 1995 to 2015

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Abstract

The aim of this study is to present a review based on the literature and proceedings from selected telemedicine conferences. The review was developed using the PRISMA framework. The Embase and PubMed (updated until July 13, 2015) literature databases were searched for telemedicine-related terms and Poland. The literature search identified 129 eligible articles in the databases and 85 in conference proceedings until July 2015. Articles measured as a number of contributions per year presented a similar rising, fluctuating and almost parallel pattern. Fifty-nine percent of the reviewed papers were published in impacted journals. Almost half of all publications presented original papers. The published articles concerned mostly cardiology (16%), family medicine (15%) and pathology (11%). Conference proceedings papers concerned orthopedics (29%, significantly more frequent; $p < 0.001$) and cardiology (14%). Scientific activity of researchers and practitioners in Poland in the field of telemedicine is not high, but it is increasing over time. There is a tendency to present the research rather in high-quality journals instead of conferences before publication. The occurrence of individual medical specialty telemedicine in Poland may reflect country-specific needs.

Key words: Poland, telemedicine, literature review, telehealth, eHealth

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Introduction

Over the last 2 decades, substantial development has been observed in telemedicine, including eHealth, mHealth and other related terms often used to describe the delivery of healthcare at a distance using a variety of telecommunication devices and systems. The terms "telemedicine" and "eHealth" are often used interchangeably, but their semantic meanings are not identical. According to the American Telemedicine Association, the term "telemedicine" means "the use of medical information exchanged from one site to another via electronic communications to improve a patient's clinical health status, including applications and services using two-way video, email, smartphones, wireless tools, and other forms of telecommunications technology."[1] The Polish Telemedicine and eHealth Society considers telemedicine to cover the entire spectrum of medical services, including liability and licensing.[2] Thus, eHealth is a broader term that also includes several aspects of medical/health or clinical information systems. The idea of performing medical procedures at a distance and/or using communications technology has a long tradition in Poland. The first example found in the literature documented the implementation of telemedicine in Lviv (currently Ukraine) in 1935. Professors Marian Franke and Witold Lipiński organized the first clinical teleelectrocardiography (tele-ECG) system, which allowed wired transmission at a distance of approx. 500 m.[3] The next documented Polish application of telemedicine occurred 60 years later. As a Central European country with a population of 38.5 million, Poland and its pattern of telemedicine development may be representative of the whole Central and Eastern European region.

Studies support the contention that telemedicine is at least as good as conventional services regarding effectiveness, cost and patient outcomes.[4] The successful integration of telemedicine into existing healthcare enterprises has been a challenge for both users and researchers, as it often tends to focus on organizational issues, neglecting the social framework and human factors. Telemedicine has become an important element of healthcare systems, particularly in western societies. Scientific publications and conference proceedings are often used to gauge the level of interest in and the implementation of new paradigms of telehealthcare. There has been no or little assessment of telemedicine research productivity and efficiency in Poland to date. The aim of this study was to review the literature systematically addressing telemedicine and eHealth in Poland, to provide a general overview of the current status and time trends in this area of research. The expected potential value of this type of review is to help find country-specific implementations and promote further research efforts not only in Poland, but also in other countries from the region.

Material and methods

A systematic review of the literature was chosen as an appropriate method to study the development of telemedicine and eHealth implementations in Poland. The study followed the PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions.[5] Investigators experienced in telemedicine research independently searched PubMed and Embase for all reports on telemedicine in Poland. Search terms were formulated using MeSH headings and included the following combination: ("eHealth" OR "telemedicine" OR "mobile health" OR "mHealth" OR "telehealth" OR "remote consultation") AND ("Poland" OR "Polish"). After the screening of titles and abstracts of all initially identified publications, duplicates were removed and the full texts of the remaining articles were obtained for further analysis. There were no language or date restrictions, and the search was last updated on July 13, 2015.

The inclusion criteria were fulfilled if publications: 1) clearly addressed the issue of telemedicine in Poland; 2) reported original findings or reviewed the literature; and 3) were published in a peer-reviewed journal.

The authors carried out a secondary literature search to identify all presentation reports and abstracts by Polish authors for the Med-e-Tel and Medicine 2.0 conferences. Med-e-Tel is the official conference of the International Society for Telemedicine and eHealth held annually since 2000. Medicine 2.0 is a world conference on social media, mobile applications and the Internet in health, medicine and biomedical research, held annually since 2008. Although there are other telemedicine meetings, Med-e-Tel is the main European telemedicine meeting Polish researchers are likely to attend. The search covered electronic proceedings through July 2015. After screening the abstracts, all of the full-text papers were reviewed by 2 investigators (MK and MK). Both reviewers had to agree on whether the paper met the inclusion criteria to include the paper in the final analysis. Disagreements were resolved by discussion with the 3rd investigator (WG).

A predefined form was used to extract the following information from the articles: 1) year of publication; 2) list of authors and their affiliations; 3) details of the publishing journal; 4) area of medicine; 5) telemedical technology; 6) type of publication, i.e., controlled study, observational or feasibility study, survey, description of functionality, review, or other non-original paper; and 7) main results. The data was extracted and checked for validity. The impact factor (IF) of each journal was obtained from Journal Citation Reports. The identified studies were very heterogeneous regarding scope and methodology, which allowed for only semi-quantitative statistics. Categorical variables are presented as the number of observations with the ratio. Due to a non-normal distribution, continuous variables are presented as the median with range. Proportions of articles and conference proceedings addressing identical

areas of telemedicine were compared using the two-tailed Fisher's exact test. Calculations were carried out using the STATISTICA v. 10.0 software package (Stat Soft Inc., Tulsa, USA). Probability values <0.05 were considered statistically significant.

Results

Out of the 632 records retrieved from the initial database search, 109 were eligible for the final review, and an additional 20 articles were identified through reference search. Only selected papers are cited here, but all of them were thoroughly read and analyzed. The details of the screening process are presented in Fig. 1.

The peer-review status of the first published paper by Franke and Lipiński remains unknown.[3] The peer-review process is obvious for the paper addressing telemedicine in Poland published in 1995.[6] The number of articles published per year fluctuated from 0 to 16, with an overall increasing trend (Fig. 2).

The descriptive statistics of the excerpts from the articles and their classifications are presented in Table 1.

Telemedicine and eHealth were mostly utilized only in selected clinical disciplines like cardiology (16%), family medicine (15%) and pathology (11%), probably due to the clinical needs, research interest or other facilitated use (Fig. 3). However, no further investigation was conducted following that observation due to the review protocol.

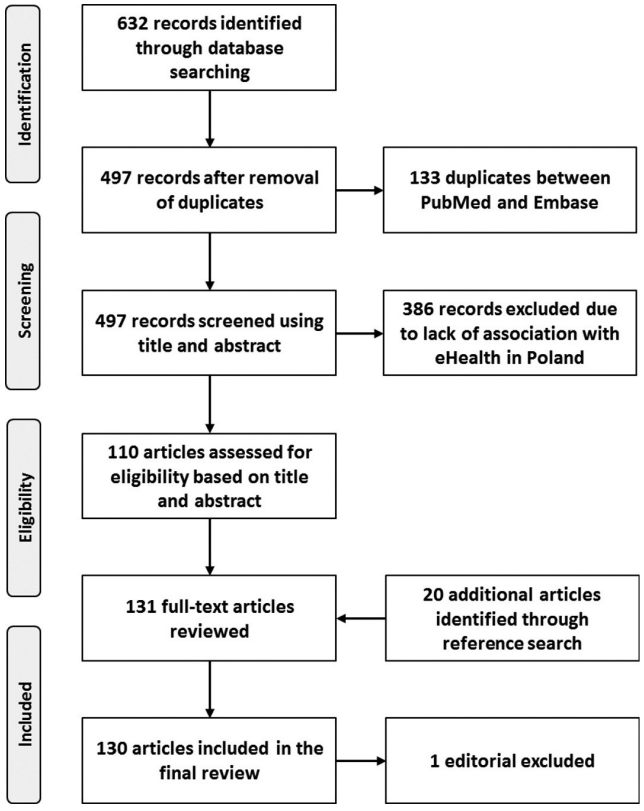

Fig. 1. PRISMA flowchart showing the selection process of eligible articles

Table 1. Summary characteristics of the reviewed articles (n = 129)

Published articles	n (%)
Authors	
international collaboration	17 (13.2)
number of authors, median (range)	3 (1–22)
1–2 authors	54 (41.9)
≥5 authors	37 (28.7)
Journal characteristics	
non-Polish journal	79 (61.2)
impact factor >0.00	77 (58.9)
impact factor (if applicable), median (range)	1.48 (0.26–7.08)
Language	
full text in English	91 (70.5)
at least abstract in English	38 (24.0)
only in Polish	7 (5.4)
Type of publication*	
controlled studies	19 (14.7)
observational or feasibility studies	31 (24.0)
surveys	18 (14.0)
descriptions of functionality	17 (13.2)
reviews and other non-original papers	51 (39.5)

* Publication may have had mixed methodology, therefore the denominator is 136.

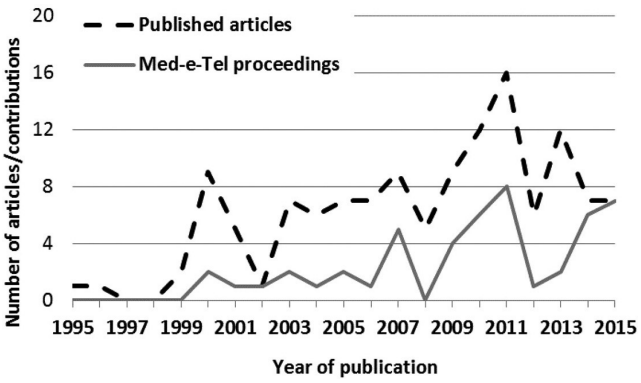

Fig. 2. Annual volume of publications and Med-e-Tel proceedings from Polish authors

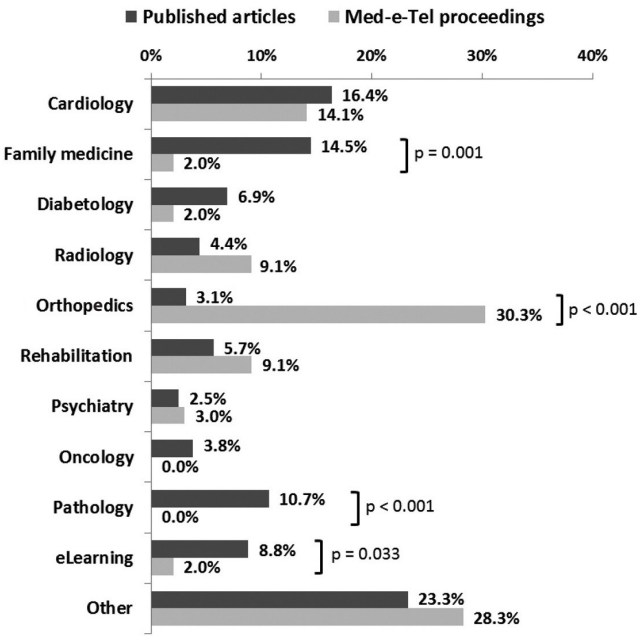

Fig. 3. Telemedicine application areas in reviewed articles and proceedings

The search for conference materials revealed 82 articles (Med-e-Tel – 82 papers and Medicine 2.0 – only 3 papers) that were relevant for a full review. Polish authors have contributed to the conferences since the first Med-e-Tel meeting in 2000, with a visible, increasing trend over the years (Fig. 2). All but 2 contributions were original (not presented elsewhere). The most frequently addressed areas of telemedicine were orthopedics (29%, significantly more frequent than in published papers; $p < 0.001$) and cardiology (14%). Other medical disciplines were significantly less often presented during telemedicine-oriented conferences (particularly pathology and family medicine) than in published papers (Fig. 3).

Discussion

The analysis of publications addressing telemedicine on a national scale systematically is rather rare in the literature, possibly because authors have more of an institutional rather than countrywide perspective. The review of country-specific peer-reviewed publications and proceedings papers provides the opportunity to find common and diverse aspects of regional (countrywide) developments in the field.

Our analysis revealed that video conferences and imaging data transmission via satellite between Polish and German clinicians from departments of radiology in Berlin and Wrocław opened the new age of telemedicine implementations in Poland in 1995.[6] Since then, the Internet and other telecommunications-based systems have become important sources of health-related information that have started to supplement conventional health services across Europe.[7,8] Research conducted in Poland and other countries clearly shows that the interest in telemedicine is growing.[2] The acceptance of telemedicine and eHealth solutions is increasing in the general population and even undergraduate medical professionals.[2,9,10]

This review did not explicitly distinguish between telemedicine and eHealth applications in order to capture the broadest picture of research in this area in Poland to date. The current review reveals a few interesting trends. Despite differences in the total annual number of papers, Polish publications and conference proceedings seem to follow a similar (almost parallel) fluctuating pattern. Both curves in Fig. 2 show peaks in 2000, 2007 and 2011, and lows in 2008 and 2012. It would be interesting to know the reason for these peaks and valleys. It is possible that these cyclic patterns might be related to the funding of telemedicine projects. However, funding information was not always provided in the searched publications.

In general, published articles were expert opinions on telemedicine, with most focused on cardiology, family medicine, and pathology.[11–24] Telecardiology seems to be a very prominent telemedicine sector in Poland, and cardiac telerehabilitation has become well-recognized due to several projects.[14,20,25–27] Specialists in family medicine, primary care and internal medicine usually implement home telemonitoring of chronic diseases and blood pressure monitoring in their studies, and there are a few research groups focused on telediabetology.[28,29] Telepathology was introduced in the 1990s in Poznań with the description of remote automatic microscope functionality, and several more papers focused on additional telepathology applications.[23,24,30–32]

We observed a significant impact of research focusing on otorhinolaryngology ("ear, nose and throat" or ENT), especially with using telemedicine for cochlear implant fitting.[33,34] Teledermatology was not frequently mentioned in Polish studies.[35] Telemedicine and eHealth implementations in psychology, psychiatry and neurology most frequently regarded psychotherapy techniques.[36–40] The daily use of teleradiology to deal with the shortage of radiologists is rarely the topic of research – it is so well integrated into medical services that it is generally no longer regarded as innovative or challenging.[25,41] Telepulmonology papers show mostly mature implementations.[15,42] Papers in teleoncology are unique.[43] Telemedicine use in orthopedics is well delineated and focuses on certain pathologies.[44–46] The knowledge and attitude of nursing students toward telenursing were surveyed.[47] Internet-based medical information use is represented well in several medical specialties.[7,8,48,49]

Other papers describe various telemedicine and eHealth technologies used in real or virtual patient care, and some even address the outdated but still operational use of the phone for teleconsultations.[11,50–52] Papers on distance learning have been presented since 1995, initially as live broadcasts over satellite, then moving to Internet-based applications.[6] Later studies have addressed many more aspects of e-learning, including barriers, virtual environments and the use of resources, such as virtual patient modules and grid medical libraries.[14,53,54]

It is not possible to directly relate the trends observed in Poland to what has been happening in other countries.[55–57] Based on the almost constant increase in the global number of similar publications indexed in PubMed (from 366 papers indexed in the year 1995 to 2946 papers indexed in the year 2015), the contribution of the activity of Polish authors may be considered lower than expected for a country of this size and population. The current review may help other researchers with similar telecommunication and healthcare infrastructures.

This paper has limitations in that it used only 2 databases for the search, and used a limited set of telemedicine-associated terms, which may have influenced the number of retrieved articles. An additional limitation was the selection of only 2 conference proceedings, Med-e-Tel and Medicine 2.0, to include. An extended and methodologically improved review would likely enable a more detailed analysis of the telemedicine and eHealth status in Poland, and provide stronger conclusions.

Conclusions

The scientific activity in the field of eHealth and telemedicine implementations in Poland did not achieve the level anticipated, given the growing use of telemedicine across Europe. However, it has increased over time. On the positive side, this search revealed that about half of the identified articles reported original contributions and more than a half were published in journals indexed in the Journal Citation Report. The relatively low number of controlled clinical studies (about 15%) represents the underexploited scientific potential in this field. The profile of disciplines represented on the selected conferences seems to be skewed toward some specialties, which may reflect the fact that they are attended only by certain groups of researchers. This type of country-specific literature review may be helpful in assessing the state-of-the-art in research for a particular country, but may also serve as an example for other countries wanting to evaluate their research activities or to establish collaboration. Understanding the research and implementation activities within a given country may also help researchers, policy makers and healthcare officials to support and promote the use of telemedicine in their country.

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Thromboprophylaxis in cancer patients in hospice

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Abstract

Advanced cancer patients in hospice are at notably increased risk of venous thromboembolism (VTE) due to age, local and distal advancement of the malignancy and bed confinement, among other factors. Asymptomatic VTE prevalence among palliative care patients has been found to reach 50%, whereas the clinically overt form occurs in 10%. Hospice patients are frequently given medications increasing VTE risk, for instance megestrol which is a drug commonly used in cancer cachexia. Many of the available guidelines encourage the implementation of thromboprophylaxis (TPX) in cancer patients, e.g., in the perioperative period or over the course of chemotherapy. However, concerning patients remaining under hospice care where the priority goal is not life extension but assurance of the best possible quality of life (QoL), the main benefit from the TPX would be a decrease in the risk of symptom burden associated with VTE, i.e., pain, edema or dyspnea. Nevertheless, studies performed on a sufficiently large study group, which could unequivocally determine the influence of anticoagulation on VTE symptom burden in hospice patients, are still lacking. VTE prophylaxis is challenging for many reasons: its unknown effect on QoL, vague risk of its discontinuation, and risk of bleeding complications which is additionally increased in conditions prevalent in hospice population, i.e., malnutrition, renal or liver insufficiency. So far, most of the guidelines issued by oncological societies do not precisely refer to the problem of TPX in hospice patients. Therefore, the decisions on the implementation of anticoagulation should be taken individually, with previous assessment of VTE risk, comorbidities and possible hemorrhagic complications.

Key words: thromboprophylaxis, venous thromboembolism, hospice, palliative care

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Introduction

Venous thromboembolism (VTE) is the second most common cause of death among cancer patients.[1] It consists of 2 entities: deep vein thrombosis (DVT) and pulmonary thromboembolism (PE). The relative risk of VTE is 6-fold higher in cancer patients compared to healthy control as, the meta-analyses have shown.[2] Considering cancer patients in general, symptomatic DVT has been demonstrated in 15%, and autopsies have revealed a 50% prevalence of PE in this group of patients.[3] Regarding palliative care units, evidence of asymptomatic DVT was found in 52% of 287 patients screened by Johnson et al.[4] In another study, clinically overt VTE was reported in nearly 10% of 712 participants.[5]

Hypercoagulability, venous stasis and endothelial damage, known as the Virchow triad, are the main risk factors contributing to thrombosis. Each of the triad's components can be affected by cancer. Tumor elaboration of tissue factor, microparticles and inflammatory cytokines result in the activation of coagulation cascade.[6] Venous stasis is caused by either vessel compression by a tumor mass or prolonged bed confinement, whereas vessel damage can occur as the consequence of e.g. chemotherapy, indwelling central venous catheters or direct tumor invasion.[2,7] Of note, activated platelets, coagulation and fibrinolytic factors are involved in angiogenesis and both local and distal progression of cancer.[8] In general, VTE predisposing factors in oncological patients could be divided into cancer-, treatment- and patient-dependent types (Table 1).

VTE is not only associated with a significant reduction in the overall survival of patients with malignancy, but also presents with burdensome symptoms including limb and/or chest pain and dyspnea. In a qualitative study by Seaman et al., cancer patients being treated for VTE found the symptoms of VTE very distressful – both physically and psychologically.[2,9] According to the findings of Johnson et al., 9% of likely venous thrombosis were symptomatic at the time of original diagnosis, but another 32% of cancer patients with likely DVT subsequently developed symptoms which included significant lower limb pain and swelling.[4]

Table 1. Factors predisposing oncological patients to venous thromboembolism

A. Cancer-dependent advanced stage or metastatic disease type and stage of cancer (particularly pancreatic, gastric, ovarian, lung)
B. Treatment-dependent recent surgery active chemotherapy (its type and duration) and radiotherapy prothrombotic drugs: erythropoiesis stimulating factors, bevacizumab, thalidomide, lenalidomide, tamoxifen, antiandrogens, megestrol central venous catheters
C. Patient-dependent advanced age race (higher risk in African Americans) obesity comorbidities, e.g. renal, liver or pulmonary disease prolonged immobilization prior history of thrombosis

A randomized control trial has demonstrated that primary thromboprophylaxis (TPX) can significantly reduce the incidence of VTE in immobilized cancer patients.[10] The data focusing on palliative care patients, however, is unsatisfactory. Weber et al. performed a prospective randomized study to determine the utility of prophylactic anti-coagulation in cancer patients hospitalized in a palliative care unit with an estimated life expectancy of 6 months or less.[11] Although neither harm nor an unequivocal benefit from the TPX was revealed, the results should be verified on a larger study group since only 20 patients were enrolled in the study.[11] Another study showed that patients without primary TPX on admission to hospice were at higher risk of developing VTE symptoms compared to patients receiving primary TPX.[12] However, the difference was not statistically significant and the proportion of patients receiving primary TPX (4% only) was probably too small to derive a firm conclusion in this matter.

Although routinely used in patients with advanced malignancy in oncology wards, primary TPX is relatively rarely administered in the palliative care setting, including hospice. Contrary to the formal and unequivocal guidelines for management of pain, vomiting and nausea, nutrition or sedation at the end of life, no unanimous evidence-based guidance on VTE prophylaxis and management in patients approaching death is available. This largely results from the lack of sufficiently powered studies concerning the factual symptom burden of VTE in this group of patients and its impact on QoL. In the absence of such data the relevance of TPX in terminal care remains vague.

Moreover, the problem is controversial in terms of ethics. According to the definition of palliative care established by the World Health Organization, dying is recognized as a natural process and the main target of palliation is not to postpone death, but to improve QoL through the alleviation of distressing symptoms.[13] It is not clearly defined whether TPX prolongs life in patients approaching death, but neither has it been investigated if and to what extent anticoagulation could relieve the burdensome symptoms associated with VTE at the terminal stage of malignancy.

Nowadays, in the era of highly developed treatment modalities and advanced supportive care, the significance of hospice not solely as a place of dying, but also as a measure of the improvement of patient condition, seems to become more and more pronounced. On average, approx. 1 in 5 hospice patients were discharged alive from hospices in the USA in 2010.[14] Therefore, although the interventions to prevent long-term complications of malignancies are frequently perceived futile and unnecessary in a hospice setting, the problem should not be neglected.

In our paper, we review the data on both physicians' and cancer patients' attitudes toward anticoagulation at the end of life, analyze available methods of thromboprophylaxis

in the view of this group of patients and review the research and recommendations of international cancer societies concerning primary TPX in terminal stage cancer patients.

Attitudes of physicians and patients to primary thromboprophylaxis in hospice

Physicians working in palliative medicine have been found to significantly underestimate the likely prevalence of VTE in hospice inpatients. One study has shown they estimated the risk to be 1–5% only, whereas the actual prevalence is most likely at least 5-fold higher.[4,15] The conviction of a low prevalence of VTE led medical directors of palliative care units interviewed by Noble et al. not to consider symptomatic VTE a clinical priority.[16] Moreover, some of them expressed the view that fatal PE is a less distressing mode of death ('quick and easy') compared to other cancer-related pathologies patients may experience, should they live longer. Simultaneously, TPX was considered not in line with the philosophy of palliative care as it was regarded as a life-prolonging intervention. Finally, TPX was perceived not to be a cost-effective intervention since it did not alter the outcome at this stage of the disease.[16]

However, physicians' approach to VTE prevention has been shown to evolve. There has been a shift from avoiding the use of low molecular weight heparin (LMWH) to its more common implementation in palliative care units. Among palliative care specialists surveyed by Noble et al. in 2000, 62% routinely stopped TPX, whereas in 2005 it was only 18%.[17] Likewise, a more individual approach is observed, which takes into consideration patients' own preferences regarding anticoagulation.

Decisions on limitation of treatment at the end-of-life period pose ethical challenges and are perceived by physicians as difficult.[18] It is still vague when, if at all, TPX should be ceased in patients approaching death. In a survey study in which experts in palliative care, oncology, intensive care and anticoagulation were enrolled, all the surveyed physicians opted to withdraw or withhold primary prophylaxis in patients with a Karnofsky Performance Status (KPS) of less than 10, defined as "moribund".[19] Whereas one quarter of doctors would have employed prophylactic anticoagulants when KPS was at least 20, most physicians (85%) declared they would have implemented primary TPX if KPS was over 40. There was an inverse relation between patient's performance status and the readiness of physicians to prescribe TPX. The study has also shown that doctors' decisions of whether to employ VTE prevention were made with a potential risk-benefit assessment.

Studies have revealed that palliative care inpatients wish to be involved in the decision-making process, particularly concerning withdrawal or non-administration of TPX.[20,21] Moreover, they expressed their concern about potential disqualification from TPX due to the advanced malignancy they were struggling with. In the study by Gartner et al., 87% of patients who had been involved in the decision process opted to receive TPX.[21]

Anticoagulation is often thought to be painful and bothersome for hospice patients due to the need for daily subcutaneous injections associated with the use of low molecular weight heparin (LMWH), one of the most commonly implemented anticoagulants in routine TPX, which could decrease QoL.[8] However, in one study, all of the 28 patients interviewed receiving palliative care, who had been given LMWH for at least 5 consecutive days, found LMWH an acceptable intervention, and many said it improved their QoL by giving them a feeling of safety and reassurance.[20] They perceived optimizing QoL as not only treating symptoms but also taking measures to prevent other symptoms. Another study on the acceptability of long-term LMWH use revealed that for terminally ill patients it was important to know that something active was being done despite the hopelessness of their clinical situation.[22]

LMWH was also found acceptable by palliative care patients receiving TPX in a long-term setting for the treatment of VTE.[9,22]

Although the aforementioned studies have demonstrated a generally positive impact of TPX on QoL, it is particularly difficult to draw generalized conclusions from studies concerning VTE prevention performed on patients under palliative care due to the often various performance statuses of the participants and the lack of a uniform tool for QoL assessment.

Selection of thromboprophylaxis method with reference to hospice practice

There are various methods of thromboprophylaxis widely used in clinical practice. Patients approaching death, however, constitute a particular subgroup of patients in whom not every method might be suitable.

The most popular forms of mechanical TPX include compression stockings and intermittent pneumatic compression (IPC). Despite the fact that it does not cause hemorrhagic complications, IPC has been poorly studied in non-surgical cancer patients. Also, little research on the use of elastic stockings is available in the literature concerning patients under palliative care. Nevertheless, the available data has shown the stockings were found uncomfortable and unacceptable by patients, causing itching, sweating and an unpleasant feeling of pressure, and therefore decreasing QoL.[10] Oral vitamin K antagonists (VKA) inhibit the synthesis of vitamin K-dependent coagulation factors. VKA have numerous interactions with food and drugs used in supportive or active oncological treatment.

They also have a narrow therapeutic window, therefore the treatment requires titration and further monitoring based on international normalized ratio (INR). Repeated blood sampling is burdensome for patients and may decrease adherence to the treatment.[8] Additionally, patients under palliative or hospice care have been shown to require more frequent INR control due to difficulties in achieving and maintaining therapeutic drug levels.[23] This mainly results from, e.g., malnutrition, diarrhea, emesis or liver failure, common in patients with advanced malignancy. As a matter of fact, cancer patients who are treated with VKA for VTE have a substantial rate of recurrent VTE.[23] VKA is also found less effective than low molecular weight heparin in patients with cancer-associated thrombosis.[23] However, it has been not established whether the conclusion of these findings could be extrapolated to patients receiving primary TPX, particularly in a hospice setting.

Unfractionated heparin (UFH), which is factor Xa and thrombin inhibitor, can be administered either intravenously or subcutaneously, has a good safety profile in terms of renal insufficiency and its effects can be reversed by protamine sulfate. UFH, however, should be given more than once daily, requires monitoring of activated partial thromboplastin time (APTT) and can cause heparin-induced thrombocytopenia (HIT). Therefore, it is used in selected patients only, especially those with significant impairment of renal function.

The new generation of heparin, low molecular weight heparin (LMWH), is currently more often implemented than UFH in TPX. Compared to UFH, it has a longer half-life and can be administered only once a day, is characterized by higher bio-availability and entails much lower risk of HIT.[24] In comparison with warfarin, LMWH is more effective in the prevention of recurrent VTE in cancer patients and safer with respect to bleeding complications.[25] In the American College of Chest Physicians (CHEST) Guidelines, LMWH is suggested as the preferred long-term treatment for VTE in cancer patients.[23] Routine monitoring of coagulation parameters is not required in most cases, although there is a need for repeated subcutaneous injections, which could adversely impact QoL. However, as mentioned above, it was shown to be a trivial intervention, acceptable by palliative care inpatients, with little or no impact on QoL and bruising as the only negative experience reported.[20] Although LMWH has been reported to increase the overall survival in cancer patients, the survival benefit was not statistically significant in the subgroup of patients in the advanced stages of malignancy.[26] Additionally, the Fragmin Advanced Malignancy Outcome Study (FAMOUS) revealed that LMWH administration did not improve 1-year survival in the final 3 months of life of advanced cancer patients.[27] Life prolongation, however, is not in line with the principles of end-of-life care and should not be considered important in a hospice setting.

Renal insufficiency is one of the contraindications to LMWH use. Given that this condition can be found in more than half of cancer patients, LMWH utilization in the prevention of VTE in this group of patients might be challenging and requires dose adjustment (with the exception of tinzaparin, which does not accumulate in patients with renal function impairment and can be administered without dose corrections).[28]

Fondaparinux is an indirect inhibitor of factor Xa. It was demonstrated to be an option for VTE prevention in cancer patients hospitalized for acute medical illness or surgery.[29] On account of the lack of a reversal agent and significant dependence on renal clearance, its employment in the palliative care setting might be limited, although there are reports on the successful use of fondaparinux in both primary and secondary VTE prevention in oncological patients.[30]

Novel oral anticoagulants (NOA) are specific inhibitors of activated factor X (apixaban, rivaroxaban and edoxaban) or thrombin (dabigatran). Less interaction with food and drugs, oral administration and no need for drug-level monitoring make its use convenient. Until recently, no antidote was available to any of the NOA. The introduction of both idarucizumab, a humanized monoclonal antibody against dabigatran, and andexanet alfa, a molecule reversing Xa inhibitor activity, was a breakthrough in this matter.[31,32]

The MAGELLAN (Multicenter, Randomized, Parallel Group Efficacy and Safety Study for the Prevention of Venous Thromboembolism in Hospitalized Acutely Ill Medical Patients Comparing Rivaroxaban with Enoxaparin) study, investigating the efficacy and safety of rivaroxaban, revealed a non-significant trend of lower efficacy of rivaroxaban compared to enoxaparin in the subgroup of cancer patients as well as significantly higher bleeding risk associated with rivaroxaban over enoxaparin administration.[8] Apixaban, on the other hand, was shown as effective as enoxaparin in primary TPX in the ADOPT (Apixaban dosing to optimize protection from thrombosis) trial, although related with significantly more relevant bleeding events.[33] In the latest CHEST guidelines, the risk reduction for recurrent VTE has not been compared between NOA and LMWH, however, indirect comparisons make it possible to assume that LMWH may be more effective than NOA in cancer patients with VTE.[23] Simultaneously, the risk reduction for recurrent VTE is similar between NOA and VKA, therefore VKA is no longer suggested over NOA in cancer patients treated for VTE.[23] Although NOA is an attractive option for TPX in oncological patients, the clinical trials designed exclusively for cancer patients – not to mention palliative care patients – are lacking.

Thromboprophylaxis in end-of-life care: Current guidelines and challenges

The National Institute for Health and Clinical Excellence (NICE) recommends the use of pharmacological VTE prophylaxis in palliative care for patients who have potentially reversible acute pathology.[34] The potential risks and benefits as well as the opinion of patients and their families should be taken into account. NICE recommends the use of fondaparinux, LMWH or UFH (for patients with renal failure). Patients admitted for terminal care or those commenced on an end-of-life care pathway should not be considered for routine pharmacological or mechanical VTE prophylaxis. Additionally, the decisions regarding TPX for patients in palliative care should be reviewed daily.

Previously, VTE prevention was supported by the American College of Chest Physicians (CHEST) in the palliative care setting in selected patients only, i.e. in whom it could be expected that TPX could prevent progressive deterioration of QoL.[35] However, the current guidelines of the CHEST on VTE prevention do not refer to the population of palliative care patients.[36] Prophylactic-dose LMWH or low-dose UFH is recommended in outpatients with solid tumors and additional risk factors for VTE (e.g. immobilization), provided they are at low risk of bleeding. On the other hand, routine use of TPX is not supported by the CHEST in chronically immobilized patients residing at nursing homes.[36]

According to National Comprehensive Cancer Network (NCCN) guidelines, no routine TPX is recommended outside of clinical trials in cancer patients after being discharged from the hospital nor in ambulatory cancer patients remaining at risk of VTE (e.g. advanced stages of cancer, poor performance status, medical comorbidities).[37] TPX use in the palliative care setting is not referred to in the guidelines.

Anticoagulation should not be used to extend the survival of cancer patients without VTE in the absence of other indications, according to American Society of Clinical Oncology (ASCO) recommendations.[10,38] Routine TPX can be considered only in selected high-risk outpatients with cancer, however the application of this recommendation to palliative or end-of-life care was not specified.[38]

The European Society for Medical Oncology (ESMO), despite recommending prophylaxis with UFH, LMWH or fondaparinux in hospitalized, bedridden cancer patients with an acute medical complication, has not referred to the problem of VTE prevention in palliative care.[39] The aforementioned guidelines are summarized in Table 2.

Since the decisions on whether to implement TPX in palliative care are often left to physicians' individual assessment, in recent years it seems to be more common for specialist palliative care units to have their own policy regarding VTE prevention. Among palliative care units in Great Britain, only 3% had a TPX policy in 2000, whereas in 2005 the number had increased to 7%.[17]

Given that evidence based on large studies is still lacking, doctors are compelled to rely on their own experience in everyday practice. The studies concerning actual symptom burden associated with VTE in end-of-life care and its cumulative effect on QoL are pending, as is research on the extent to which TPX could reduce VTE symptom burden and improve QoL. Certainly, not the survival rates but the quality of the remaining lifetime should be the most important measure of outcome in the research regarding hospice patients. For that reason, the results of studies involving hospitalized cancer patients in general are not necessarily applicable to the hospice setting. Unfortunately, a reliable tool for QoL assessment is still lacking.

The use of anticoagulants in hospice patients should be also verified in terms of bleeding, since the population of advanced cancer patients is frequently at hemorrhagic risk due to renal and liver failure, malnutrition or the metastatic process involving organs participating in hemostasis (liver, bone marrow). In one study on TPX in palliative care units, contraindications for TPX were present in 25% of all cancer patients and in 35% of the bedridden ones.[21] The rate was even higher in the study by Johnson et al., where primary TPX was contraindicated in 42.6% out of 1164 hospice patients.[12]

Table 2. Summary of guidelines for thromboprophylaxis in the palliative care setting

Recommendation	Author	References
TPX not recommended at the end-of-life care pathway. May be considered in patients admitted with reversible acute pathology	National Institute for Health and Clinical Excellence (NICE)	34
No guidelines on TPX in palliative care. TPX recommended in immobilized outpatients with solid tumors, but opposed in immobilized patients at nursing homes	American College of Chest Physicians (CHEST)	36
No guidelines on TPX in palliative care. Routine TPX use should be limited to clinical trials only	National Comprehensive Cancer Network (NCCN)	37
No guidelines on TPX in palliative care. TPX should not be the life-prolonging procedure. Can be considered in selected high-risk cancer outpatients	American Society of Clinical Oncology (ASCO)	10, 38
No guidelines on TPX in palliative care setting	European Society for Medical Oncology (ESMO)	39

The economic aspect of TPX in hospice should also be considered. The costs of drugs and nursing time may be considerable, and for some hospice, mainly financed with donations, this may be a barrier difficult to overcome. Chambers calculated that the drug costs of one particular hospice would increase by 28% if LMWH were administered to all immobile cancer patients.[40] Hopefully, the use of generic versions of LMWH would be a less expensive alternative. Moreover, as calculated, LMWH should be administered to 190 patients with advanced cancer to prevent one symptomatic VTE. Statistically, preventing less than one episode of VTE annually would be accompanied by 3.5 additional bleeding complications.[40] The cost-effectiveness calculations are particularly challenging in the population of hospice patients since an increased risk of VTE is often accompanied by high risk of bleeding events. Since the treatment of hemorrhagic episodes generates additional costs, this should be also taken into account.

Finally, the vast majority of research and recommendations focus on palliative care as a whole, without the distinction of a hospice setting. Palliative care units provide care to a wider group of patients, including those who are not dying. In other words, palliative care is not limited to end-of-life care and can be applied in any stage of the disease, in contrast to hospice. A hospice patient and palliative patient may significantly differ in performance status and life expectancy, therefore it would be reasonable for the authors of future studies and guidelines to refer to these 2 groups of patients rather separately.

Conclusions

As long as there is no clear evidence from large, randomized studies supporting the use of thromboprophylaxis in hospice, it seems that TPX in this group of patients should not be a routine practice. Patients ought to be involved in the decision-making process concerning TPX. Studies on the relations between VTE symptom burden, TPX and QoL should be performed on a hospice population, not palliative in general.

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The importance of antiangiogenic effect in multiple myeloma treatment

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Abstract

Angiogenesis plays a significant role in oncogenesis, and thus it has become an attractive target for cancer treatment. It is the formation of new blood vessels that occurs physiologically as well as under pathological conditions, and may influence cancer proliferation and survival. The current therapeutic approach in oncology includes conventional chemotherapy in combination with biologically-based treatment in various perspectives, targeting not only the malignant cells, but also its microenvironment. Target treatment might be less toxic than conventional chemotherapy. In multiple myeloma, there is a close connection between bone marrow stroma, myeloma cell growth and their ability to survive. It has been reported in many clinical observations that the more advanced the multiple myeloma, the more increased the angiogenesis, and this might correlate with the treatment response. There are several angiogenesis inhibitors already registered or in clinical trials in cancer treatment. Despite the continuous research on the development of prognostic factors and introduction of new agents in the treatment, multiple myeloma still remains an incurable and debilitating disease. Some antiangiogenic agents have already been introduced in multiple myeloma treatment, but there is still a need to search for new antiangiogenic drugs and the exploitation of angiogenesis in a clinical approach.

Key words: multiple myeloma, angiogenesis, treatment, angiogenesis inhibitors

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Introduction

Angiogenesis is the formation of new blood vessels, occurring physiologically in various stages of human life. Under pathological conditions, it plays a significant role in the inflammation process, and proliferation and metastasis of most malignant neoplasms. Recent studies have shown a role of angiogenesis and angiogenic factors in the development and course of hematological malignancies.[1]

Multiple myeloma (MM) is a hematological disorder, derived from B lymphocytes that are in the final stage of differentiation. This accounts for approx. 10% of hematologic malignancies. The essence of multiple myeloma is clonal proliferation of plasma cells in the bone marrow, monoclonal protein production and release of cytokines responsible for the destruction of bone tissue. Symptoms of multiple myeloma depend mainly on the stage of the disease.[2] Despite the continuous research on the development of prognostic factors and introduction of new agents in the treatment, multiple myeloma still remains an incurable and debilitating disease.[3]

The field of neovascularization was first evaluated in solid tumors. In 1997, Folkman et al. observed an increased amount of microvessels in the bone marrow of children with acute lymphoblastic leukemia.[4] Angiogenesis is a multifactorial process, associated with the formation of new blood vessels on the basis of the existing vascular network.[5] It begins with an increase of permeability and widening the lumen of blood vessels. Subsequently, endothelial sprouts are formed, and this process is regulated by several proangiogenic and antiangiogenic factors.[6,7] In fact, the process of pathologic angiogenesis associated with cancer activity differs from physiological blood vessel formation. The new blood vessel network is chaotic and might be associated with blood flow alteration and increased permeability of the vessels. The proangiogenic growth factors are produced mainly by endothelial cells, but also mast cells and pericytes. In the case of cancer, the "angiogenic switch" is induced. Cancer cells have the potential to produce proangiogenic cytokines, but also to stimulate the environment. Under normal conditions, there is a balance between proangiogenic and antiangiogenic factors. When it is switched, there is an increased angiogenesis potential. Several factors associated with angiogenesis may provide autocrine and paracrine effects to cancer cells. Importantly, the "angiogenic switch" may occur at any stage of cancer, but it is dependent on the cancer type and its interaction with the microenvironment.[8]

Angiogenesis in multiple myeloma

In multiple myeloma, inducement of proangiogenic and proinflammatory cytokine production is associated with the interaction of myeloma cells and the bone marrow stroma that includes fibroblasts, stromal cells, and also osteoblasts and osteoclasts, monocytes, macrophages, mast cells, and T lymphocytes. In general, in MM there is a tight connection between bone marrow stroma, myeloma cell growth and their ability to survive. The MM cells directly produce some proangiogenic molecules, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), interleukin-8 (IL-8), osteopontin (OPN), metalloproteinases and angiopoetin-1 (Ang-1), but can also stimulate other cells of the bone marrow microenvironment to secrete different growth factors, and finally endothelial cells to originate the angiogenic process.[5] The involvement of selected genes associated with angiogenesis in MM disease is described in Table 1.

The increased angiogenesis in MM was first described by Vacca et al., who conducted in vitro research and revealed increased activity of isolated plasma cells from active myeloma patient cells to produce the proangiogenic factors, in comparison with plasma cells derived from patients with monoclonal gammopathy of undetermined significance (MGUS) or inactive MM.[9] This observation was followed by continuous research that showed increased bone marrow

Table 1. Involvement of selected genes and proteins associated with angiogenesis in MM disease[5,16,17,19]

Gene and protein name	Impact on angiogenesis	Expression by MM cells
VEGF	proangiogenic	expressed
HGF	proangiogenic	aberrantly expressed
ANG	proangiogenic	aberrantly expressed
IL-8	proangiogenic	upregulated (vs normal plasma cells)
ANGPT1	proangiogenic	upregulated (vs normal plasma cells)
MMP-9	proangiogenic	expressed
TSP-1	antiangiogenic	expressed (potential prognosis factor)
ADAMTS9	antiangiogenic	aberrantly expressed
LAMA5	antiangiogenic	downregulated (vs normal plasma cells)

VEGF – vascular endothelial growth factor; HGF – hepatocyte growth factor; ANG – angiogenin; IL-8 – interleukin-8; ANGPT1 – angiopoetin-1; MMP-9 – matrix metalloproteinase-9; TSP-1 – thrombospondin-1; ADAMTS9 – a disintegrin and metalloproteinase with throbospondin motifs 9; LAMA5 – laminin alpha 5.

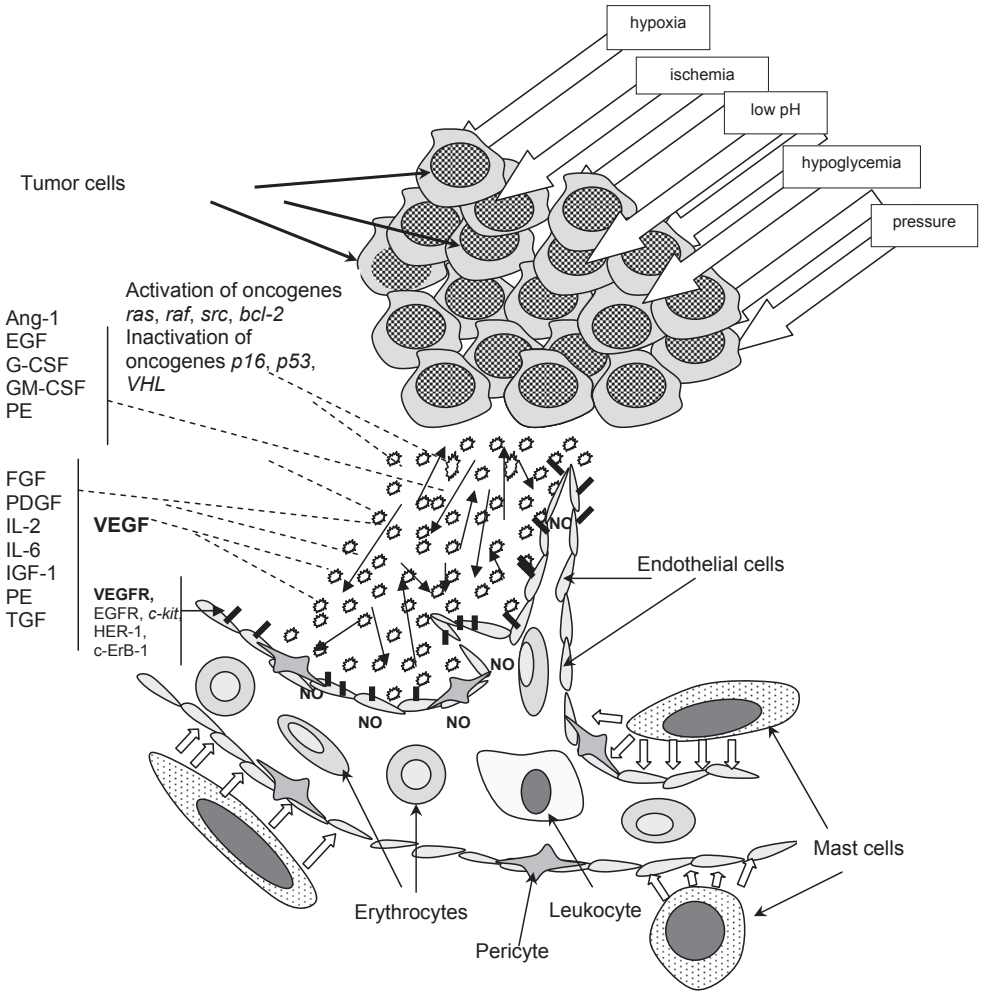

Fig. 1. Regulatory factors and mechanisms for angiogenesis in multiple myeloma[18]

VEGFR – vascular endothelial growth factor; PDGF – platelet-derived growth factor; FGF – fibroblast growth factor; EGFR – epidermal growth factor receptor; HER – human epidermal growth factor receptor; IGF-1 – insulin-like growth factor; Ang-1 – angiopoetin-1; EGF – epidermal growth factor; G-CSF – granulocyte colony-stimulating factor; GM-CSF – granulocyte-macrophage colony-stimulating factor; PE – plasma endostatin

angiogenesis in patients with advanced MM. It has been revealed that bone marrow microvessel density (MVD) assessed in histopathological samples was substantially higher in these patients than in health controls. In a study by Rajkumar et al., angiogenesis was assessed in bone marrow from 400 patients with multiple myeloma, MGUS and primary amyloidosis. It was found that the degree of angiogenesis is lower in patients with MGUS, and significantly increased in patients with multiple myeloma.[10] The greater intensity of blood vessel formation, the higher the proliferation of plasma cells seems to be, so it could indicate that angiogenesis might be associated with progression of the disease.[10] The assessment of bone marrow MVD might be an independent prognostic factor at the moment of diagnosis as well as in the response assessment.[11–13]

Several studies have been conducted on the relationship between the levels of angiogenic factors in the blood and MVD. The increased levels of proangiogenic molecules might also be associated with the treatment and prognosis.[14,15] The proangiogenic factors differ in respect of the activity. VEGF-A has the highest proangiogenic potential, and it is the main factor in angiogenesis and vasculogenesis. When the release of VEGF-A is inhibited, a regression of existing and formation of new blood vessels might be observed. A study conducted by Mileshkin et al. has shown that VEGF levels decreased significantly in MM patients who responded to thalidomide therapy.[16] On the other hand, the research by Cibeira et al. has not confirmed any correlation between VEGF level and response rate.[17]

Apart from releasing and stimulating angiogenic factors, the MM cells might also influence new blood vessel formation by direct interaction with matrix cells and fibronectin (Fig. 1).[18] Endothelial cells located in the bone marrow microenvironment may also produce angiogenic factors that act in a dual way (autocrine and paracrine) on both MM and endothelial cells. Thus, there is a mutual stimulation between endothelial cells and malignant plasma cells.[19]

Angiogenesis inhibitors in cancer treatment

The current therapeutic approach in oncology includes conventional chemotherapy in combination with biologically-based treatment in various perspectives, targeting not only the malignant cells but also its microenvironment, so new therapeutic targets are available nowadays. Angiogenesis seems to be a key factor in malignant tumor growth and survival, so there are some antiangiogenic agents already registered and others being investigated in

Table 2. Selected angiogenesis inhibitors in cancer treatment[19]

Drug	Signaling pathway	Molecular target	Indication (registered or in clinical trial)
Thalidomide	RTKs	VEGFR	multiple myeloma
Bevacizumab	RTKs	VEGFR-A	non-small lung cancer breast cancer glioblastoma colorectal cancer renal cell carcinoma
Aflibercept	RTKs	VEGFR-1 and -2	
Sunitinib	RTKs	VEGFR PDGFR c-kit FLT-3 CSF-1R	gist renal cell carcinoma breast cancer
Crizotinib	RTKs	c-Met HGFR	lung cancer breast cancer
Sofarenib	RTKs	Raf-kinase (B-Raf, C-Raf) VEGFR-2 and -3 PDGFR-β c-kit	colon cancer pancreatic cancer breast cancer
Semaxanib	RTKs	VEGFR	colorectal cancer (clinical development stopped due to severe thromboembolic adverse events)
Erlotinib	RTKs	EGFR/HER-1	nscl pancreatic cancer
Imatinib	RTKs	PDGFR c-kit	antiangiogenic inhibition in vitro

RTKs – receptor tyrosine kinases; VEGFR – vascular endothelial growth factor; PDGFR – Platelet-derived growth factor receptors; HGFR – hepatocyte growth factor receptor; CSF-1R – Colony stimulating factor 1 receptor; EGFR – epidermal growth factor receptor; HER – human epidermal growth factor receptor.

clinical trials. It has been reported in animal models that target therapy might be less toxic than conventional chemotherapy. Angiogenesis is a complex pathway, involving a number of factors, so there is a possibility to inhibit it on different levels.[18] It is crucial to distinguish between agents that impact angiogenesis and vascular targets. Antiangiogenic drugs mainly affect new vessel growth and formation in cancer tissue. Vascular targeting agents may destroy the existing vasculature of the tumor. The actions of both kinds of agents may synergize. There are several molecules of interest as potential therapeutic targets that have a regulatory or signaling function involved in angiogenesis, such as growth factors (e.g., VEGF, fibroblast growth factor – FGF, epidermal growth factor – EGF), transcription factors like hypoxia-inducible factor (HIF), and receptor tyrosine kinases. The same applies to molecules involved in phosphatidylinositol-4,5-biosphosphate-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling.

Bevacizumab is the humanized monoclonal antibody VEGF, and is approved in combination with chemotherapy for the treatment of some cancers (non-small lung cancer, breast cancer, glioblastoma, colorectal cancer, and renal cell carcinoma). This acts by inhibition of VEGF receptors, thereby also interfering with the autocrine and paracrine mechanisms of malignant cell survival mediated by VEGFR-1 and VEGFR-2. Bevacizumab is also in clinical trials in metastatic breast cancer and colorectal cancer.[20]

The most important agents with antiangiogenic potential in clinical trials or already registered in cancer treatment are presented in Table 2.

Angiogenesis inhibitors in MM

As angiogenesis seems to be strongly associated with MM development and prognosis, there have been increasing efforts to search for treatment with antiangiogenic potential. There are some drugs already registered in MM treatment that have demonstrated an antiangiogenic effect. These drugs might be administered alone or along with chemotherapy.

Immunomodulatory drugs

Thalidomide was the first agent introduced to MM treatment because of its potential antiangiogenic action. The drug was synthetized in the early 1950s and prescribed because of its sedative effect; finally it was withdrawn due to serious teratogenic side effects. In the 1990s, a study of rabbit model corneal neovascularization induced in response to bFGF demonstrated the antiangiogenic activity of thalidomide.[21] Afterwards, the clinical efficacy of thalidomide in MM patients was reported. Singhal et al.

showed a response to thalidomide monotherapy in a clinical phase II trial that included previously treated patients with refractory disease.[22] Partial response was achieved in almost 1/3 of patients and 14% achieved a complete or nearly complete remission.[22] That was the beginning of subsequent clinical studies and, in consequence, thalidomide was approved by the United States Federal Drug Administration (FDA) for the therapy of newly-diagnosed MM patients in combination with dexamethasone. The results of a phase III trial showed that the response rate in patients treated with thalidomide and dexamethasone was 63% in comparison to 41% in patients treated with dexamethasone in monotherapy.[23] The mechanism of the antiangiogenic effect associated with thalidomide is actually unknown. It has been observed that thalidomide might have important immunomodulatory effects associated with a decreasing synthesis of TNF-α and shifting the T cell population toward T helper. Thalidomide also decreases the expression of intercellular and vascular cell adhesion molecules (intercellular adhesion molecule 1 – ICAM-1 and vascular cell adhesion molecule – VCAM), and thus attenuates the interaction between stromal and malignant plasma cells.[24] In a study by Gupta et al., thalidomide and the newer immunomodulatory drugs like lenalidomide have been reported to significantly decrease the expression of proangiogenic factors, mainly VEGF and interleukin-6 (IL-6), in MM patients.[25] However, as has been reported in some other studies, the thalidomide action seems not always to be associated with a decrease of bone marrow angiogenesis and the levels of cytokines related to angiogenesis.[17] Moreover, in spite of high vascularity of extramedullary plasmocytoma, it has been observed that it does not respond to thalidomide treatment.[26]

Lenalidomide is derived from a modified thalidomide chemical structure and it also shows the immunomodulatory effects with lower rates of adverse events. In patients that were previously treated and had relapsed or refractory MM, lenalidomide in combination with dexamethasone showed a significant increase in the response rate, from 22.5% to 59.2%, in comparison to dexamethasone alone.[27] Lenalidomide was previously approved by the FDA for second-line therapy in MM and, further, in 2015 for newly diagnosed patients. The mechanisms of action mainly seen in MM may comprise induction of cell cycle arrest by an increase in expression of the cyclin-dependent kinase inhibitor p21 and decrease in expression of interferon regulatory factor 4, and also induction of apoptosis and attenuation of angiogenesis.[28]

Lu et al. conducted in vitro research and reported that lenalidomide may inhibit the formation of microvessels in a dose-dependent manner.[29] The inhibitory effect of lenalidomide may derive from the associations between cadherin 5, β-catenin and CD31. Moreover, lenalidomide was shown to attenuate PI3K–Akt pathway signaling induced by VEGF, which is known to modulate adherence junction formation.[29] In a Dredge et al.'s study, it was observed that le antiangiogenic effect of lenalidomide might not be related to a decrease of endothelial cell proliferation, still the migration of endothelial cells is inhibited significantly.[30] The study has been extended and it has been reported that lenalidomide may inhibit the action of some proangiogenic molecules in an animal model.[31]

Pomalidomide is a novel anti-myeloma agent among the immunomodulatory class drugs. Preclinical studies have shown that pomalidomide is active against MM cell lines in cases of bortezomib and lenalidomide resistance, which has been confirmed in a number of further clinical trials with a relatively tolerable safety profile.[32,33] Aside from these mechanisms of actions, pomalidomide inhibits stromal cell adhesion in bone marrow and has been shown to significantly inhibit angiogenesis by targeting VEGF and hypoxia-inducible factor-1α (HIF1-α), a transcription factor regulating angiogenesis by induction of VEGF transcription.[34]

Proteasome inhibitors

Bortezomib, a modified boronic acid dipeptide, is a selective inhibitor of nuclear factor-κB (NFκB) and has been approved for clinical use for the treatment of MM patients, but also shows activity in other hematological malignancies. It blocks very specifically the ß-subunit of the 26S proteasome.[35] The anticancer activity of bortezomib might also be associated with angiogenesis inhibition. It has been previously reported that proteasome inhibitors have antiangiogenic potential in animal models.[36] Inhibition of NFκB mediated by bortezomib is supposed to be involved in targeting HIF-1α-mediated VEGF expression.[37] Roccaro et al. examined the activity of bortezomib on the angiogenic phenotype of multiple myeloma patient-derived endothelial cells (MMEC). The authors reported that bortezomib induced a dose-dependent inhibition of VEGF and IL-6 production, and confirmed reverse transcriptase-PCR related to drug downregulation, IL-6, insulin-like growth factor-I (IGF-1), angiopoietin 1 (Ang1), and angiopoietin 2 (Ang2) transcription.[38]

Carfilzomib is a second-generation proteasome inhibitor, which has been approved by the FDA for clinical use in relapsed or refractory MM patients. In preclinical trials, carfilzomib demonstrated more potent antimyeloma activity than bortezomib.[39] It has got a similar antiangiogenic potential like bortezomib.

Agents targeting VEGF

In the study by White et al. (AMBER trial), bevacizumab was administered in combination with bortezomib for the treatment of relapsed/refractory MM patients. There was no significant increase of progression free survival (PFS) when bevacizumab was given along with bortezomib vs

bortezomib in monotherapy.[40] Similar unsatisfactory observations have been reported about other VEGF receptor-targeted agents, such as semaxanib (SU5416), zactima (ZD6474), and pazopanib (GW786034).[41–43] Although anti-VEGF antibodies might suppress a pathway involved in malignant myeloma cell growth, there are other factors associated with the disease activity and progression that these agents might not add any clinical benefits to in the treatment.

Novel antiangiogenic strategies

It has been suggested that modifications in multicellular eukaryotic messenger RNA (miRNA) expression may participate in the pathogenesis of most cancers in humans.[44] MicroRNAs are actually the class of non-coding RNAs (22-nucleotide) that operate in RNA silencing and post-transcriptional regulation of gene expression. They maintain biological events in various settings, such as cell growth, differentiation and apoptosis, but also metabolism of fat and viral infection. It has been observed that some miRNAs may be involved in controlling the production of angiogenic molecules, and therefore the angiogenesis process. Roccaro et al. have demonstrated that miR-15a and miR-16 are significantly diminished or lacking in relapsed/refractory MM patients. In vitro, as the regulators of MM pathogenesis, miR-15a and miR-16 attenuate the formation of capillaries and MM cell-associated endothelial cell growth. When pre-miR-15a and pre-miR-16 were transfected into malignant plasma cells, this contributed to significant inhibition of VEGF secretion. The authors have concluded that the field of regulation of VEGF by these miRNAs in MM patients' needs to be investigated.[45] Furthermore, Sun et al. conducted a study concerning the miR-15a and miR-16 expression levels and their association with the advanced stage of MM patients. The miRNAs were found to be downregulated in malignant plasma cells, and a correlation between downregulation and the MM stage was established. The results also confirmed the previous observations that miR-15a and miR-16 expression may inhibit the proangiogenic activity of malignant plasma cells.[46] Currently, there have been several reports that hypoxia is involved in miRNA expression in cancer. Another miRNA that is downregulated in MM is miR-199a-5p, and this has become an important issue of concern, as it directly targets HIF1-α, a transcription factor regulating angiogenesis, by induction of VEGF transcription.[47] A Raimondi et al.'s study has revealed that enforced expression of miR-199a-5p contributed to downregulation of HIF-1α expression and, moreover, some other proangiogenic factors such as FGFb, VEGF-A and IL-8 in hypoxic malignant plasma cells in vitro.[48]

Summary

Multiple myeloma is a unique hematological cancer wherein abnormal plasma cells, through interaction, subordinate the microenvironment for their own growth and expansion. MM might be a metastatic as well as a localized disease. Every single time it remodels the environment, destroying the bone structure and forcing angiogenesis, and thus provides a supply of substances essential to its survival. Awareness of these mechanisms may enable the development of a strategy for prolonged treatment based on specific inhibitors that could stabilize the treatment effect, and thus extend patients' survival. Research on the regulatory mechanisms of angiogenesis in MM according to their complexity and usefulness for treatment should be continued.

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