Advances in Clinical and Experimental Medicine

MONTHLY ISSN 1899-5276 (PRINT) ISSN 2451-2680 (ONLINE) www.advances.umed.wroc.pl

2018, Vol. 27, No. 10 (October)

Impact Factor (IF) – 1.262
Ministry of Science and Higher Education – 15 pts.
Index Copernicus (ICV) – 155.19 pts.

Wroclaw Medical University

Advances
in Clinical and Experimental Medicine

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MONTHLY 2018
Vol. 27, No. 10
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Advances in Clinical and Experimental Medicine is a peer-reviewed open access journal published by Wroclaw Medical University. Its abbreviated title is Adv Clin Exp Med. Journal publishes original papers and reviews encompassing all aspects of medicine, including molecular biology, biochemistry, genetics, biotechnology, and other areas. It is published monthly, one volume per year.

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Publisher
Wroclaw Medical University
Wybrzeże L. Pasteura 1
50-367 Wrocław, Poland

Online edition is the original version of the journal

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Indexed in: MEDLINE, Science Citation Index Expanded, Journal Citation Reports/Science Edition, Scopus, EMBASE/Excerpta Medica, Ulrich's™ International Periodicals Directory, Index Copernicus

Typographic design: Monika Kolęda, Piotr Gil
DTP: Wydawnictwo UMW, TYPOGRAF
Cover: Monika Kolęda
Printing and binding: EXDRUK

Advances
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MONTHLY 2018, Vol. 27, No. 10 (October)

ISSN 1899-5276 (PRINT)
ISSN 2451-2680 (ONLINE)
www.advances.umed.wroc.pl

Contents

Original papers

Reviews

Cord blood-derived mesenchymal stem cells with hepatogenic differentiation potential ameliorate chronic liver affection in experimental models

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med.* 2018;27(10):1329–1339

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Funding sources
Science and Technology Development Funds (STDF), Cairo, Egypt

Conflict of interest
None declared

Received on January 6, 2017
Revised on March 23, 2017
Accepted on April 12, 2017

Abstract

Background. The liver is one of the major target organs for which cell-based therapies are very promising. The limitations of various cellular therapies, including bone marrow (BM)-derived mesenchymal stem cells (MSCs), urges the exploration of stem cell sources more suitable for transplantation. Human umbilical cord blood (HUCB) can overcome these drawbacks with a favorable reparative outcome.

Objectives. The aim of this study was to evaluate the therapeutic potential of MSCs in 2 groups of chronic liver injury experimental models.

Material and methods. Propagation and characterization of MSCs isolated from cord blood (CB) samples were performed and differentiation into osteogenic, adipogenic and hepatogenic lineages was induced. The 1st experimental model group (80 mice) included a negative control, a pathological control and 60 mice infected with Schistosoma mansoni (S. mansoni) and transplanted with MSCs. The 2nd experimental model group (30 hamsters) included 10 healthy hamsters serving as a negative control and 20 hamsters injected with repeated doses of carbon tetrachloride (CCl_4) to induce liver fibrosis; 10 of them were treated with an intrahepatic (IH) injection of 3×10^6 MSCs and the other 10 were untreated pathological controls. Mice and hamsters were sacrificed 12 weeks post-transplantation and their liver sections were stained immunohistochemically for the detection of human hepatocyte-like cells. Moreover, the sections were examined for the levels of fibrosis.

Results. In both models, the transplantation of CB-derived MSCs (CB-MSCs) resulted in the engraftment of the fibrotic livers with newly formed hepatocytes, as evidenced by positive immunohistochemistry staining with human Hepatocyte Paraffin 1 (Hep Par 1), alpha-fenoprotein (AFP), cytokeratin 18 (CK18), cytokeratin 7 (CK7), and OV6 monoclonal antibody. The transplanted liver sections showed markedly reduced hepatic fibrosis with a significantly lower fibrotic index, as well as significantly improved liver functions compared to the pathological control ($p < 0.001$).

Conclusions. This data provides hope that human CB-MSCs can be utilized as multipotent stem cells with unlimited potentiality in regenerative medicine and supports the concept of cellular therapy for the cure of hepatic fibrosis.

Key words: cord blood, mesenchymal stem cells, carbon tetrachloride, liver fibrosis

DOI
10.17219/acem/70430

Introduction

Liver fibrosis is the wound-healing reaction of hepatogenic cells to chronic liver injury.[1] Following repetitive injury, the liver undergoes a remarkable tissue remodeling and develops fibrosis. This fibrosis is characterized by an extensive accumulation of the extracellular matrix, with the disposition of scar tissue encapsulating the region of injury. Although liver transplantation is undoubtedly the most efficient therapy for cirrhosis, broad clinical application of this procedure is limited by the scarcity of donor organs.[2] Thus, it is crucial to investigate the efficacy of various treatments for hepatic cirrhosis.

The liver is one of the major target organs for which cell-based therapies are very promising. The limitations of various cellular therapies, including bone marrow (BM)-derived mesenchymal stem cells (MSCs), urges the exploration of stem cell sources more suitable for transplantation. Human umbilical cord blood (HUCB) can overcome these drawbacks with a favorable reparative outcome.[3,4] Human umbilical cord blood stem cells remain viable even after long periods of cryopreservation.[5–7] These cells are an ideal option for clinical applications due to their ready availability from cord blood (CB), ease of expansion in an in vitro culture, simple isolation via plastic adherence, ability to evade rejection, and their multipotentiality for differentiation.[8,9]

Over the past 2 decades, HUCB has been well established as a valued source of hematopoietic stem and progenitor cells. Besides hematopoietic cells, CB also contains cells with various stromal properties.[10,11] From the point of view of clinical practice, it is very important to select a model of liver fibrosis that is close to the human disease in order to evaluate the effect of stem cells on the fibrosis. In the present study, we employed 2 models of fibrosis, induced by Schistosoma mansoni (S. mansoni) infection in mice and by carbon tetrachloride (CCl_4) in hamsters, to evaluate the effect of the MSCs injected through diverse routes and doses.

Material and methods

Isolation and culture of mesenchymal stem cells

Cord blood samples were collected from full-term babies delivered by cesarean section after informed written consent was given by the mothers, as approved by the institutional review board at Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Samples were processed according to the procedure described by Broxmeyer et al.[10] Mononuclear cell fraction was isolated by the density gradient separation method and cultured according to the procedure described by Kögler et al.[11] The MSCs were initiated in cultures, passaged and propagated according to Kögler et al.[5]

Immunophenotyping

Cells from the 3rd passage were washed twice in phosphate-buffered saline buffer (PBS). Fluorescein isothiocyanate (FITC) mouse anti-human CD44, CD45 and HLA-DR, as well as FITC mouse anti-human CD105 coupled with PE (phycoerythrin) mouse anti-human CD90, and FITC mouse anti-human CD34 coupled with PE mouse anti-human CD73, were added to flow cytometry tubes.

One hundred microliters of the cell suspension was added to each tube and incubation was done for 20 min at 4°C. The cells were washed twice with PBS and then resuspended in PBS. The final analysis was done by flow cytometer.

Gene expression analysis

Molecular analysis of the MSCs was performed according to the protocols described by Zaibak et al. and Demerdash et al.[12,13] Total RNA was extracted from trypsinized cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Its concentration and purity were assessed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Reverse transcripts were prepared using a high capacity cDNA kit (Applied Biosystems, Foster City, USA). The expression of the following genes was detected by real-time PCR using a QuantiTect SYBR Green PCR kit (Qiagen): *Oct4* (5' TCTCGCCCCCTCCAGGT; 3'GCCCCACTCCAACCTGG), *Sox2* (5' AGCTACAGCATGATGCAGGACC; 3'CTGGTCATGGAGTTGTACTGCAGG); and *GAPDH* (5'ATGGAGAAGGCTGGGGCTC, 3'AAGTTGTCATGGATGACCTTG).

Osteogenic differentiation

Following the guidelines of Kögler et al., the MSCs were seeded at a density of 6×10^3 cells/cm^2 into 6-well plates and cultured in complete Dulbecco's modified Eagle's medium (DMEM).[5] When the cells reached approx. 80–90% confluency, the medium was replaced with osteogenic differentiation medium (Lonza, Basel, Switzerland). Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine was added to negative control wells. The media were replaced every 3–4 days. On the 14th day, calcium deposition was assessed by alizarin red staining.

Adipogenic differentiation

Following the guidelines of Kögler et al., the MSCs were seeded at a density of 5×10^3 cells/cm^2 into 6-well plates and cultured in complete DMEM.[5] When the cells reached approx. 80–90% confluency, they were cultured in the adipogenic induction medium for 3 days, followed by an adipogenic maintenance medium for 3 days. Three cycles of induction/maintenance were carried out for optimal adipogenic differentiation. Dulbecco's modified Eagle's

medium supplemented with 10% FBS 1% penicillin/streptomycin, and 1% L-glutamine was added to negative control wells. On the 21st day, adipogenic differentiation was assessed by Oil Red O staining.

Hepatogenic differentiation

The induction of hepatogenic differentiation was done according to the protocol described by Waclawczyk et al.[14] Briefly, the MSCs of passage 3 were seeded at a density of 5×10^3 cells/cm^2 and cultured in 30% FBS/DMEM until reaching 80% confluency. The medium was then replaced with hepatogenic differentiation medium I (HDM I), consisting of hepatozyme supplemented with 0.5% FBS, hepatocyte growth factor (HGF) (50 ng/mL) (R&D Systems, Minneapolis, USA), fibroblast growth factor-4 (FGF-4) (20 ng/mL) (R&D Systems, Minneapolis, USA), epidermal growth factor (EGF) (20 ng/mL) (R&D Systems, Minneapolis, USA), 1% penicillin/streptomycin, and 1% L-glutamine. The cells were cultured for 2 weeks before exchanging the medium with HDM II, consisting of DMEM supplemented with HGF (50 ng/mL), FGF-4 (20 ng/mL), oncostatin-M (50 ng/mL), insulin transferrin selenium (ITS) (5 µL/mL), 10^{-7} dexamethasone, 1% penicillin/ streptomycin, and 1% L-glutamine. The HDM II was left for another 2 weeks. Cells were scraped, collected and slide-fixed for cytopathological diagnosis of hepatocyte markers (glycogen, alpha-fetoprotein (AFP) and cytokeratin 18 (CK18)) using periodic acid-schiff (PAS) staining (Sigma-Aldrich, St. Louis, USA) for glycogen detection and immunohistochemistry staining for AFP and CK18 detection.

Experimental model

All animals were raised and maintained at the animal house in TBRI in barrier units with a defined and regularly monitored health status. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Induction of an experimental model for hepatic fibrosis in mice by infection with Schistosoma mansoni cercariae

The S. mansoni-infected model consisted of 80 BALB/c mice, 8 weeks old and weighing 20–25 g, which were divided into the following groups:

- normal control group – 10 mice,
- infected control group – 10 mice infected with S. mansoni cercariae (60 cercariae/mouse) using the tail immersion method and left for 12 weeks to induce liver fibrosis, and
- MSCs-transplanted group – 60 mice infected with S. mansoni. This group was subdivided according to the method of MSCs transplantation into 2 subgroups (30 mice each): intravenous (IV) and intrahepatic (IH).

Both the IV and IH subgroups were further subdivided, according to the dose of transplanted MSCs, into 3 subgroups; IV1, IH1, IV2, IH2, IV3, and IH3, transplanted with 3×10^5 & 6×10^5 & 1×10^6 MSCs/mouse, respectively.

Mice from each group were sacrificed 4, 8, and 12 weeks after MSCs transplantation, respectively.

Induction of experimental model for hepatic cirrhosis by carbon tetrachloride

The CCl_4 model consisted of 30 hamsters weighing 60–65 g and divided into 3 groups:

- normal control group – 10 healthy hamsters;
- CCl_4 control group – 10 hamsters injected with CCl_4 at a dose of 100 µL/hamster (Al-gamhuria, Cairo, Egypt) mixed with olive oil (100 µL CCl_4 + 900 µL olive oil) intraperitoneally (IP) twice a week for 2 months.[15] Healthy control hamsters were injected with the same volume of olive oil;
- MSCs-transplanted group – 10 hamsters injected with CCl_4 as above and treated with MSCs (3×10^6 cells/hamster) IH 2 months after CCl_4 injection.

The cells were injected via the IH route because hamsters have no tail and the IV route was not feasible. The hamsters were sacrificed 12 weeks after MSCs transplantation.

Liver function analysis

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), and bilirubin were measured using a Synchron CX5 (Beckman Coulter, Brea, USA) analyzer.

Liver pathology and immunohistochemistry analysis

Liver sections from sacrificed animals were stained with hematoxylin and eosin (H&E) for histologic assessment and with Sirius red for assessment of fibrosis.[16] The CK18, cytokeratin 7 (CK7), Hepatocyte Paraffin 1 (Hep Par 1), AFP, and OV6 monoclonal antibodies were used (Santa Cruz Biotechnology Company, Santa Cruz, USA). In addition, negative controls in which the primary antibody was omitted and replaced by PBS were also used. Livers known to express markers were used as positive controls.

The intensity, distribution and pattern were analyzed and immunoexpression was evaluated. The percentage of positively stained cells was determined semiquantitatively by assessing the whole section.

Liver fibrosis analysis

Hepatic sections, 20 µm in thickness, were prepared from paraffin sections and stained with Picrosirius red. For the quantification of the collagen content, an Automatic Computer Image Analysis System (Zeiss, Oberkochen, Germany) was used. Image analysis was performed using

the computer software AxioVision v. 4.8, supplied by the manufacturer of the system. The sectional area of the red-stained fibrous tissue was measured in 5 consecutive microscopic fields (×5 magnification) to yield the fibrotic area (μm^2) and the proportion of fibrotic area relative to the total area examined was then calculated (fibrotic index in %).

Statistical analysis

The data were analyzed using the SPSS package v. 18.0 for Windows (SPSS Inc., Chicago, USA). Laboratory data of different groups were compared with one-way analysis of variance (ANOVA). At a p-value ≤0.05 differences were considered statistically significant.

Results

Immunophenotyping

The MSCs showed high expression levels of adhesion marker CD44, typical mesenchymal markers (CD90 and CD73), the endoglin receptor CD105, and dual expression of CD105/90; they were negative for HLA-DR, hematopoietic lineage marker CD34 and leukocyte common antigen CD45 expression (Table 1).

Gene expression analysis

The MSCs expressed *Oct4* and *Sox2*, which are considered to be core transcription factors that regulate the maintenance of the pluripotent state in embryonic and adult stem cells.

Osteogenic differentiation

When HUCB-MSCs were induced to differentiate into osteogenic lineage, the spindle shape of the HUCB-derived cells became less elongated and more polygonal in shape with the formation of aggregates (Fig. 1A).

Adipogenic differentiation

Under the influence of adipogenic differentiation conditions, the MSCs became large and rounded with an accumulation of neutral lipid vacuoles indicated by the Oil Red O stain (Fig. 1B).

Hepatogenic differentiation

Morphology

The fibroblastic-like morphology of the MSCs was lost. A broadened, flattened shape developed at first, while a round shape with a cuboidal morphology developed later. Slides coated with MSCs-derived cells and stained with H&E are shown in Fig. 1C.

Periodic acid-schiff staining

Smears were examined using a Zeiss Axio Scope A1 (Zeiss), under a magnification of ×400. Glycogen storage was determined by PAS staining. Positively stained glycogen granules were detected in the cytoplasm of differentiated MSCs, while undifferentiated MSCs were negative for PAS staining (Fig. 2).

Immunocytochemistry

Smears were examined using a Zeiss Axio Scope A1 (Zeiss), under ×400 magnification. With hepatocyte markers (AFP and CK18) being cytoplasmic markers, both AFP and CK18 were expressed in the cytoplasm of differentiated MSCs (Fig. 3 A,B), while undifferentiated MSCs were negative for both markers.

Schistosoma mansoni-induced fibrogenesis model

Only the results of transplantation with 1×10^6 MSCs after 3 months are presented here, as they were the most promising results.

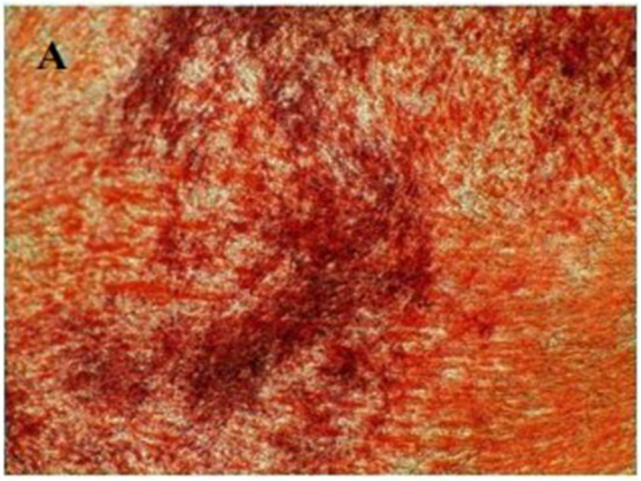
Effect of intravenous transplantation of 1×10^6 MSCs/mouse on Schistosoma mansoni-induced fibrogenesis

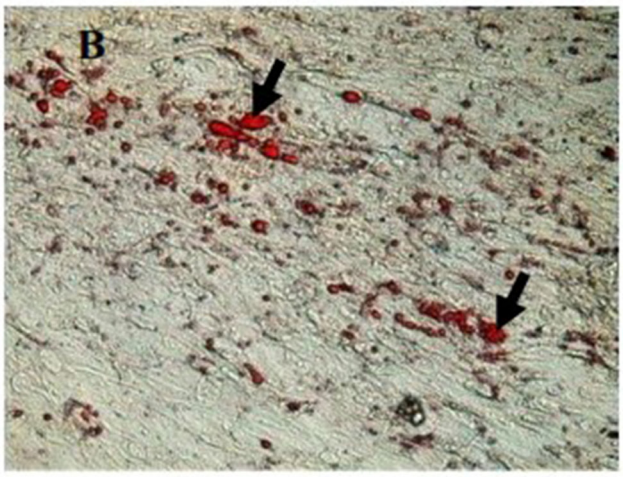
Intravenous transplantation of 1×10^6 MSCs in S. mansoni-infected mice revealed a high, significant reduction ($p < 0.001$) in fibrotic index 3 months after MSCs transplantation, compared to the corresponding index in the infected control group (Table 1).

Table 1. Results of liver functions – aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin (ALB) levels – and % fibrosis in different studied groups of murine Schistosoma mansoni-infected mice (mean ±SE)

Parameter	Studied groups of murine Schistosoma mansoni-infected mice			
	negative control (n = 10)	infected control (n = 10)	infected & treated with IV 1×10^6 MSCs (n = 30)	infected & treated with IH 1×10^6 MSCs (n = 30)
Albumin [g/dL]	4.36 ±0.049	0.73 ±0.18	5.2 ±0.167	3.2 ±0.096
ALT [U/L]	24.66 ±0.421	136 ±3.65	51 ±0.96	66.82 ±3.06
AST [U/L]	68.67 ±0.76	350.67 ±19.33	79.67 ±7.02	101.76 ±0.66
% fibrosis	0.2 ±0.04	27.48 ±0.745	5.37 ±0.21	7.58 ±0.38

MSCs – mesenchymal stem cells; IV – intravenous; IH – intrahepatic; SE – standard error.





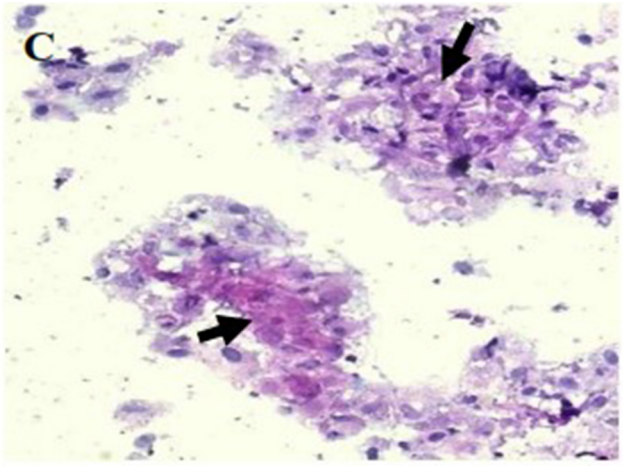


Fig. 1. Mesenchymal stem cells (MSCs) of P3 were induced to osteogenic, adipogenic and hepatogenic differentiation, which was confirmed A) by the formation of a mineralized matrix as evidenced by alizarin red staining, B) by the accumulation of neutral lipid vacuoles evidenced by the oil red O stain, and C) by the morphological changes towards hepatocyte-like cells showing large polyhedral cells with rounded nuclei in small sheets or separate hepatocyte-like cells

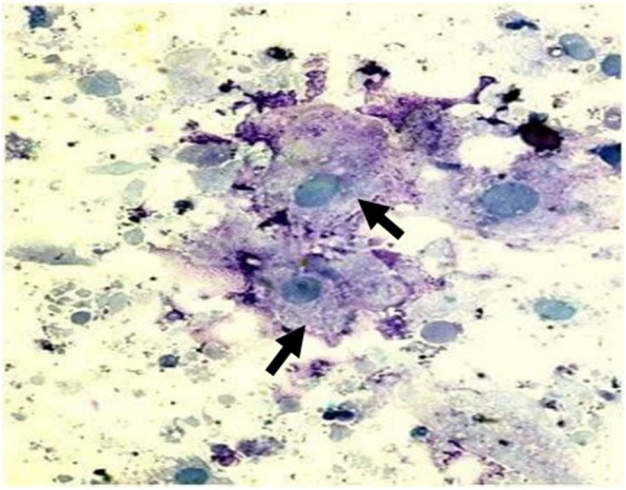


Fig. 2. Positively periodic acid-Schiff (PAS)-stained glycogen granules in the cytoplasm of differentiated mesenchymal stem cells (MSCs) showing large polyhedral cells with rounded nuclei in small sheets or separate hepatocytes-like cells positive for PAS stain (×400 magnification)

There was a significant improvement ($p < 0.001$) in the ALT, AST and ALB (Table 1) levels of the MSCs-transplanted group 3 months post-transplantation, when compared to the corresponding level in the infected control. It was observed that ALB reached a nearly normal level after the 3rd month.

In mice transplanted IV with 1×10^6 MSCs and sacrificed 12 weeks after transplantation, liver sections stained with H&E (Fig. 4 A–C) and Sirius red (Fig. 4 D–F) showed diminished granuloma size and a relative decrease in hepatic fibrosis. The cells were able to engraft into the fibrotic livers with some signs of regeneration, i.e., newly formed hepatocytes of human origin.

Livers of the MSCs-transplanted group showed engraftment with human hepatocyte-like cells, as proven by the cytoplasmic expression of AFP, Hep Par 1, CK18, CK7, and OV6 (Fig. 5 A–E) and by the negative staining of hepatocytes for desmin and vimentin. In addition, the livers of the MSCs-transplanted group showed less fibrosis than the pathological control group.

Effect of intrahepatic transplantation of 1×10^6 MSCs/mouse on Schistosoma mansoni-induced fibrogenesis

Almost same results were obtained as in IV route groups with less reduction in the fibrotic index (Table 1).

There was a significant improvement in ALT, AST and ALB levels (Table 1) 3 months after IH MSCs transplantation, compared to the levels in the infected control group. This improvement was less marked than that with the IV route groups.

In mice transplanted intrahepatically with 1×10^6 MSCs, liver sections stained with either H&E or Sirius red showed relatively diminished granuloma size and a relative decrease in hepatic fibrosis (Fig. 4), compared to the IV-transplanted group.

Immunoperoxidase staining of the liver sections from BALB/c mice infected with S. mansoni and injected IH

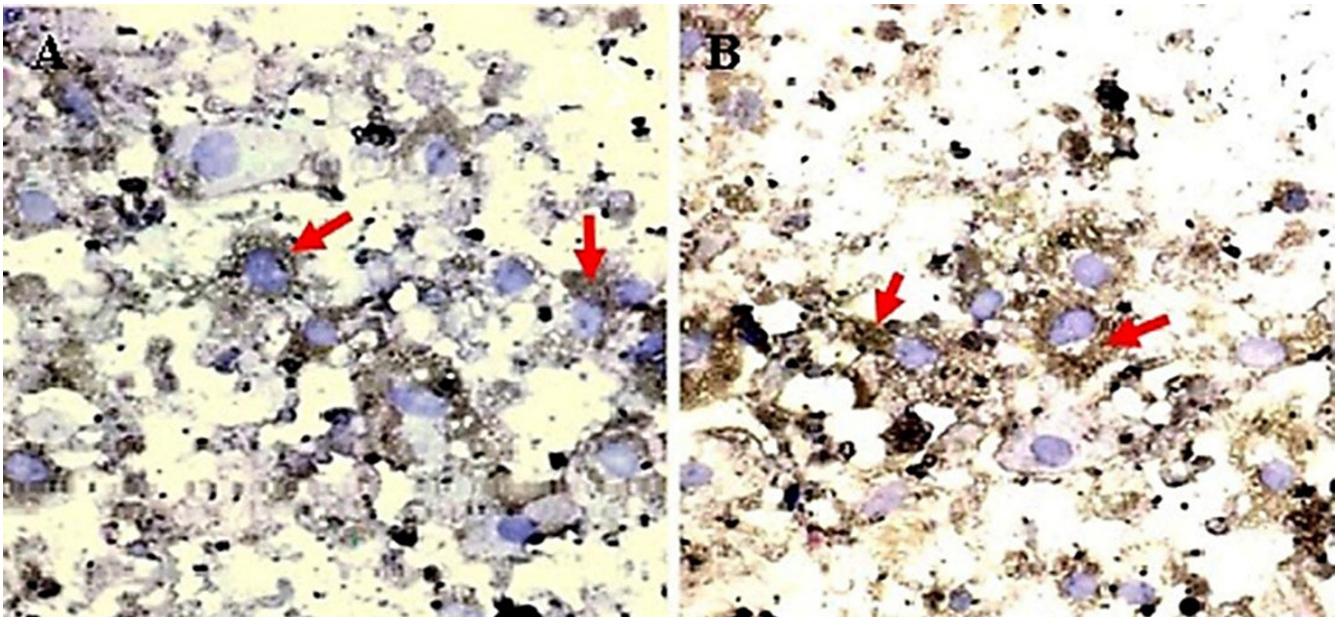


Fig. 3. Mesenchymal stem cells (MSCs)-differentiated hepatocyte-like cells positive for A) alpha-fetoprotein (AFP) and B) cytokeratin 18 (CK18), presented as cytoplasmic brownish stain (immunohistochemistry (IHC) ×400 magnification)

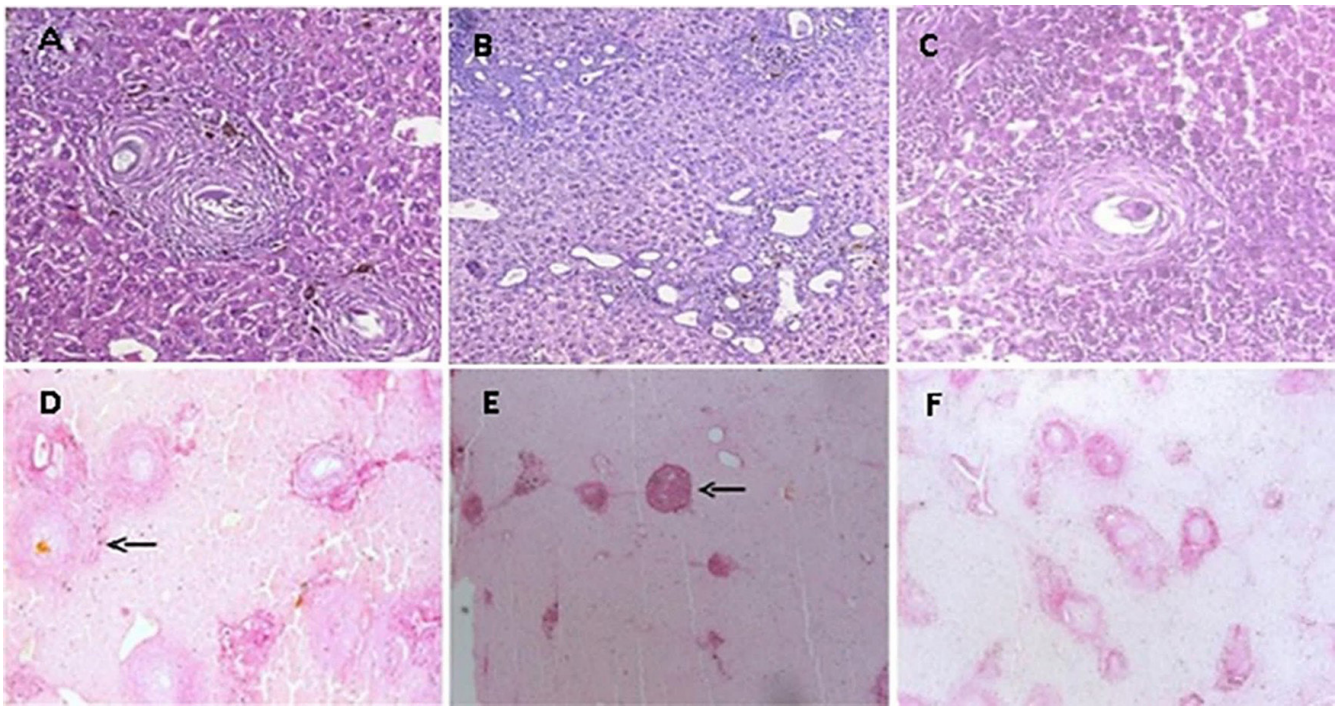


Fig. 4. Liver sections from BALB/c mice (hematoxylin and eosin (H&E) staining, ×400 magnification): A) infected control mouse showing a large fibrocellular granuloma 8 weeks post-infection; B) infected & intravenously (IV) 1×10^6 mesenchymal stem cells (MSCs)-treated mice 12 weeks post-treatment, showing a small regressed fibrocellular granuloma; C) infected and and intrahepatically (IH) 1×10^6 MSCs-treated mice 12 weeks post-treatment, showing moderate collagen fibrous bundles forming moderate size fibrocellular bilharzial granuloma. Liver sections from BALB/c mice infected with Schistosoma mansoni (Sirius red staining), showing: D) a large untreated fibrocellular granuloma 8 weeks post-infection; E) IV 1×10^6 MSCs-treated mice 12 weeks post-treatment with small regressed granuloma; F) IH 1×10^6 MSCs-treated mice 12 weeks post-treatment, showing moderate size fibrocellular bilharzial granuloma

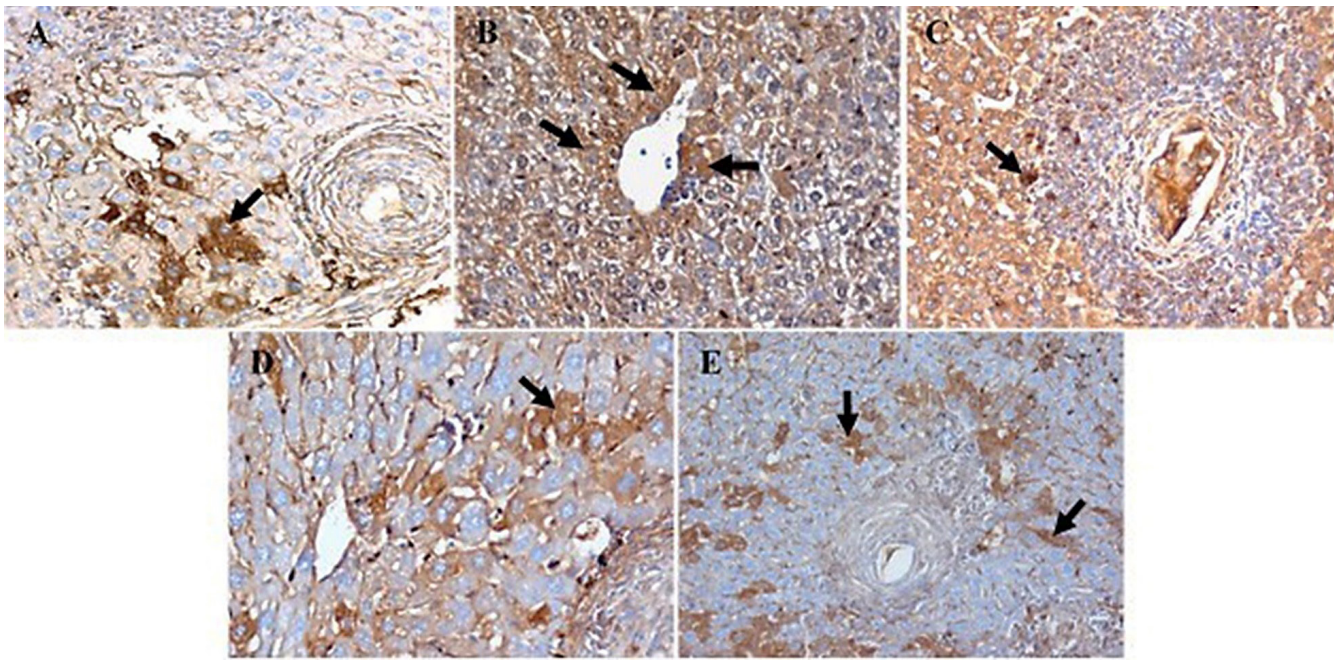


Fig. 5. Immunoperoxidase staining (×400 magnification) of liver sections of Schistosoma mansoni-infected BALB/c mice 12 weeks post-intravenous (IV) treatment with 1×10^6 mesenchymal stem cells (MSCs), showing: A) 50% new hepatocytes positive for alpha-fetoprotein (AFP), as a brownish cytoplasmic stain; B) 50% positivity for hepatocyte paraffin 1 (Hep Par 1); C) 50% positivity for cytokeratin 18 (CK18); D) 40% positivity for cytokeratin 7 (CK7); and E) 45% positivity for OV6

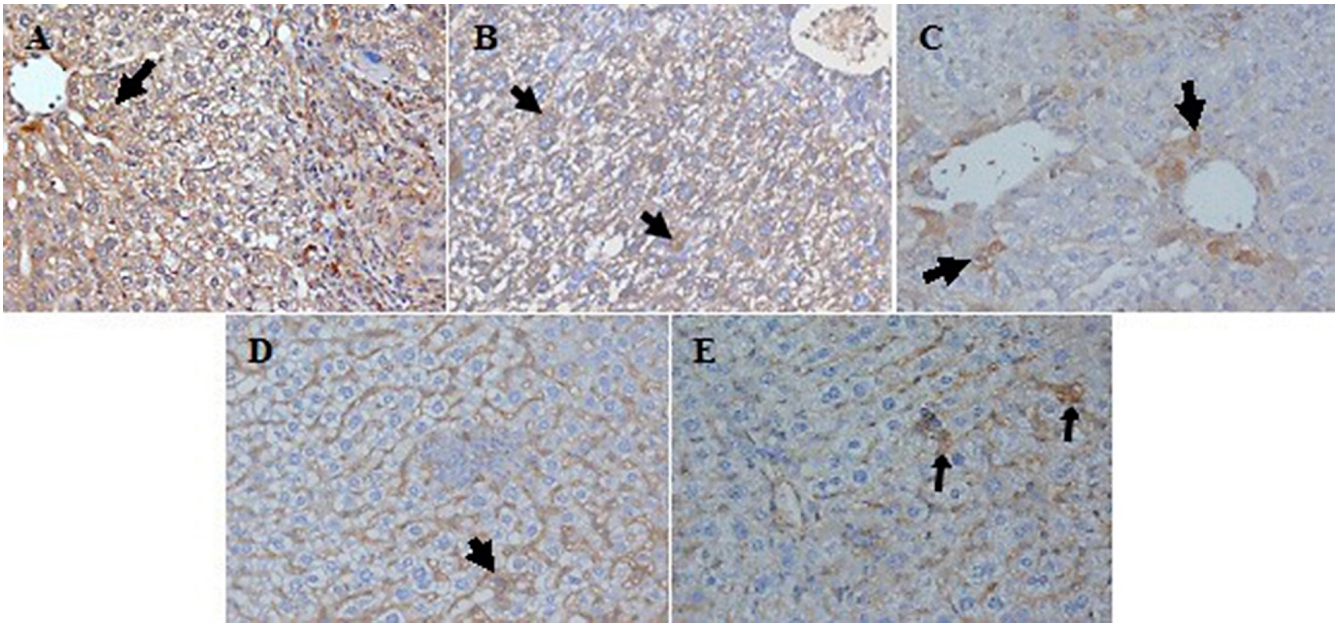


Fig. 6. Immunoperoxidase staining (×400 magnification) of liver sections of Schistosoma mansoni-infected BALB/c mice 12 weeks post-intrahepatic (IH) treatment with 1×10^6 mesenchymal stem cells (MSCs) showing: A) 10% new hepatocytes positive for alpha-fetoprotein (AFP), as a brownish cytoplasmic stain; B) 15% positivity for hepatocyte paraffin 1 (Hep Par 1); C) 20% positivity for cytokeratin 18 (CK18); D) 15% positivity for cytokeratin 7 (CK7); and E) 10% positivity for OV6

with 1 × 10^6 MSCs 12 weeks post-treatment showed new hepatocytes with brownish cytoplasmic stain positive for AFP, Hep Par 1, CK18, CK7, and OV6 (Fig. 6 A–E). Cells were able to engraft into the fibrotic liver with evidence of regeneration in the newly formed hepatocytes of human origin.

Carbon tetrachloride-induced liver cirrhosis model

Six trials over 3 months were performed to optimize the protocol for developing a model of cirrhosis. Two models were subjected to different doses and concentrations of CCl_4 to reach a sub-lethal dose which induced liver cirrhosis, yet avoided exposing the liver to severe toxicity ending in the death of the animal.

BALB/c mice were used as the 1st model. Carbon tetrachloride was dissolved in olive oil in ratios of 1:1, 1:2 and 1:5. The animals were then injected IP twice a week with 0.5 mL/kg, 0.5 mL/kg and 0.1 mL/kg of the prepared concentrations, respectively. Severe liver affection and death occurred within 1, 2 and 3 weeks, respectively.

The 2nd model used was hamsters. Carbon tetrachloride was dissolved in olive oil in ratios of 1:20, 1:5 and 1:10. The animals were injected IP twice a week with 0.01 mL/kg, 0.1 mL/kg and 0.0016 mL/kg of the prepared concentrations, respectively.

Liver specimens obtained from the animals injected with 0.01 mL/kg of the emulsion at a ratio of 1:20 did not show any evidence of liver affection or fibrosis after 1 month, while the hamsters subjected to a dose of 0.1 mL/kg at a ratio of 1:5 died as a result of severe liver affection within 2–3 weeks.

Histopathologic studies of liver specimens obtained from hamsters injected with 100 µL/hamster of the emulsion at a ratio of 1:10 showed that fibrosis started to be evident within 1 month and complete cirrhotic nodules were found after 3 months.

Carbon tetrachloride-induced fibrogenesis in hamsters transplanted intrahepatically with 3 × 10^6 mesenchymal stem cells

Transplantation of 3 × 10^6 MSCs in CCl_4-injected hamsters revealed a significant reduction ($p < 0.01$) in the fibrotic index 3 months after MSCs transplantation, compared to the corresponding index in the CCl_4 control group (Table 2).

There was a high significant improvement ($p < 0.001$) in ALT, AST and bilirubin levels (Table 2) 3 months after MSCs transplantation, compared to the corresponding levels in the CCl_4 control group.

The liver sections of the CCl_4 control hamsters stained with either H&E or Sirius red showed micro- and macrocirrhotic nodules (Fig. 7 A,B). Diminished cirrhotic nodules and a relative decrease in hepatic fibrosis were observed after MSCs transplantation. Cells were able to engraft into the fibrotic livers with newly formed hepatocytes of human origin as an evidence of some regeneration (Fig. 7 C,D).

The livers of the MSCs-transplanted group showed engraftment with human hepatocyte-like cells as proven by cytoplasmic expression of human AFP, Hep Par 1, CK18, OV6, and CK7 (Fig. 8 A–E).

Discussion

In the current study, CB-MSCs were isolated and expanded, and were positive for MSC phenotypic markers (CD73, CD105, CD90, and CD44), while being negative for HLA-DR and the hematopoietic stem cell phenotypic markers (CD34 and CD45). They showed high expression levels of *Oct4* and *Sox2* transcription factors, which regulate the maintenance of the pluripotency state in embryonic and adult stem cells.[17,18] Moreover, MSCs showed multilineage differentiation potential, being able to differentiate in vitro into mesodermal lineage cells (osteoblasts and adipocytes), and into the hepatogenic lineage of an endodermal origin. The expression of hepatic parenchymal markers – ALB, AFP, CK18, and glycogen – showed that our MSCs cultures contained functional hepatocytes, similar to the outcomes of the studies by Liang et al. and Li et al.[19,20]

We assessed the therapeutic effect of the transplantation of MSCs in 2 different experimental models: mice with S. mansoni infection (either IV or IH) and CCl_4-injected hamsters. In cell-based therapies, the dose of the transplanted cells is a key factor, as an appropriate cell count is vital for the survival of the injured experimental animals. In this study, we tested 3 doses (3 × 10^5, 6 × 10^5 and 1 × 10^6) of MSCs/mouse and a dose of 3 × 10^6 MSCs/hamster, in 0.5 mL DMEM. The recipient mice tolerated all doses and the best results were obtained when using the highest dose of 1 × 10^6/mouse. It was previously reported that the therapeutic effects of MSCs on liver cirrhosis gradually improved with increased cell dose.[21] A dose

Table 2. Results of liver functions (ALT, AST and total bilirubin) and % fibrosis in different studied groups of the CCl_4 model (mean ±SE)

Parameter	Studied groups of CCl_4 model		
	negative control (n = 10)	CCl_4 group (n = 10)	CCl_4 & treated with IH 3 x 10^6 MSCs (n = 10)
Total bilirubin [mg/dL]	0.28 ±0.02	5.97 ±0.23	0.52 ±0.01
ALT [U/L]	31.3 ±2.03	687.67 ±13.3	127.67 ±1.76
AST [U/L]	74.3 ±2.3	901.7 ±6.6	102.3 ±1.45
% fibrosis	1.27 ±0.19	17 ±0.82	2 ±0.21

AST – aspartate aminotransferase; ALT – alanine aminotransferase; CCl_4 – carbon tetrachloride; IH – intrahepatic; MSCs – mesenchymal stem cells; SE – standard error.

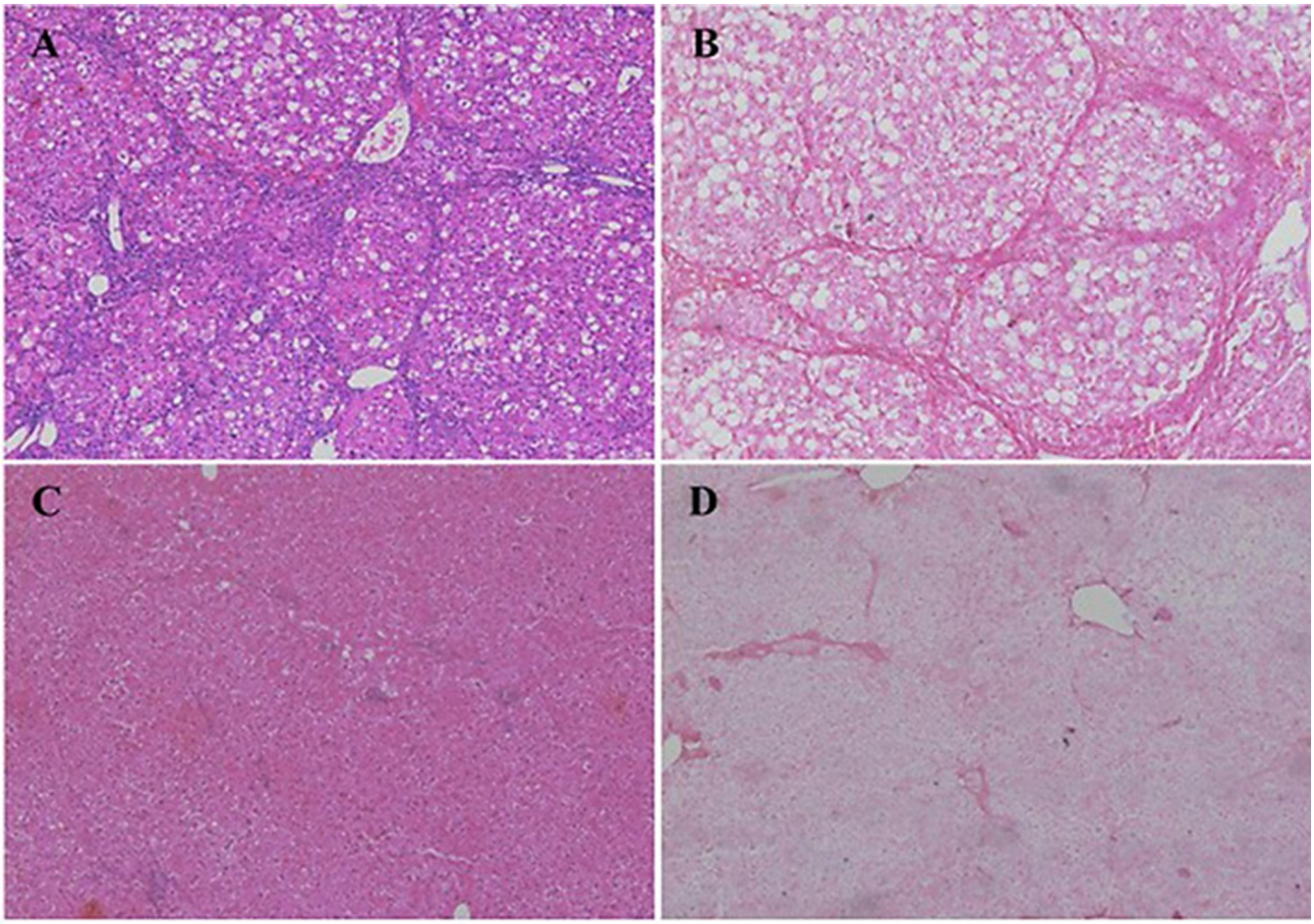


Fig. 7. Liver sections of a carbon tetrachloride (CCl_4) control hamster showing micro- and macrocirrhotic nodules: A) hematoxylin and eosin (H&E) staining, ×200 magnification; B) Sirius red, ×200 magnification. Liver sections of CCl_4 hamster 12 weeks post-intrahepatic (IH) treatment with 3×10^6 mesenchymal stem cells (MSCs), showing few fibrous bundles, liver with almost intact architecture; C) H&E, x100 magnification; and D) Sirius red, ×200 magnification

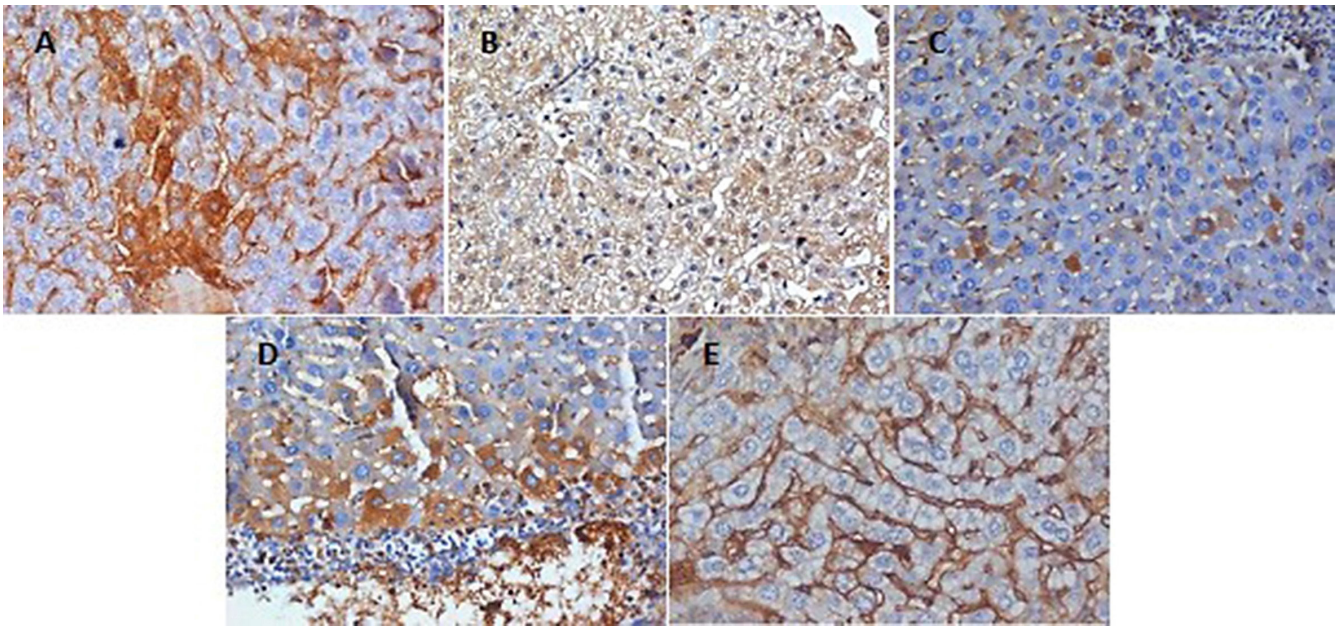


Fig. 8. Immunoperoxidase staining (×400 magnification) of liver sections of carbon tetrachloride (CCl_4) hamster 12 weeks post-intrahepatic (IH) mesenchymal stem cells (MSCs)-treatment, showing positive brownish cytoplasmic staining for A) alpha-fetoprotein (AFP), B) hepatocyte paraffin 1 (Hep Par 1), C) cytokeratin 18 (CK18), D) OV6, and E) cytokeratin 7 (CK7)

of 1×10^6 MSCs/mouse has been used in several mice experiments.[22,23] However, Yang et al. found that 1×10^6 cells/mouse dose was lethal for their mice, attributing that fact to vein embolism, so they used the 5×10^5 cells/mouse dose, while Park et al. recommended the use of an even higher dose – 2×10^6 MSCs/mouse.[24,25] We used triple maximum dose of MSCs/mouse to inject in a single hamster, according to their weight.

After transplantation, the livers of the MSCs-injected groups of both animal models showed engraftment with human hepatocyte-like cells, as proven by the cytoplasmic expression of human AFP, Hep Par 1, CK18, CK7, and OV6). Liver engraftment with human hepatocyte-like cells denoted the homing of MSCs to injured livers and the ability of MSCs to differentiate into hepatocytes.[24,25]

Salem and Thiemermann stated that the therapeutic potentialities of stem cells were based on their tendency to home to the sites of inflammation following tissue injury when introduced IV.[26] While the mechanisms driving this property were not fully understood, Ley et al. found that the injured cells were induced to express specific receptors or ligands that could facilitate trafficking, adhesion and infiltration of MSCs to the damaged sites, in a way very similar to leukocytes.[27] Furthermore, it has been demonstrated that MSCs express receptors for chemokines and ligands involved in leukocyte migration during inflammation, including SDF-1 and CXCR4.[28,29] Hepatic growth factor (HGF) secreted by liver-engrafted MSCs could promote their trans-differentiation into parenchymal hepatocytes.[30]

Our results showed a significant reduction in fibrotic index and improvement in liver function tests following MSC transplantation in both animal models in comparison to the corresponding pathological controls, which was more evident 12 weeks after injection. Many experimental reports showed a promising outcome of MSCs transplantation, as they have the potential to almost completely restore liver function, ameliorate symptoms and enhance survival rates in many hepatic disorders. [24,31]

Mesenchymal stem cells can prevent the hepatocytes from undergoing fibrogenesis by secreting a variety of cytokines, such as HGF, IL-6 and IL-10.[32] It has been proven that HGF has anti-apoptotic activity in hepatocytes and plays an essential role in liver regeneration.[33,34] Additionally, MSCs were found to have the potential to attenuate fibrosis in a CCl_4-induced liver fibrogenesis animal model by directly suppressing hepatic stellate cells.[35]

In the current study, the liver sections obtained from S. mansoni-infected mice showed large fibrocellular granuloma. Transplantation with 1×10^6 MSCs after S. mansoni infection showed a diminished granuloma size and a relative decrease in hepatic fibrosis, starting from the 4th week post-transplantation, and reaching its maximum improvement level 12 weeks after MSCs injection. Similar to our findings, Abdel Aziz et al. reported a significant reduction in the hepatic collagen content of hepatic fibrosis 4 weeks post-administration of MSCs and attributed this finding to a modulation in the expression of the *MMP* and *TIMP* encoding genes.[36] Several animal studies and clinical trials stated that MSCs have the potential to reverse the fibrogenesis process by inhibiting collagen deposition and TGF-β1 production.[9] Four weeks post-MSCs transplantation in the Schistosoma model, we detected the engraftment of MSCs into the fibrotic livers with evidence of some signs of regeneration, such as the appearance of newly formed hepatocytes, while the most prominent improvement in mice liver functions came 12 weeks after MSC injection. We found that the IV route was more effective in reducing the fibrosis index and improving liver function than the IH route, as did Kuo et al., who demonstrated that IV injection was more effective in rescuing liver failure than intrasplenic transplantation.[37]

It was observed that the improvement in both fibrosis index and liver function was more obvious in the CCl_4 model than in the S. mansoni-infected model. This could be attributed to the underlying pathology in each model, as CCl_4, a metabolite produced by cytochrome P-450 in hepatocytes, leads to lipid peroxidation and membrane damage, which results in a reversible acute centrilobular liver necrosis.[38] Meanwhile, in the S. mansoni model, most of the pathology is attributed to the host's reaction to the eggs, which reaches its peak by the 8th week of infection, and to the mice not receiving any treatment, so cumulative damage could have occurred. This balance is influenced by the varying competence of the host to kill worms, to inhibit worm fecundity and to destroy eggs and repair tissue damage.[39,40]

In conclusion, CB-MSCs transplantation succeeded in ameliorating liver fibrosis in both Schistosoma and CCl_4 chronic liver injury experimental models, as evidenced by the engraftment of the fibrotic livers with newly formed hepatocytes, diminished hepatic fibrosis and fibrotic index of the liver sections, and improvement in liver functions. Our results provide hope that CB-MSCs could be introduced as multipotent stem cells with great potentiality in cell-based therapy of liver fibrosis.

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The assessment of the integrated antioxidant system of the body and the phenomenon of spa reaction in the course of radon therapy: A pilot study

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1341–1346

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Funding sources
The study was financed within the framework of the Polish Radon Cluster.

Conflict of interest
None declared

Received on November 12, 2016
Reviewed on December 6, 2016
Accepted on March 13, 2017

Abstract

Background. Spa reaction is an adaptive response of an organism, emerging as a result of external factors. It is a positive element of spa therapy leading to metabolic changes, which are important for the body. The effect of these changes is an increase in immune activity and regenerative reactions of the body. The mechanism of the response is not fully known.

Objectives. The aim of the study was to evaluate the changes observed in the field of the integrated antioxidant system of the body in the course of radon therapy, especially in reference to spa reaction.

Material and methods. The study was conducted in the health resort in Świeradów-Zdrój. The observation regarded patients undergoing treatment with radon water. Before the treatment, after 5 and 18 days of treatment, the total antioxidant status (TAS) was evaluated with the use of a standard colorimetric assay. The study group consisted of 35 patients with degenerative joints and disc disease. The control group consisted of 15 people selected from the employees of the spa, also suffering from osteoarthritis, who did not undergo radon therapy (without contact with radon).

Results. On the 5th day of the treatment, in both groups, the TAS increase was observed with significantly worse results in the control group. After the treatment, in the study group, an increase in TAS was observed, whereas in the control group, a significant decrease in the TAS concentration was noted.

Conclusions. A beneficial effect of radon treatments on the growth of TAS in the body of the patients treated in the spa was demonstrated. The increase in the TAS concentration on the 5th day of treatment may indicate the relationship between these changes and the phenomenon of spa response. The changes are a result of low doses of ionizing radiation originating from radon dissolved in medicinal water, used in the course of the therapy.

Key words: balneotherapy, radiation hormesis, total antioxidant status, radon water, spa reaction

DOI
10.17219/acem/69450

Introduction

In living organisms, oxygen reacts with organic compounds oxidizing them without undergoing total reduction due to the action of various, both external and internal, factors. The consequence of these shortcomings is the formation of reactive oxygen species (ROS). Among ROS, which are also called free radicals, there can be: singlet oxygen $^1O_2$, superoxide anion, hydrogen peroxide H_2O_2, and a hydroxide radical OH responsible for intracellular damage and, in consequence, apoptosis. In the state of health, the level of free radicals is strictly controlled in the process of maintaining a balance between their generation and removal.[1–3] The generation of ROS or decreased possibility to remove free radicals is defined as oxidative stress.[4,5] The consequence of these effects is cell damage and the development of many diseases, such as atherosclerosis, Alzheimer's disease, Parkinson's disease, inflammation, degenerative diseases of the skeletal system, allergies, cancer, diabetes, macular degeneration.[6–8] The human body can be protected from the harmful effects of ROS by producing various metabolic reactions, both regulating and blocking the production of free radicals. The antioxidant defense system includes:

– endogenous antioxidants – produced by the body:
  - enzymatic – antioxidant enzymes: superoxide dismutase, glutathione peroxidase, catalase;
  - non-enzymatic: linolenic acid, polyamides, albumin, bilirubin, glutathione, uric acid, ceruloplasmin, transferrin, coenzyme Q10 (these compounds have different points of binding);[9]
– exogenous antioxidants – delivered from the outside: vitamins C, A, E, carotenoids, xanthophylls, polyphenols.

These compounds are directly involved in free radical reactions; they affect both the transmission of cellular signals and the activity of enzymes or genes involved in the processes of cell death and DNA repair.[10–12]

The evaluation of the antioxidant system can be performed with the use of a variety of methods, by assessing the total antioxidant status (TAS) or its individual components, especially in the first studied group. The present study concerned the total antioxidant capacity of plasma, TAS. The integrated antioxidant system embracing all biological components active in preventing oxidation was assessed.

Radon therapy in Europe has been used for over 100 years. Obviously, therapeutic programs were modified throughout the last century due to actively developing hydrotherapy and spa therapy. In Polish conditions, radon therapy can be ordained medically only by a doctor after considering the indications and contraindications, and then the standard number of treatments is prescribed. The number of treatments, their duration and type, such as inhalations, baths or mouthwashes (especially periodontal rinses) are defined. Radon health resorts use water from natural sources, from drilling performed in accordance with the law of mining, with the approval of the Ministry of Environment and under the supervision of the Ministry of Health. Medicinal water has to meet certain criteria of bacteriological and balneochemical tests in order to be considered curative.

Radon in large doses has a negative impact on health, and its adverse effects are connected with damaging enzymes and nucleic acids, which leads to the formation of neoplastic cells. Therefore, while using radon as a medicinal material one should have these effects in mind. The basis for a rational therapeutic effect of radon water is the so-called hypothesis of radiation hormesis. According to the theory, small doses of ionizing radiation activate life processes. Currently, there are no scientific reports explaining the mechanisms of radiation hormesis. Among the mechanisms at the level of the cell control system there are: stimulation of DNA repair processes, protein synthesis, activation of genes, production of stress proteins, detoxification of radicals, activation of membrane receptors, proliferation of splenocytes, and stimulation of the immune system, resulting in a lower risk of mutations or tumorigenesis.[13]

Radon water is specific water containing small quantities of unstable radioactive elements – radon and its decay products. The water is used in medicine when the content of radon exceeds 74 Bq/L (2 nCi/L), and when it meets operational and hygienic requirements. Radon is a chemical element formed in the process of disintegration of radioactive uranium and thorium. It is a noble, colorless and odorless gas, soluble in water, especially in weakly mineralized or acidified water. There are many radon isotopes, among which there is a precursor – radon-222 derived directly from radium-226 through alpha decay. The emitted alpha particles have low penetrating power, but high ionizing ability. The half-life is 3.8 days. The concentration of radon in natural conditions changes constantly throughout the day, as well as seasonally, in connection with precipitation. In the course of therapy, radon loss is also observed for technical reasons: while collecting water in reservoirs, transmission pipes, heating, chilling, and during intense exploitation. The decrease of radon content varies from 40% to 80%. This large variation in the concentration of radon at a collecting place makes calculating the dose impossible; thus, it is not practiced.[14] The absorption of radon in 95% occurs in the lungs, whereas the excretion from the body occurs in 90% through the lungs and the remaining part – through the kidneys and skin. During radon baths, the absorption occurs mainly through inhalation, because radon and its derivatives accumulate in large quantities above the water. The lungs are exposed to radon to a significant extent due to the deposition of the degradation products in the alveoli. The radioactive residue is deposited on the skin surface and kept there for several hours. Radioactive decay in the body is very diverse and largely depends on the amount of body fat.

In this process, the adrenal cortex, liver and muscles also play an important role. The anti-inflammatory, desensitizing and analgesic action of radon can be explained by the stimulation of the adrenal cortex and an increased production of steroid hormones. In the study, there was observed an increase in the concentrations of luteinizing hormone and growth hormone in serum, accompanied with an increase in cortisol, testosterone, estradiol, and estriol. Radon treatments improve peripheral circulation, reduce swelling, arthritis, tendon and muscle pain, and improve performance mobility. Lowered blood pressure, cholesterol and triglycerides, decreased red blood cell sedimentation reaction, increased hemoglobin levels and red blood cells, increased levels of ionized calcium, parathyroid hormone and calcitonin, accelerated removal of harmful products of metabolism were also observed.[15–18]

Spa (balneological) reaction is the body's adaptive response, created as a result of external factors (stimuli). It is a positive element of spa therapy, leading to metabolic changes, which are important for the body. Spa reaction is often the result of the use of a medicinal stimulus exceeding the body's compensation capacity. It is followed by excessive secretion of histamine, stimulation of the autonomic nervous system, burst of adrenal hormones by endocrine glands. The effect of these changes is an increase in the immune system and regeneration reactions. The mechanism of spa reaction is not yet fully examined. It is assumed, however, that in the abovementioned process it is mainly the autonomic nervous system that reacts to the applied stimulus.

The intensity of spa reaction depends on the type and quantity of natural medicinal materials used in the conducted study of radon water. The factors that determine the intensity of spa reaction are also the patients' age and state of health. In the image of spa reaction there may appear specific variable symptoms depending on the type of disease and non-specific ones, related to the reactivity of the organism treated with medical stimuli. These are mostly common ailments not previously observed in the patient. The symptoms can also be generalized in nature, have a local character and different levels of intensity. General symptoms include most commonly: increased erythrocyte sedimentation rate (ESR) and leukocyte number, an increase in body temperature, blood pressure, muscle pain, a decrease in overall physical performance. Local symptoms concern most commonly skin symptoms in the form of erythema, pruritus or urticaria. The reaction can affect even 70–80% of patients. The peak of spa reaction falls on the 2nd–4th day of the stay in a spa, increasing after about 7 days. On about the 14th day of the stay at a sanatorium, the symptoms of spa reaction start to decrease. The exacerbation of the underlying disease and the activation of inflammatory reactions often appear. The clinical symptoms of the reaction are characterized with the intensity of muscle and joint pains, headaches, coronary pain, swelling of joints, loss of appetite, fatigue, irritability, depression, and sleep disorders. A characteristic feature of spa reaction is its division into phases: the initial phase I (lasts from 2 to 7 days) – labile reactions; the ergotrop phase II (lasts for about 1 week) – a period of an increased response; the trophop phase III (lasts until the end of treatment) – a relief reaction, a period of recovery and adaptation.

In phase III, the clinical improvement is achieved, often noticeable especially after returning home for a period of 8–10 months. Spa reaction usually occurs in patients who have used sulfur or radon baths and holistic mud peloid treatments. During the severe phase of spa reaction, it is advisable to reduce the intensity of the medicine stimulus, and sometimes it is recommended to temporarily discontinue the treatment.[19,20]

The aim of the study was to evaluate the changes in the scope of the integrated antioxidant system of the body in the course of radon therapy in terms of spa reaction.

Material and methods

The study was conducted in the health resort Świeradów-Zdrój. The observation included patients undergoing treatment in the spa within 21-day sanatorium stays. Venous blood was collected from patients before the treatment and after 18 days of treatment in the spa. Heparin plasma was used. To evaluate the TAS, a standard colorimetric assay by Randox Laboratories Ltd. (Warszawa, Polska) was applied. The obtained compound was characterized by a relatively stable blue-green color denoted with the use of light having a wavelength of 600 nm. The presence of antioxidants in the sample weakens in the emergence of color in proportion to their concentration. The reference range of plasma in the sample was 1.30–1.77 mmol/L and it was determined for the European working population. The reference range is variable depending on genetic factors, and it is also characterized by local variability. The studies were non-randomized. The study group consisted of 35 patients with joints and spine pains caused by osteoarthritis or a discectomy. The age of patients ranged from 47 to 63 years. The mean age of patients was 56.5 years. Among the respondents there were 23 women and 11 men. The essential criteria for the selection of patients were the presence of degenerative joints and/or disc disease, the age range of 45–65 years, the consent to participate in the study, and no contraindications to comprehensive treatment in the spa. The exclusion criteria were the lack of consent to participate in the research, age below 45 years and over 65 years, the presence of diseases constituting a contraindication to treatment (compatible with a standard list of indications and contraindications to spa therapy), and the presence of metabolic diseases. The patients were on a normal or light diet dominated by dishes

prepared with low fat content. Both diets were standard calorie diets and there were no vitamin supplements used. Medicinal radon water was used in the therapy. Among the types of treatments applied there were: comprehensive radon bath – at a temperature of 37°C, with a duration of 15 min, the treatments performed every other day; radon inhalations – at a temperature of 37°C, with a duration of 15 min, the treatments performed every other day. Baths and inhalations were performed interchangeably and the total number of radon treatments during 1 stay was 15. In addition, the following forms of therapy were used: kinesitherapy – with a duration of 30–45 min; physiotherapy – in limited extent due to the possibility of activating oxidation processes. The conducted study involved curative radon water from Świeradów-Zdrój that has been used in treatments for more than 100 years. The natural water with low mineralized content plays a major therapeutic role with the parameters of Rn 303.1–441.5 Bq/L. In the treatment rooms (an inhalation room, cabins with baths and a swimming pool), the alpha radiation was 184.4–450.0 MeV. This evaluation allows one to determine the exposure of the patient. In contrast, the calculation of the absorbed dose of radiation is not carried out, because it is a variable value. It depends both on the body anatomy, especially the fat content and the surface of absorption, and the presence of some diseases, as well as on radon loss dependent on its exploitation, as mentioned in the Introduction. The measurements were performed in a room on a daily basis with the use of certified detectors. The measurements were analyzed every 3 months at the Institute of Occupational Medicine in the Department of Radiation Protection in Łódź.

The study was designed with the participation of the control group. The control group consisted of 14 people selected from the employees of the spa, including 9 women and 5 men, aged from 50 to 62 years, with the average age of 54.2 years. The control group included persons suffering from osteoarthritis of the musculoskeletal system who were not given treatments with radon. The essential criteria in the selection were the presence of degenerative joints and disc disease, the age range of 45–65 years, the consent to participate in the research, and lack of diseases which are a contraindication to treatment. The exclusion criteria were the lack of consent to participate in research, age below 45 years and over 65 years and the presence of diseases which constitute a contraindication to treatment. The selection and exclusion criteria in the control group were the same as in the study group.

The study was conducted within the framework of the Polish Radon Cluster. The initiative Polish Radon Cluster was incorporated on 12 November, 2014. It is an organization which unites Polish radon spas that offer radon treatments on a large scale. Among the partners of the Radon Polish Cluster there are: Wroclaw Medical University, the Association Forum of Local Activity and Sudecki Business Incubator, Świeradów-Czerniawa Spa Ltd. – PGU Group, Lądek-Długopole SA Spa. The mission of the Polish Radon Cluster is to promote the use of the unique therapeutic and rehabilitation potential, which is at the disposal of spa management in mining areas, with the deposits of radon water, in the form of a voluntary agreement of entrepreneurs, individuals, legal entities, and research groups. The aim and the initiative of the Cluster relies in creating in Poland, particularly in the region of Lower Silesia, a modern national center for radon treatment based on the results of research and know-how of the members of the Polish Radon Cluster.

The study was approved by the Bioethics Committee of Wroclaw Medical University, Poland (No. 135/2015). A written consent was obtained from the President of the Świeradów-Czerniawa Spa and individual written consents were also obtained from the patients; they were prepared in accordance with the model recommended by the Bioethical Committee of Wroclaw Medical University. The documentation is held by the authors of the work.

For statistical analysis, STATISTICA v. 12 program (Randox Laboratories Polska, sp. z o.o. Warszawa, Poland) was used. Arithmetic means (a measure of the position), standard deviations (a measure of variability) and a range of variability (extreme values) were calculated for measurable variables. All the tested quantitative variables were checked with the Shapiro-Wilk test to determine the type of distribution. The comparison between the results of measurements in each group was performed using the Friedman ANOVA and post-hoc test. For all the comparisons, the level of $\alpha = 0.05$ was assumed and the obtained p-values were rounded to 4 decimal places.

Results

In the study group, an increase in the level of TAS in trials II and III was noted. In the control group, in test II, the level of TAS increased, whereas in trail III, a decrease of TAS below the output value was noted. The Friedman ANOVA analysis of variance showed statistically significant changes in the test group and a slightly lower result in the control group. A multiple comparison with the use of the Friedman post-hoc test ANOVA of measurements I and II, II and III showed a statistically significant change in relation to the study group (an increase in the TAS concentration). In the case of the control group, an increase in the concentration of TAS in test II was statistically significant. In contrast, the comparison of measurements II and III showed a decrease in the TAS concentration and exhibited the characteristics of significance. The p-value in the comparison of measurement I and III for the 2 groups did not meet the target level of significance (Table 1, Fig. 1).

Table 1. Comparison of results in both groups

Parameters	TAS [mmol/L]	N	$\bar{x}$	Min	Max	SD	p-value*	p-value**
Study group	measurement I	35	1.82	1.55	2.06	0.14	0.0000	I vs II – p = 0.0001 I vs III – p = 0.3788 II vs III – p = 0.0001
	measurement II	35	2.07	1.80	2.53	0.20		
	measurement III	35	1.87	1.71	2.13	0.12		
Control group	measurement I	15	1.83	1.65	2.09	0.11	0.0013	I vs II – p = 0.0071 I vs III – p = 0.9444 II vs III – p = 0.0030
	measurement II	15	1.97	1.81	2.21	0.12		
	measurement III	15	1.81	1.62	2.10	0.14		

N – number of trials; $\bar{x}$ – arithmetic mean (a measure of position); Min – minimum; Max – maximum; SD – standard deviation (a measure of variability); * the Friedman analysis of variance (ANOVA); ** multiple comparisons in the Friedman post-hoc ANOVA.

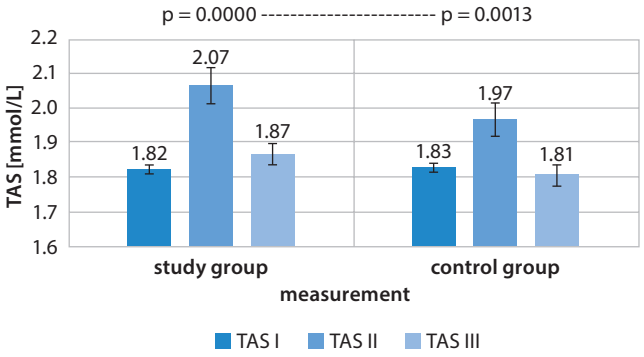

Fig. 1. Comparison of the means in both groups for 3 measurements – variance analysis (Friedman ANOVA)

Discussion

In the study group, an increase in the TAS concentration after the treatment was noted, whereas in the control group, a decrease in TAS of the system was noted, which can indicate and determine the effectiveness of the spa therapy. In the conducted observation, an important issue was the assessment of the level of TAS on the 5th day of the treatment (measurement II). In both groups, an increase in TAS of the system was found. In the study group, there was a statistically significant change in the concentration of TAS (p = 0.0001), whereas in the control group it was p = 0.007 (Table 1). The obtained TAS results may indicate the sensitivity of the antioxidant system to radon therapy. The increase in the TAS concentration in the study group was due to the activation of the antioxidant system of the body as a result of the conducted therapy. However, in the control group, the increase in TAS on the 5th day is unclear, and the change in value is less significant. In addition, the end result indicates a decrease in the concentration of TAS in the control group, which could ultimately indicate the lack of a factor stimulating the antioxidant system. The activation of the antioxidant system may be affected by a number of metabolic changes, which were discussed in the Introduction. Currently, it is difficult to assess the extent to which ionizing radiation stimulates the antioxidant system. There are not many publications that would match the results of the conducted observation. Most articles published in the 60s, 70s and 80s of the 20th century, as well as studies on animals conducted in Japan, discussed the effects of radon therapy in very general terms. The studies demonstrated mainly the analgesic effects, decreased swelling, improvement in physical fitness. Years later, the studies on the subject matter showed that radon decreased the release of ROS in patients with Bechterew's disease and acted as an anti-proliferative agent to tumor cells, stimulating, i.a., the activity of superoxide dismutase.[21–25] Most of the publications discuss the health effects of ionizing radiation exposure, but only in reference to a specific case. Some of the reports refer to the studies of the populations living in areas with naturally high radiation or populations inhabiting the areas after a nuclear explosion, and those after occupational exposures. These studies showed that low doses of radiation had not only a neutral but also a positive impact on health.[26] Spa reaction is an important criterion of the body's reactivity to medicinal stimuli in the course of therapeutic spa treatment. When evaluating the results, it can be assumed that the human body receives a balneological stimulus as a factor causing free radical reactions. The process reveals a variety of symptoms and conditions. In a further step, by the accumulation of stimuli one can observe a change in the metabolism and an activation of immune reactions. It is followed by an increase in the production of antioxidants. The end result is a higher level of TAS of the body, the start of the adaptation and regeneration processes, and the clinical improvement after the treatment. The use of balneological stimuli should be individualized and adjusted to the adaptive capacity of the patient. The use of inappropriately strong stimuli may cause adverse effects in the form of more or less serious disequilibrium in homeostasis. On the other hand, the use of a medicinal stimulus with inadequately low intensity will not cause the expected results in terms of improving the health status of the patient. Spa reaction as a specific body response finally resulting in the improvement of the patient status (which occurs in the last phase of spa treatment) may express a controlled disturbance of homeostasis through a complex, lasting several days, activity with an exposure to the stimulus. The stimuli used in a spa express the same

character as adaptive stress. In the case of radon water used in the study, as a result of ionizing radiation (stimulus), used with specified time intervals, there was observed an increase in the resistance to a stress-applied stimulus. The result of these reactions will be adaptation, body hardening and habituation. A possible positive effect is an increase of the body's immune reactions due to little known mechanisms of temporary, functional or morphological changes dependent also on the stimulation of the autonomic nervous system. These mechanisms may give rise to positive changes determining the systemic improvement after the spa treatment.[27,28] In the case of radon water with low doses of radiation, it is relatively difficult to find statistically reliable material. Much of the work in which the conclusions are drawn is not based on the observations of the effects of radiation, but on their absence. A big problem is the absorbed dose of radiation, the scope of systemic changes and the loss of radiation during radon water distribution. The main element believed to activate the transformation are low doses of absorbed ionizing radiation used in accordance with the theory of radiation hormesis. Irradiation of a large mass of tissue brings more pronounced systemic effects than focusing all radiation energy on an isolated part of the body. A possible positive effect is habituation.[29,30] It can be assumed that an increase in immunity, physical fitness, pain relief, the normalization of blood pressure, a decreased heart rate are the effects of small doses of ionizing radiation, resulting from the stimulation of the antioxidant system. A clear indicator of such changes is the increase in TAS of the body in the study group after the treatment.

The assessment of the positive impact of radon therapy and other forms of spa therapy treatment in reference to the phenomenon of spa reaction requires conducting multidisciplinary research with a specific explanation of the significance of the phenomenon of radiation hormesis. It is desirable to design randomized studies conducted in large groups of patients.

Conclusions

A beneficial effect of radon treatments on the growth of TAS in the body of the patients treated in the spa was demonstrated.

The increase in the TAS concentration on the 5th day of treatment may indicate the relationship between these changes and the phenomenon of spa response.

The changes are a result of low doses of ionizing radiation originating from radon dissolved in medicinal water used in the course of the therapy.

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Dietary acid load and cardiometabolic risk in the Polish adult population

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1347–1354

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Funding sources
WOBASZ II project was financed from the financial resources at the disposal of the Minister of Health within the framework of the health program entitled: National Program of Equalization the Accessibility to Cardiovascular Disease Prevention and Treatment for 2010–2012 POLKARD – goal: Analyses and epidemiology – "Monitoring of the epidemiological situation in Poland in the field of cardiovascular diseases".

Conflict of interest
None declared

Acknowledgements
Authors express special thanks to the research team and collaborating persons from the field centers in 16 voivodeships, and to all participants of the WOBASZ and WOBASZ II studies.

Received on January 10, 2017
Reviewed on February 5, 2017
Accepted on March 24, 2017

DOI
10.17219/acem/69733

Abstract

Background. The potential influence of disorders of acid/base homeostasis on cardiovascular risk factors has been suggested.

Objectives. The aim of the study was to estimate the relationship between dietary acid load and the prevalence of cardiovascular disease and the prevalence and intensity of cardiovascular risk factors (i.e., hypertension, diabetes, overweight and obesity, dyslipidemia) in the Polish adult population.

Material and methods. Data was derived from a cross-sectional survey of a random sample of 6170 Polish residents aged 20+ (Multi-Center National Population Health Examination Survey, WOBASZ II study), including anthropometric and laboratory measurements, and estimates of nutrient intakes by 24-hour recall. Dietary acid/base load was assessed as potential renal acid load (PRAL) and net endogenous acid production (NEAP).

Results. The median PRAL and NEAP values for the whole study population were: PRAL –3.85 mEq/day and NEAP 39.79 mEq/day. The prevalence of overweight and obesity, both in males and females, tended to decrease across tertiles of PRAL and to increase across tertiles of NEAP. In females, the values of several metabolic characteristics differed across tertiles of NEAP. After adjustment for age and waist circumference, these relationships did not persist, but the prevalence of diabetes was found to increase across tertiles of PRAL (p for trend <0.05) in females.

Conclusions. The dietary acid load in the Polish adult population was relatively low. There was no independent relationship between dietary acid load and cardiovascular disease and its risk factors in the population under study, except for the positive association between the PRAL value and diabetes prevalence in females.

Key words: cardiovascular risk factors, Polish population, dietary acid load

The body's acid/base balance is one of the essential factors that influence the human metabolism and thus also health.[1,2] Its disturbances may negatively affect the organism. Metabolic acidosis seems to foster osteoporosis and renal calculosis. It was also suggested that it may contribute to the development of arterial hypertension, atherosclerosis, diabetes, and cancer.[3–8]

It is widely accepted that diet may influence the body's acid/base balance as consumed nutrients are precursors to nonvolatile acids (hydrogen sulphate that arises from sulfur-containing amino acids, and phosphoric acid from phospholipids) and alkaline substances (cations of potassium, magnesium and calcium). Meat, fish, cheese, cereals, and rice have a relatively high acid-forming potential, while fruits, legumes, vegetables, red wine and potatoes have a base-forming potential.[9] The Western dietary pattern is characterized by a big share of products of high acid load, which impedes the maintenance of the optimal body acid/base balance, i.e., the lack of acidosis or mild metabolic acidosis.[10]

It is possible to calculate the potential renal acid load (PRAL) of every food item or diet as a whole. A negative PRAL value indicates a base-forming potential of the ingested food, while a positive value points to its acid-forming potential.[11,12]

The daily PRAL, along with a relatively constant 24-hour amount of organic acids excreted with urine, which is related to the individual body surface area, contributes to the daily renal net acid excretion. There are 2 methods that enable the evaluation of renal net acid excretion. One of them is a direct measurement in the 24-hour urine collection. The 2nd, more practical to be applied in epidemiological studies, relies on calculations using validated formulae that take into account nutrient intake data. There are several available algorithms created for the prediction of the daily net endogenous acid production (NEAP).[13]

Until now, no large epidemiological study that would evaluate the link between the dietary acid load and cardiovascular risk in the Polish population has been performed. The aim of our investigation was to estimate the relationship between dietary acid load and the prevalence and intensity of cardiovascular disease risk factors (i.e., hypertension, diabetes, overweight and obesity, dyslipidemia) and the prevalence of cardiovascular disease (CVD) in the Polish population.

Material and methods

Study procedure

The data that was used in the present study came from the Multi-Center National Population Health Examination Survey (WOBASZ II study). The project was carried out by the Institute of Cardiology (Warszawa, Poland), together with 5 medical universities in Poland, in 2013–2014. The study was accepted by the Bioethics Committee of Institute of Cardiology in Warszawa.

Study population

The survey included a random sample of inhabitants of the whole Poland aged 20 and above. A total number of 6170 respondents participated in the study; 45.5% of the sample of available randomized subjects. The details concerning the randomization protocol and participant selection were presented elsewhere.[14]

Laboratory measurements

Laboratory tests were performed in the Central Laboratory "Diagnostyka" at the Institute of Cardiology in Warszawa, which holds the CDC certificate (Center for Disease Control – Lipid Standardization Program) in Atlanta and the European certificate of quality RIQAS (Random International Quality Assessment Scheme). Measurements of cholesterol and triglycerides, LDL- and HDL-cholesterol, and glucose concentrations were performed. The details concerning blood sampling, transport, and methodology of measurements were described elsewhere.[14]

Assessment of hypertension/ anthropometric measurements

Blood pressure measurements were performed 3 times at the right arm by means of automatic AND UA-631 devices (A&D Company Ltd.; R&D Technical Centre, Saitama, Japan), approved by AAMI (Association for the Advancement of Medical Instrumentation). For the assessment of the blood pressure value, the average from the 2nd and 3rd measurements was taken.

Dietary assessment

Dietary assessment was performed by skilled nurses using a quantitative interview on the consumption of products and dishes in the period of 24 h prior to the examination. For a precise assessment of the amount of food consumed, an album developed by the Food and Nutrition Institute in Warsaw with over 200 photographs of the most commonly consumed dishes, products and drinks was used. Additionally, information about taking dietary supplements was collected. In the dietary analysis data from 5690 respondents (2554 males and 3136 females) were used, after the rejection of 459 cases (197 males and 262 females) because of a lack or unreliability of the interview. Based on the conducted interviews, energy and nutritional values of the diets were calculated, using the Polish food composition database.[15] In the case of vitamins and minerals intakes both with food and dietary supplements were taken into account.

Calculation of dietary acid load

Values of PRAL and NEAP were calculated from estimated nutrient intakes[3]:

1. PRAL [mEq/day] = (0.49 × protein [g/day]) + (0.037 × × phosphorus [mg/day]) – (0.021 × potassium [mg/day]) – – (0.026 × magnesium [mg/day]) – (0.013 × calcium [mg/day]);
2. NEAP [mEq/day] = PRAL [mEq/day] + OA_{est} [mEq/day].

OA_{est} denotes estimated urinary organic anions, calculated according to the formula:

$$OA_{est}\ [mEq/day] = \text{individual body surface area} \times 41/1.73$$

$$\text{body surface area}\ [m^2] = 0.007184 \times \text{height}\ [cm]^{0.725} \times \times \text{weight}\ [kg]^{0.425}.$$

Statistical analysis

The results were analyzed in 2 steps. In the 1st, the tertiles of PRAL and NEAP were defined and the type of distribution of continuous variables were determined (the Shapiro-Wilk test). The majority of the variables were non-normal. Differences of medians between men and women (Table 1) were tested by the Mann-Whitney-Wilcoxon test. To compare the medians of variables in tertiles of PRAL and NEAP the nonparametric Kruskal-Wallis test for continuous and the χ^2 test for categorized variables were used. In the case of significance, or borderline significance of differences of medians between tertiles, linear trends were analyzed using the Cochran-Armitage test (Tables 2,3). In the 2nd step, comparison of means or frequency, adjusted by age and waist circumference, were tested by using the multivariate analysis of covariance and regression. Likewise, for significantly different adjusted means or frequencies, the linear trends were tested (Tables 4,5). P-values of less than 0.05 were considered statistically significant. Data analyses were processed using Statistical Analysis System (SAS, v. 9.2, SAS Institute Inc., Cary, USA).

Table 1. Median values of the main characteristics of the study participants

Characteristic	Total n = 6169	Men n = 2760	Women n = 3409	p-value
Age [years]	n = 6169 51.00	n = 2760 49.00	n = 3409 52.00	<0.01
BMI [kg/m^2]	n = 5849 26.57	n = 2631 27.08	n = 3218 26.03	<0.0001
Waist circumference [cm]	n = 6005 92.00	n = 2684 97.00	n = 3321 87.00	<0.0001
PRAL [mEq/day]	n = 5690 –3.85	n = 2554 0.88	n = 3136 –7.10	<0.0001
NEAP [mEq/day]	n = 5394 39.79	n = 2434 47.55	n = 2960 34.83	<0.0001

BMI – body mass index; NEAP – net endogenous acid production; PRAL – potential renal acid load.

Results

The median PRAL and NEAP values for the whole study population were: PRAL –3.85 mEq/day and NEAP 39.79 mEq/day. Both PRAL and NEAP values were significantly lower in the female than in the male group, similarly to the BMI and waist circumference values (Table 1).

Characteristics of participants, stratified by tertiles of PRAL, are presented in Table 2. There were no significant differences across tertiles regarding the prevalence of smoking habits, CVD, arterial hypertension, diabetes, and lipid abnormalities, as well as laboratory and clamp measurements, except for that of HDL-cholesterol level. Among males there were significantly lower age medians in higher tertiles of PRAL (p-value for trend across tertiles <0.0001) and in females significantly the lowest medians of BMI and waist circumference in the 3rd tertile of PRAL. The prevalence of overweight and obesity in both males and females tended to decrease across tertiles, with the p for trend <0.05 and <0.01, respectively.

Characteristics of participants, stratified by tertiles of NEAP, are shown in Table 3. No significant differences were found across tertiles concerning education level, prevalence of smoking habit, cardiovascular disease, arterial hypertension, and lipid abnormalities, except for that of hypertriglyceridemia in females, with the lowest prevalence in the 1st tertile. In males, HDL-cholesterol level was the lowest, and diastolic blood pressure value the highest (at the borderline of statistical significance) in the highest NEAP tertile. In females the values of systolic and diastolic blood pressure, and also glucose and triglyceride levels were significantly the lowest, and HDL-cholesterol level the highest in the 1st than in the higher tertiles of NEAP.

There were significant differences across tertiles of NEAP concerning age in males and body mass in the both sexes. In males, the age tended to decrease (p for trend <0.0001) with the number of tertiles. Both in males and females BMI tended to increase with tertiles of NEAP (p for trend <0.0001and <0.001 respectively), as well as waist circumferences (p for trend <0.0001). In both sexes, across tertiles of NEAP, the prevalence of overweight and obesity tended to increase (p for trend <0.05 and <0.001 respectively). In females, the prevalence of diabetes also tended to increase (p for trend <0.05).

When adjusted for age and waist circumference, there were no significant differences across tertiles, either of PRAL and NEAP, concerning the prevalence of CVD, hypertension, and lipid abnormalities (Tables 4,5). In females, the prevalence of diabetes was significantly the highest in the highest tertile of PRAL (p for trend <0.05). In males the median value of HDL-cholesterol was significantly the lowest in the 1st tertile of PRAL and the mean value of triglycerides the highest in the 2nd tertile of NEAP. The further adjustment for MUFA, PUFA, SFA, and fiber intakes did not reveal any additional significant differences in lipid characteristics across PRAL and NEAP tertiles.

Table 2. Characteristics of the men and women under study by tertiles of PRAL

Characteristic	Men				Women			
	PRAL tertiles			p-value	PRAL tertiles			p-value
	1 (–182.80; –8.06)	2 (–8.05; 9.78)	3 (9.79; 133.06)		1 (–105.58; –13.94)	2 (–13.93; 0.27)	3 (0.28; 67.93)	
median values								
PRAL [mEq/day]	–19.55	0.87	21.75	<0.0001	–22.36	–7.11	9.27	<0.0001
NEAP [mEq/day]	27.31	47.79	70.46	<0.0001	18.53	34.94	51.20	<0.0001
Age [years]	52.00	51.00	46.00	<0.0001 for trend < 0.0001	51.00	52.00	51.00	ns
BMI [kg/m^2]	27.24	26.86	27.08	ns	26.30	26.54	25.70	<0.01 for trend <0.05
Waist circumference [cm]	97.00	96.50	96.00	ns	87.00	87.00	86.00	<0.05 for trend <0.05
Prevalence [%]								
Overweight or obese[a]	71.65	68.49	66.63	0.09 for trend <0.05	60.60	59.61	56.31	0.1 for trend <0.01
Current smokers	26.09	30.54	30.24	ns	19.33	19.33	17.32	ns
Education elementary school secondary school university or equivalent	 46.24 33.65 20.12	 46.53 37.25 16.22	 40.71 39.41 19.88	 <0.05	 35.60 38.76 25.65	 38.79 36.21 25.00	 34.42 39.98 25.60	 ns
Cardiovascular disease[b]	15.98	14.34	13.38	ns	10.72	11.58	12.33	ns
Hypertension[c]	47.96	51.07	52.25	ns	59.13	56.33	59.48	ns
Diabetes[d]	10.36	13.19	11.12	ns	9.24	8.23	11.26	0.06
Hypercholesterolemia[e] High LDL-cholesterol[f] Hypertriglyceridemia[g] Low HDL-cholesterol[h]	54.87 57.00 36.86 20.44	55.72 55.70 35.22 16.69	55.87 56.68 35.97 18.94	ns ns ns ns	57.21 51.34 21.81 21.99	54.84 51.94 22.95 21.83	53.93 49.19 21.47 18.55	ns ns ns ns
Laboratory and clamp measurements (median values)								
Systolic blood pressure [mm Hg]	132.00	131.00	131.00	ns	124.00	124.00	123.50	ns
Diastolic blood pressure [mm Hg]	80.50	80.50	81.50	ns	78.50	79.00	78.50	ns
Glucose [mmol/L]	5.36	5.36	5.33	ns	5.09	5.15	5.12	ns
Total cholesterol [mmol/L]	5.14	5.15	5.17	ns	5.18	5.12	5.10	ns
LDL-cholesterol [mmol/L]	3.17	3.13	3.15	ns	3.03	3.03	2.97	ns
HDL-cholesterol [mmol/L]	1.24	1.31	1.26	<0.01	1.50	1.48	1.53	<0.05
Triglycerides [mmol/L]	1.43	1.34	1.37	ns	1.17	1.20	1.16	ns

BMI – body mass index; NEAP – net endogenous acid production; ns – not statistically significant; PRAL – potential renal acid load; [a] BMI ≥25.0 kg/m^2; [b] the subject declared hospitalization because of heart infarction, coronary heart disease, heart failure, stroke, arterial fibrillation or other arrhythmias, coronary artery angioplasty or bypass, or if a pacemaker was used; [c] systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or antihypertensive drug treatment; [d] fasting glucose ≥7 mmol/L and/or previous diagnosis of diabetes; [e] total cholesterol ≥5.0 mmol/L; [f] LDL-cholesterol ≥3.0 mmol/L; [g] triglycerides >1.7 mmol/L; [h] HDL-cholesterol <1.0 mmol/L for men, HDL-cholesterol <1.2 mmol/L for women or hipolipidemic drug treatment.

Discussion

Similar to our findings were those obtained in the prospective cohort study performed in 2241 older Dutch adults (aged ≥55 years), where the median dietary acid load ranged from –14.6 to 19.9 mEq/day across categories of PRAL, and from 31.7 to 40.5 mEq/day across categories of NEAP.[12] However, a higher dietary acid load was found in the E3N-EPIC cohort study (66,485 women) where median PRAL was –3.0 mEq/day (ranged from –23.0 to 14.3 mEq/day) and NEAP ranged from 31.5 to 58.2 mEq/day,[5] as well as in the prospective cohort study of 87,293 women (The Nurses' Health Study II) where dietary acid load ranged from 34.4 to 76.0 mEq/day across categories of NEAP.[16] Also, in the studies performed in the Japanese populations the dietary acid load was more acidic than in our subjects. In a group of female students of dietetics (n = 1136) the median of PRAL was 10.4 mEq/day (ranged from –0.8 mEq/day to 19.5 mEq/day),[17] and in the Furukawa employees (n = 2028) the median PRAL was 9.0 mEq/day (3.6–13.6 mEq/day) and NEAP 52.1 (45.3–60.0 mEq/day).[3]

Table 3. Characteristics of the men and women under study by tertiles of NEAP

Characteristic	Men				Women			
	NEAP tertiles			p-value	NEAP tertiles			p-value
	1 (–133.630; 38.556)	2 (38.566; 57.480)	3 (57.481; 184.800)		1 (–66.445; 27.480)	2 (27.481; 42.040)	3 (42.041; 115.110)	
Median values								
PRAL [mEq/day]	–19.83	0.49	22.01	<0.0001	–22.45	–7.12	9.28	<0.0001
NEAP [mEq/day]	27.18	47.53	70.38	<0.0001	18.46	34.83	51.20	<0.0001
Age [years]	52.00	51.00	45.00	<0.0001 for trend <0.0001	51.00	52.00	51.00	ns
BMI [kg/m^2]	26.73	26.87	27.62	<0.0001 for trend <0.0001	25.39	26.60	26.49	<0.0001 for trend <0.001
Waist circumference [cm]	96.00	97.00	98.00	<0.001 for trend <0.0001	85.00	87.00	88.00	<0.0001 for trend <0.0001
Prevalence [%]								
Overweight or obesity[a]	66.71	68.56	71.55	0.1 for trend <0.05	53.55	60.79	62.21	<0.001 for trend 0.001
Current smokers	27.62	31.56	28.36	ns	20.69	18.26	17.83	ns
Education elementary school secondary school university or equivalent	 47.78 32.59 19.63	 45.25 37.98 16.77	 39.01 40.25 20.74	ns	 34.79 39.45 25.76	 38.03 37.02 24.95	 35.37 39.43 25.20	ns
Cardiovascular disease[b]	16.52	13.44	13.18	ns	10.95	11.55	12.66	ns
Hypertension[c]	48.81	50.69	51.74	ns	61.21	56.70	56.70	ns
Diabetes[d]	11.15	11.51	11.50	ns	8.47	8.95	11.33	0.07 for trend <0.05
Hypercholesterolemia[e]	54.60	56.65	55.64	ns	55.94	56.56	54.96	ns
High LDL-cholesterol[f]	57.49	55.84	56.56	ns	49.95	52.71	50.05	ns
Hypertriglyceridemia[g]	34.44	36.19	37.44	ns	18.82	24.53	22.33	<0.01 for trend = 0.06
Low HDL-cholesterol[h]	18.41	17.46	19.28	ns	19.14	22.40	20.42	ns
Laboratory and clamp measurements (median values)								
Systolic blood pressure [mm Hg]	131.00	131.50	131.00	ns	122.50	125.00	124.00	<0.05 for trend <0.05
Diastolic blood pressure [mm Hg]	80.00	80.50	82.00	ns	77.50	79.00	79.00	<0.05 for trend <0.05
Glucose [mmol/L]	5.37	5.33	5.33	ns	5.07	5.15	5.14	<0.01 for trend <0.01
Total cholesterol [mmol/L]	5.14	5.20	5.14	ns	5.17	5.16	5.11	ns
LDL-cholesterol [mmol/L]	3.17	3.15	3.14	ns	2.99	3.06	3.00	ns
HDL-cholesterol [mmol/L]	1.28	1.30	1.25	<0.05	1.54	1.48	1.51	<0.05
Triglycerides [mmol/L]	1.36	1.37	1.39	ns	1.13	1.22	1.18	<0.05 for trend = 0.06

BMI – body mass index; NEAP – net endogenous acid production; ns – not statistically significant; PRAL – potential renal acid load; [a] BMI ≥25.0 kg/m^2; [b] the subject declared hospitalization because of heart infarction, coronary heart disease, heart failure, stroke, arterial fibrillation or other arrhythmias, coronary artery angioplasty or bypass, or if a pacemaker was used; [c] systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or antihypertensive drug treatment; [d] fasting glucose ≥7 mmol/L and/or previous diagnosis of diabetes; [e] total cholesterol ≥5.0 mmol/L; [f] LDL-cholesterol ≥3.0 mmol/L; [g] triglycerides >1.7 mmol/L; [h] HDL-cholesterol <1.0 mmol/L for men, HDL-cholesterol <1.2 mmol/L for women or hipolipidemic drug treatment.

In our study, higher values of PRAL were accompanied by a higher consumption of acidifying foods, and lower consumption of alkalinizing foods. In the additional analysis, performed for the requirement of this discussion, the level of consumption of acid-forming foods across PRAL tertiles tended to increase as follows: meat and meat products (men: from 116.0 to 160.0 g/day), eggs (men: from 5.75 to 12.25 g/day; women: from 3.0 to 6.75 g/day), grains (men: from 148.0 to 204.82 g/day; women: from 107.9 to 138.0 g/day), and alcohol (only in males from 4.35 to 6.65 g/day). Simultaneously, consumption of vegetables and fruits tended to decrease. In males, the intake

Table 4. Prevalence of cardiovascular disease, hypertension, lipid abnormalities, and diabetes and median values of cardiovascular risk factors by tertiles of PRAL in males and females, adjusted for age and waist circumference

Characteristic	Men				Women			
	PRAL tertiles			p-value	PRAL tertiles			p-value
	1 (–182.80; –8.06)	2 (–8.05; 9.78)	3 (9.79; 133.06)		1 (–105.58; –13.94)	2 (–13.93; 0.27)	3 (0.28; 67.93)	
Cardiovascular disease[a] [%]	14.8	13.1	14.9	ns	10.4	11.0	12.8	ns
Hypertension[b] [%]	50.3	47.4	50.8	ns	41.1	42.8	42.0	ns
Diabetes[c] [%]	9.7	12.7	12.2	ns	9.1	8.0	11.7	0.01 for trend <0.05
Hypercholesterolemia[d] High LDL-cholesterol[e] Hypertriglyceridemia[f] Low HDL-cholesterol[g]	54.5 56.8 36.6 20.2	54.9 55.7 35.8 17.0	56.8 57.5 35.0 18.3	ns ns ns ns	57.0 51.1 21.2 21.9	54.7 51.6 22.2 21.8	53.6 49.2 21.6 19.0	ns ns ns ns
Systolic blood pressure [mm Hg]	134.1	134.7	134.5	ns	127.4	127.6	128.2	ns
Diastolic blood pressure [mm Hg]	81.2	81.3	82.0	ns	79.3	79.4	79.2	ns
Glucose [mmol/L]	5.58	5.70	5.64	ns	5.35	5.38	5.45	ns
Total cholesterol [mmol/L]	5.16	5.23	5.20	ns	5.21	5.15	5.19	ns
LDL-cholesterol [mmol/L]	3.19	3.16	3.22	ns	3.13	3.07	3.10	ns
HDL-cholesterol [mmol/L]	1.30	1.36	1.33	<0.05	1.53	1.52	1,55	ns
Triglycerides [mmol/L]	1.70	1.87	1.70	ns	1.35	1.35	1.35	ns

ns – not statistically significant; PRAL – potential renal acid load; [a] the subject declared hospitalization because of heart infarction, coronary heart disease, heart failure, stroke, arterial fibrillation or other arrhythmias, coronary artery angioplasty or bypass, or if a pacemaker was used; [b] systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or antihypertensive drug treatment; [c] fasting glucose ≥7 mmol/L and/or previous diagnosis of diabetes; [d] total cholesterol ≥5.0 mmol/L; [e] LDL-cholesterol ≥3.0 mmol/L; [f] triglycerides >1.7 mmol/L; [g] HDL-cholesterol <1.0 mmol/L for men, HDL-cholesterol <1.2 mmol/L for women or hipolipidemic drug treatment.

Table 5. Prevalence of cardiovascular disease, hypertension, lipid abnormalities, and diabetes and median values of cardiovascular risk factors by tertiles of NEAP in males and females, adjusted for age and waist circumference

Characteristic	Men				Women			
	NEAP tertiles			p-value	NEAP tertiles			p-value
	1 (–133.630; 38.556)	2 (38.566; 57.480)	3 (57.481; 184.800)		1 (–66.445; 27.480)	2 (27.481; 42.040)	3 (42.041; 115.110)	
Cardiovascular disease[a] [%]	15.3	12.8	15.1	ns	11.0	11.3	12.6	ns
Hypertension[b] [%]	50.0	48.2	50.6	ns	41.2	42.7	42.6	ns
Diabetes[c] [%]	10.9	11.0	12.5	ns	9.5	8.7	10.7	ns
Hypercholesterolemia[d] [%] High LDL-cholesterol[e] [%] Hypertriglyceridemia[f] [%] Low HDL-cholesterol[g] [%]	53.7 57.3 36.3 19.7	56.2 55.8 36.2 17.3	56.5 56.8 35,2 17.8	ns ns ns ns	55.9 50.5 20.6 20.5	56.3 52.5 24.0 22.2	54.0 49.6 20.9 19.5	ns ns ns ns
Systolic blood pressure [mm Hg]	134.1	134.6	134.5	ns	126.9	128.1	127.9	ns
Diastolic blood pressure [mm Hg]	81.2	81.5	82.0	ns	78.9	79.7	79.2	ns
Glucose [mmol/L]	5.60	5.69	5.63	ns	5.36	5.37	5.43	ns
Total cholesterol [mmol/L]	5.15	5.26	5.18	ns	5.20	5.19	5.19	ns
LDL-cholesterol [mmol/L]	3.18	3.20	3.19	ns	3.12	3.10	3.11	ns
HDL-cholesterol [mmol/L]	1.31	1.35	1.34	ns	1.53	1.52	1.54	ns
Triglycerides [mmol/L]	1.68	1.91	1.70	<0.05	1.33	1.38	1.34	ns

NEAP – net endogenous acid production; ns – not statistically significant; [a] the subject declared hospitalization because of heart infarction, coronary heart disease, heart failure, stroke, arterial fibrillation or other arrhythmias, coronary artery angioplasty or bypass, or if a pacemaker was used; [b] systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or antihypertensive drug treatment; [c] fasting glucose ≥7 mmol/L and/or previous diagnosis of diabetes; [d] total cholesterol ≥5.0 mmol/L; [e] LDL-cholesterol ≥3.0 mmol/L; [f] triglycerides >1.7 mmol/L; [g] HDL-cholesterol <1.0 mmol/L for men, HDL-cholesterol <1.2 mmol/L for women or hipolipidemic drug treatment.

of vegetables declined from 300.0 to 188.9 g/day, and of fruits from 200.0 to 50.0 g/day, while in females consumption of vegetables dropped from 268.0 to 50.8 g/day and that of fruits from 250.0 to 129.4 g/day.

It was found that males in higher tertiles of PRAL were significantly younger (p for trend <0.0001). This suggests that younger males tend to consume a less healthy diet. Nevertheless, a detailed analysis of food consumption in age groups has not been performed yet.

We observed that females in the higher tertiles of PRAL tended to be characterized by lower adiposity, measured by both BMI and waist circumference values. The prevalence of overweight and obesity tended to decrease with tertiles of PRAL in both sexes. It may be assumed that this resulted from a higher intake of protein in the higher tertiles of PRAL. In males it tended to increase from 68.1 to 100.1 g/day, and in females from 53.3 to 68.2 g/day, ($p < 0.0001$). Protein is known as a dietary factor that may suppress appetite and contribute to weight loss.[18] Additionally, males in the higher PRAL tertiles were younger, which may also explains the tendency towards a lower prevalence of overweight and obesity in higher PRAL male groups.

The probable influence of acid-base homeostasis on cardiovascular risk was suggested by other investigators. It seems that diet-induced acidosis may contribute to the elevation of blood pressure. The proposed mechanisms are: increased cortisol production, increased calcium, and decreased citrate excretion. The raised cortisol secretion related to mild metabolic acidosis was postulated to exacerbate also other metabolic risk factors.[17]

To our knowledge, this is the 1st study to examine the relationships between dietary acid-base load measures and cardiovascular risk factors in the Polish population. We did not observe any relevant differences regarding metabolic characteristics across tertiles of either PRAL and NEAP in males. Likewise, there were no essential findings in females, except of the observation that prevalence of diabetes was independently associated with PRAL. It tended to increase across tertiles of PRAL (p for trend <0.05).

Similarly as in our investigation, in the Rotterdam Study there was no relationship between dietary acid-base potential and prevalence of arterial hypertension and also other cardiovascular risk factors. This could be explained by a relatively low dietary acid-forming potential in both populations.[12] On the other hand, in the Nurses' Health Study II, it was shown that high values of NEAP were independently associated with a higher risk of hypertension. The association between estimated NEAP and the risk of hypertension was stronger among lean women (BMI <25 kg/m^2).[16] Likewise, in the investigation preformed in female Japanese dietetic students, higher dietary acid load, expressed by PRAL, was associated with higher systolic and diastolic blood pressure after adjustment for possible confounding factors. It was also shown that there were independent positive associations between PRAL and total and LDL serum cholesterol level, as well as with BMI and waist circumference values.[17]

Our finding concerning the lack of the relationship between the dietary acid load and prevalence of diabetes in males is in accordance with the results of the observational prospective study, done by Hu et al. that involved 911 non-diabetic Swedish men aged 70–71 years, and did not show any link between dietary acid load and insulin sensitivity, beta cell function or risk of type 2 diabetes.[19] In our study the prevalence of diabetes was independently associated only with PRAL in females. Also, in the investigation performed by Fagherazzi et al., it was shown in a large sample of 66,485 women that high values of PRAL, and also NEAP, were linked to increased risk of type 2 diabetes.[5]

The mechanism underlying the putative association between dietary acid load and type 2 diabetes is not fully understood as there are few studies dedicated to this problem. Anyway, there are some mechanisms that could explain this possible relationship. Chronic metabolic acidosis, resulting from high dietary acid load, may probably contribute to insulin resistance and metabolic syndrome, which was suggested by cross-sectional studies, an also confirmed by an interventional study in humans.[20–23] Furthermore, in an animal model, metabolic acidosis impaired the binding of insulin to its receptors, which supported the hypothesis that metabolic acidosis may foster insulin resistance.[24] Moreover, in experimental studies, acid/base alterations were associated with decreased insulin secretion.[25] Inhibition of the insulin signaling pathway and increased hepatic gluconeogenesis are also discussed as possible mechanisms linking metabolic acidosis with diabetes risk.[26] However, the evidence for both, the existence of the relationship between dietary acid load and risk of type 2 diabetes, and also mechanisms underlying this potential association, is very weak.

Our study has some strengths and limitations. Among strengths we can list the following: the large (over 6000 participants), cross-sectional, randomly selected sample of the Polish population, inclusion of nutrient intake from dietary supplements into the analysis, and anthropometric and clamp measurements that were not self-reported, but performed by a professional staff. However, there were also several limitations. First of all, the cross-sectional design does not allow for the assessment of causality owing to the uncertain temporality of the association. Dietary data came from 24-hour recall, which is not considered to be representative for habitual diet at the individual level. Moreover, the misreporting of dietary intake, especially in overweight subjects, is a serious problem for precise dietary assessment.[27] There was no information about renal function, which plays a pivotal role in acid-base homeostasis. And lastly, PRAL and NEAP values were not measured directly, but estimated from self-reported dietary 24-hour intake. Nevertheless, dietary PRAL and NEAP scores are widely used in large sample studies and highly correlate with acid load measured from 24-hour urine.[1,28]

Conclusions

The dietary acid load in the adult Polish population was relatively low. It was lower in females than in males. There was no independent relationship between dietary acid load and CVD or cardiovascular risk factors in the population under study, except for the positive association between the PRAL value and diabetes prevalence in females.

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Benign paroxysmal positional vertigo in patients after mild traumatic brain injury

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1355–1359

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Funding sources
A grant from the Medical University of Lodz
(No. 503/2-036-02/503-21-002).

Conflict of interest
None declared

Received on December 5, 2016
Reviewed on February 9, 2017
Accepted on March 23, 2017

Abstract

Background. Post-traumatic vertigo, dizziness and balance disorders following head trauma range from 15% to 78% in the general population. Benign paroxysmal positional vertigo (BPPV) is the most common vestibular disorder in such patients.

Objectives. The aim of the study was to assess the occurrence of BPPV in patients with mild traumatic brain injury (MTBI) and determine the outcome of treatment.

Material and methods. A group of 179 patients, with a mean age of 45.2 years, complaining of vertigo/ /dizziness and balance instability after MTBI, was enrolled into the study. All these patients were diagnosed and treated in the Department of Otolaryngology (Medical University of Lodz, Poland) between the years 2012 and 2014. Anamnesis and otoneurological examination were conducted in each patient. The diagnosis was based on the medical history, the Dix-Hallpike test or the rollover test. The treatment comprised the Epley, barbecue and particle repositioning (RM) maneuvers.

Results. Nineteen patients (10.6%) complained about attacks of vertigo elicited by positional changes. The diagnosis of BPPV was confirmed in 9 (47.4%) patients: 8 cases with a positive Dix-Hallpike test and 1 with the roll test. In 10 cases, a high probability of BPPV was diagnosed based on the medical history. Eight patients were treated by a single Epley maneuver and 1 patient by the barbecue roll. In 4 (44.4%) patients, the maneuvers were repeated. On the follow-up examination, the patients were not found to have vertigo.

Conclusions. Benign paroxysmal positional vertigo should be diagnosed and treated successfully in patients after head trauma.

Key words: rehabilitation, benign paroxysmal positional vertigo, mild traumatic brain injury

DOI
10.17219/acem/69708

Introduction

Traumatic brain injury (TBI) is increasingly becoming more commonplace, with one of the most common subtypes being mild traumatic brain injury (MTBI).[1–4] Although patients with severe TBI are more likely to seek medical attention at the outpatient ward, many patients with MTBI report to general practitioners or primary healthcare units at varying times after injury, or fail to report it at all.[5] The diagnostic criteria for MTBI used by the American Congress of Rehabilitation Medicine comprise loss of consciousness, the presence of headaches, dizziness or changes in mental status, feeling dazed, disorientated or confused, and experiencing persistent post-traumatic amnesia, as well as cognitive and/or behavioral/emotional symptoms following the injury.[6]

In literature, the incidence of post-traumatic vertigo, dizziness and balance disorders following MTBI is estimated to range from 15% to 78% in the general population and to be approx. 14% in hospitalized patients. Although traumatic disequilibrium or vertigo is not a clinical entity, it refers to the etiology for the heterogeneous presentation of peripheral or central vestibular system dysfunction. The duration of post-traumatic balance system disturbances varies. While 53% of patients report dizziness occurring within 1 week after MTBI, 18% report symptoms persisting for at least 2 years. Post-traumatic balance dysfunction signs may occur in 10–15% of patients experiencing head trauma for 1 year or longer, and in 28% of those classified as MTBI.[4,7]

Benign paroxysmal positional vertigo (BPPV) is a disorder of the inner ear characterized by sudden episodes of severe rotatory vertigo triggered by rapid changes in head position relative to gravity. The provoked situations are lying down, tipping the head up or down, sitting up from a supine position, particularly after awaking in the morning, or turning over in bed. In addition, a reaction can be elicited by the patient looking upward, for example, to place an object on a shelf above the head, or bending forward to tie shoelaces. The majority of patients experience 1 or more seconds of latency following the movement before experiencing violent attacks of vertigo lasting 10–30 s and linear-rotatory nystagmus: the fast phase beating toward the undermost ear, or upward when the gaze is directed to the uppermost ear.[7–9]

A population-based study estimated BPPV to have a lifetime prevalence of 2.4% and accounts for 8% of individuals with moderate to severe dizziness and vertigo.[10] The severity and frequency of the occurrence of BPPV differ and are unpredictable. Most BPPV sufferers experience the "benign" form, where the symptoms resolve spontaneously within weeks or months; however, the complains persist in 20–30% of untreated patients, recurring over a period of years in about 30% of cases.[7,8,11]

Benign paroxysmal positional vertigo is thought to be caused by micro-sized calcium crystals called otoconia, which become dislodged from their normal location in the utricle, one of the sensory organs of the inner ear. These detached otoconia may aggregate into larger clumps in the fluid-filled spaces of the inner ear. Since they are heavier than the endolymph, in 85–95% of cases, they become trapped in the most dependent part, the posterior semicircular canal, when the patient is in a supine position. This causes inertial changes in the endolymph and unwanted fluid flow in the semicircular canal, resulting in ampullofugal cupula deflection, whenever head motion is along the plane of the canal. In 5–20% of cases, this debris could be displaced into the horizontal canal or, in 2–3% of cases, into the anterior canal.[8,9]

In several publications based on large numbers of BBPV cases, the cause of otoconial detachment could not be established in 34–66% of idiopathic or primary BPPV cases, such as in elderly patients.[7,8,12] Although some scanning electron microscopy studies have found otoconia to have degenerated, more than 1 explanation of BPPV is possible, even in elderly patients.[7,8,11] The most common causes of acquired or secondary form of BPPV are thought to be head and labyrinth trauma, inner ear diseases such as viral neurolabyrinthitis or vestibular neuritis, Meniere's disease, vestibulobasilar ischemia and anterior inferior cerebellar artery (AICA) syndrome, or prolonged bedrest due to unrelated disease. Occasionally BPPV is experienced following general surgery or procedures on the inner ear, such as stapes surgery.[7,8]

Many authors found that BPPV was the most common vestibular disorder in patients after head trauma.[12–22] The onset typically appears just after trauma, or a few days or weeks after MTBI, and can be unilateral or bilateral. Even mild head injury could result in the detachment of otoconia in the endolymph. Post-traumatic development of BPPV can also occur as the result of a degenerative process following labyrinth concussion, where after some time, an otoconial clump will accumulate, which could flow to an inappropriate site in the inner ear.[8]

A diagnosis of posterior semicircular canal BPPV can be established by the Dix-Hallpike positional test. The patient, sitting on a couch, is abruptly tilted with the head turned and hanging in the lateral lying position. This elicits a typical attack of vertigo and nystagmus mentioned above. The nystagmus is best seen in Frenzel glasses. The signs and symptoms of BPPV commonly come and go, and episodes of vertigo can disappear for some time and then recur. Effective methods for treating posterior canal BPPV are the Epley and Semont repositioning maneuvers, which are designed to move the endolymphatic debris from the posterior semicircular canal into the vestibule. For horizontal BPPV, roll maneuvers such as the Lampert maneuver or barbecue roll are the most commonly employed.[23–25]

The aim of the study was to assess the occurrence of BPPV in patients with MTBI and determine the outcome of treatment.

Material and methods

A group of 179 patients complaining of vertigo/dizziness and balance instability after MTBI were enrolled into the study. All were diagnosed and treated in the Department of Otolaryngology (Medical University of Lodz, Poland), between the years 2012 and 2014. The study group comprised 98 women and 81 men, with a mean age of 45.2 (±11.8 years). The inclusion criteria were as follows: MTBI with or without short loss of consciousness according to the recommendations for MTBI diagnosis, an absence of meningeal and pathological focal signs in clinical neurological examination, and an absence of organic lesions under radiological imaging examination.[6] All participants were fully informed about the aim of the study and the test procedure, and gave their informed consent to their participation. The study design was approved by the Ethics Committee (No. RNN/87/12KE).

The 1st examination took place from 14 to 30 days following head trauma. Anamnesis and otoneurological examination were conducted in each patient. The diagnosis was based on a review of a medical history and the presence of nystagmus induced by the Hallpike-Dix test, confirming posterior semicircular canal BPPV, or the rollover test, for lateral canal BPPV, as given in consensus document of the Committee for the Classification of Vestibular Disorders of the Bárány Society.[26]

The balance system was evaluated by videonystagmography (VNG) (Ulmer SYNAPSYS 2008, Marseille, France), which recorded spontaneous ocular movements with eyes open and closed, as well as positional tests and a number of ocular-motor tests including the smooth pursuit, optokinetic and saccadic tests. Kinetic stimulation was also evaluated with the torsion swing test and the Fitzgerald-Hallpike caloric test.

The treatment comprised the Epley, barbecue and particle repositioning (RM) maneuvers. The Epley maneuver was performed specifically in the case of posterior semicircular canal (PSC) BPPV.[25,27] In the Epley maneuver, the patient sat on the examination table and the examiner performed the Dix-Hallpike test to the affected side, which elicited the typical nystagmus and vertigo. This position was maintained for 1–2 min after the nystagmus subsided. The patient's head was then rotated 90° toward the opposite side and held in this position for 30 s.

The barbecue maneuver was performed specifically in the case of horizontal canal BPPV. Beginning in the supine position, the patient was asked to roll 90° towards the unaffected side, and then turn the head 90° to the unaffected side, thus positioning the head at an angle of almost 90° to the body. This nose-down position was maintained for another 1–2 min, after which the patient was helped to sit up.

The symptoms occurring during each RM and at the end of the treatment session were recorded. The patients were instructed to avoid bending over and stay in an upright position for the remainder of the day. Patients were re-examined 2 and 4 weeks after the treatment to determine the efficacy of RM.

Results

Nineteen of the 179 studied patients with MTBI (10.6%), comprising 8 female and 11 male patients (mean age: 40.4 ±10.6 years, ranging from 16 to 57 years), complained about attacks of vertigo elicited by positional changes or physical exertion which were relieved by rest. All complained about other sensations of persistent dizziness such as light-headedness, floating and drunkenness. None complained of vertigo before the trauma or had any history of the pre-existing inner ear disease that can cause BPPV. In 11 patients, the head trauma was the consequence of a motor vehicle accident, 4 were due to fighting, 2 due to accidents at work, 1 at home, and 1 at a sporting event. The diagnosis of BPPV was confirmed in 9 of these 19 (47.4%) patients: in 8 cases by a positive Dix-Hallpike test (posterior canal BPPV) and in 1 by the roll test (lateral canal BPPV). In 3 patients, BPPV was bilateral: both ears were found to be simultaneously positive in the Dix-Hallpike test (Table 1).

In 10 out of 179 studied cases (5.6%), BPPV was diagnosed based on a medical history, which recorded a regular occurrence of vertigo episodes provoked by changes in head position relative to gravity. These observations indicated a high probability of BPPV; 8 of these patients presented posterior BPPV and 2 presented lateral BPPV. No symptoms were observed during the examination. For those with dizziness, the Brandt-Daroff exercises and the roll test were recommended.[7,8] Videonystagmography compensated unilateral weakness was confirmed in only 1 patient. Eight patients with posterior BPPV confirmed by the Dix-Hallpike test were immediately treated by a single Epley maneuver, while 1 patient with lateral BPPV was treated

Table 1. Clinical data: gender, age and clinical findings in patients (n = 9) with post-traumatic BPPV confirmed by maneuvers

Patient No., sex and age [years]	Semicircular canal(s)	Repositioning maneuver	Recurrence
1. F, 38	RP + LP	Epley	1 × RP
2. F, 28	RP	Epley	–
3. M, 60	LP + LH	Epley + roll	2 × LP
4. M, 27	RP	Epley	–
5. M, 57	LP + RP	Epley	1 × RP
6. F, 66	LP	Epley	–
7. F, 62	RP	Epley	1 × RP
8. M, 20	RP	Epley	–
9. M, 23	LH	roll	–

BPPV – benign paroxysmal positional vertigo; F – female; M – male; RP – right posterior; LP – left posterior; RH – right horizontal; LH – left horizontal.

using the barbecue roll. The examination was performed 2 weeks later to determine the efficiency of the treatment. In 4 (44.4%) patients, the maneuvers were repeated. On the follow-up examination, the patients were not found to have vertigo. All patients were urged to contact the physician if the positional vertigo recurred. In 1 patient, recurrence was confirmed after 2 years by the Dix-Hallpike test. The Epley maneuver was performed with consequent relief of symptoms.

Discussion

The rapid growth in the occurrence of motor accidents and their ensuing injuries over the last few decades have forced general practitioners, and specialists in laryngology and neurology to consult ever more victims suffering from post-traumatic vertigo or disequilibrium following the accident. In the USA, 1.7 million people sustain a TBI each year, and an analysis of multiorgan injuries found that head injury was confirmed in 72% of cases. It can be concluded that the head is the region of the human body most susceptible to trauma. In the European Union ("old" Member States), approx. 7.7 million people who have experienced an MTBI have disabilities.[1–5]

Although head trauma is considered a common cause of BPPV, few studies have examined the clinical results and outcome of traumatic BPPV.[7,12–14,17,18] In addition, due to the wide inclusion criteria concerning the type of head trauma, its severity, time of examination and analysis used when recruiting patients after head trauma, considerable variation can be seen in the results. Few articles present the outcome of treating traumatic BPPV.[8,24]

In the present study, BPPV was diagnosed in 10.6% of the patients with vertigo and disequilibrium after head trauma. The incidence, duration, frequency, and intensity of BPPV symptoms vary, perhaps due to the frequent occurrence of spontaneous recovery. Katsarkas identified post-traumatic etiology in 6.2% of 1644 BPPV patients.[12] Baloh et al. noted the presence of BPPV in 240 patients, and attributed this to traumatic head injury in 17%.[18] Ernst et al. diagnosed BPPV in 50% of patients on the 1st day after head trauma, but only 14% were confirmed using the Dix-Hallpike test.[17] Hoffer et al. identified BPPV in 29% of patients complaining of moderate and severe head trauma.[22] Such variability in BPPV diagnosis could be attributable to differences in inclusion criteria, variability in the patients who need medical care, and because this type of vertigo may appear at different times following injury, from 1 day to a few months.

As these studies do not include data concerning moderate injuries, for example those occurring in the home, any inferences regarding the "true" incidence of BPPV should therefore be made with caution.[14,28] Our results confirm those of Katsarkas, who note that the prevalence of post-traumatic BPPV was independent of sex.[12] Also, our findings indicate the presence of bilateral BPPV in 33% of all BPPV cases, compared to 14.3% according to Katsarkas,40% by Motin et al., 55% by Liu and only 6.3% by Marzo et al.[9,12,14,19]

The present study diagnoses BPPV in patients from 1 to 2 months after injury, but this criterion differs in other studies: Liu evaluated patients after 3 months, Ernst et al. from 3 weeks to 3 months, and Motin et al. examined the subjects over a period of 18 months following head trauma.[9,17,19] In addition, considerable variation in diagnostic procedures and treatment options regarding the management of BPPV can be seen between different medical specialties and disciplines.[8] Repeated testing is recommended on separate occasions to avoid missing a diagnosis. Failure to diagnose BPPV may lead to costly and potentially unnecessary diagnostic testing and/or radiological imaging.[8,11]

In 8 of our 19 patients (42.1%), the diagnosis of BPPV was confirmed by a positive Dix-Hallpike test. In the study including 100 patients with vertigo or BPPV after head trauma, Davies and Luxon established a diagnosis of anamnesis in 61% of patients but only 25% were confirmed by a positive Dix-Hallpike test result.[20] Nguyen-Huynh reported that even in a specialty clinic, 11% of patients did not give a positive Dix-Hallpike result on initial examination.[11] The Dix-Hallpike test is considered the gold standard for the diagnosis of posterior canal BPPV and it is the most common diagnostic criterion required in clinical trials.[10,11] Based on a review of published literature, some authors estimate the sensitivity of the Dix-Hallpike test to range from 48% to 88%, but with poor specificity.[27,29] Guidelines specify that a positive test result is sufficient for diagnosis of BPPV but a negative test result should not rule out BPPV completely.[8,11]

In the current study, 44.4% of patients with BPPV reported complete symptom resolution after a single repositioning maneuver. The other patients reported resolution after repeated procedures performed over the next 2 weeks, followed by re-examination. During a 2-year follow-up period, only 1 patient has reported a recurrent attack.

Motin et al. reported the resolution of vertigo after the 1st treatment in 40% of patients and Liu in 39% of patients.[9,19] These results are not as good as those observed for idiopathic BPPV.[29,30] Some authors suggest that trauma may lead to a loss of otolith quantity by microscopic hemorrhage in the inner ear or possibly a wide range of vestibular pathologies, such as blood and tissue degeneration with slight loosening. Otoconia are detached by trauma and microscopic hemorrhages, or by tissues shearing. Experimental head injury in guinea pigs showed a disarrangement of the vestibular system with lithic, exfoliate vacuolization of the sensory epithelia, and exfoliated membranes in the utricular and saccular maculae with otolith separation from the saccular maculae.[7]

Laryngologists, neurologists and primary care physicians are involved in the identification, monitoring and management of patients with BPPV. Establishing a diagnosis

of BPPV is important since it is treated by relatively simple physical maneuvers without the need for additional investigations or drug therapy. Some authors report that early education is beneficial for recovery in patients after MTBI.[5] Misunderstanding the reason of dizziness and vertigo in BPPV may influence the emotional state of the patient. In the present study, patients were informed of the treatment options, as well as the risks associated with symptomatic BPPV, including those of temporary dizziness or disorientation, and the possible recurrence of BPPV.

Conclusion

Despite the fact that the occurrence of BPPV in patients with MTBI is not frequent, BPPV should be diagnosed and treated successfully without any medication, no surgery and no special equipment.

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Retrospective analysis of cases with an ectopic opening of the common bile duct into duodenal bulb

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1361–1364

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Funding sources
None declared

Conflict of interest
None declared

Received on September 7, 2016
Reviewed on September 22, 2016
Accepted on March 22, 2017

Abstract

Background. Ectopic opening of the common bile duct (EOCBD) is a very rare entity. It has been reported in the 3rd or 4th portion of the duodenum, pyloric canal, duodenal bulb, and the stomach.

Objectives. The aim of this study was to evaluate the clinical characteristics, laboratory values and imaging studies of patients with EOCBD into the duodenal bulb retrospectively.

Material and methods. The files of patients who underwent endoscopic retrograde cholangiopancreatography (ERCP) between January 2003 and November 2015 were reviewed. The demographic data, presentations, abdominal ultrasonography, computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP), and ERCP findings of patients with EOCBD into the duodenal bulb were evaluated retrospectively.

Results. Ectopic openings of the CBD into the duodenal bulb were found in 20 out of 3270 patients who had undergone ERCP. Twenty patients (15 males and 5 females) with a median age of 59 (40–88) years were included in the study. Ectopic opening of the CBD into the duodenal bulb were found in 20 patients (0.61%). Laboratory test abnormalities included: hyperbilirubinemia in 20 (100%) patients, leukocytosis in 14 (70%) patients, and an elevated serum alkaline phosphatase and gamma-glutamyl transferase level in 20 (100%) patients. Indications for ERCP were CBD dilatation and extrahepatic cholestasis (n = 20), cholangitis (n = 12), only choledocholithiasis (n = 7), and acute pancreatitis (n = 2).

Conclusions. In patients with recurrent duodenal ulcers and/or apical stricture with accompanying CBD dilatation, extrahepatic cholestasis and cholangitis, EOCBD into the duodenal bulb should be considered.

Key words: ectopic opening of the common bile duct in the duodenal bulb, duodenal ulcer, apical stricture, biliary disease

DOI
10.17219/acem/69691

Introduction

Congenital anomalies of the common bile duct (CBD) are usually detected in childhood, but it might be undetected until adulthood. Congenital anomalies include accessory biliary ducts, aberrant cystic duct, bile duct cysts, double CBD, and ectopic opening of the CBD (EOCBD).[1] The etiology of EOCBD has been referred to errors in embryogenesis. Ectopic opening of the CBD is a very rare entity. However, it has been reported in the 3rd or 4th portion of the duodenum, pyloric canal, duodenal bulb, and the stomach.[2–5] The clinical and endoscopic features, and the significance of EOCBD are not sufficiently known. It is associated with benign biliary diseases, but usually manifests with recurrent duodenal ulcer, bulbus deformity with accompanying dilatation of the extra- and intrahepatic bile ducts with pneumobilia and stone.[5] Cases presented with gallbladder and stomach cancer with EOCBD into the duodenal bulb have also been reported.[6–8]

We retrospectively evaluated the clinical characteristics of patients with EOCBD into the duodenum (EOD), as well as their transabdominal ultrasonography (TUS), computerized tomography (CT), endoscopy, magnetic resonance cholangiopancreatography (MRCP) and endoscopic retrograde cholangiopancreatography (ERCP) findings of this rare anomaly.

Material and methods

In this study, we retrospectively evaluated the files of patients who underwent ERCP in a time frame of January 2003–November 2015 in the Department of Gastroenterology of Adana Numune Training and Research Hospital (Turkey). Patients with EOD were found in our ERCP records. The demographic data, presentations, medical records, TUS, CT, endoscopy and ERCP findings of patients with EOD were evaluated retrospectively. The Ethics Committee of Adana Numune Training and Research Hospital approved the study.

An ectopic opening of the duodenum was defined as the failure to demonstrate the papilla in its original location in the 2nd part of the duodenum and papilla is observed as an orifice in the bulb which was proved to be CBD in cholangiogram either with MRCP or ERCP. The following ERCP findings were analyzed: the presence or absence of dilated bile ducts, configuration of the CBD, the presence of biliary stones.

Patients with infected bile duct stones typically presented with fever, abdominal pain and jaundice, and in severe cases – hypotension and mental confusion.[9] In patients with duodenal ulcer and apical stricture, gastric biopsies were taken from the antrum to exclude *Helicobacter pylori* (*H. pylori*) gastritis by histopathological examination. Eradication of *H. pylori* was performed with 14-day of tetracycline-lansoprazole-metronidazole-bismuth subsalicylate containing regimen therapy. After *H. pylori* eradication, ERCP was repeated.

Statistical analyses were performed using PASW Statistics v. 18 (SPSS Inc., Chicago, USA).

Results

Twenty patients diagnosed with EOD were included in the study. There were 15 men and 5 women with a median age of 59 (40–88) years. Out of 3,270 patients who had undergone ERCP, EOD was found only in 20 patients (0.61%).

All patients presented with episodic biliary pain (epigastric and/or right upper quadrant). Twelve patients (60%) had fever, abdominal pain and jaundice. Two patients had acute renal failure because of nausea and vomiting, as well as partial apical stricture. Four cases had previous bleeding episodes. Twelve patients were admitted to emergency department with severe suppurative cholangitis. Four out of 12 patients were in septic shock with multiple organ failure due to liver abscess shown on CT and they received prompt medical treatment. After their clinical condition improved, ERCP was performed. We found duodenal ulcer and partial apical stricture in 16 patients with endoscopy. Proton pump inhibitors were given to patients with duodenal ulcers and partial apical stricture. *Helicobacter pylori* infection was diagnosed through histologic examination in 4 patients and *H. pylori* eradication was done by 14-day tetracycline-lansoprazole-metronidazole-bismuth subsalicylate containing regimen therapies. Afterward, ERCP was performed. General characteristics, clinical and laboratory findings of the patients on admission to emergency department and applied treatments of the patients are presented in Table 1.

Laboratory test abnormalities included: hyperbilirubinemia in 20 (100%) patients, an elevated serum alkaline phosphatase and gamma-glutamyl transferase level in 20 (100%) patients, and leukocytosis in 14 (70%) patients.

Indications for ERCP were CBD dilatation and extrahepatic cholestasis (n = 20), cholangitis (n = 12), only choledocholithiasis (n = 7), acute pancreatitis (n = 2). A total of 44 ERCP sessions were performed in these patients.

There was a history of simple cholecystectomy in 8 (0.4%) patients (gallbladder stones). These patients did not have any problems after ERCP.

A major duodenal papilla was found in a slit-like opening in the duodenal bulb (2 patients). Various stages of ulceration, bulbar deformity and partial apical stricture were found in the duodenal bulb of 16 (80%) patients.

Transabdominal ultrasonography and MRCP findings were extrahepatic bile duct dilatation, with or without intrahepatic duct dilatation (20 patients). Transabdominal ultrasonography revealed choledocholithiasis in 6 patients, and MRCP revealed choledocholithiasis in 18 patients. Computed tomography revealed liver abscess in 4 patients.

Table 1. General properties, characteristics of patients admitted to emergency department and indications for ERCP

Patient characteristic	Number (%)
Men	15 (75)
Women	5 (25)
Median age [years]	59
ERCP	3270
EOPCD	20
Clinical characteristics	
Biliary pain	20 (100)
Cholangitis (fever, abdominal pain and jaundice)	12 (60)
Acute renal failure	2 (10)
Previous bleeding	4 (20)
Liver abscess	4 (20)
Apical stricture	16 (80)
Indications for ERCP	
CBD dilation and extrahepatic cholestasis	20 (100)
Cholangitis	12 (60)
Choledocholithiasis	7 (35)
Acute pancreatitis	2 (10)
Total number of ERCPs	44

ERCP – endoscopic retrograde cholangiopancreatography; EOPCD – ectopic opening of the common bile duct; CBD – common bile duct.

In the ERCP findings, the extrahepatic bile duct was dilated (CBD >10 mm diameter in patients with cholecystectomy, other patients with CBD >7 mm diameter) in 20 (100%) patients, with or without intrahepatic bile duct dilatation. Eighteen (90%) patients had choledocholithiasis; 7 patients had cholelithiasis.

Surgical treatment for bulbar stricture and biliary ectopic opening was performed in 14 patients. These patients did not have any problems after the operation. Patients who rejected surgical treatment underwent biliary balloon dilatation. The patients underwent follow-ups for a mean of 12 months. There were no other clinical problems that occurred during 12 months.

Discussion

In our study, we found that the patients with EOCBD into the duodenal bulb might be undetected until adulthood and it may present with recurrent duodenal ulcer, apical stricture and severe biliary complications. We found the incidence of EOCBD into the duodenal bulb in patients who underwent ERCP as 0.61%.

The true incidence of patients with an EOCBD into the duodenal bulb is unknown. In a study by Lee et al., 18 out of 16,541 patients had an EOCBD into the duodenal bulb.[4] In 0.1% of patients who had undergone ERCP, EOCBD into the duodenal bulb was diagnosed.[4] In a study by Sezgin et al., the diagnosis of EOCBD into various sites of the upper digestive tract was detected in 11 patients out of 1,040 patients who underwent ERCP (1.05%) and EOCBD into the duodenal bulb was observed only in 4 patients out of 1,040 patients who underwent ERCP (0.38%).[2] Disibeyaz et al. reported EOCBD into the duodenal bulb was found in 53 cases out of 12,158 patients who underwent ERCP (0.43%).[10] Saritas et al. reported the frequency of ectopic biliary drainage in 2% of patients who underwent ERCP (10 out of 400 ERCPs).[11] In the present study, EOCBD into the duodenal bulb was found in 20 patients out of 3,270 patients who underwent ERCP (0.61%).

Ectopic opening of the CBD into the duodenal bulb may be undetected until adulthood. In the study by Lee et al., the median age was 51 years, in the study by Sezgin et al., the median age was 59.2 years. In the study of Disibeyaz et al., the median age of the group was 55 years. Saritas et al. reported an EOCBD in patients with a median age of 54 years.[2,4,10,11] In our study, the median age was 51 years.

An ectopic opening of the CBD is usually associated with biliary tract illness.[12] In the study by Lee et al., 10 patients (56%) had bile duct stones, 15 patients (83%) had abnormal liver function tests, 11 patients (73%) had obstructive jaundice, and 7 patients (39%) had cholangitis.[4] Another study by Sezgin et al. reported choledocholithiasis (7 patients), acute pancreatitis, CBD dilatation, extrahepatic cholestasis, and cholangitis.[2] In the study by Disibeyaz et al., 25 patients (64.1%) had cholecystectomy. Two of them had acalculous cholecystitis. Recurrent cholangitis was evident in 10 patients (25.6%).[10] In our study, indications for ERCP were CBD dilatation and extrahepatic cholestasis (20 patients), cholangitis (12 patients), only choledocholithiasis (7 patients), and acute pancreatitis (2 patients).

An important feature in EOCBD is its association with duodenal ulcers. Lee et al. reported that 13 (72%) of the 18 patients had a history of duodenal ulcer.[4] Sezgin et al. reported that 2 out of 4 patients (50%) had duodenal ulcers and resultant apical stricture.[2] Recurrent duodenal ulcer was found in 24 (61.5%) patients and 4 subjects (20.5%) underwent gastric bypass operation due to gastric outlet obstruction related to peptic ulcer in the study by Disibeyaz et al.[10] In our study, we found duodenal ulcers and partial apical stricture in 16 patients with an endoscope. Bile reflux into the duodenal bulb causes ulcer formation and increases pH. In high pH, bile acids may induce gastric and duodenal bulb mucosal damage. Gastric metaplasia may develop in the mucosa of the duodenal bulb.[5–13]

In conclusion, the opening of the CBD into the duodenal bulb may be associated with biliary and duodenal diseases. Particularly, in patients with recurrent duodenal ulcers and/or apical stricture associated with CBD dilatation, extrahepatic cholestasis and cholangitis, EOCBD into duodenal bulb should be considered.

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The use of antioxidant vitamin supplements among oncological patients

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1365–1369

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Funding sources
None declared

Conflict of interest
None declared

Received on October 26, 2016
Reviewed on January 19, 2017
Accepted on March 30, 2017

Abstract

Background. Dietary supplementation is becoming more and more common among both healthy and unhealthy people. The use of supplements is often unjustified, though in some groups of patients it is a necessary management for providing the required vitamins and minerals.

Objectives. The aim of the study was to assess the frequency of using antioxidant vitamin supplements (A, C and E) among the patients of the oncology ward.

Material and methods. The study group included 78 patients aged 19–83 years. The dietary intake of vitamins as well as the intake of supplements was assessed based on the data from the Food Frequency Questionnaire (FFQ).

Results. It was observed that 46.2% of patients used some kind of a dietary supplement and 77.8% of them used antioxidant vitamins. Among those taking vitamin A, C or E supplements, 72.2% of women and 80% of men used multivitamins. It was reported that the average fulfillment of the recommended daily intake for vitamin A was 303 ±136%, for vitamin C it was 282 ±166% and for vitamin E it was 199 ±80%. More than 25% of the patients whose diets contained at least the same level of vitamins as dietary recommendations were using antioxidant vitamin supplements at the same time.

Conclusions. Although the average dietary intake of antioxidant vitamins among the patients was not insufficient, the use of dietary supplements in different forms was common in our study. The results of other studies concerning the safety of using dietary supplements by cancer patients are not conclusive. Dietary supplementation in oncological patients should always be used after a medical consultation with a doctor and a dietician.

Key words: cancer, oxidative stress, antioxidant vitamins, supplementation

DOI
10.17219/acem/70030

Introduction

A tumor is an abnormal mass of tissue that has the capacity for proliferation and unregulated growth. It can develop anywhere in the body and can spread to other organs. Cancer is the leading cause of death worldwide, accounting for 8.2 million deaths in 2012. The mortality rate of the disease in Poland is 150 deaths per 100,000 people.[1]

Although some studies have found that the use of selected supplemental vitamins and minerals decreased the risk of cancer, other studies did not confirm such a relationship or they even demonstrated the opposite effect.[2–4] It is further noted that the use of β-carotene supplementation was associated with an increased risk of carcinogenesis in heavy smokers.[5] Thus, the mechanism of action of the individual substances is complex and may depend both on the origin of the dietary component and on other risk factors for cancer.

Antioxidants are compounds that interact with and neutralize reactive oxygen species (ROS). The balance between the production of ROS and the mechanism of action of antioxidant defense is essential to maintaining homeostasis in the body. The most common dietary antioxidants include vitamins A, C and E, and polyphenols. Antioxidant deficiency may contribute to the development of diseases resulting from the action of ROS, such as cancer, cardiovascular diseases, neurodegenerative diseases, and diabetes. Due to the ability of antioxidants to regulate the cell cycle and to limit DNA damage, studies on their potential use in cancer treatment have been carried out.[6]

Supplementation as a form of adjuvant therapy in cancer is common in oncological patients. Boon et al. showed that patients with breast cancer most commonly supplemented vitamin C and vitamin E in the form of pharmaceutical preparations.[7] Patients used complementary therapy to alleviate the symptoms of the disease and to improve their health. Such activities are often made without their doctor's knowledge, and the recommendations come from forums and untested websites.

The aim of the study was to assess the use of antioxidant vitamin supplements (A, C and E) among the patients of the oncology ward and to discuss the efficacy and safety of using those dietary supplements in cancer therapy.

Material and methods

The study group included 78 patients (48 women and 30 men) from the Department of Clinical Oncology, Wroclaw Medical University, Poland, aged 19–83 years (59.6 ±11.3 years) and diagnosed with cancer. Nearly 1 in 4 participants was hospitalized due to colorectal cancer and in every other woman, breast cancer was diagnosed. Other patients were diagnosed with lymphoma, bladder, ovarian, prostate, kidney, pancreatic, liver, lung, urinary tract, or testicular cancer (Table 1).

Table 1. The incidence of various types of cancers in the study population

Type of cancer	Overall, n = 78		Women, n = 48		Men, n = 30	
	n	%	n	%	n	%
Colorectal cancer	30	38.5	15	31.2	15	50.0
Breast cancer	23	29.5	23	47.9	0	0.0
Other cancers	25	32.0	10	20.9	15	50.0

The dietary intake of antioxidant vitamins (A, C and E) and supplements as well as the period of their use was assessed based on the data from the Food Frequency Questionnaire (FFQ). The questionnaire is a checklist of 154 food items classified into 8 groups: milk and dairy products; fruits; vegetables; meat and eggs; cereal products; mixed dishes; beverages; and snacks. The frequency of consumption was related to the preceding year and was reported as 1 of 9 different categories (0–1/month; 1–3/month; 1/week; 2–4/week; 5–6/week; 1/day; 2–3/day; 4–5/day; >5/day). The portion sizes of the consumed foods were determined by using the "Album of Photographs of Food Products and Dishes" [in Polish].[8] The food intake in g/day was calculated based on the consumption frequency and the given portion size. The average content of each antioxidant vitamin in daily food rations (DFR) was estimated using the database of the Food and Nutrition Institute from 2008 and was compared to the Polish recommendations from 2012.[9,10] The analysis of antioxidant vitamin content in DFR was rated at the level of Recommended Dietary Allowances (RDA) or, for vitamin E, at the level of Adequate Intake (AI).

The results of the study were analyzed statistically using STATISTICA v. 12.0 PL software (StatSoft Inc., Tulsa, USA). All statistical analyses were performed using the Shapiro-Wilk test, Student's t-test, the Mann-Whitney U test, and the χ^2 test. The level of statistical significance was set at $\alpha = 0.05$.

The study was approved by the Bioethics Committee of Wroclaw Medical University, Poland (No. KB – 362/2014).

Results

The use of dietary supplements was declared by 46.2% (n = 36) of patients, of whom 77.8% were taking antioxidant vitamin supplements. The latter were chosen by oncological patients significantly more often than other vitamin or mineral supplements. Among the patients taking antioxidant vitamin supplements, multivitamins were used by 72.2% of women and 80% of men, while vitamin C supplements were used by 33.3% of women and 50.0% of men. Vitamin E and A supplements were used only by women (11.1% and 5.6%, respectively) (Fig. 1).

Nearly half of the study group using antioxidant vitamins were patients with colorectal cancer. The frequency of the use of supplements containing antioxidant

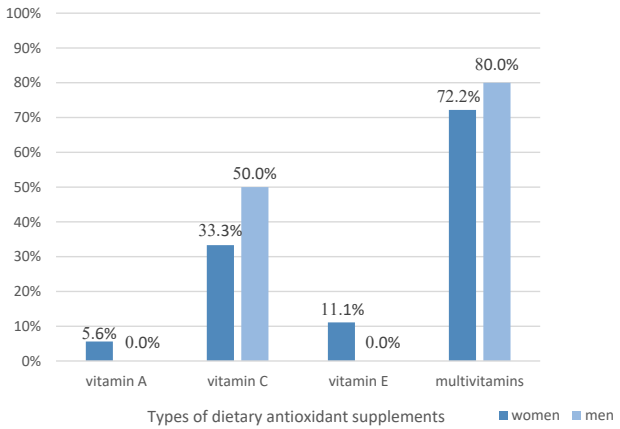

Fig. 1. The intake of selected antioxidant vitamin supplements in the group of women (n = 18) and men (n = 10) who declared using antioxidant supplements

vitamins among people with colorectal cancer was similar in the group of women and men. Moreover, there was no statistically significant difference between the intake of vitamin A, C or E supplements or multivitamins and gender and the type of cancer. The percentage of patients taking antioxidant vitamins was comparable in the group of patients using supplements for less than 1 year and those who supplemented their diet for more than 1 year.

Table 2 demonstrates the average daily dietary intake of antioxidant vitamins and the fulfillment of the recommended intake of these components. The estimated daily antioxidant vitamin content in the diet was similar in the group of men and women. The average fulfillment of the recommended daily intake for vitamin A was 303% ±136%, for vitamin C it was 282% ±166% and for vitamin E it was 199% ±80%. In the group of women, the average fulfillment of the recommendation for vitamin A was significantly higher than in the group of men. It was demonstrated that the intake of vitamins A, C and E was at least at the same level as the recommended level (≥90% of daily requirement) among 97.4% of the patients, 94.9% of the patients and 96.2% of the patients, respectively (Fig. 2).

Overall, antioxidant vitamin supplements were used by 36% (n = 28) of the participants. More than 25% of the patients who fulfilled their requirements for vitamins A and E used antioxidant vitamin supplements at the same time. The supplements of vitamin C were used by 1/3 of the respondents who fulfilled their recommended daily intake for this vitamin.

Discussion

According to the American Institute for Cancer Research (AICR), there is no sufficient evidence to assess the safety of using dietary supplements in cancer. Particular caution is advised when using dietary supplements with antioxidant properties. Recommendations of AICR allow the use of moderate amounts of multivitamins, at a dose which does exceed the recommended Dietary Reference Intakes in order to complement the daily intake of these nutrients.[11]

Despite this, the use of dietary supplements by patients who have been diagnosed with cancer is common. In a systematic review of 32 studies, Velicer and Ulrich reported that 64–81% of the patients used vitamin or mineral supplements, and 26–77% used multivitamins.[12] There were statistically significant differences in the frequency of dietary supplement use depending on the type of cancer, gender and education level of the participants.

The majority of the diets of the study participants were not insufficient in the analyzed nutrients. Despite the fact that the dietary intake of antioxidant vitamins A, C and E fulfilled the RDA, this group of supplements was the most frequently used by the subjects. Taking antioxidant supplements cannot be explained by a lack of the adequate dietary intake of those vitamins.

Vitamin A is a group of chemical compounds called retinoids. The most common retinoid in animal food sources is retinol, whereas in plant-based foods it is β-carotene.

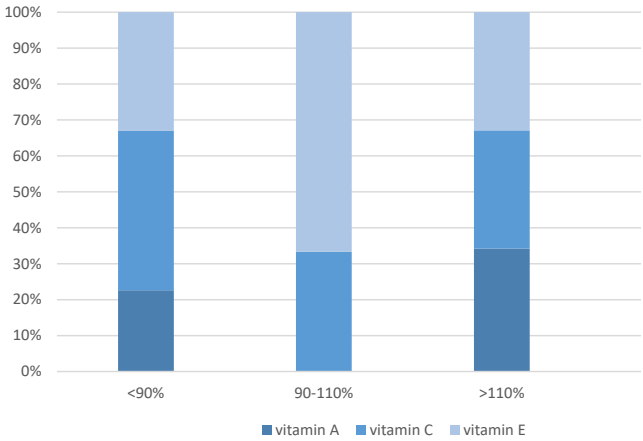

Fig. 2. The percentage of diet with antioxidant vitamin intake below (<90%), at (90–110%) and above (>110%) the dietary recommendation

Table 2. Mean dietary intake of antioxidant vitamins in the study population and the fulfillment of the recommended intake [%] of these compounds

Nutrient	Women, n = 48	Fulfillment of the recommended intake [%] (A)	Men, n = 30	Fulfillment of the recommended intake [%] (B)	A vs B p-value
	$\bar{x}$ ±SD		$\bar{x}$ ±SD		
Vitamin A [μg]	2301.6 ±1050.8	328.8 ±150.1	2359.4 ±901.9	262.2 ±100.2	0.03
Vitamin C [mg]	230.9 ±140.3	307.8 ±187.0	217.1 ±103.8	241.2 ±15.4	ns
Vitamin E [mg]	16.8 ±6.4	209.7 ±79.9	18.2 ±7.9	181.7 ±79.4	ns

$\bar{x}$ ±SD – mean ± standard deviation (SD); ns – no statistically significant differences in the fulfillment of the recommendation between gender groups.

Dietary supplements may contain different forms of vitamin A, such as retinol, retinyl palmitate, retinyl acetate, and β-carotene.[13]

Koay et al. revealed that retinoids in conjunction with trastuzumab or tamoxifen, drugs used in the treatment of breast cancer, inhibited cell growth and induced apoptosis.[14] However, Jiménez-Lara et al. showed that 9-cis-retinoic acid inhibited the apoptosis of breast cancer cells in the presence of anticancer drugs, such as doxorubicin, etoposide and camptothecin.[15] The probable mechanism of action of retinoids was the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an anti-apoptotic agent.

Vitamin E is common in a variety of food products, like plant oils and margarines, nuts, milk, whole-grain foods, and leafy vegetables.[9] In dietary supplements, vitamin E may occur in a few chemical forms, but the most common of them are d-α-tocopherol, d-α-tocopheryl acetate and d-α-tocopheryl succinate.[13]

The results of the study conducted by Li et al. showed that the potential anticancer properties of vitamin E varied depending on its chemical form.[16] The authors observed a very weak ability of α-tocopherol to inhibit the promotion of lung cancer in comparison to δ-tocopherol.

In an experimental animal study, Takahashi et al. showed that δ-tocopherol inhibited the promotion of prostate cancer in proportion to the dose.[17] The observed mechanism of action of vitamin E was the modulation of the activity of caspase-3 and caspase-7 – enzymes controlling apoptosis.

Vitamin C, or L-ascorbic acid, is a water-soluble vitamin. Its antioxidant properties are used to prevent the oxidation of food. Chemical forms of vitamin C used in dietary supplements are L-ascorbic acid, L-ascorbyl 6-palmitate, and sodium, magnesium, calcium, and zinc L-ascorbate.[13]

An intravenous infusion of high doses of vitamin C may cause the apoptosis of cancer cells. Uetaki et al. observed important changes in the metabolism of human MCF7 breast cancer and HT29 colon cancer cells after treatment with vitamin C.[18] The vitamin concentration required to eliminate 50% of MCF-7 cells amounted to 2.3 mM, whereas for HT29 it was more than 10 mM. The cytotoxicity of vitamin C toward MCF-7 cells was inhibited in the presence of antioxidants – N-acetylcysteine and glutathione.

Frömberg et al. observed an improved sensibility of cancer cells treated with vitamin C toward anticancer drugs: docetaxel, epirubicin, irinotecan, and fluorouracil.[19] However, Heaney et al. reported that vitamin C reduced the cytotoxity of doxorubicin, cisplatin, methotrexate, and imatinib, through suppressing the depolarization of the cell membrane.[20]

Padayatty et al. concluded that the results of studies concerning the effectiveness of vitamin C therapy in cancer treatment are not conclusive.[21] What is more, their methodology varied depending on the route of administration of the preparations. Vitamin C plasma concentration, after the administration of its maximum safe oral doses (3 g every 4 h), was dozens of times lower than after an intravenous injection of 50 g of ascorbate. Only the parenteral administration increased the concentration of vitamin C in the blood high enough to result in a possible anticancer action of this substance.

The reason for the rising interest in the possibility of using antioxidants in the treatment of cancer is the evidence-based role of ROS in carcinogenesis. Chronic oxidative stress may induce mutations in genetic material and is involved in the development of cancer. Increased ROS levels in cancer cells are thought to be the reason for tumor progression and for resistance to treatment with anticancer drugs.[22] At the same time, it was shown that excessive amounts of ROS inhibited tumor growth and promoted apoptosis.[23] So if the mechanism of action of antioxidative agents is linked to ROS depletion, their use in cancer patients is highly questionable.

The safety of antioxidant vitamins in the form of dietary supplements remains highly controversial during radiotherapy and chemotherapy. Daily supplementation with 400 IU of α-tocopherol and 30 mg of β-carotene reduced the side effects of radiotherapy in head and neck cancer patients. However, the applied treatment has been found to be less effective, including an increase in mortality, among participants who received vitamin E.[24,25] The results of the study conducted by Meyer et al. showed that taking antioxidant supplements is particularly unfavorable in smokers during radiotherapy.[26] The authors reported more than a 2-fold increased risk of all-cause mortality or recurrence as well as more than a 3-fold increased risk of death from cancer of the head or neck in smokers compared to nonsmokers.

On the other hand, Suhail et al. observed that oral supplementation of 500 mg of vitamin C and 400 mg of vitamin E was associated with lower levels of DNA damage in breast cancer patients during chemotherapy.[27] It may indicate the usefulness of these vitamins in reducing the side effects of treatment.

Moreover, Poole et al. reported a 16% reduced risk of death linked to the intake of multivitamins, vitamin E or vitamin C in a group of women who had completed breast cancer treatment.[28]

The use of antioxidant vitamins might be beneficial in terminal patients for improving their quality of life. In the study conducted by Fuchs-Tarlovsky et al., the supplementation of antioxidant vitamins in patients with cervical cancer increased quality of life measured by the Quality of Life (QoL) scale.[29] It also decreased protein damage caused by oxidative stress. However, it had no effect on the increase in food intake by the participants of the study.

Summing up the possibility of the use of oral antioxidants as part of cancer treatment, Yasueda et al. reported that so far there has been no sufficient evidence

of the harmful effects of antioxidants on oncological patients.[30] The authors emphasized that the exception was tobacco smokers during radiotherapy. Based on scientific research, it is not possible to determine whether supplements influence the effectiveness of the applied cancer therapy or decrease the side effects of the treatment.

Conclusions

Patients participating in the study were hospitalized in the oncology ward during anticancer therapy or shortly before or after its termination. Although the diet of most patients contained adequate amounts of vitamins A, C and E, the use of antioxidant supplements was common. According to the current knowledge, antioxidant vitamin supplementation during anticancer treatment not only increases the effectiveness of therapy, but may even reduce it. Making the decision to use a complementary therapy alongside conventional medical treatment should always be supported by evidence in medicine, pharmacology and dietetics.

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Endovascular treatment of iatrogenic arteriovenous fistula of the iliac vessel

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1371–1375

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Funding sources
None declared

Conflict of interest
None declared

Received on November 17, 2016
Reviewed on December 7, 2016
Accepted on March 28, 2017

Abstract

Background. Iatrogenic vascular injuries, due to the particular nature of such pathology, are associated with high morbidity and mortality in the postoperative period.

Objectives. The objective of this study was to present a case of non-classic approach to the therapy of iatrogenic arteriovenous fistula.

Material and methods. We present a case of a 17-year old female patient admitted to the Department of Vascular, General and Oncologic Surgery (Copernicus Memorial Hospital, Łódź, Poland) due to an iatrogenic injury to the common iliac vein and artery, following neurosurgical intervention on the spine. Two weeks prior to admission, the patient underwent surgery in the Neurosurgery Clinic for herniated nucleus pulposus and lumbar spine scoliosis. The imaging diagnostic revealed the presence of a pseudoaneurysm of the right common iliac artery and arteriovenous fistula between the right common iliac vessels. The patient was qualified for endovascular treatment. Two self-expanding covered stents were successfully deployed. The clinical and radiological outcome of the procedure was good. The postoperative period was uneventful. The patient was discharged home on the 3rd postoperative day.

Results. The control examinations (directly after the procedure and 6, 12, 24, and 32 months thereafter) revealed full patency of the iliac vessels, as well as no recurrence of arteriovenous fistula, nor a pseudoaneurysm of the right common iliac artery. No symptoms of either chronic limb ischaemia or venous insufficiency were observed.

Conclusions. Iatrogenic vessel injury, being a complication of neurosurgical and orthopedic surgeries, may be overlooked and remain undetected both in intra- and postoperative period. Modern imaging techniques allow for an adequate diagnosis of the injury and planning the treatment of arteriovenous fistula. The endovascular procedures are the method of choice in patients with arteriovenous fistulas of iliac vessels, alternative to open surgery.

Key words: iatrogenic vessel injury, arteriovenous fistula, pseudoaneurysm of the common iliac artery, endovascular treatment

DOI
10.17219/acem/69859

Introduction

Iatrogenic vascular injuries, due to the particular nature of such pathology, are associated with high morbidity and mortality in the postoperative period.[1,2] This dangerous and sometimes life-threatening complication is being increasingly reported in the literature. It results mainly from the large number of diagnostic and therapeutic interventions requiring vascular access, as well as complex and extensive surgical repair procedures. The most dangerous early and late consequences of iatrogenic vessel injuries include intra- and postoperative hemorrhages from the large arteries and veins.[3] The group of early complications consists of a pulsating hematoma, arterial and venous thrombosis, acute ischaemia of tissues and organs, whereas late complications include chronic ischaemia, post-thrombotic syndrome, pulmonary embolism, pseudoaneurysm formation and arteriovenous fistulas.[1] Due to their local and systemic consequences, vascular fistulas pose a significant diagnostic and therapeutic issue. The size and localization of the fistula determine the clinical image, with typical vascular murmur, steal symptoms below the level of the fistula and venous overload leading to right ventricular failure.[4–6] Early identification of the fistula and its successful treatment would allow us to avoid significant consequences for the patient's health. Constant development and the use of endovascular methods present a valuable opportunity for treating vascular injuries and mitigating their consequences, including arteriovenous fistulas.[2,7–10] Vascular injury may complicate the course of other procedures and their serious consequences during specialist training of prospective surgeons.[4] The early and proper diagnosis, as well as correct therapy, increases the patient's chances of surviving vascular injury and avoiding complications.

Case report

A 17-years old female patient (OHX) of Asian origin was referred to the Department of Vascular, General and Oncologic Surgery (Copernicus Memorial Hospital, Łódź, Poland) due to suspected arteriovenous fistula of the iliac vessels. Two weeks prior to admission, she was underwent surgery in the Neurosurgery Clinic for herniated nucleus pulposus and lumbar spine scoliosis. The discharge summary revealed that hemilaminectomy of L4, removal of L4/L5 discus, intravertebral stabilization using Capstone Medtronic and posterior L4/L5 stabilization using Legacy Medtronic were performed (Medtronic, Memphis, USA). The patient was discharged home on the 5th postoperative day. The follow-up visits in the Neurosurgery Clinic (7 days after discharge) and Outpatient Clinic for Neurosurgery (in 6 weeks) were recommended, as well as permanent rehabilitation and physiotherapy. The patient reported unspecified pain in the right iliac area during her 1st hospital stay, which was reported to the attending physician. During her follow-up visit in the Neurosurgery Clinic 7 days after being discharged, she reported the feeling of "ringing" within the abdominal cavity. The attending neurosurgeon ordered a next visit in the Outpatient Clinic for Neurosurgery in 2 weeks and referred her for ultrasound examination of the iliac and femoral vessels. The next day, the patient, accompanied by her mother, reported to the Outpatient Clinic for Vascular Surgery. The physical examination revealed good general condition in a circulatory and respiratory stable patient. Her heart rate was normal with 74 bpm, heart tones loud and clear, and blood pressure was 115/75 mm Hg. The abdominal palpation and auscultation revealed increased muscle tonus in the right iliac area over the pathological mass showing slight machinery murmur. The pulse over the arteries of the lower extremities was normal and symmetric. There was no edema of the lower extremities. The Doppler ultrasound revealed the hypoechogenic mass over right the iliac vessels with turbulent, high-amplitude flow (color and spectral Doppler). The accelerated, high-amplitude, machinery flow, typical for arteriovenous fistula was observed in the right iliac vein. The patient was urgently referred to the Department of Vascular, General and Oncologic Surgery (Copernicus Memorial Hospital, Łódź, Poland). The laboratory findings performed on admission revealed anemia (RBC: 2.51 T/L, Hb: 7.4 g/dL, Ht: 24.1%, MCV: 96.0 fL, MCH: 29.5 pg, MCHC: 3.7 g/dL), leukocytosis (WBC: 13.51 G/L) and thrombocytosis (PLT: 508 G/L). The abdominal angio-MRI showed the presence of pseudoaneurysm of the right common iliac artery (Fig. 1). The patient was qualified for an iliac vessels angiography the next day, which confirmed the presence of large aneurysm of the right common iliac artery and arteriovenous fistula between right common iliac vessels (Fig. 2). The decision was made to treat the patient using endovascular method. The right femoral artery was punctured and self-expanding nitinol coated Fluency® 9 × 40 mm stent was implanted (Bard Incorporated, Karlsruhe, Germany). Due to the observed leak to the aneurysm sack, the second Fluency® stent (10 × 40 mm) was introduced. The control angiography revealed neither leak into

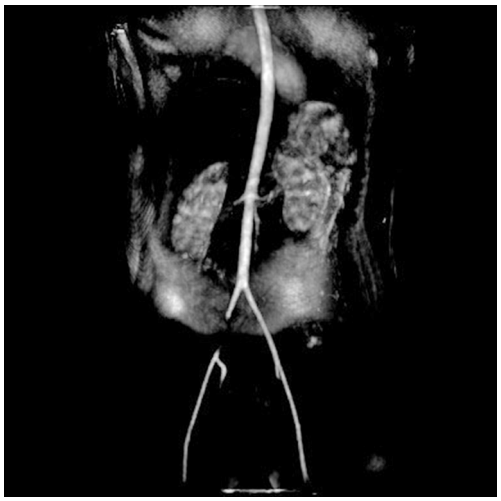

Fig. 1. Magnetic resonance imaging (MRI) angiography of the abdominal aorta and iliac arteries. The injury of right common iliac artery is clearly visible

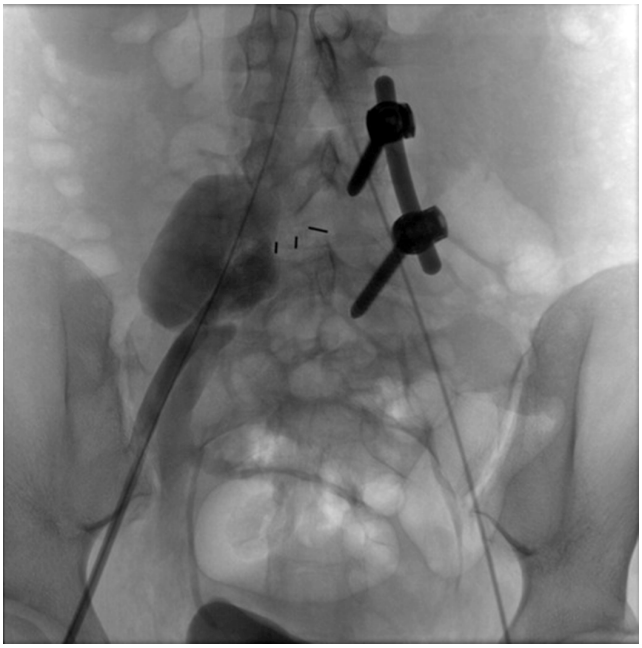

Fig. 2. Angiography of iliac vessels. A pseudoaneurysm and fistula between the right common iliac artery and common iliac vein is visible

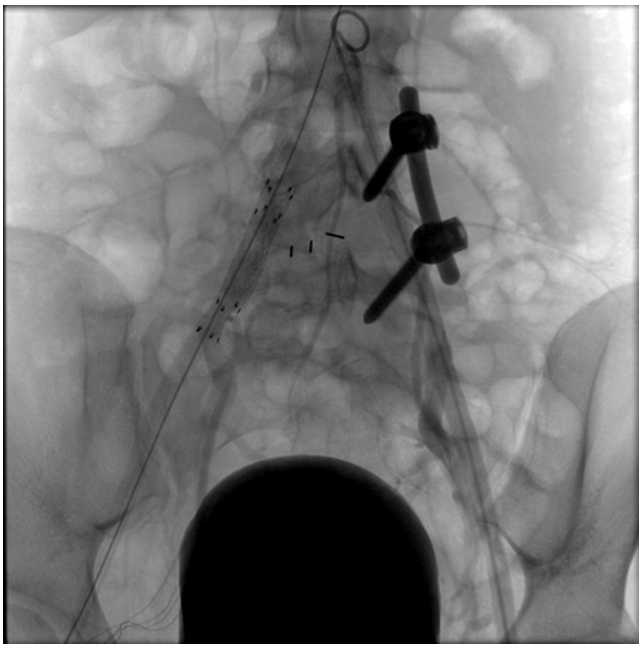

Fig. 3. Angiography of the iliac vessels after the implantation of 2 covered stents into the right common iliac artery

the aneurysm nor an arteriovenous fistula between the right common iliac artery and vein. A good flow through the right common, internal and external iliac arteries were observed (Fig. 3). Due to the identified anemia the patient received 2 units of group-matched red blood cell concentrate, which led to the normalization of morphotic parameters. In the postoperative period, the patient complained of slight pain in the right iliac region, which subsided after the administration of analgesics. The pulse over lower extremities arteries was normal and symmetric. The skin was normally colored and warm, with no edema. The postoperative period was uneventful. Once the compression dressing was removed the patient was mobilized and discharged in good general condition on the 3rd postoperative day.

Results

Prior being discharged, the patient had the Doppler ultrasound of iliac vessels performed, which revealed the patency of implanted stents and normal flow through iliac veins and arteries. Neither a pseudoaneurysm nor an arteriovenous fistula were revealed. Both veins and arteries of the right lower extremity were patent and showed normal flow. The patient was followed up in the out-patient clinic for vascular surgery. The control examinations revealed neither ischaemia of the extremity nor impaired patency of the stents (after 6, 12, 24 and 32 months; Fig. 4–6). No recurrence of arteriovenous fistula was observed. The patient is well and remains active.

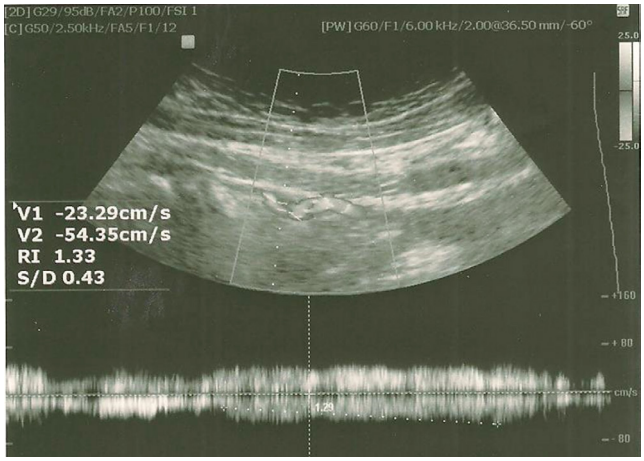

Fig. 4. Doppler ultrasound of the right iliac vein. Normal flow pattern in the right common iliac vein

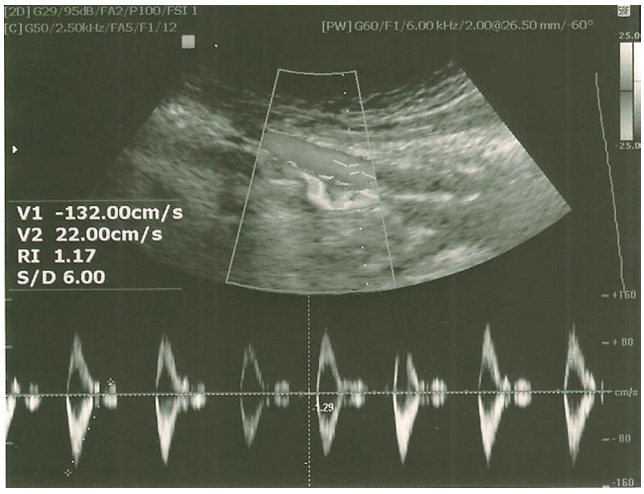

Fig. 5. Doppler ultrasound of the right iliac artery. Normal flow pattern in the right common iliac artery

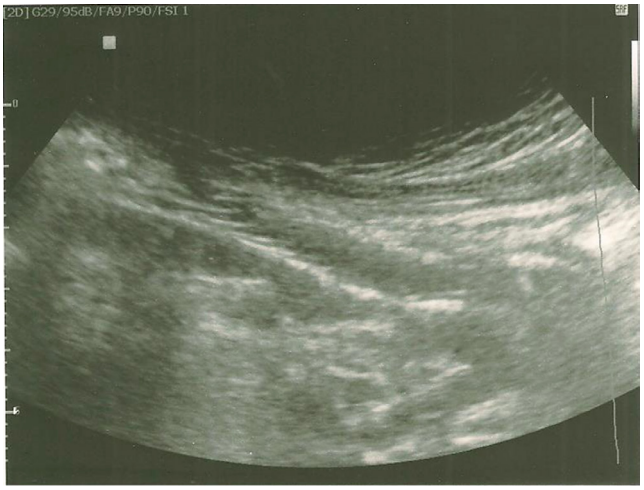

Fig. 6. Pelvis ultrasound. The arrow indicates stents implanted into the right common iliac artery

Discussion

Iatrogenic injury of the large pelvic vessels is one of the complications of neurosurgical and orthopedic surgeries.[11,12] It is responsible for 0.05–0.3% of all complications occurring in such patients.[12–14] The associated morbidity and mortality results from large vessels hemorrhage leading to hypovolemic shock.[15] If undetected in the course of the primary operation, this may lead to the patient's death during or after the surgery.[1,2,16,17] The possibility to overlook vessel injury in the perioperative period results from its frequent faint clinical manifestation and the lack of knowledge concerning the possible consequences of performed surgeries showed by the physicians.[18] It is not uncommon that they become detected incidentally, many years after the injury.[6,15,19,20] Due to the associated early and late complications, each such complication requires urgent diagnostic and adequate, specialist treatment.[15,18,21] The diagnostic methods allow us to diagnose the patient with iatrogenic vessel injury include Doppler ultrasound, CT-angiography, MRI-angiography or angiography.[11,21–23] The vessel injuries, including arteriovenous fistulas, are often detected intraoperatively, which is associated with high mortality rates.[24] The therapy of vascular complications of neurosurgical and orthopedic procedures includes both open surgery and endovascular procedures.[3,15,16,18] As immediate surgical treatment due to life-threatening condition, is a 1st line treatment, the benefits of minimal invasive approach in the form of endovascular procedure may result in lower morbidity and mortality.[2,3,5,7–10,21,23,25,26] We, therefore, suggest establishing a complications' register.[27]

Conclusions

Iatrogenic vessel injury following neurosurgical and orthopedic procedures may be overlooked and remain undetected in the intraoperative and postoperative course. Modern imaging techniques allow for the adequate diagnosis of the injury and planning of the treatment of arteriovenous fistula. The endovascular procedures are the method of choice in patients with arteriovenous fistula of iliac vessels, alternative to open surgery.

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The best time for orthodontic treatment for Polish children based on skeletal age analysis in accordance to refund policy of the Polish National Health Fund (NFZ)

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

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1377–1382

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Funding sources
None declared

Conflict of interest
None declared

Received on June 14, 2016
Reviewed on July 12, 2016
Accepted on March 29, 2017

Abstract

Background. The ability to estimate the growth potential correctly is an important factor in taking effective actions with respect to orthodontic diagnosis, suitable treatment protocol and optimal timing of commencing the therapy.

Objectives. The aim of this study was to compare skeletal maturity between the groups depending on the malocclusion as well as to define the optimal timing for particular orthodontic treatment procedures in children with miscellaneous types of malocclusion and compare it with the duration of treatment proposed by the National Health Fund (NFZ).

Material and methods. The delivery of the objectives of this study has been divided into 4 stages: the selection of the lateral head films (n = 180) of patients with malocclusion without congenital defect – diagnosing an orthodontic defect using Angle's classification, noting the chronological age (7–16 years) at the moment of taking the radiograph; the evaluation of the patients' skeletal maturation stages determined by the cervical vertebrae; comparison of the skeletal maturity between the groups considering the calendar age and the type of malocclusion, and analysis of 45 cephalometric RTG projections of patients with different malocclusions.

Results. The average age for children with malocclusion was significantly higher for class III as compared to class II or I in pubertal peak group (CS3 and CS4), and for class III as compared to class I or II after the puberty peak (CS5 and CS6). Our findings of the average age according to Angle's classification revealed significant differences between class II and class III at CS2, CS3, CS4, and CS5, between class I and class III at CS3 and CS5, between class I and class II at CS4, between class III and I at CS5, and between all classes at CS6 maturity stage.

Conclusions. Based on our research, it was concluded that the time of the refund of orthodontic treatment in Poland should be extended to 13.6 years of age for class II malocclusion and to 14.7 years of age for class III defects.

Key words: orthodontic treatment, malocclusion, Angle's classification, skeletal age, cervical vertebral maturation method

DOI
10.17219/acem/69976

Introduction

The ability to estimate the growth potential correctly is an important factor in taking effective actions with respect to orthodontic diagnosis, suitable treatment protocol and optimal timing of commencing the therapy.[1] The assessment of skeletal maturity in patients is essential to avoid complications related to early discontinuation of class III malocclusion therapy as well as to carry out an effective treatment with the use of removable functional appliances in children with class II deviations.[2] Moreover, it has been stated that clinical effects of the therapy are observed the soonest in patients whose treatment commenced at the right time, i.e., during the peak growth.[3]

The most popular methods of evaluating bone age include the analysis of the cervical vertebrae in lateral head films and the radiographs of hand and wrist bones.[4] Warmeling et al. also believe that, although the correlation between both methods is very high, the popularity of the method using lateral head radiographs has been growing in the recent years.[5]

Taiwanese scientists published a report about the significance of the biological factors (breaking of voice in boys, the pace of the bodily growth, menstruation in girls) in monitoring the growth. Based on an analysis of 304 girls, they proved that girls started menstruating ca. 1 year after the peak growth defined by means of the analyses of the hand and wrist X-rays as well as lateral head radiographs.[6]

The relation between skeletal maturity (relying on the maturity of the vertebrae) and dentition development has also been described in the literature. By evaluating the mineralization stages for specific teeth, the highest correlation between the shapes of certain vertebrae and the 2nd lower molar in girls and the lower canine in boys was identified. The mineralization of teeth was also correlated with the skeletal age.[7]

Based on an evaluation of the shape of the cervical vertebrae and subsequent stages of the dental age in teenage patients, Gupta et al. presented treatment protocols for various malocclusion defects depending on the timing of the therapy.[8] They assessed the dental age by means of Demirjian's method and the skeletal age was estimated with the use of the cervical stage method. The authors emphasized that the treatment of various types of occlusal defects should be commenced depending on the patient's skeletal age.

On the basis of our clinical experience in the field of orthodontic treatment of children and the observation of rules for granting refunds by NFZ, we came to the conclusion that the relationship between the length of the duration of the growth spurt and the type of malocclusion should be carefully investigated.

The assumption of the work was that the stages of accelerated growth (CS3–CS4) may occur at a different calendar age depending on the existing malocclusion.

The aim of this study was to compare skeletal maturity between the groups depending on the malocclusion as well as to define the optimal timing for particular orthodontic treatment procedures in children with miscellaneous types of malocclusion and compare it with the duration of treatment proposed by the National Health Fund (NFZ).

Material and methods

Study population

Research material consisted of 180 lateral head films of patients (124 women, 56 men) with different malocclusions, selected from the medical records of 350 patients treated in an orthodontic clinic between 2014 and 2016 (Table 1).

Table 1. Distribution of the patients according to stages of the skeletal development

Stages of skeletal maturity		Age of patients with malocclusions [years]	
CS	n	mean	SD
CS1	30	8.4	1.8
CS2	30	9.5	1.2
CS3	30	11	1.6
CS4	30	12.6	1.7
CS5	30	12.9	0.8
CS6	30	14.8	1.1
Total	180	11.5	1.9

Study protocol

According to Angle's definition, in class I malocclusions, the mesiobuccal cusp of the maxillary 1st molar occludes with the buccal grove of the mandibular 1st molar. Irregularity consists in the disorders in the anterior section relative to the midline or horizontal plane; it may also involve Angle's class II malocclusion, which covers distal occlusion and includes 2 divisions. In class II div. 1, the mesiobuccal cusp of the maxillary 1st molar is located anteriorly to the buccal grove of the mandibular 1st molar. The interincisal angle is decreased, which is related to the proclination of the incisors (protrusion). In this group, the increase of the overjet above 3 mm is also observed. In class II div. 2, the interincisal angle is increased due to the retrusion of the incisors, which may compensate for the increased overjet. In Angle's class II, the radiographs of patients with incisal protrusion and retrusion were analyzed collectively.

In class III, the mesiobuccally cusp of the maxillary 1st molar is located posteriorly to the buccal grove of the mandibular 1st molar.

The study has been divided into 4 stages:

I. The selection of lateral head films of patients with malocclusion without congenital defects:
   – diagnosed Angle's I, II or III class orthodontic defect;
   – chronological age of 7–16 years at the moment of taking the radiograph.

II. The assessment of skeletal maturity in 3 groups.

III. The comparison of the skeletal maturity between the groups considering the calendar age and the type of malocclusion – statistical analysis.

IV. The analysis of 45 cephalometric RTG projections of patients at the calendar age of 7–16 years with different malocclusions to assess skeletal maturity. The study used the CS method presented by Franchi et al. and also described by Flieger et al.[3,9]:
   – CS1: the bottom edges of the 2nd and 3rd vertebrae are straight, the 3rd and 4th vertebrae have a trapezoidal shape – growth acceleration will occur in 2 years;
   – CS2: the bottom edge of the 2nd vertebra becomes concave, the inferior edges of the of 3rd and 4th vertebrae are straight; a trapezoid shape of the 3rd and 4th vertebrae can be noticed – growth acceleration will occur in ca. 1 year;
   – CS3: the inferior edges of the 2nd and 3rd vertebrae are concave, a more rectangular shape of the 2nd and 3rd vertebrae occurs – the growth peak began last year;
   – CS4: the concavity is visible in the bottom edges of the 2nd, 3rd and 4th vertebrae, a rectangular shape of the 3rd and 4th vertebrae begins to be seen in a horizontal plane – the growth peak has begun;
   – CS5: the concavity is visible in the bottom edges of the 2nd, 3rd and 4th vertebrae, a square shape of the 3rd and 4th vertebrae begins to be seen – the growth peak ended a year before that stage;
   – CS6: strongly visible concavities on the bottom edges of the 2nd, 3rd and 4th vertebrae can be noticed, a rectangular shape of the 3rd and 4th vertebrae begins to be seen in a vertical plane – the growth peak ended 2 years before that stage.

Statistical analysis

The statistical analysis was conducted using STATISTICA v. 9.0 software (StatSoft Inc., Tulsa, USA). The average chronological age of the patients with malocclusions and their skeletal age was compared between the groups using the one-way ANOVA test. Pair comparisons were carried out based on the Tukey's post hoc test at a significance level $p = 0.05$.

Results

An analysis of the average age according to Angle's classification revealed no significant differences between class II and class III at CS1 and CS2 maturity stages (Table 2).

Table 2. An analysis of the average age according to the Angle's classification at CS1 and CS2 maturity stages

CS1 and CS2			Age	
Angle's class	n	%	mean	SD
I	0	0.0	–	–
II	29	48.3	8.3	0.74
III	31	51.7	8.1	0.34
Total	60	100	8.2	0.57

II vs III: $p > 0.05$.

The mean age of the subjects with class III malocclusion in pubertal peak group (CS3 and CS4) was significantly higher as compared to class II or class I (Table 3).

Table 3. An analysis of the average age according to the Angle's classification at CS3 and CS4 maturity stages

CS3 or CS4			Age	
Angle's class	n	%	mean	SD
I	22	36.6	11.8	1.33
II	23	38.3	11.2	1.27
III	15	25.1	12.8	0.48
Total	60	100	11.8	1.29

II vs III: $p < 0.05$; I vs III: $p < 0.05$; I vs II: $p > 0.05$.

Our findings show that patients with class III malocclusions as compared to class I according to Angle's classification commence the CS5 and CS6 phase of the skeletal development significantly later.

Table 4. An analysis of the average age according to the Angle's classification at CS5 and CS6 maturity stages

CS5 and CS6			Age	
Angle's class	n	%	mean	SD
I	21	35	12.9	0.88
II	19	32	13.6	1.28
III	20	33	14.7	0.95
Total	60	100	13.8	1.30

II vs III: $p < 0.05$; I vs III: $p < 0.002$; I vs II: $p > 0.05$.

An analysis of the average age according to Angle's classification revealed significant differences between class II (mean 9.2 ±0.29 years) and class III (mean 8.53 ±0.17 years) at CS2 maturity stage (Fig. 1).

An analysis of the average age according to Angle's classification revealed significant differences between class III (mean 10.87 ±1.07 years) and class I (mean 10.44 ±0.98 years) or class II (mean 12.37 ±0.14 years) at CS3 maturity stage (Fig. 2).

An analysis of the average age according to Angle's classification revealed significant differences between

class I (mean 13.25 ±0.31 years) and class II (mean 12.10 ±0.95 years) or class II and class III (mean 13.29 ±0.10 years) at CS4 maturity stage (Fig. 3).

An analysis of the average age according to Angle's classification revealed significant differences between class I (mean 12.46 ±0.61 years) and class III (mean 13.56 ±0.26 years) or class II (mean 12.63 ±0.61 years) and class III at CS5 maturity stage (Fig. 4).

Furthermore, significant differences in the patients' average age at CS6 maturity stage between all classes of malocclusion according to Angle's classification were noted. The average age of the subjects with class III malocclusions was significantly higher as compared to class II and class I, as well as for class II as compared to class I.

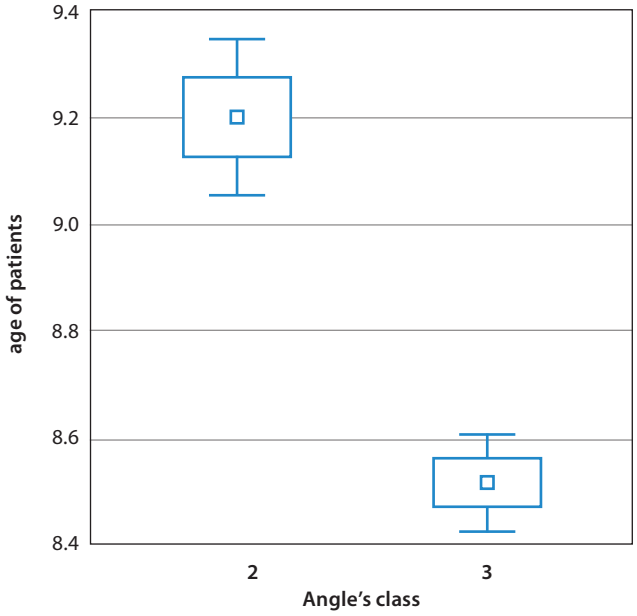

Fig. 1. An analysis of an average age according to the Angle's classification at CS2 maturity stage

II vs III: $p < 0.0002$.

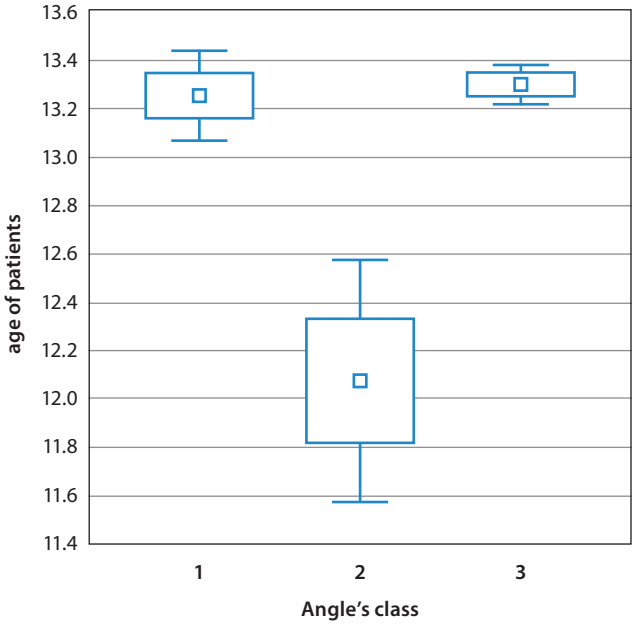

Fig. 3. An analysis of an average age according to the Angle's classification at CS4 maturity stage

II vs III: $p < 0.0001$; II vs III: $p < 0.0001$.

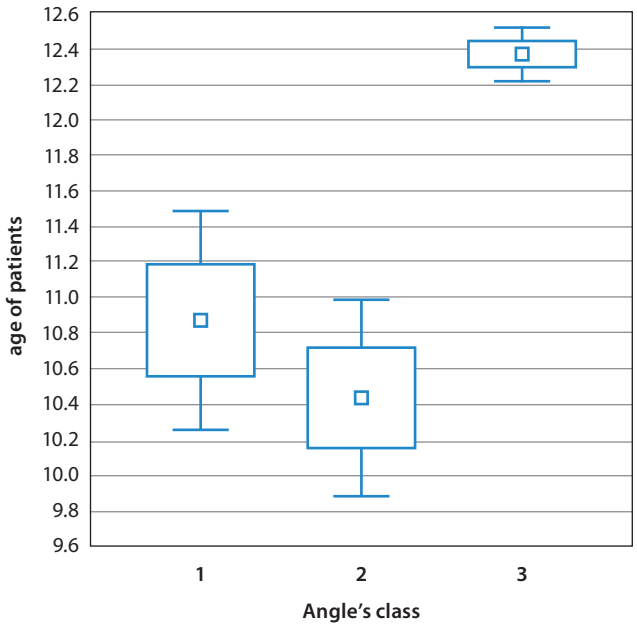

Fig. 2. An analysis of an average age according to the Angle's classification at CS3 maturity stage

II vs III: $p < 0.0004$; I vs III: $p < 0.0004$.

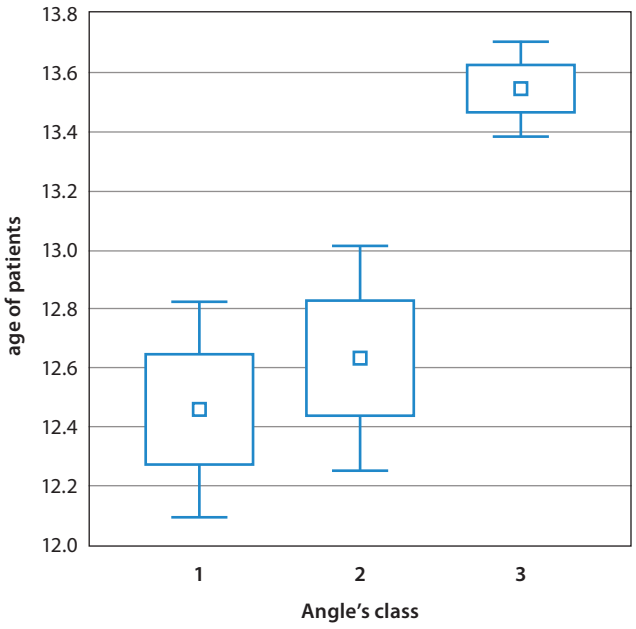

Fig. 4. An analysis of an average age according to the Angle's classification at CS5 maturity stage

I vs III: $p < 0.0003$; II vs III: $p < 0.002$.

Discussion

The analysis of the mandibular growth potential, in particular as regards the diagnosis of Angle's class III malocclusion, is an extremely important factor in the effective planning of the orthodontic therapy at different levels of an individual's development.[10] Chen et al. evaluated the change in the length of the mandible at particular stages of the skeletal development based on the analysis of the shape of the cervical vertebrae.[11] The length of the mandible was examined by them on lateral head films by measuring the distance between the points Ar and Pog at stages CS1 and CS4, i.e., from 2 years before the peak growth to the total completion of the growth. Researchers observed that the distance obtained at stage CS4 was taken away from the distance obtained at stage CS1. In that manner, the authors created a system allowing us to predict the actual value of the mandibular growth based on the evaluation of the maturity of the cervical vertebrae and, therefore, make better use of specific types of orthodontic appliances in the treatment of various types of malocclusions.

Our study also allows for effective planning of orthodontic treatment in Polish children with various malocclusions based on ascertaining the skeletal age in terms of the chronological age and reimbursement needs.

Kuc-Michalska and Baccetti studied the length of the peak growth in patients with Angle's class I and III defects.[12] Based on the analysis of 218 lateral head films (93 women and 125 men) taken between stages CS3 and CS4 of the peak growth, the authors demonstrated that the peak growth occurs at a similar chronological age in patients with Angle's class I and III. However, they also observed that the time interval between stages CS3 and CS4 in patients with class I was 11 months vs 16 months for class III patients.

According to our research, the average age of the patients with class I and III malocclusions during the growth spurt demonstrate the difference of 12 months in favor of class III. The time interval was not tested, because patients with the skeletal age at stages CS3 and CS4 were counted as 1 group.

Other authors suggested that a greater growth of the mandible length in patients with Angle's class III malocclusions was related to a longer duration of adolescence.[13] Other sources indicate that the treatment of class III malocclusions should start as early as possible, optimally right after the diagnosis.[14]

By measuring the difference between the chronological age of the patients after the puberty peak (CS5 and CS6) and the patients before the puberty peak (CS1 and CS2), we showed the highest difference amounting to 6.6 years, recorded for children with class III malocclusions. Thus, we demonstrated that the adolescence period in this type of malocclusion takes the longest and ends at around 14 years of age. Furthermore, we showed that the patients with class III defects appeared in the treatment with an average age of 8.1 years, which seems to be a good time to start orthodontic treatment of this malocclusion.

Baccetti emphasized the importance of predicting the growth peak for higher effectiveness of treating Angle's class II malocclusion.[15] He demonstrated that functional therapies of distal occlusion (e.g., with a Twin-block appliance) are the most effective when they commence at the end of stage CS2 or at the beginning of stage CS3. In his opinion that allows for the reduction of the treatment time to the necessary minimum.

In our study group, we noticed that the average age for children with class II malocclusion in pubertal peak group (CS 3 and CS4) was 11.2 years. By measuring the difference between the chronological age of class II patients after the puberty peak (CS5 and CS6) and patients before the puberty peak (CS1 and CS2), we showed a difference amounting to 5.3 years. This means that in patients with this type of malocclusion, adolescence lasts about 1 year shorter than in patients with class III malocclusions.

Simultaneously, the average of the chronological age for class II and III malocclusion patients was similar at stages CS1 and CS2, while being higher at stages CS5 and CS6. This suggests that the growth acceleration lasts longer in patients with diagnosed class III malocclusions than in patients with class II.

Klimas et al. conducted a study which aimed to estimate the bone age by the analysis of the cervical vertebrae on the cephalograms (CVM) in Polish children with standard occlusion and children with hypodontia.[16] The patients were classified into 2 groups: a group of patients with standard occlusion and a group with shortages of permanent tooth buds. The skeletal age of children from both groups was estimated. Then, skeletal maturity was compared between the groups, taking into consideration the similar calendar age of the patients. The authors proved that the CVM method enables an effective assessment of the skeletal age and a demonstration of the differences between the calendar age and bone maturity. Furthermore, they observed a delay in reaching skeletal maturity by children with congenital missing teeth compared to children with standard occlusion.[16]

Our assessments and analysis of the skeletal age enabled us to successfully determine the growth stage of the patients with different types of malocclusion, which allows for a highly effective orthodontic therapy in Polish children, and may be an indication of the National Health Fund's refund rules.

Conclusions

It was shown that the accelerated growth stage (CS3–CS4) can appear at a different age depending on the type of malocclusion. This information is very important in regards to the beginning of the treatment of this type of malocclusions.

Accelerated growth (the best for class II therapy) occurs at the average age of 11.2 years. In the same period, class III patients show the average age of 12.8. A period

of accelerated growth takes longer in patients with class III than in patients with class II. According to Angle, the difference in time reaches about 1 year and 6 months. The expiry of the growth spurt occurs around the age of 13 years for all types of malocclusions.

Based on our research, it is concluded that the time of the refund of orthodontic treatment in Poland should be extended to 13.6 years of age for class II malocclusions and to 14.7 years of age for class III defects.

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Iron status and dietary iron intake in vegetarians

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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

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1383–1389

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Funding sources
None declared

Conflict of interest
None declared

Received on January 16, 2017
Reviewed on March 16, 2017
Accepted on April 19, 2017

Abstract

Background. Iron is one of the nutrients that require special consideration in a plant-based diet. The widespread belief is that meat is the best source of iron and a vegetarian diet increases the risk of its deficiency. This conviction has been the subject of analysis in a growing number of scientific reports.

Objectives. The aim of this study was to assess the iron intake and iron metabolism in vegetarians and vegans compared to a control group.

Material and methods. A total of 55 vegetarians and 36 healthy volunteers were studied. The following parameters were measured in serum: iron, ferritin, transferrin, transferrin receptor, and hepcidin-25, using the enzyme-linked immunosorbent assay (ELISA) method. The dietary iron intake was assessed using a 24-hour dietary recall.

Results. The mean daily intake (DI) of iron was significantly higher in the female vegan group compared to the control group. Iron, hepcidin-25, ferritin and transferrin receptor in serum remained within their normal ranges. The ferritin concentration was significantly decreased and that of transferrin significantly higher in both female groups and in the male vegan group.

Conclusions. The obtained results show that the studied parameters, excluding transferrin, remained within normal ranges. However, the ferritin concentration was significantly decreased in the female vegetarian group and also in both vegan groups. This may indicate low iron storage.

Key words: iron, ferritin, transferrin, vegetarianism, hepcidin-25

DOI
10.17219/acem/70527

Introduction

Iron is involved in various bodily functions, including one of the most important, which is the transportation of oxygen from the lungs to all cells of the body. Additionally, this element is a component of hemoproteins, where it takes part in the electron transfer and oxygen reduction. Iron is also necessary in erythropoiesis, in plenty of immunological reactions and in the production of leukocytes, nucleic acids, neurotransmitters, collagen, hormones, and neurolemma.[1] Iron is essential for proper cell metabolism and cellular respiration. Cell iron overload results in toxicity related to the increased production of free radicals and lipid peroxidation, which can lead to cell death; therefore, iron homeostasis requires strict regulations.[1]

Food is the only source of iron for the human body, delivering it in both the heme and non-heme form. It is assumed that only 1/3 of the iron in meat is heme, while the remaining 2/3 and all the iron content of plant products is non-heme. Vegetarianism is the practice of eliminating meat and the by-products of animal slaughter from one's diet.[2] Vegetarian diets contain no heme iron.[3] Non-heme iron primarily exists in an oxidized form (Fe^{3+}), which is not bioavailable and first requires reduction to the Fe^{2+} form to be transported across the intestinal epithelium.[4]

Studies conducted by Hallberg and Rossander-Hulthén showed that the amount of iron that is absorbed from a mixed diet (containing meat) ranges from 14% to 17%. However, the amount of non-heme iron absorbed from a vegetarian diet is between 5% and 12%.[5] Another study has revealed similar results: 18% of iron from a mixed diet and 10% from a plant-based diet.[6] Moreover, the amount of non-heme iron absorbed from a 1-product meal is 6 times smaller than the amount of heme iron absorbed from a 1-product meal. On the other hand, in mixed meals, non-heme iron bioavailability is just 2 times smaller.[7] This is the reason why the recommendation of iron intake is 1.8-fold greater compared with non-vegetarians. However, this recommendation was based on a test diet that was low in vitamin C and high in factors that reduce iron absorption.[5]

Factors enhancing iron absorption are: iron deficiency (pregnancy, lactation, growth), increased erythropoiesis, the malfunction of protein synthesis, or hemochromatosis. Other components manifesting the aforementioned effect are: amino acids, monosaccharides, animal proteins, ascorbic acid, organic acids, carotenoids, hydrochloric acid, fermented products, products made from fermented soy beans, and internal factor IF12.[8]

Non-heme iron absorption is inhibited by zinc, copper, manganese, cobalt, nickel, dietary fiber, carbohydrates, phosvitin, egg yolk albumins, oxalic acid, phosphate, phytic acid, salicylic acid, tannin, polyphenols, barbiturate, soporific drugs, and major calcium supplementation.[8] Previously, it was surmised that also soy protein had a negative effect on iron bioavailability, but other studies showed that iron contained in soy beans is in the ferritin form, which shows high bioavailability.[9]

Previously, researchers focused intently on diet components that enhanced or inhibited non-heme iron bioavailability, which has been proven by more recent studies to be less important than iron status. This is the reason why iron bioavailability is underestimated.[10] It is acknowledged that iron status in the human body is more important than its bioavailability, and that in women, menstrual blood loss (rather than their diet) is a major determining factor in iron metabolism. This means that the amount of iron absorbed depends on iron status of the body – people with low iron levels absorb more and excrete less iron. Additionally, non-heme iron absorption in people with a low iron level is as effective as the absorption of heme iron. Therefore, it is proven that the human body can adapt to new conditions.[7,9,11–13] Iron deficiency related to food intake patterns develops slowly over months or even years. Deficiency symptoms are most often detectable by laboratory diagnostic methods.[14]

The aim of this study was to assess the iron intake based on a 24-hour dietary recall and to estimate iron metabolism (the concentration of iron, ferritin, transferrin, transferrin receptor, and hepcidin-25) in groups of vegetarians and vegans compared to a control group.

Material and methods

This study was performed between February and May 2013 in the Department of Clinical Nutrition at the Medical University of Gdańsk, Poland. Participants aged over 18 years were recruited from Gdańsk, Gdynia and Sopot in Poland via information on websites. This study was part of the nutritional status of vegetarians research (No. MN 01-0098/08). The study received all the necessary approvals, including one from a bioethics review committee of the Medical University of Gdańsk, Poland.

The data was segregated according to gender into:

- a vegetarian female group (n = 21), abbreviated as VEG_1;
- a vegetarian male group (n = 11), abbreviated as VEG_2;
- a vegan female group (n = 18), abbreviated as VEG_3;
- a vegan male group (n = 5), abbreviated as VEG_4.

Thirty-six omnivores were divided by gender and included in 2 control groups. All participants from both groups were healthy and without any acute or chronic diseases, with a good nutritional status. The subjects were not taking any medication. The inclusion criterion for the study was a minimum of 1 year on a vegetarian diet.

The basic characteristic of the study groups is presented in Table 1.

Diet assessment

A retrospective method of diet assessment – a 24-hour dietary recall (24HR) – was used. This method, in the form of a structured interview, aimed to capture detailed

Table 1. The basic characteristic of the study groups

Group	Number	Age [years]	BMI [kg/m^2]
VEG_1	21	29.95 ±7.26	22.2
VEG_2	11	29.64 ±4.06	22.3
VEG_3	18	32.1 ±10.01	21.5
VEG_4	5	34.8 ±5.26	23.0
Control F/M	36 31/5	F: 27.4 ±9.8 M: 32.6 ±12.9	F: 20.8 M: 24.7

BMI – body mass index; VEG_1 – vegetarian female group; VEG_2 – vegetarian male group; VEG_3 – vegan female group; VEG_4 – vegan male group; F – females; M – males.

information about all foods and beverages consumed by the respondent over the previous 24-hour period. The interview was conducted by a trained nutritionist. The data was analyzed by a computer program called Cronometer® (Cronometer Software Inc., Revelstoke, Canada).[15] This software enables the user to analyze single foods and beverages and estimate their nutrient values. Additionally, the daily iron intake was compared to the Recommended Dietary Allowance (RDA) values established by the National Food and Nutrition Institute in Poland.

Laboratory parameters

Venous blood was collected after a 12-hour period of fasting, centrifuged and stored at –80°C.

The following biochemical parameters were measured in serum:

– iron, by the in vitro assay method, with Roche Diagnostic (Mannheim, Germany) reagents and a Hitachi 704 device (Roche Diagnostics, Basel, Switzerland);

– ferritin, transferrin receptor and hepcidin-25, by the enzyme-linked immunosorbent assay (ELISA) method and an ELIZAMAT 3000 device (DRG Diagnostics, Marburg, Germany);

– transferrin, by the ELISA method (Assaypro, St. Charles, USA) and an ELIZAMAT 3000 device.

Statistical analysis

The data is expressed as the mean with standard deviation (SD) and median. The Kolmogorov-Smirnov test was used to verify whether the variable distribution was normal. The differences between the means were evaluated by an independent Student's t test, and the Mann-Whitney U test was used when the distribution of the variables was not normal. Spearman's rank correlation coefficient (R) was used to evaluate the relationships between the variables. Statistical analysis was performed using Microsoft Office Excel 2007 (Microsoft, Redmond, USA) and STATISTICA v. 12.0 (StatSoft Inc., Tulsa, USA). A p-value <0.05 was considered as statistically significant.

Results

Iron intake

The results of the daily iron intake are presented in Table 2 and Fig. 1 for women, and in Table 3 and Fig. 2 for men. The mean daily iron intake was significantly lower in the control group in comparison to the VEG_3 group ($p = 0.002$). In the vegan female group (VEG_3), a significantly higher daily intake of iron in comparison to vegetarian female group (VEG_1) ($p = 0.0256$) was observed. Among the female participants, no one (0%) from the VEG_1 group and only 2 subjects (18%) from the VEG_3 group implemented the iron intake recommendations for vegetarians. Among the male participants, 67% from the VEG_2 group and 75% from the the VEG_4 group implemented those recommendations.

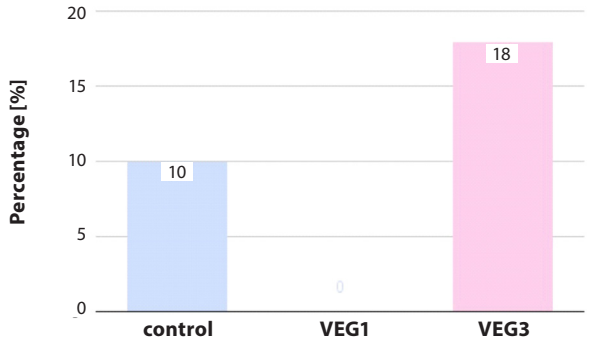

Fig. 1. The percentage of women who met the recommendation for the iron intake

VEG_1 – vegetarian female group; VEG_3 – vegan female group.

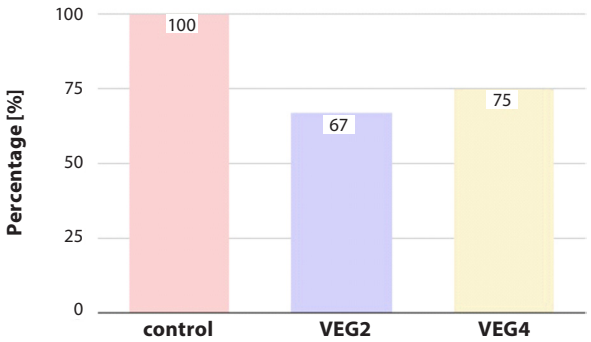

Fig. 2. The percentage of men who met the recommendation for the iron intake

VEG_2 – vegetarian male group; VEG_4 – vegan male group.

Iron status

The iron concentration was within a normal range in all studied groups (Tables 2,3). There were no significant differences in the iron concentration between any groups.

Table 2. Women: the iron intake and serum concentrations of iron, hepcidin-25, ferritin, transferrin, and transferrin receptor (values are arithmetic mean ± standard deviation (SD), median and range)

Values	VEG_1 group	VEG_3 group	Control group	p-value*	p-value**	p-value***
Iron intake [mg][1]						
Mean ±SD Median Range	14.22 ±5.75 12.7 5.1–27.6	19.86 ± 8.87 18.1 5.1–41.5	13.25 ±4.78 11.6 7.7–25.9	0.524	0.002	0.0256
Iron [µg/dL][2]						
Mean ±SD Median Range	110.71 ±43.85 106 44–177	91.28 ±47.14 79 36–182	111.68 ±38.32 109 41–170	0.933	0.1056	0.191
Hepcidin-25 [ng/mL][3]						
Mean ±SD Median Range	11.73 ±4.15 11.0 5.40–21.36	12.35 ±5.91 11.53 1.21–26.10	15.82 ±6.16 17.12 5.04–25.70	0.0174	0.0899	0.699
Ferritin [ng/mL][4]						
Mean ±SD Median Range	12.16 ±10.85 8.1 5.0–45.3	11.21 ±13.78 6.4 5.0–62.3	15.55 ±11.75 9.8 5.0–42.3	0.349	0.3085	0.8098
Transferrin [g/L][5]						
Mean ±SD Median Range	3.93 ±1.45 4.03 1.1–6.0	3.87 ±1.52 3.16 1.91–6.00	3.19 ±1.15 3.1 1.1–5.3	0.085	0.133	0.903
Transferrin receptor [µg/mL][6]						
Mean ±SD Median Range	0.70 ±0.43 0.55 0.35–1.85	0.55 ±0.24 0.43 0.35–1.15	0.57 ±0.2 0.5 0.35–1.05	0.211	0.831	0.188

[1] 18 mg (× 1.8 for vegetarians and vegans); [2] 37–145 µg/dL; [3] 1.0–39.3 ng/mL; [4] 10–120 n/mL; [5] 2.0–3.6 g/L; [6] 0.378–1.513 µg/mL; VEG_1 – vegetarian female group; VEG_3 – vegan female group; p-value * – VEG_1 group vs control group; p-value ** – VEG_3 group vs control group; p-value *** – VEG_1 group vs VEG_3 group.

Additionally, a positive correlation between the concentration of iron and ferritin (R = 0.19; p = 0.023) in the VEG_1 group was observed.

Hepcidin-25

The mean hepcidin-25 level was lower in the VEG_1 group in comparison to the control group (p = 0.0174) (Table 2). However, the mean hepcidin-25 concentration was maintained within the references range (1.0–39.3 ng/mL) in all groups. In the VEG_1 group, a positive correlation between the hepcidn-25 and ferritin concentration (R = 0.60; p = 0.025) was observed.

Ferritin

The mean ferritin concentration was maintained within normal limits (for men: 20–250 ng/mL, for women: 10–120 ng/mL) in all groups; the results are presented in Tables 2 and 3.

Transferrin and transferrin receptor

The mean transferrin concentration was above the reference range in the VEG_1 (3.93 ±1.45 g/L), VEG_2 (3.87 ±1.52 g/L) and VEG_4 (4.19 ±0.66 g/L) groups. In both control groups, the mean transferrin level was maintained within a normal range (Tables 2, 3). There were no significant differences in the transferrin concentration between any groups.

The mean transferrin receptor concentration was maintained within normal limits. There were no significant differences between any of the vegetarian groups and the control groups.

Discussion

The mean intake of iron among the vegetarians in our study was 18.53 ±8.23 mg; only 20% of participants met the recommended daily iron intake for vegetarians. However, our results showed that the mean daily iron intake in the female vegan group was significantly higher (19.86 ±8.87 mg) compared to the control group (13.25 ±4.78 mg). The National Food and Nutrition Institute in Poland recommends a daily intake (DI) of iron of 10 mg for men and 18 mg for women, and a 1.8-fold greater intake for vegetarians and vegans.[16] The recommended iron intake for men is 18 mg/day and for women 33 mg/day, at which point meeting the demand exclusively by food consumption becomes difficult and requires considerable knowledge in this area. Nevertheless, 100% of female vegetarians and 82% of female vegans, along with 33% of male vegetarians and 25% of male vegans, did not reach the daily recommendation for the iron intake.

Table 3. Men: the iron intake and serum concentrations of iron, hepcidin-25, ferritin, transferrin, and transferrin receptor (values are arithmetic mean ± standard deviation (SD), median and range)

Values	VEG_2 group	VEG_4 group	Control group	p-value*	p-value**	p-value***
Iron intake [mg][1]						
Mean ±SD Median Range	22.98 ±7.85 21.2 14.4–38.6	24.4 ±8.72 22.5 16.0–36.6	17.67 ±2.36 17.9 15.2–19.9	0.2879	0.2586	0.7755
Iron [µg/dL][2]						
Mean ±SD Median Range	124.00 ±36.80 134 66–170	121.80 ±40.46 127 71–166	130 ±26.71 127 95–162	0.75	0.715	0.916
Hepcidin-25 [ng/mL][3]						
Mean ±SD Median Range	18.12 ±7.96 17.87 7.22–33.06	13.02 ±3.79 14.95 7.89–16.57	20.89 ±7.52 19.43 12.63–32.15	0.523	0.07	0.2
Ferritin [ng/mL][4]						
Mean ±SD Median Range	36.29 ±24.75 40.5 6.0–74.5	21.22 ±15.38 16.0 8.5–46.3	55.66 ±39.28 78.0 12.1–88.7	0.246	0.105	0.234
Transferrin [g/L][5]						
Mean ±SD Median Range	3.39 ±1.60 3.05 1.6–6.0	4.19 ±0.66 4.4 3.02–4.60	3.03 ±1.11 3.16 1.4–4.2	0.658	0.08	0.307
Transferrin receptor [µg/mL][6]						
Mean ±SD Median Range	0.52 ±0.18 0.45 0.35–0.85	0.71 ±0.46 0.45 0.35–1.35	0.64 ±0.22 0.6 0.35–0.95	0.249	0.767	0.2367

[1] 10 mg (× 1.8 for vegetarians and vegans); [2] 59–158 µg/dL; [3] 1.0–39.3 ng/mL; [4] 20–250 n/mL; [5] 2.0–3.6 g/L; [6] 0.378–1.513 µg/mL; VEG_2 – vegetarian male group; VEG_4 – vegan male group; p-value* – VEG_2 group vs control group; p-value** – VEG_4 group vs control group; p-value*** – VEG_2 group vs VEG_4 group.

Results of other studies also proved that vegetarians did not follow the recommendations established for them. Lee et al. analyzed the 3-day menus of 54 Buddhist vegetarian nuns.[17] The mean iron intake in this group was 14.1 mg/day, while in non-vegetarian Catholic nuns and students, it was 15.2 mg/day and 10.0 mg/day, respectively. It is assessed that among vegetarian females, the iron intake ranges from 11 to 18 mg/day.[7] Additionally, among non-vegetarian girls, a low intake of iron was observed. Broniecka et al. studied 159 non-vegetarian girls at the age of 17–18 years. The median quantity of iron amounted to 6.4 ±2.8 mg/day, which is below the Estimated Average Requirements (EAR), i.e., 8 mg/day.[18] Wolnicka and Taraszewska presented similar results having surveyed 193 non-vegetarian girls aged 11–13 years. The iron content of their diet was 7.4 ±3.1 mg/day.[19] The studies showed that a vegetarian and vegan diet can be associated with an increased risk of nutrient deficiencies and anemia.[20] The elimination of meat and the intake of plant-based products, which contain only non-heme iron, are associated with decreased iron bioavailability. Therefore, the recommendations for the iron intake are 1.8-fold higher for vegetarians. Iron bioavailability is enhanced by ascorbic acid, resistant starch, oligosaccharides, vitamin A, and β-carotene.[21] Observational studies showed that a vegetarian diet is associated with a higher intake of vitamin C, vitamin E, fibre, magnesium, and β-carotene than a non-vegetarian diet. At the same time, a plant-based diet leaves vegetarians with a lower intake of vitamin B_{12}, vitamin D and zinc.[2]

It has been proven that iron absorption depends on the composition of the diet and its preparation. However, in subjects whose diet contains only non-heme iron, the absorption mechanisms adapt to new conditions.[3] Roughead and Hunt showed that both heme and non-heme iron absorption was increased in people with low iron levels. However, non-heme iron bioavailability increased even 10–15-fold compared to the heme form, for which the increase was only 2–3-fold. The heme iron study involved subjects who ate meals containing beef, which is rich in highly bioavailable iron.[21] The British 2003 National Diet and Nutrition Survey has shown that a plant-based diet does not provide less iron compared to a diet that contains meat.[22] Several studies showed that a well-balanced vegetarian diet provides the required iron amounts and sometimes even exceeds the references[9,20,22] Similar results were obtained in an EPIC-Oxford cohort study, published in 2003. A total of 33,883 meat-eaters and 31,546 non-meat-eaters took part in that study. The mean intake of iron was 12.6 mg/day among vegetarian women and 13.9 mg/day among vegetarian men. Moreover, the intake of this mineral was 14.1 mg/day among vegan women and 15.3 mg/day among vegan men, which makes it the highest intake rate of all groups.[23]

Our study showed no deficiency in iron, hepcidin-25, ferritin, and transferrin receptors in vegetarians and vegans.

The transferrin level was significantly higher in female vegetarian and vegan groups and in the male vegan group, while the ferritin level was lower in the female vegetarian and vegan groups and also in the male vegan group.

In 1989, the US Food and Nutrition Board declared that anemia caused by iron deficiency occurs in vegetarians as often as in non-vegetarians, and this claim has upheld until now.[6] Bhatti et al. showed the iron levels in children and adults among Hindu Brahmins (who observe a strict vegetarian diet) compared to vegetarians from other communities, considered a control group. The mean iron concentration in serum was 95.6 μg/dL among Brahmin boys (control group – 85.7 μg/dL) and 90.7 μg/dL among Brahmin girls (control group – 87.0 μg/dL). The mean iron level was 85 μg/dL among Brahmin men (control group – 83.1 μg/dL) and 72.5 μg/dL among Brahmin women (control group – 65.5 μg/dL). The results show that the mean iron concentration in the Brahmin community was maintained within a normal range and was higher compared to the control group, which may mean that the daily iron intake was adequate.[24] Obeid et al. assessed the iron concentration in vegans, lacto-ovo-vegetarians and occasional meat-eaters (semi-vegetarians). These results show that the mean iron level in all groups was maintained within a normal range and any differences between those groups were insignificant – in the vegan group, the mean iron concentration in blood serum was 80 μg/dL, in vegetarians 91 μg/dL and in semi-vegetarians 93 μg/dL.[25]

In our study, the mean hepcidin-25 concentration in all groups was maintained within the normal range. But in the female vegetarian group, hepcidin-25 was significantly lower than in the control group. Hepcidin-25 synthesis and secretion by the liver is controlled by iron stores within macrophages, inflammation, hypoxia, and erythropoiesis. In response to an increased iron concentration, hepcidin-25 synthesis is upregulated in the liver, which regulates iron absorption, its storage and recycling from macrophages. Hepcidin-25 is a direct inhibitor of ferroportin, a protein that transports iron out of the cells that store it.[26,27] The production of hepcidin-25, depending the content of iron in the body, is carried out by high Fe human hemochromatosis protein (HFE), transferrin receptor 1 and 2 (TfR1 and TfR2), bone morphogenetic protein 6 (BMP6), hemojuvelin, and transferrin. In response to the expanding resources of iron, the liver produces hepcidin-25, which inhibits the intestinal absorption and prevents further excessive accumulation. A decreased level of hepcidin-25 observed in our study among vegetarian women can cause an increased intestinal iron absorption and iron release from macrophages.[28]

In our study, the mean ferritin concentration in all groups was maintained within the normal range. However, its level was significantly lower among female vegetarians and particularly among female and male vegans compared to omnivores. Subjects on a plant-based diet, even if the iron intake was adequate, exhibited a lower ferritin concentration (but still maintained within normal limits) in comparison to non-vegetarians. However, the mechanism underlying this effect has not been elucidated.[11] Lower ferritin levels mainly concern lacto-ovo-vegetarian women, although only 3 out of 11 have a lower hematocrit and hemoglobin concentration.[3] There have been many studies showing a similar association. Kim and Bae examined 107 postmenopausal vegetarian women. The ferritin level in this group was significantly lower than in the control group, but it was maintained within the normal range.[29] In another study, Obeid et al. noticed no differences in the ferritin level between vegans, lacto-ovo-vegetarians and semi-vegetarians.[25] A reduced ferritin level is always related to iron depletion.[8] Contrarily, some authors suggested that a high ferritin concentration is associated with an increased risk of metabolic syndrome or chronic degenerative diseases, such as Alzheimer's disease. In 1998, Fernández-Real et al. revealed that there is a strong correlation between a high ferritin level and the development of insulin resistance contributing to an increased risk of type 2 diabetes.[30,31]

In our study, the mean transferrin concentration was shown to be elevated in both the female vegetarian and vegan groups, and also in the male vegan group. An increased transferrin concentration can be associated with iron deficiency and might be due to multiple reasons, such as inadequate food processing, insufficient amount of vitamin C in the diet, low iron content in the diet and other reasons, e.g., malabsorption or blood loss.[32] An increased transferrin concentration, similarly to a decreased ferritin level in this study, may result from the fact that vegetarian food provides only non-heme iron, which manifests lower bioavailability than heme iron. Therefore, the preparation of plant-based meals requires techniques that increase iron bioavailability, including soaking, cooking, sprouting, fermentation, consumption of products rich in vitamin C along with products rich in non-heme iron, and others.[8] The results obtained in this study vary from the former research results. In a study by Deriemaeker et al., the mean transferrin level among elderly Dutch vegetarians was higher compared to the control group; however, contrary to our study, it remained within the normal range.[33] Furthermore, Obeid et al. showed that the transferrin concentration in blood serum among lacto-ovo-vegetarians was 2.4 g/L, among vegans 2.3 g/L and among semi-vegetarians 2.3 g/L – there was no real variation in the results between all these groups and all were within the normal range.[25]

In our study, the transferrin receptor was within the normal range in all the groups studied, and there were no significant differences between them. Furthermore, other studies show no differences in the level of transferrin receptor. Walls et al. examined 2 groups of men aged from 59 to 78 years. Those in the 1st group were put on a plant-based diet for 14 weeks, while the men in the 2nd group were on a diet including beef for the same period of time. The transferrin receptor concentration among the meat-eaters

decreased from 4.9 ±2.5 μg/mL to 4.6 ±2.5 μg/mL in the 5th week of the study, and thereafter to 4.3 ±1.9 μg/mL in the 12th week of the study. In the vegetarian group, the transferrin receptor concentration increased from 4.6 ±1.7 μg/mL to 4.9 ±2.0 μg/mL in the 5th week of the study and it maintained the same level until the 12th week of research. The differences in the transferrin receptor level are associated with the iron bioavailability level. A diet that includes only non-heme iron is associated with an increased transferrin receptor level, as observed in the vegetarian group. However, food rich in heme iron caused a decrease in the transferrin receptor concentration, as observed in the group of meat-eaters.[34]

The limitation of our study is the lack of measurement of hemoglobin and other morphology parameters. Moreover, a single 24-hour dietary recall does not show that habitual intake (because it is less accurate method compared to 72-hour dietary recall); additional methods of evaluating food habits should be used in further studies.

Summary

Some authors have shown that the risk of developing anemia amongst vegetarians who follow a well-balanced diet (rich in vitamin C and iron) is not higher than in the case of non-vegetarians.[35]

Our results suggested a low storage of iron in the vegetarian and vegan groups studied (low ferritin, high transferin levels). However, the decreased level of hepcidin-25 observed in our study among vegetarian women is one of the mechanisms possibly increasing the absorption of iron. We conclude that periodic monitoring of basic biochemical parameters is recommended, especially among vegans. Also, exhaustive information about vegan and vegetarian diet is necessary.

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The importance of serum interleukin-20 levels in patients with Behçet's disease

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1391–1395

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Funding sources
None declared

Conflict of interest
None declared

Received on November 24, 2016
Reviewed on January 14, 2017
Accepted on April 19, 2017

Abstract

Background. Behçet's disease (BD) is a complex multisystemic disease with an unknown origin, which presents with aphthous and genital ulcers, cutaneous lesions, arthritis, ocular lesions, and defects in the gastrointestinal and central nervous systems.

Objectives. In this study, we examined the relationship between serum interleukin-20 (IL-20) levels and disease activity in BD patients.

Material and methods. A total of 45 BD patients diagnosed according to the BD diagnosis criteria determined by the International Study Group for Behçet's Disease were included in the study. Out of 45 patients, 17 had inactive BD and 28 had active BD. The control group consisted of 25 healthy subjects. The IL-20 levels of all the groups were detected and compared with each other. Serum IL-20, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels were examined.

Results. The IL-20 levels of the active BD patient group were significantly higher than in the control group ($p < 0.001$) and in the inactive BD patient group ($p < 0.001$). No statistically significant difference was detected between the IL-20 levels of the control group and the inactive BD patient group ($p = 0.2$).

Conclusions. Higher IL-20 levels in active BD patients, when compared to inactive BD patients and healthy controls indicate that the disease is an inflammatory one and IL-20 plays a role in the disease pathogenesis. Moreover, it can be concluded that IL-20 might have a role in the complex process of the settlement and activation of the disease.

Key words: cytokines, inflammation, Behçet's disease, interleukin-20

DOI
10.17219/acem/70523

Introduction

Behçet's disease (BD) is a chronic, recurrent inflammatory disease capable of affecting multiple systems; it is mostly presented with mucocutaneous, ocular, articular, vascular, and neurological symptoms. Although the etiology of the disease is not yet fully understood, it has been related to viral, bacterial, genetic, environmental, psychological basis; toxic, coagulative and immune factors have also been suspected.[1,2] Neutrophil hyperfunction, vasculitis and autoimmune response are the 3 major pathophysiological changes that play an important role in the disease pathogenesis.[3] After the publication of studies which showed an increase in immunoglobulins, immune complexes, complement, and acute phase reactants in BD, the disease was classified among autoimmune disorders.[4] Cytokines predominate in most immune mechanisms. These proteins are synthesized from several cell types which are important mediators in different immune mechanisms. Proinflammatory cytokines and mediators can be effective in BD prognosis.[5] Many studies have been carried out on serum cytokine levels to enlighten the etiopathogenesis of BD. These include interleukins (IL): IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, and also interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α).[1,4–7]

Interleukin-20 (IL-20) is a cytokine that belongs to the IL-10 family with an amino acid sequence very similar to that of IL-10. It is expressed from the active monocytes, keratinocytes and endothelial cells.[8] Interleukin-20 exerts its biological effects via type I and type II receptors composed of IL-20 R1 and IL-20 R2 complex chains, respectively. Intracellular domains of both receptors are in contact with Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2).[9,10]

The importance of IL-20 receptors has been shown in several inflammatory diseases, but the mechanism of action and pathophysiological significance is not yet fully understood. T cells and cytokines play an important role in immunological disorders. A variety of proinflammatory cytokines are expressed, including IL-1, IL-2, IL-6, IL-8, IL-12, IFN-γ, and TNF-α. Among these proinflammatory cytokines, IL-1β and TNF-α have proven to induce IL-20 secretion, especially from cells like macrophages and synoviocytes. Interleukin-20 itself increases the contribution of neutrophiles, monocytes and T cells to inflammation by directly inducing monocyte chemoattractant protein 1 (MCP-1) and IL-8 production. Several studies have demonstrated that IL-20 plays a role in inflammatory diseases, such as rheumatoid arthritis, psoriasis and atherosclerosis.[11–13]

In the study on atherosclerotic plaques, IL-20 was shown to increase the synthesis of fibroblast growth factor (β-FGF), vascular endothelial cell growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2), which are related to chronic inflammation and atherosclerotic angiogenesis. In the study conducted in the patients with rheumatoid arthritis, IL-20 has been shown to increase the synthesis of TNF-α, IL-1β, IL-6, IL-8, and MCP-1.[14]

In this study, we concentrated on serum IL-20 levels. We compared serum IL-20 levels with levels of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), which are known to be elevated in inflammation. We aimed to detect the role of IL-20 in BD pathogenesis and its relation with disease activity.

Material and methods

A total of 45 patients (20 male, 25 female) diagnosed with BD according to the diagnosis criteria determined by the International Study Group for Behçet's Disease, admitted to the Clinic of Immunology in the Dışkapı Yıldırım Beyazıt Training and Research Hospital (Ankara, Turkey) were included in the study (17 inactive and 28 active patients). Patients having any other acute or chronic inflammatory disease which might affect IL-20 levels were excluded from the study. The control group was composed of 25 healthy individuals (16 male, 9 female) with no acute or chronic illness. Behçet's disease activity was determined by patients filling out the Behçet's Disease Current Activity Form (BDCAF).[15,16]

Study groups were designed as BD patients (active and inactive groups) and healthy controls. Demographic features of all participants were recorded. Venous blood samples were collected from the participants after 12 h of fasting. Blood samples collected for IL-20 analysis were centrifuged at 4000 rpm for 10 min. Separated sera were aliquoted into Eppendorf tubes and stored at –80°C until the time of analysis. USCN Human IL-20 ELISA kit (USCN Life Science Inc., Wuhan, China) was used for serum IL-20 level detection. Prior to the study, the frozen specimens were allowed to melt at room temperature. Apart from IL-20 levels, also CRP, ESR and white blood cell (WBC) parameters, which are known to be related with disease activity, were detected from the same samples. C-reactive protein was studied with the immunoturbidimetric method, while ESR was studied with the Westergren method, and WBC analysis was made by the volume, conductivity, scatter (VCS) technique.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the ethical committee (No. 02.04.2010/16).

Informed consent was obtained from all individual participants included in the study.

Statistical analysis

Data analysis was made with Statistical Package for the Social Sciences (SPSS) for Windows v. 11.5 software (SPSS Inc., Chicago, USA). The normality tests were made with the Shapiro-Wilk test. Descriptive statistics of normally distributed continuous variables are illustrated as mean with standard deviation (SD). Descriptive statistics of non-normally distributed continuous variables are illustrated as median (interquartile range) values. Differences of normally distributed variables were analyzed with Student's t-test, while non-normally distributed variables were analyzed with the Mann-Whitney U test. The Kruskal-Wallis test was used for more than 2 groups. The nonparametric multiple comparison test was used when the Kruskal-Wallis test results appeared to be significant.

Nominal variables were analyzed with Pearson's χ^2 test. A correlation between continuous variables was detected with Spearman's correlation test. Probability value $p < 0.05$ was accepted as statistically significant for all tests.

Results

The patient group consisted of 45 patients, 20 males (44%) and 25 females (55.6%), while the control group consisted of 16 males (64%) and 9 females (36%). The mean age of the patient and control groups was 41.5 ±11 years and 29.2 ±5.3 years, respectively (Table 1). Among the patients diagnosed with BD, 18 (40%) had aphthous ulcers, 9 (20%) had genital ulcers, 6 (13.3%) had uveitis, 5 (11.1%) had acneiform skin lesions and erythema nodosum, 3 (6.7%) had neurological findings, 2 (4.4%) had pulmonary thromboembolism, and 9 (20%) had arthralgia (Table 2). When compared with the control group, the IL-20 levels of the active patient group were significantly higher ($p < 0.001$). Moreover, the IL-20 levels of the active patient group were higher than the IL-20 levels of the inactive patient group, and the difference was statistically significant ($p < 0.001$). There was no statistically significant difference between the IL-20 levels of the inactive patients and the control group ($p = 0.2$) Erythrocyte sedimentation rate was higher in the patient group ($p < 0.001$) (Table 3). Also, serum CRP levels were significantly higher in the patient group when compared to the control group ($p < 0.001$) (Table 3).

In symptomatic BD patients, a moderate positive correlation between serum IL-20 levels and symptoms was detected ($r = 0.604$; $p < 0.001$). No significant correlation was found between serum IL-20 levels and ESR and CRP parameters ($r = 0.268$; $p = 0.075$ and $r = 0.001$; $p = 0.995$, respectively) (Table 4).

The IL-20 levels in patients with aphthous ulcers were significantly higher than the in patients without aphthous ulcers ($p = 0.006$) (Table 5). The IL-20 levels of patients with or without genital ulcers, uveitis, skin lesions, and arthralgia did not show any significant difference (p-values: 0.232, 0.181, 0.207, and 0.181, respectively).

Table 1. Demographical features of BD group and control group

Feature	Control group	BD group
Age [years]	29.2 ±5.3	41.5 ±11.0
Gender, n (%) male female	 16 (64) 9 (36)	 20 (44.4) 25 (55.6)

BD – Behçet's disease.

Table 2. Distribution of clinical findings among active BD patients (n = 28)

Clinical findings	Number of patients (%)
Aphthous ulcers	18 (40.0)
Genital ulcers	9 (20.0)
Uveitis	6 (13.3)
Skin lesions	5 (11.1)
Neurological findings	3 (6.7)
Pulmonary thromboembolism	2 (4.4)
Arthralgia	9 (20.0)

BD – Behçet's disease.

Table 3. Laboratory parameters of the study groups

Parameter	Control group	BD group	p-value
IL-20 [pg/mL]	16.5 (0.0–140.8)	133.4 (0.0–1000.0)	<0.001
ESR [mm/h]	8.0 (1.0–11.0)	22.0 (1.0–100.0)	<0.001
CRP [mg/L]	3.2 (3.2–3.2)	3.4 (3.2–108.0)	<0.001

BD – Behçet's disease, IL-20 – interleukin-20; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein.

Table 4. ESR, CRP levels and clinical findings within BD group, their correlations with serum IL-20 levels and significance levels

Variable	Correlation coefficient	p-value
ESR	0.268	0.075
CRP	0.001	0.995
Number of clinical findings	0.604	<0.001

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; BD – Behçet's disease.

Table 5. Serum IL-20 levels of patients with and without aphthous ulcers

Variable	Aphthous ulcers (+)	Aphthous ulcers (–)	p-value
IL-20 [pg/mL]	223.7 (82.9–1000.0)	90.8 (0.0–608.0)	0.006

IL-20 – interleukin-20.

Discussion

Behçet's disease is a chronic, recurrent inflammatory disease which is presented with mucocutaneous, ocular, articular, vascular, and neurological symptoms. Neutrophil hyperfunction, vasculitis and autoimmune response are

the 3 major pathophysiological changes that play an important role in the disease pathogenesis. Although the etiology of the disease is not yet fully understood, it has been related to viral, bacterial, genetic, environmental, psychological, toxic, and immune factors. The most common hypothesis is that in patients with a genetic tendency to the disease, an uncontrolled immune response occurs, which in turn is triggered by infective (viral, bacterial, etc.) agents, heat shock proteins or autoantigens, and results in the occurrence of vasculitis.[4]

After the publication of studies which showed increases in immunoglobulins, immune complexes, complement, and acute phase reactants in BD, this disease was classified among autoimmune disorders.[5,6] Cytokines are known to be predominant in several immune mechanisms. These proteins are synthesized in different cell types which are important in immune-inflammatory reactions. Proinflammatory cytokines may also have a role in BD prognosis.[13]

To enlighten the etiopathogenesis of BD, several studies on serum cytokines, including IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IFN-γ, TNF-α, and leptin, have been carried out.[7] Interleukin-20 is a member of the IL-10 family of cytokines. It is expressed from the monocytes, keratinocytes, synoviocytes, and endothelial cells.[8] The family of IL-10 cytokines are a group of cytokines encoded in the same genomic region, showing similarities in protein structures and in the receptors they use. Interleukin-20 exerts its effects over type-1 and type-2 receptors. These receptors have shown to be expressed from 25 different tissues, including the brain, kidneys, heart, lungs, small intestine, and skin.[17]

In many inflammatory diseases, the importance of IL-20 and its receptors have been proven. But the mechanism of action and pathophysiological significance remains uncertain. Cytokines and T cells play an important role in disorders with immunological basis. Several proinflammatory cytokines are exposed, including IL-1, IL-2, IL-6, IL-8, IL-12, IFN-γ, and TNF-α. Among these cytokines, IL-1β and TNF-α are known to induce IL-20, especially from cells like macrophages and synoviocytes. Indeed, IL-20 is known to induce the activity of neutrophiles, monocytes and T cells in inflammation by increasing MCP-1 and IL-8 expression.[11–13] Inflammatory cytokines like TNF-α, IL-6 and IL-8 have been shown to increase in BD and correlate positively with the disease activity.[8] That is why IL-20 may also play a role in the pathogenesis of BD. When the skin samples of psoriasis patients and healthy controls were compared, IL-20 expression was shown to be elevated clearly in the areas of skin lesions; in another study with polymerase chain reaction (PCR), IL-20 levels were found to be 100 times higher than IFN-γ levels in the psoriatic skin.[12,17–20] In a study conducted in 2004, IL-20-IL-20 receptor complex expression was shown in both the synovial membranes and synovial fibroblasts of patients with rheumatoid arthritis. That study demonstrated that IL-20 induces the expression of proinflammatory cytokines, like TNF-α and IL-1, in the synovial fibroblasts, which indeed plays an important role in the pathogenesis of rheumatoid arthritis.[21] In 2008, Li et al. claimed that IL-22, IL-19 and IL-20 played a role in the pathogenesis of autoimmune uveitis and autoimmune intraocular inflammatory disease with increased gene expressions. The study was performed on the RNA samples of 50 patients with noninfectious autoimmune uveitis.[22]

In this study, we compared the IL-20 serum levels of BD patients with those of healthy controls. Besides this, we compared IL-20 levels in active and inactive disease states to verify if IL-20 can be an activation marker, like CRP, ESR and WBC parameters. Serum IL-20 levels were significantly higher in the patient group when compared to the control group. Especially in the active BD patients, serum IL-20 levels were significantly higher than in the control group. The reasons underlying this situation might be that IL-20 is a proinflammatory cytokine and it contributes to the settlement and maintenance of chronic inflammation, and many other unknown factors. There was no statistically significant difference between the serum IL-20 levels of the control and inactive patient groups. This may be due to suppressed immunological reaction and inflammation in the inactive patient group under the influence of medication. Besides this, CRP, ESR and WBC levels were detected to be higher in the patient group when compared to the control group.

Conclusions

Higher IL-20 serum levels in BD patients in comparison to the control group and were found. Also, higher IL-20 levels in the active group, when compared to the inactive group, and the correlation of IL-20 levels with standard activation markers, like CRP and ESR, present the possibility that IL-20 plays a role in the complex process of disease formation and activation. However, more detailed and large population-based studies need to be carried out to confirm the direct role of IL-20 in BD pathogenesis.

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The relationship between oral hygiene level and gingivitis in children

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1397–1401

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Funding sources
None declared

Conflict of interest
None declared

Received on January 9, 2017
Reviewed on January 17, 2017
Accepted on April 12, 2017

Abstract

Background. The condition of the teeth, periodontium and oral mucosa determines directly and indirectly the general health of the organism and, therefore, requires to be monitored. Childhood, especially the first years of life, is the time when proper health-promoting behaviors are formed, allowing the future prevention of such diseases as dental decay and gingivitis. Studies concerning the gingival status and bleeding in children are scarce, although such knowledge can be useful in planning preventive programs.

Objectives. The aim of the study was to determine the relationship between oral hygiene level and the likelihood of gingival bleeding.

Material and methods. The examined group comprised 2856 children aged 7 years, living in an urban area in western Poland. The hygiene status was evaluated according to the simplified Debris Index (DI-S) criteria and the periodontal status was evaluated according to the Community Periodontal Index (CPI) criteria. Logistic regression analysis was used to determine the predictors of gingival bleeding.

Results. The average value of DI-S was 0.91; it was not significantly different between girls and boys. In more than a half of the studied subjects (59.10%), oral hygiene was fair, in 12.46% – poor. Clinically healthy periodontium was observed in 91.32% of cases, bleeding on probing was present in 7.46% of cases. Calculus was detected in 1.22% of the children. Logistic regression analysis of the predictors of gingival bleeding showed that children with fair and poor oral hygiene were respectively 6 and 25 times more likely to show bleeding on probing compared to the children with very good oral hygiene.

Conclusions. In the studied group, it was noted that there is a need to promote oral hygiene, as it contributes to the fall in gingivitis prevalence among schoolchildren.

Key words: gingivitis, oral hygiene, Polish children

DOI
10.17219/acem/70417

Introduction

The condition of the teeth, periodontium and oral mucosa determines directly and indirectly the general health of the organism and, therefore, requires to be monitored. Epidemiological studies indicate that in children and adolescents the most often observed gingivitis is dental plaque-induced gingivitis, which is a reversible and non-destructive form of periodontal disease.[1,2] Poor oral hygiene and the accumulation of bacterial plaque is a known and important predisposing factor of gingivitis. However, the prevalence of destructive forms of periodontal disease is lower in young individuals than in adults. Still, dental plaque accumulation in childhood and adolescence can be associated with the development of periodontal disease in later life.[3] Children with healthy gingival tissues most likely progress to adult life with good periodontal health. Therefore, the key concern is periodontal health and good oral hygiene in childhood.[1–5]

Childhood, especially the first years of life, is the time when proper health-promoting behaviors are formed, allowing the future prevention of such diseases as dental decay and gingivitis. A fundamental element of prevention are effective daily prophylactic-hygienic procedures to remove dental plaque. Studies concerning the gingival status and bleeding in children are scarce, although such knowledge can be useful in planning preventive programs.

The aim of the study was to evaluate the hygiene and periodontal status among children living in an urban area in western Poland, and to determine the relationship between oral hygiene and the likelihood of gingival bleeding.

Material and methods

A total of 2856 generally healthy 7-year-olds entered the study (1408 girls and 1448 boys), representing 70% of the population attending the 1st year of elementary public schools in Poznań (about 550,000 inhabitants), Poland. The schools were randomly selected; all 7-year-old children who were at school on the days of examination were examined. Children with medically compromising conditions which could affect periodontal health were excluded from the study. Parents or caregivers were asked to sign a written statement of consent for their child's participation in the study. The studies were performed in the morning (9–11 a.m.). In order to minimize extra brushing efforts, the children were not informed in advance about the exact date of the oral examination.

The oral hygiene status was assessed using the simplified Debris Index (DI-S) of Green and Vermillon.[6] The teeth were examined as recommended by the index (the labial surfaces of teeth 11, 16, 26, and 31, and the lingual surfaces of teeth 36 and 46). The criteria for classifying DI-S scores of 0–3 were as follows: oral cleanliness was considered "very good" if the DI-S score was ≤0.2; "good" if the DI-S score was 0.3–0.6; "fair" when it showed 0.7–1.8; and "poor" when the score ranged between 1.9 and 3.0.[6]

The periodontal status was assessed using the Community Periodontal Index (CPI) according to the World Health Organization (WHO) basic methods of oral health surveys.[7] Recording of CPI was done in accordance with the WHO guidelines for the population group under 15 years of age. To avoid recording false pockets, which are common around erupting teeth, 3 codes were used: code 0 – healthy (no bleeding on probing and no calculus); code 1 – bleeding on probing (no calculus); code 2 – dental calculus present. The evaluation was performed on 6 index teeth (teeth 16, 11, 26, 36, 31, 46). According to the WHO classification, the highest CPI code was applied.[8,9]

The oral cavity was examined by pediatric dentists in the school nurse consultation room, under artificial light, using a dental mirror and a probe, according to the recommendations for oral epidemiological surveys by the WHO.[7] The same test conditions were kept for all examined children. The practitioners were calibrated prior to the study in order to control reliability. Calibration was performed by the clinical examination of children who were not included in the study. The kappa value was 0.85 for the oral hygiene status and 0.8 for the periodontal status, thus representing a satisfactory level of concordance. The obtained data was recorded in specially designed charts.

The statistical analyses were performed using STATISTICA v. 12 (StatSoft Inc., Tulsa, USA) and CytelStudio v. 10.0 (StatXact, Cambridge, USA) programs. Average values for quantitative data and the percentage for category data were determined. The χ^2 test was used to determine whether gender is related to the CPI value, oral cleanliness (the DI-S value) and bleeding, as well as whether bleeding is related to DI-S. Logistic regression analysis was used to identify independent risk factors, such as gender and oral hygiene (DI-S) for gingival bleeding (present or absent) expressed in odds ratios (OR) and 95% confidence intervals (CI). The level of significance was set at 0.05 (Fig. 1).

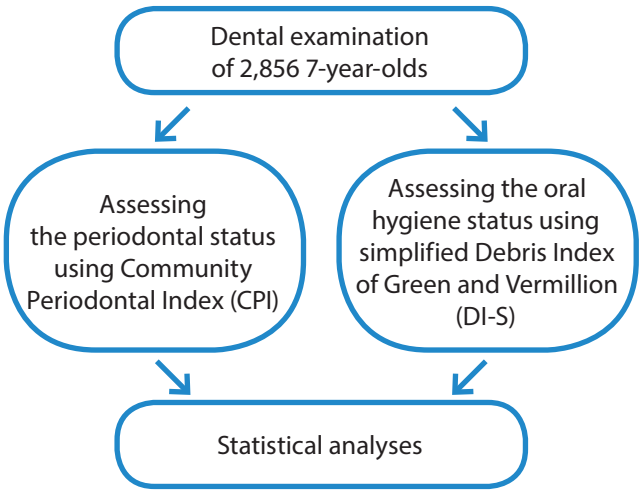

Fig. 1. Methodology of the study

The study was reviewed and approved by the Ethics Committee at Poznan University of Medical Sciences, Poland; it has been conducted in full accordance with the World Medical Association Declaration of Helsinki (No. 466/10).

Results

The average DI-S value in the studied group was 0.91 and it was not significantly statistically different in girls (DI-S = 0.87) and in boys (DI-S = 0.95). Very good oral hygiene was noted in 23.14% of the children, fair oral hygiene in more than a half (59.10%) of the studied group and poor in 12.46% of the children. No statistically significant dependence was observed between the distribution of the oral hygiene status and gender (p = 0.066) (Table 1).

Table 1. Oral hygiene status (DI-S)

Score	Female [%]	Male [%]	Total [%]
0.0–0.2	24.86	21.4	23.14
0.3–0.6	5.40	5.18	5.28
0.7–1.8	58.52	59.67	59.10
1.9–3.0	11.22	13.67	12.46

DI-S – simplified Debris Index.

Clinically healthy periodontium was observed in 91.32% of the studied children. Bleeding on probing was present in 7.46% of cases and supragingival dental calculus in 1.22% of cases. Statistical analysis did not show a dependence between the CPI value and gender (p = 0.669) (Table 2).

Table 2. Periodontal status (CPI)

Score [%]	Female [%]	Male [%]	Total [%]
0	91.55	91.09	91.32
1	7.31	7.60	7.46
2	1.14	1.31	1.22

CPI – Community Periodontal Index.

A statistical analysis performed using the χ^2 test confirmed a dependence between bleeding and the oral hygiene status (p < 0.0001). Among those children who showed bleeding, only 3.76% presented very good oral hygiene on the day of the examination and 2.35% presented good oral hygiene. However, in more than a half of them (54.93%), hygiene was classified as fair, and in 38.96% as poor.

Gender was not significantly associated with gingival bleeding. Therefore, in this model, the only significant variable was a range of the DI-S values (p < 0.0001). Logistic regression analysis of the predictors of gingival bleeding showed that children with poor oral hygiene were 4 times more likely to have gingival bleeding compared to children with fair oral hygiene (p < 0.0001; OR: 4.0; 95% CI: 2.95–5.61). Furthermore, children with fair and poor oral hygiene were respectively 6 (p < 0.0001; OR: 6.08; 95% CI: 2.96–14.49) and 25 (p < 0.0001; OR: 24.82; 95% CI: 11.77–60.02) times more likely to have bleeding on probing compared to children with very good oral hygiene. There was no significant dependence between very good and good hygiene (p = 0.15) (Table 3).

Table 3. Logistic regression output with regard to the predictors of bleeding on probing

Predictor	OR	95% CI	p-value
hygiene level* (DI-S)			
Good (0.3–0.6)	2.80	0.71–9.83	0.150
Fair (0.7–1.8)	6.08	2.96–14.49	<0.0001
Poor (1.9–3.0)	24.82	11.77–60.02	<0.0001

Predictors of bleeding on probing were determined as present vs absent; OR – odds ratio; CI – confidence interval; DI-S – simplified Debris Index; * reference category: very good oral hygiene level (DI-S ≤ 0.2).

Discussion

Dental literature reports concerning oral hygiene in early school children are scarce. In Poland, children start compulsory education at 7 years of age. At this age, the first permanent teeth usually erupt. Therefore, this age seems to be the best to evaluate oral hygiene and the periodontal status in children.

In the studies carried out among children in Iran, the average DI-S value was 1.19, among children from Minsk (Belarus) it was 1.64, and in Kuwait, the DI-S value was 1.5.[1,10,11] Moreover, the inclusion of gender data into the study provided a conclusion that oral hygiene in boys was significantly better than in girls.[11] In the quoted publications, the average value of DI-S showed worse oral hygiene than in the studied population of western Poland, where it was 0.91 for the population, with similar values for boys and girls.

Numerous epidemiological studies show similar oral hygiene status in children all over the world. In the studies by de Almeida et al., 799 Portuguese 6-year-olds were examined for oral hygiene, of which 7.6% showed very good oral hygiene status; good oral hygiene status was present in 16.5%, fair in 72.6% and poor in 3.3% of the studied children.[12] On the other hand, Al-Mutawa et al. did not note very good oral hygiene in the group of 3294 children – the DI-S value in that case was below 0.3. In the majority of the studied population (67%), oral hygiene was assessed as fair, and in 29.1% of cases – as poor.[11] Krishnam et al. evaluated oral hygiene in 5129 children, aged 5–12 years, of the Eastern Ghats region of southern India, using the simplified oral hygiene index (OHI-S) by Green and Vermillion (good: 0.0–1.2; fair: 1.2–3.0; poor: 3.1–6.0).[13] Good oral hygiene was present in 34.27% of the children, 50.98% showed fair hygiene status and 14.7% were evaluated as having poor oral hygiene. Anupriya et al. evaluated

oral hygiene using the OHI-S in 1787 5–8-year-olds of Himachal Pradesh in the northern India.[14] Good hygiene was noted in 9.4%, fair in 63.6%, and poor in 26.8% of cases. In our own studies, similarly to other authors', the most numerous group of patients consisted of the students presenting fair oral hygiene status (59.1%).

The literature concerning the periodontal status in 7-year-old children presents varied results. The percentage of healthy periodontium at that age ranges between 9% and 80%.[5,14–16] Składnik-Jankowska and Kaczmarek evaluated the periodontal status of 7-year-olds from southern Poland and found healthy periodontium in 80% of them, with no statistically significant difference between genders.[15] A relatively high percentage of children with healthy periodontium (70.43%) was observed by Varas et al. in children aged 6–8 years (Santiago Metropolitan Region in Chile), while markedly lower values – 22.5% – were reported by Dini et al. in Brazilian 7-year-olds.[5,16] A still lower percentage was noted by Anupriya et al.: in only 9.4% of the children aged 5–8 years.[14] Our own studies showed healthy periodontium to be present in 91.32% of the studied population of the 7-year-olds from a big city. Similarly to the quoted findings of other authors, no statistically significant difference was found here between girls and boys.

Different values concerning bleeding are reported in some other studies. In the paper by Składnik-Jankowska and Kaczmarek, bleeding on probing was present in 14.5% of the 7-year-olds and this value was nearly 2 times higher than that obtained in our own studies (7.46%). Still higher values were quoted by Varas et al., Anupriya et al. and Dini et al., who observed gingival bleeding in 27.49%, 71.9% and 76.8% of children, respectively.[5,14,16]

Contrary to our study, in the research by Arnlaugsson and Magnusson, as well as Hemadneh and Ayesh, the gingival bleeding index (GBI) was used.[2,4] Among Icelandic 6-year-olds of Reykjavik examined by Arnlaugsson and Magnusson, healthy gums were noted in 26% of the children.[2] Similar values were obtained by Hemadneh and Ayesh in Jordanian children, out of whom 30.6% had healthy gums.[4] However, the studies by Bossnjak et al., comprising Croatian children aged 6–11 years, revealed that healthy gums were present in 43% of the studied subjects.[17] In the quoted studies, as well as in our own ones, there were no statistically significant differences between girls and boys.

Dental calculus occurs less often in children than in adults. In adolescent patients, supragingival deposit is mainly present. Składnik-Jankowska and Kaczmarek and Varas et al. noted this deposit in a small percentage of the examined children, 4% and 2.08%, respectively.[5,15] A higher percentage was reported by Anupriya et al. in 5–8-year-old Indian children and Varenne et al. in 6-year-old African children – 10.2% and 58%, respectively.[14,18]

Based on epidemiological studies performed in Poland in 1987 and 1995, Banach compared the prevalence of dental calculus among 7- and 12-year-olds.[19] The percentage of Polish 7-year-olds with dental calculus dropped from 3.1% to 0.6% over 8 years, and the values for 12-year-olds decreased from 33.4% to 21%.[19] In the studies by Chłapowska, carried out in the city of Poznań in 1995, dental calculus was observed in 3.7% of children aged 7 years and in 26.7% of those aged 12 years.[20] However, in the study undertaken 4 years later by Pawlaczyk-Kamieńska, which included 521 children aged 12 years from the same area (Poznań), dental calculus was found in 26.3% of the children.[21] In the same educational setting of Poznań, 20 years after the study made by Chłapowska, dental calculus was noted in 1.22% of children aged 7 years. The presented data displays a drop in the percentage of children with dental calculus from 3.7% to 1.22%.[20,21] Moreover, the data confirms the former reports of an increase in the percentage of people with dental calculus along with their age.

The statistical analysis of our own results did not reveal any correlation between gender and bleeding, although the research done by Gopinath et al. showed that males were 2.11 times more likely to develop gingivitis than females.[22] However, in our study, a correlation was found between the amount of bacterial plaque and the likelihood of bleeding, which confirms a relationship of gingivitis etiology and the oral hygiene status. In children with poor oral hygiene, the likelihood of bleeding on probing was 4 times higher than in children with fair oral hygiene. When children with fair oral hygiene were compared to those with very good hygiene, it was revealed that the likelihood of bleeding was 6 times higher in the fair oral hygiene group. Still, the children with poor hygiene were 25 times more likely to develop bleeding on probing than those with a very good hygiene status. In 71.56% of the studied 7-year-old children living in Poznań, oral hygiene was evaluated as fair (59.1%) or poor (12.46%). Therefore, they have a 6 or 25 times lower chance to maintain healthy periodontium in the future, compared to their counterparts showing very good hygiene (23.14% of the children). The study proved a correlation between the oral hygiene status and gingival bleeding. Therefore, an improvement of daily hygienic procedures to remove bacterial dental plaque seems of greatest importance in reducing the likelihood of periodontal disease in adult age.

Epidemiological studies provide the necessary knowledge to formulate and implement programs of prophylaxis, which will bring about an improvement of oral health. It seems that the provision of dental education to children with mixed dentition should also be a priority in order to teach better skills of bacterial plaque removal.

Limitations of our study comprise the sample size and participants. It might be possible that children with poor oral hygiene were not given the consent for the examination to avoid any unpleasant comments. Furthermore, some children probably did not participate in the research because of strong dental fear. We also did not study the socioeconomic status and educational level of the subjects, which could have led to a deeper analysis. Nevertheless, we believe that our findings closely represent the population values.

Conclusions

Among the examined children living in a big city, the majority show fair or poor oral hygiene.

Unsatisfactory oral hygiene status significantly increases the likelihood of bleeding on probing. The performed study has shown the need to treat and prevent periodontal pathologies. Therefore, the implementation of educational programs to improve oral health promoting behaviors seems necessary.

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Characteristics and risk factors of bike-related accidents: Preliminary analysis

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1403–1409

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Funding sources
None declared

Conflict of interest
None declared

Received on March 6, 2017
Reviewed on April 1, 2017
Accepted on April 26, 2017

Abstract

Background. The growing popularity of cycling not only enhances self-efficacy, physical well-being and improves quality of life, but it also leads to the increasing number of bike-related injuries.

Objectives. The aim of this study was to characterize the population of cyclists in Poland, assess the occurrence of safety behaviors among them and to precise the risk factors for bike accidents. Additionally, we analyzed bike-related injuries.

Material and methods. The survey was based on a 39-item questionnaire created for this study. We collected data concerning the demographical status of respondents, their cycling preferences, attitude toward safety behaviors during cycling, and the characteristics of bike-related injuries. Our research covered 729 people who declared themselves as cyclists (302 women – average age: 31.5 ±10.3 years; 427 men – average age: 32.6 ±10.7 years).

Results. In the study, 71.3% of the interviewees declared that they did not always wear helmets. Women more often than men claimed that they did not always use head protection ($p < 0.001$). Among 729 people, 277 suffered from bike-related injuries. In this group, we noted 870 accidents that resulted in 1671 different injuries. In our study, wearing helmets did not influence the region of trauma nor its type ($p < 0.05$). We noted 811 injuries of the upper extremities and 541 of the lower extremities.

Conclusions. Young men constitute the main group of cyclists in Poland. The risk factors for bike-related accidents in our study proved to be: educational level (secondary education and incomplete higher education), number of children (having at least 3 children), frequency of using a bike (the more often, the more injuries), type of bike used (mountain bikes and fold-up bikes), and frequency of using a helmet (using helmets was correlated with a higher rate of injuries). The upper and lower extremities were the most common location of injuries.

Key words: bike-related accidents, wounds, injuries, cycling, maxillofacial fractures

DOI
10.17219/acem/70695

Introduction

The growing popularity of cycling as a means of transport, a part of a certain lifestyle and a simple and healthy way of spending time creates the possibility not only to reduce air pollution and traffic, but also to improve public health. A shift from motorized transportation to cycling could significantly contribute to the reduction of traffic congestion, noise, carbon dioxide emission, and air pollution.[1–3] The benefits of cycling are truly impressive and include physical and psychosocial aspects.

It has been proven that cycling enhances self-efficacy, physical well-being and improves quality of life.[1,4,5] By following the recommended 30 min of daily physical activity, the reduction of risk for cardiovascular diseases can be obtained. The researchers also hypothesized that it could have a positive, preventive effect against certain types of cancer.[1,6] Regular cycling also improves lipid profile, normalizes blood pressure, helps to control weight, and decreases insulin resistance.[6–8] This beneficial influence on health makes this kind of sport especially advisable for patients with type 2 diabetes.[8] It has been shown that cycling positively affects the musculoskeletal system, also in terms of rehabilitation.[9,10] Application of cycling leg exercise has shown beneficial effects on motor abilities in patients suffering from subacute and chronic diseases.[10,11] Moreover, cycling has a positive influence on instrumental activities of daily living and social function in elderly people with mobility limitations. Unfortunately, the elderly are the group which incur more accidents than adults.[12,13] New research reports that the loss of the ability to cycle can be a new warning sign for atypical parkinsonism.[14] Some authors indicated that cycling in big cities might be erroneously assessed as unfavorable for health, especially for the respiratory system because of exposure to air pollution. However, it has turned out that the benefits of active travel outweigh the harm caused by air pollution in all regions except for the most extreme air pollution concentrations.[2,15]

Considering the safety aspects during cycling, several factors of bike-related accidents should be mentioned. First of all, the environment and the infrastructure built may be correlated with bicycle injuries.[1,3] Cyclists are often forced to share the same road with cars, buses and trucks, and they are more vulnerable to accidents than motorized drivers.[2] Modification of the existing infrastructure is also important, e.g., replacing signal-controlled intersections with roundabouts.[4] Also demographic factors, and traffic speed and density influence the bike-related accident rate.[3,5] Different behaviors among cyclists, such as wearing helmets and reflective elements, using headphones, cell-phones or other devices, form a very differential group of factors.

The aim of this study was to characterize the population of cyclists in Poland, assess the occurrence of safety behaviors among them and to precise the risk factors for bike accidents. Additionally, we analyzed bike-related injuries.

Material and methods

This survey was based on a 39-item questionnaire created for this study, covering a wide spectrum of issues related to cycling. It included 2 parts. In most questions, the participants were allowed to choose 1 answer, unless stated otherwise. A few questions additionally had an option for including comments. The 1st part consisted of 22 questions related to the demographical status of respondents (age, sex, professional activity, education, marital status, having children), their cycling preferences and attitude toward safety behaviors during cycling (e.g. type of bike, frequency of riding, season of cycling, wearing helmets). The 2nd part concerned only people who had at least 1 cycling injury. It included 17 detailed questions about the circumstances of the accident.

The survey was prepared in a digital version, using online service for creating surveys and tests – www.ankietka.pl. It was available on the website http://www.ankietka.pl/ankieta/206347/ankieta-dla-rowerzystow.html between November 20, 2015 and February 10, 2016. Our research covered 729 people who declared themselves as cyclists. To distribute the survey to the wide spectrum of respondents, link to the survey was published on Facebook fan pages devoted to cycling, active lifestyle or traveling, and on bicycling websites.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normality of data distribution. The χ^2 and Mann-Whitney U tests were done to determine the relationship among variables. Univariate and multivariate logistic regression analyses were performed. The p-value <0.05 was considered statistically significant.

Results

Most respondents were male (58.6%). As shown in Fig. 1, almost half of the group were between 19 and 29 years old (43.8%), and around 1/3 was between 30 and 29 years old (31.3%). Cyclists younger than 19 years and older than 39 years accounted for <25% of the studied sample.

Important differences between men and women were observed during data analysis. The following graphs show these diverse patterns of using a bike by both sexes. Figure 2 illustrates that despite the fact that both men and women prefer spring and summer as the most suitable seasons for cycling, men significantly more often use bikes in spring (by 6.4 percentage points; $\chi^2 = 10.170$; $p = 0.002$), autumn (by 19.4 percentage points; $\chi^2 = 46.469$; $p < 0.001$) and winter (by 25.4 percentage points; $\chi^2 = 46.0234$; $p < 0.001$). No statistically significant difference among male and female groups in the frequency of riding a bike in summer was observed.

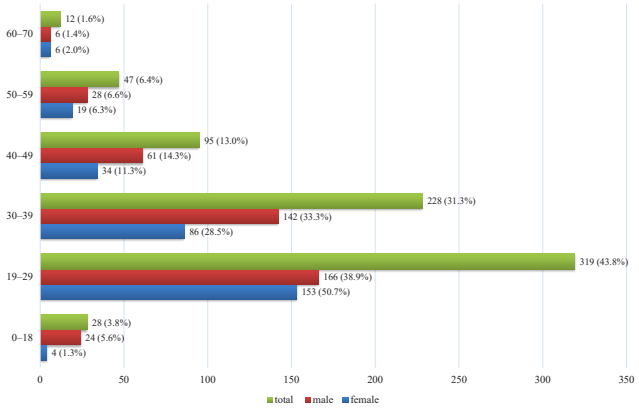

Fig. 1. Age of the respondents

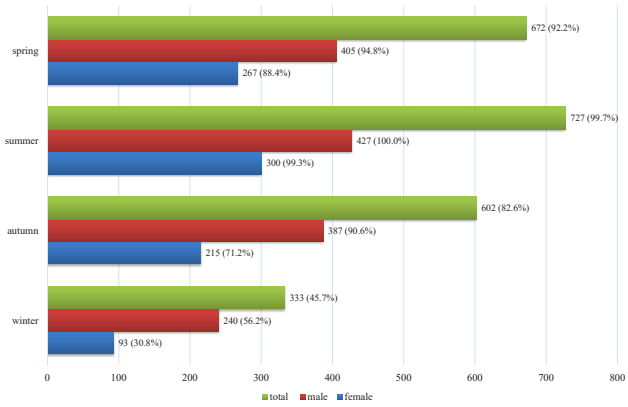

Fig. 2. Seasons of cycling

Generally, the respondents declared that they rode a bike a few times a week (45.5%) or every day (26.9%). But data presented in Fig. 3 confirms the fact that women used bikes less frequently than men ($\chi^2 = 51.082$; $p<0.001$). Female respondents usually declared that they rode a bike a few times a week (36.8%) or from time to time (27.5%), while more than half of male respondents stated that they used a bike a few times a week (51.8%, i.e., 15 percentage points more than women) and around 1/3 claimed that they used a bike every day (30.4%).

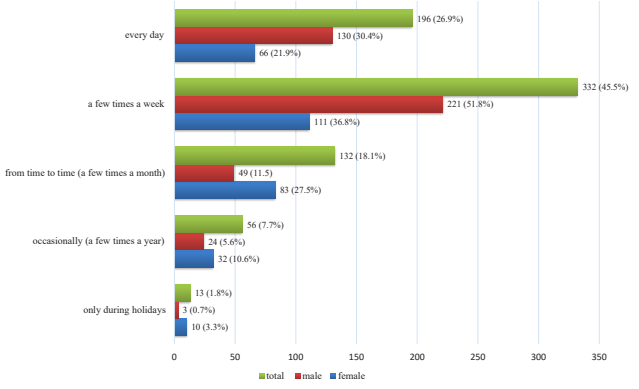

Fig. 3. Frequency of using a bike

According to the survey results, 3 types of bikes were the most popular – mountain bikes (40.9%), touring bikes (25.4%) and city bikes (24.3%). But here significant gender differences were also identified. For women, the most popular type of bike was a city bike (39.7%), while men more often preferred mountain (45%) and touring (28.6%) bikes. The popularity of city bikes among men (13.4%) was close to the popularity of road bikes (11.5%) ($\chi^2 = 51.082$; $p < 0.001$) (Fig. 4).

About 71.3% of the interviewees declared that they did not always wear helmets. Despite stereotypes about attitudes of men and women toward risk, women more often than men claimed that they did not always use helmets (19 percentage points more; $\chi^2 = 31.175$; $p < 0.001$) (Fig. 5). This can be explained by the fact that women usually used different type of bikes and also they used them less often than men.

To determine the risk factors, the logistic regression analysis was conducted. In the first step, 19 variables with possible impact on the probability of having an accident were identified and then used in binary logistic regressions with single categorical predictors. These variables were: gender, age group, professional activity, educational level, marital status, number of children and their age, time of the year when a bike is used, the frequency of using a bike, using reflectors, reason for cycling, listening to music, opinions about the obligatory use of helmets and about the usefulness of helmets, frequency of using a helmet, having a driving license and a cycling license, type of bike, and alcohol consumption. It turned out that 10 of these variables were statistically significant in these models ($p < 0.05$).

In the next step, these 10 variables were used as independent variables in a multivariable logistic regression model. The outcome of this model is presented in Table 1.

Out of 10 variables used in a multivariable logistic regression, 5 remained statistically significant ($p < 0.05$): educational level, number of children, frequency of using a bike, type of bike used, and frequency of using a helmet. People with secondary education ($p < 0.01$) or with an incomplete higher education ($p < 0.05$) were almost 2 times less likely to have an accident than people with a university degree. Another significant predictor was the number of children. Having at least 3 children increased the probability of accidents by 3 times ($p < 0.01$). What is quite intuitive, cyclists using a bike every day were more exposed to accidents than others. The probability of an accident for them was 3 times higher than the probability of an accident for people who used a bike a few times a month or only during holidays ($p < 0.01$). The type of bike used also strongly affected the probability of an accident. In comparison with people using city bikes, those riding mountain bikes and fold-up bikes were more prone to accidents – 2 times ($p < 0.01$) and 8 times ($p < 0.05$), respectively. The last significant variable was the frequency of using a helmet. People who did not always use head protection had less probability of having an accident than people always using helmets during their bike rides.

Table 1. Coefficients of logistic regression model

Variable	OR	95% CI	p-value
Gender			
female	1.000	ref.	–
male	0.721	(0.498–1.044)	0.08303
Age [years]			
<30	1.000	ref.	–
30–39	1.457	(0.928–2.288)	0.10137
40–49	1.398	(0.791–2.469)	0.24783
50–70	1.717	(0.863–3.418)	0.12305
Professional activity			
student	1.000	ref.	–
employed	0.636	(0.377–1.0754)	0.09069
unemployed	1.111	(0.498–2.479)	0.79727
Educational level			
university degree	1.000	ref.	–
incomplete higher education	0.533	(0.321–0.883)	0.01451*
secondary education	0.428	(0.282–0.650)	0.00006*
elementary education	0.383	(0.142–1.036)	0.05825
Number of children			
<3	1.000	ref.	–
≥3	3.064	(1.395–6.727)	0.00520*
Time of the year when a bike is used			
only summer	1.000	ref.	–
other seasons as well	2.282	(0.849–6.130)	0.10122
Frequency of using a bike			
a few times a month or only during holidays	1.000	ref.	–
occasionally / a few times a year	1.283	(0.543–3.033)	0.56930
a few times a week	1.204	(0.723–2.002)	0.47497
every day	2.160	(1.202–3.882)	0.00988*
Type of bike used			
city bike	1.000	ref.	–
road bike	1.894	(0.929–3.859)	0.07819
touring bike	1.318	(0.793–2.191)	0.28602
mountain bike	2.079	(1.2773–3.383)	0.00318*
fold-up bike	7.525	(1.1956–47.4)	0.03129*
electric bike	0.621	(0.109–3.519)	0.58943
Reason for cycling			
leisure	1.000	ref.	–
way of spending free time with a family	0.760	(0.273–2.117)	0.59865
means of transport	1.366	(0.8695–2.146)	0.17550
exercising	1.235	(0.775–1.968)	0.37286
Does the interviewee use a helmet?			
always	1.000	ref.	–
not always	0.588	(0.402–0.860)	0.00615*

OR – odds ratio; CI – confidence interval; * statistically significant.

In our cohort, 277 out of 729 respondents suffered from bike-related injuries. In this group, we noted 870 accidents that resulted in 1,671 different injuries. The most common injuries were related to the upper and lower extremities (48.5% and 32.4% of all injuries, respectively). Injuries of the upper extremities were more often observed in the male group and they constituted 50.1% of all injuries in men ($p < 0.025$). Wounds of the upper extremities also appeared more frequently in males (17.3% of injuries; $p < 0.005$). Women more often suffered from injuries of the lower extremities in comparison to men (37.7% vs 30.6%; $p < 0.01$). This observation was similar for abrasions of lower limbs (24.6% vs 18.8%; $p < 0.02$). The female group was also at a higher risk of facial skeleton fractures in comparison to the male group (1.9% vs 0.2%; $p < 0.001$) (Table 2).

In our study, wearing helmets did not influence the region of trauma nor its type ($p < 0.05$). Detailed data regarding injuries in the helmeted and non-helmeted groups of cyclists was presented in Table 3.

Discussion

Bike-related accidents are becoming a high interest topic in the medical press, mainly due to the growing popularity of cycling and an observed peak of injuries among cyclist.[16,17] This research article examined the population of cyclists – their habits, cycling behavior in relation to potential risk factors of bike-related accidents – and also described the injury pattern.

In the analyzed literature, it was proven that demographic data like age and gender affected the injury rate and cycling patterns.[18–26] Most articles concerned the study population of adolescences with a small sample of elderly people. In the abovementioned articles, males were the main group of cyclists. However, bicycle users are not a homogenous group. On the basis of studies conducted all over the world, researchers divided cyclists into groups, taking into consideration their cycling behaviors.[4,27–29] One of the features being the basis for distinguishing bike users is the reason for cycling. For some people, this activity is only a form of recreation, for others, bicycle is a very effective and cheap means of transport (commuters), and lastly there is a group which treats it as a sport, also in its extreme version.[4,28,29] Additionally, the differences among cyclists are correlated with the frequency of cycling and the level of their experience.[22] Different classifications of cyclists have been developed, but none of them is widely accepted. The proportion of cycling population differs among different countries. In Australia and the United States, bicycles are used by a relatively small group of inhabitants; cycling is treated mostly as a form of recreation and rarely as a means of transport.[30,31] In the European countries, such as the Netherlands, Denmark or Germany, cycling is much more popular and mainly used as a mainstream mode of transport.[31] Researchers observed that cyclists

Table 2. Characteristic of injuries among the female and male groups of cyclists

Variable	Female	Male	Total	Statistical analysis	p-value
Alcohol consumption*	4 (0.46%)	5 (0.57%)	9 (1.03%)	1.63779	>0.05
Head injuries	52 (12.56%)	174 (13.84%)	226 (13.52%)	0.43770	>0.05
brain concussion	4 (0.97%)	13 (1.03%)	17 (1.02%)	0.01431	>0.05
injuries of the cerebral part of the head	4 (0.97%)	29 (2.31%)	33 (1.97%)	2.89251	>0.05
Face injuries	44 (10.63%)	132 (10.50%)	176 (10.53%)	0.00532	>0.05
abrasions	21 (5.07%)	80 (6.36%)	101 (6.04%)	0.91526	>0.05
wounds	15 (3.62%)	49 (3.90%)	64 (3.83%)	0.06393	>0.05
fractures	8 (1.93%)	3 (0.24%)	11 (0.66%)	13.66112	<0.001**
Upper extremity injuries	181 (43.72%)	630 (50.12%)	811 (48.53%)	5.10609	<0.025**
abrasions	118 (28.50%)	357 (28.40%)	475 (28.43%)	0.00158	>0.05
wounds	45 (10.87%)	218 (17.34%)	263 (15.74%)	9.84027	<0.005**
fractures	18 (4.35%)	55 (4.38%)	73 (4.37%)	0.00057	>0.05
Lower extremity injuries	156 (37.68%)	385 (30.63%)	541 (32.38%)	7.07530	<0.01**
abrasions	102 (24.64%)	237 (18.85%)	339 (20.29%)	6.44102	<0.02**
wounds	53 (12.80%)	135 (10.74%)	188 (11.25%)	1.32624	>0.05
fractures	1 (0.24%)	13 (1.03%)	14 (0.84%)	2.35526	>0.05

* The percentage was calculated with regard to the total number of accidents (n = 870). For the rest of the factors, the percentage was calculated with regard to the total number of injuries in each group (female = 414, male = 1257, total = 1671); ** statistically significant.

Table 3. Characteristic of injuries among the helmeted and non-helmeted groups of cyclists

Variable	With a helmet	Without a helmet	Total	Statistical analysis	p-value
Alcohol consumption*	5 (0.57%)	4 (0.46%)	9 (1.03%)	0.91689	>0.05
Head injuries	102 (14.76%)	124 (12.65%)	226 (13.52%)	1.53996	>0.05
brain concussion	9 (1.30%)	8 (0.82%)	17 (1.02%)	0.95106	>0.05
injuries of the cerebral part of the head	15 (2.17%)	18 (1.84%)	33 (1.97%)	0.23358	>0.05
Face injuries	78 (11.29%)	98 (10.00%)	176 (10.53%)	0.71343	>0.05
abrasions	47 (6.80%)	54 (5.51%)	101 (6.04%)	1.19034	>0.05
wounds	26 (3.76%)	38 (3.88%)	64 (3.83%)	0.01452	>0.05
fractures	5 (0.72%)	6 (0.61%)	11 (0.66%)	0.07683	>0.05
Upper extremity injuries	335 (48.48%)	476 (48.57%)	811 (48.53%)	0.00134	>0.05
abrasions	197 (28.51%)	278 (28.37%)	475 (28.43%)	0.00402	>0.05
wounds	110 (15.92%)	153 (15.61%)	263 (15.74%)	0.02875	>0.05
fractures	28 (4.05%)	45 (4.59%)	73 (4.37%)	0.28258	>0.05
Lower extremity injuries	216 (31.26%)	325 (33.16%)	541 (32.38%)	0.67118	>0.05
abrasions	134 (19.39%)	205 (20.92%)	339 (20.29%)	0.58370	>0.05
wounds	78 (11.29%)	110 (11.22%)	188 (11.25%)	0.00164	>0.05
fractures	4 (0.58%)	10 (1.02%)	14 (0.84%)	0.95096	>0.05

* The percentage was calculated with regard to the total number of accidents (n = 870). For the rest of the factors, the percentage was calculated with regard to the total number of injuries in each group (female = 691, male = 980, total = 1671).

who used bicycles as a means of transport tended to be younger and travel more frequently (more days per week), in the morning and evening peak hours, than those who rode for recreational purposes. Recreational cyclists treated cycling as physical exercise that helped them maintain a good physical condition; they more frequently used road bikes, while commuters spent more time on hybrid, city, cruiser and comfort bikes.[26]

In our article, we additionally included other sociological factors, such as professional activity, education, marital status, having children, cycling preferences, and attitude toward safety behaviors during cycling, as potential risk factors for bike-related injury. Our cohort included mostly adult cyclists at the age 19–29 years and only a small group of very young or old people, similarly to the major part of research considering cycling patterns. Also, in our study,

like in most publications, males constituted the main group of respondents. Our interviewees most frequently declared that they treated cycling as a form of recreation or a means of transport. Women declared riding a bike a few times a week or from time to time, and they more often chose city bikes. Men on the other hand stated that they used a bike more often, i.e., a few times a week or every day, and they usually preferred riding a mountain bike. Other studies also indicated that men had larger experience in cycling. When it comes to safety considerations, women more often than men claimed that they did not always wear helmets. This can be explained by the fact that women used bikes less often than men. However, more than half of the cyclists declared to have reflective elements on their clothes or bikes.

Bike accidents are a growing public health problem worldwide. Risk factors of these incidents were discussed in many medical publications. Researchers around the world confirmed that the age of cyclist is correlated with the number of accidents – children, adolescents and people older than 65 years of age take part in more accidents than adults.[20,22,23,25] Taking into consideration the number of accidents that take place, the exposure indicator should not be omitted. Subjectively, a higher number of bike crashes noted in the urban areas is strongly correlated with a greater number of bike users. Some authors compared the number of cyclists and the number of bike-related injuries in and outside the city and it turned out that off-road cycling was much more unsafe.[18,20] There is little information in the literature about the correlation of the bicycle type and bike-related injuries. It is probably related to the belief that many kinds of bicycles are used not necessarily in line with their main purpose, e.g., mountain bikes are often used as a means of transport in the city and city bikes can be seen on short off-road trips.

Our study recognized several groups of cyclists with a higher probability of undergoing bike-related accidents. They included people with secondary education and incomplete higher education, parents with at least 3 children, people who used a bike every day (higher exposure), people riding city bikes, mountain bikes and fold-up bikes. People who declared always wearing helmets during cycling proved to be more prone to accidents. This seems counterintuitive, but there can be 2 possible explanations. The first one is similar to the conclusion made by Peltzman, who suggested that the probability of risky behaviors increase along with the increase in perceived safety.[32] Some studies also indicate that drivers can act less carefully toward cyclists wearing helmets than toward those without.[33] It should be stated that both these hypotheses are controversial. Several studies have shown that head injuries are more common and more severe in cyclists that do not wear helmets.

Some epidemiological data indicates that head injuries are the most common traumas among cyclists.[34,35] It was estimated that among victims of fatal accidents, around

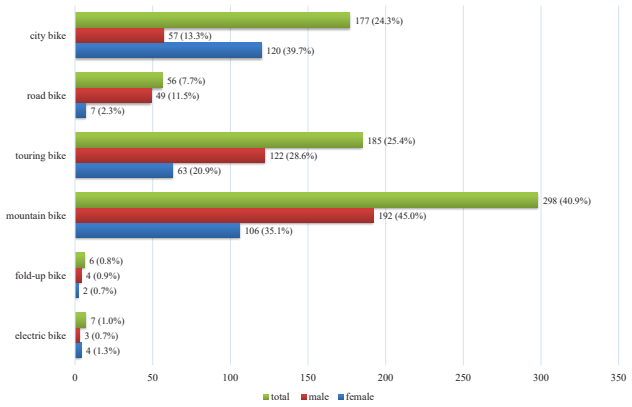

Fig. 4. Type of bike used

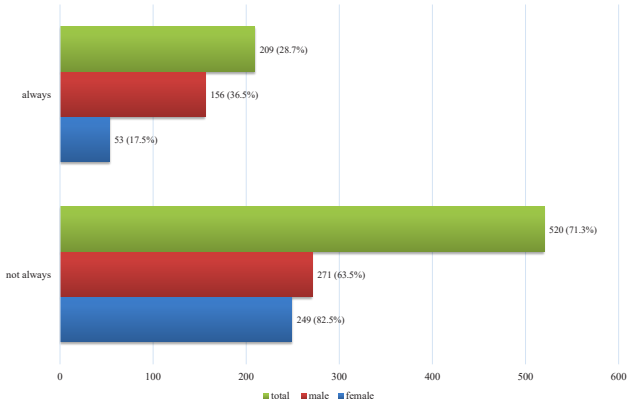

Fig. 5. Protective helmet usage among respondents

2/3 of them sustained head traumas.[36] The most frequent head injuries in bike-related accidents are skull fractures and cerebral contusions.[35] Cyclists are also at a higher risk of undergoing facial fractures.[37] The studies on the impact of wearing helmets on injuries of the facial region led to controversial conclusions.[38–40] Some authors emphasized that wearing bicycle helmets did not reduce the incidence of mid-facial fractures and was even associated with an increased risk of mandibular fractures.[37]

Contrary to the abovementioned articles, in our studies, the most common injuries were those of the upper extremities (48.53% of all injuries) and lower extremities (32.38%). Injuries of the upper extremities, including wounds, were more often observed in the male group. Women more often suffered from injuries of the lower extremities (37.68%), comprising abrasions of the lower limbs. The female group was also at a higher risk of facial skeleton fractures in comparison to the male group. In our study, wearing helmet did not influence the region of trauma nor its type.

In the cited articles, the injuries of the upper and lower extremities were the second most common type of traumas. No correlations between type and location of injuries with regard to cyclists' gender were reported.[34,38–40]

Conclusions

Young men prevailed among cyclists in Poland. Gender-related dependencies can be observed in preferences for bike type, and cycling characteristics and pattern. For women, the most popular type of bike is a city bike, while men more often prefer mountain and touring bikes. The overwhelming majority of bike users admitted to not wearing protective helmets. In our study, we identified 5 risk factors for bike-related accidents. People with secondary education and incomplete higher education were at a higher risk of injuries as well as those who have at least 3 children. Frequency of using a bike (the more often, the more injuries), type of bike used (mountain bikes and fold-up bikes) and frequency of wearing helmets constituted the other group of factors correlated with a higher rate of injuries. The most common locations of injuries proved to be the upper and lower extremities.

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A non-inferiority study to analyze the safety of totally tubeless percutaneous nephrolithotomy

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

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1411–1416

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Funding sources
None declared

Conflict of interest
None declared

Received on January 18, 2017
Reviewed on March 28, 2017
Accepted on May 9, 2017

Abstract

Background. Totally tubeless percutaneous nephrolithotomy (ttPCNL) becomes increasingly frequently utilized in the treatment of kidney stones. This procedure emerged as an answer for patients' needs to minimize hospitalization time, pain intensity and discomfort due to nephrostomy tube. However, ttPCNL may be less safe for patients, as without nephrostomy tube bleeding from renal vessels is potentially more severe.

Objectives. The purpose of our study was to retrospectively evaluate the safety parameters of ttPCNL collected in a prospective manner.

Material and methods. This was a single tertiary care center, non-inferiority study with 2 arms (55 patients in each arm). The 1st group consisted of patients who underwent ttPCNL with the application of TachoSil® (Takeda, Osaka, Japan) as sealing material, while in the 2nd group, conventional PCNL with nephrostomy tube (cPCNL) was utilized. The primary goal was to prove that hemoglobin drop after surgery, as equivalent of safety, was not inferior than 1 g/dL. The secondary endpoints comprised visual analogue scale (VAS) of pain, additional pain treatment and hospital stay.

Results. The mean hemoglobin drop after ttPCNL was insignificantly lower in comparison with cPCNL group (mean: −0.35 g/dL; confidence interval (CI): −0.8, 0.21). Visual analogue scale of pain and pain treatment were comparable between groups. Hospital stay was significantly shorter in the ttPCNL group.

Conclusions. Totally tubeless PCNL can be considered a safe option after uncomplicated lithotripsy – what is important, it is characterized by a shorter hospitalization time. Postoperatively, pain intensity is comparable between both groups.

Key words: nephrolithiasis, lithotripsy, kidney stones, totally tubeless percutaneous nephrolithotomy

DOI
10.17219/acem/71196

Introduction

Percutaneous nephrolithotomy (PCNL) is considered a standard procedure in the treatment of kidney stones larger than 2 cm in diameter.[1] It is also recommended in the case of smaller stones in patients with contraindications for shockwave lithotripsy (SWL), such as shockwave resistant stones and anatomical malformations, or when a patient elects PCNL as a procedure of higher efficacy.

Classic PCNL consists of 4 major steps, including the insertion of ureteral catheter, percutaneous access to appropriate kidney, lithotripsy, and protection of controlled kidney injury with nephrostomy tube. However, many variants of classic approach have been introduced recently, including supine position PCNL, tubeless PCNL (tPCNL) and totally tubeless PCNL (ttPCNL). The 1st variant makes the procedure faster (as there is no need to rotate the patient on a table) and easier to evacuate all debris after lithotripsy. Tubeless PCNL and ttPCNL were proposed to minimize the inconvenience due to nephrostomy tube and ureteral catheter. However, there are certain contraindications for ttPCNL, including the presence of residual stones and the need for a second look, significant intraoperative bleeding, urine extravasation, ureteral obstruction, persistent bacteriuria due to infected stones, solitary kidney, bleeding diathesis, or planned percutaneous chemolitholysis.[1]

In order to assess the safety of ttPCNL in comparison with conventional PCNL with nephrostomy tube (cPCNL), we conducted a non-inferiority study. The aim of such a trial was to show that new treatment is not (much) worse than the standard treatment.

Material and methods

Compliance with ethical standards

The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

Inclusion and exclusion criteria

Retrospective analysis of patients' records, which have been collected prospectively, was conducted; it included the period between February 2014 and December 2016. A total of 110 patients were analyzed (Fig. 1). The inclusion criteria consisted of:

– patients with stone or stones over 2 cm in diameter in a kidney;

– patients with stones 1.5–2 cm in diameter who wished to have PCNL procedure instead of SWL or retrograde intrarenal surgery;

– patients with stones 1.5–2 cm in diameter with contraindications for SWL.

All patients had a contrast-enhanced computed tomography (CT) performed before planned surgery. Based on the CT results, a detailed preoperative analysis of anatomical characteristics was conducted and surgery was planned, including punctured calyx, number of accesses, inter/infra costal entry, and potential injury of adjacent organs.

Exclusion criteria consisted of:

– presence of residual stones after lithotripsy and the need for a second look;

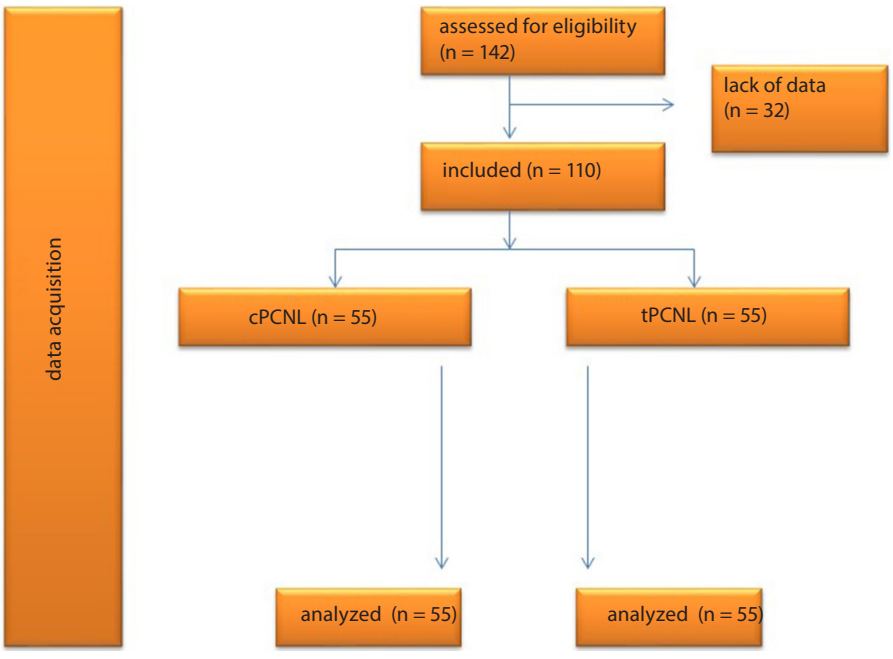

Fig. 1. Study design

Table 1. Demographic and clinical characteristics of the analyzed groups of patients

Parameter		cPCNL (n = 55)	ttPCNL (n = 55)	p-value
Age [years], median (IQR)		49.5 (45.2–62.8)	52.6 (42.4–58.3)	0.32
Sex, n (%)	female male	25 (45.4) 30 (54.5)	27 (49) 28 (50.9)	0.7
BMI [kg/m^2], mean (SD)		23.1 (4.3)	22.3 (4.5)	0.45

cPCNL – classic percutaneous nephrolithotomy; ttPCNL – totally tubeless percutaneous nephrolithotomy; BMI – body mass index; IQR – interquartile range; SD – standard deviation.

– massive bleeding requiring termination of the procedure;
– urine extravasation after completion of lithotripsy in fluoroscopy (renal pelvis rupture);
– persistent bacteriuria due to infected stones;
– ureteral obstruction;
– solitary kidney;
– contrast, paracetamolum and tramadol allergy;
– lack of any relevant data in our data system.

Endpoints and sample size estimation

Our primary endpoint was postoperative hemoglobin drop. The secondary endpoints comprised hospitalization time, degree of pain postoperatively and additional pain treatment. According to previous studies, an average hemoglobin drop after classic and tubeless PCNL is 0.97 g/dL and 0.82 g/dL (standard deviation (SD) = 1.54), respectively.[2,3] This data is taken from studies where PCNL is conducted in a different way than in the present study (e.g., TachoSil® and re-entry Malecot catheter (Coloplast, Humlebæk, Denmark) are not applied. In order to estimate the sample size for non-inferiority study, one must choose the largest difference ("delta") between means that are clinically acceptable. The upper limit of one-sided 97.5% confidence interval (CI) for non-inferiority test ("delta") was arbitrary set for 1 g/dL. The sample size was estimated to be 47 patients per arm. The total sample size required 94 patients to achieve 95% power with 0.025 type I error.[4–6]

Classic and tubeless procedure

Patients were admitted to the hospital the day before the procedure. Kidney stones were diagnosed based on contrast-enhanced CT, which was performed 1 month before the planned surgery. Basic serum and urine analyses were performed, as well as an X-ray of abdomen. On the day of the surgery, patients were given prophylactic antibiotics (1 dose of 2nd generation iv. cefalosporin). Patients were operated in general anesthesia in prone position. Access to pyelocalyceal system was conducted under fluoroscopy by urologist with Amplatz dilators (Cook Medical, Bloomington, USA). The access sheath had a diameter of 28 Fr (French catheter scale) and the nephroscope was 26 Fr. After lithotripsy, in cPCNL, re-entry Malecot nephrostomy was inserted into pyelocalyceal system and ureteral catheter was withdrawn.[7] In ttPCNL variant, rolled TachoSil® was inserted through access sheath and served as a sealing material.[8] After cPCNL, nephrostomy tube was withdrawn at day 5 after the operation and if there was no significant urine leakage, patients were discharged home. After ttPCNL, if there were no complications (such as pain, fever or significant hematocrit drop), patients were discharged home on day 2. Antinociceptive treatment included 1 g of iv. paracetamolum every 6 h on the day of surgery. In addition, 100 mg of tramadol was given intramuscularly on demand.

Statistical analysis

The study was designed as a non-inferior one to see whether the hemoglobin drop after ttPCNL (as equivalence of safety) is not higher in comparison with cPCNL.[9–11] To prove non-inferiority, it must be shown that the CI of the difference between means does not include predefined "delta". In other words, if there is truly no difference between the cPCNL and ttPCNL, then 94 patients are required to be 95% sure that the upper limit of a one-sided 97.5% CI (or equivalently a 95% 2-sided CI) will be below the non-inferiority limit of 1 g/dL. Usually, non-inferiority trials are conducted prospectively, but a recent study indicated that data collected retrospectively is equally valid.[12,13] Therefore, we decided to perform a non-inferiority study of data collected retrospectively, but in a prospective way. In addition, we decided that if non-inferiority had been proven, we would have conducted superiority analysis. As this was the case, superiority testing comprised Student's t-test for continuous variables with normal distribution. For continuous variables without normal distribution, a Mann-Whitney U test was used. For categorical variables, a χ^2 test was used. A p-value <0.05 was considered significant. All analyses were conducted using STATISTICA v. 13 (StatSoft Inc., Tulsa, USA).

Results

Demographic and clinical characteristics are given in Table 1. Patients did not differ significantly in terms of preoperative characteristics (Table 2). In the analysis, the difference between means of hemoglobin drop in 2 groups was −0.35 g/dL (CI: −0.8, 0.21). As the upper

Table 2. Preoperative characteristics of the analyzed groups of patients

Parameter		cPCNL (n = 55)	ttPCNL (n = 55)	p-value
Stone diameter [cm], mean (SD)		2.3 (0.8)	2.4 (0.7)	0.36
Side, n (%)	right left	24 (43.6) 31 (56.3)	29 (52.7) 26 (47.2)	0.34
Hemoglobin [g/dL], mean (SD)		14.4 (1.5)	14.7 (1.3)	0.9
Hematocrit [%], median (IQR)		42.1 (31.8–52.3)	42.2 (32.2–53.8)	0.76
Creatinine [umol/L], mean (SD)*		73.1 (16.2)	80.6 (15.7)	0.16
VAS [points], median (IQR)*		1.2 (0.7–1.6)	1.1 (0.7–1.5)	0.65

cPCNL – classic percutaneous nephrolithotomy; ttPCNL – totally tubeless percutaneous nephrolithotomy; SD – standard deviation; IQR – interquartile range; VAS – visual analogue scale; *assessed on the day of submission to the hospital.

Table 3. Postoperative characteristics of the analyzed groups of patients

Parameter		cPCNL group (n = 55)	ttPCNL group (n = 55)	p-value
OR time [min], mean (SD)		63.4 (23.3)	66.2 (24.8)	0.43
Hemoglobin [g/dL], mean (SD)*		12.9 (2.1)	13.6 (2.2)	0.51
Hematocrit [%], median (IQR)		35.6 (28.2–42.9)	37.1 (27.9–45.6)	0.31
Hemoglobin drop, mean (SD)		1.5 (1.2)	1.1 (0.9)	0.62
Hematocrit drop [%], median (IQR)		6.5 (4.3–7.9)	5.1 (4.5–8.2)	0.41
Creatinine [umol/L], mean (SD)*		77.7 (17.2)	84.7 (18.2)	0.24
Blood transfusion, n (%)	yes no	3 (6) 47 (94)	2 (4) 48 (96)	0.64
VAS [points], median (IQR)**		5.6 (3.1–6.2)	3.5 (3.4–6.8)	0.12
Tramadol [mg], mean (SD)***		312.3 (202.5)	277.4 (166.5)	0.07
Fever >38°C, n (%)	yes no	5 (10) 45 (90)	2 (4) 48 (96)	0.23
Hospital stay [days], median (IQR)		5.3 (3.2–7.3)	2.4 (1.1–3.8)	0.02
Supracostal access, n (%)	yes no	36 (72) 14 (28)	37 (74) 13 (26)	0.82
Number of accesses, n (%)	>1 1	9 (18) 41 (82)	7 (14) 43 (86)	0.58
Residual stones in renal pelvis, n (%)****	yes no	5 (10) 45 (90)	3 (6) 47 (94)	0.46
DJ insertion, n (%)*****	yes no	3 (6) 47 (94)	6 (12) 44 (88)	0.29

cPCNL – classic percutaneous nephrolithotomy; ttPCNL – totally tubeless percutaneous nephrolithotomy; OR – operating room; SD – standard deviation; IQR – interquartile range; VAS – visual analogue scale; DJ – double-J ureteral catheter; * blood sample collected 5 h after the procedure; ** obtained 1 day after the procedure; *** throughout hospital stay; **** abdominal X-ray the day after the procedure – stone debris <3 mm also classified as "yes"; ***** in ttPCNL – after the procedure, in cPCNL – after nephrostomy tube removal.

limit of CI (0.21) is lower than the predefined "delta" (1 g/dL), the non-inferiority of ttPCNL was proven.

In superiority analysis, there were no differences between groups in terms of safety (Table 3). However, hospitalization time was shorter in case of ttPCNL groups. The multivariate analysis of serum creatinine levels (Hotelling's test) did not show differences before and after the procedure between groups ($p < 0.37$). Other parameters, such as postoperative visual analogue scale (VAS) of pain and additional tramadol treatment, were comparable between groups.

In both groups, small debris was diagnosed in abdominal X-ray the day after the procedure, though fluoroscopy at the end of procedure revealed stone-free status. It is worth mentioning that all these residual stones were <4 mm in diameter.

Complications occurred only in a few patients. We observed arteriovenous fistula in 1 patient in the ttPCNL group. Pleural injury occurred in 1 patient in the cPCNL group. There were no colon injuries in our cohort.

Discussion

Conventional PCNL is the standard treatment for kidney stones over 2 cm in diameter. It is a highly effective treatment with a success rate over 90%. Because such a high efficacy was achieved, the next step for cPCNL was to limit

the trauma associated with procedure rather than further increase success rates. One of these attempts was to eliminate nephrostomy tube as it contributes to flank pain during hospitalization. Hence, other methods were sought to compensate for nephrostomy tube.[14] Among them are sealing materials, including human fibrinogen – TachoSil®.[15] After lithotripsy, it may be located within the tract in kidney parenchyma. It assures very good hemostasis and prevents urine leakage after a tubeless procedure. However, its safety was not established (so far) after each procedure; therefore, it may be utilized only without contraindications (above-mentioned). The 2nd step to decrease the pain after PCNL was to eliminate ureteral catheter.[16,17] The reason to utilize them was to prevent renal colic after the procedure as small debris or blood clots may fall into ureter. On the other hand, it also contributes to flank discomfort. As we have shown, 12% of patients may require double-J ureteral catheter (DJ) insertion due to renal colic after lithotripsy. It is highly probable that after lithotripsy, small debris or blood clots from kidney move to the ureter and cause flank pain. In our opinion, it is better to insert DJ catheter when it is really necessary than leave it in place after PCNL routinely, especially because after excluding patients with renal colic (n = 9), VAS was favorable for ttPCNL (Table 3). Six (12%) patients had renal colic the day after lithotripsy in comparison with only 3 (6%) patients in the cPCNL group; hence, the median of VAS was not favorable for the ttPCNL group. Colic in cPCNL group occurred due to nephrostomy obstruction with clots, which required irrigations or nephrostomy replacement. We have to emphasize that after both cPCNL (where re-entry Malecot nephrostomy is utilized) and ttPCNL we always remove ureteral catheter at the end of the procedure.

Many trials were publicized with an effort to prove safety of tPCNL and ttPCNL. Thanks to those pioneer authors, both of these procedures are today commonly conducted in many urological centers. However, the quality of evidence that both these new treatments are equally safe as cPCNL is still unsatisfactory. All those studies were superiority trials with the aim to prove that experimental treatment is superior to standard treatment.[18–20] In statistical language, the aim was to reject null hypothesis (experiment is not better than standard) and adopt an alternative one (experiment is better than standard). In the above-mentioned trials, statistical significance was not shown ($p > 0.05$); hence, the authors stated that ttPCNL are equally safe as cPCNL. Although this statement is true (which we proved in our trial), it is also based on the misconception of statistical results. It is worth mentioning that the phrase "experiment is not better than standard" is not the same as "experiment and standard are equal".[6] To prove that the new treatment is not (much) worse than the standard treatment, a non-inferiority trial has to be conducted. The major flaw of these trials is the need to adopt the smallest difference between interventions that is clinically significant. In our study, it was the difference between mean hemoglobin drop after 2 procedures. It is very difficult to say what difference would be clinically significant. Hemoglobin drop which leads to blood transfusion might be considered clinically significant. However, we perform transfusions when the hemoglobin concentration is lower than 8 g/dL. The average hemoglobin concentration after PCNL was 13.4 g/dL, so the clinically significant difference should be 5.4 g/dL. Such a huge difference would be unacceptable and it surely could not be stated that both treatments are equally safe. On the other hand, it is acceptable when the difference in hemoglobin drop after the 2 procedures is within the limit of 1 g/dL.

If non-inferiority was proven, it is reasonable to ask whether ttPCNL is safer than cPCNL, especially because direct difference is favorable for ttPCNL (hemoglobin drop 1.1 g/dL vs 1.5 g/dL). Such a small difference, even if superiority was proven, would bring nothing to the practice. However, hospitalization time was favorable for ttPCNL group.

Conclusions

1. We are at least 95% sure that in the whole population of patients after ttPCNL, the mean difference in hemoglobin drop in comparison with cPCNL is smaller than 1 g/dL. That makes this procedure equally safe as cPCNL.
2. We could not prove that ttPCNL has a favorable outcome in terms of intensity of pain postoperatively.
3. Occasionally, ureteral catheter insertion may be needed after ttPCNL. After excluding subjects with renal colic, patients from ttPCNL group experience lower pain postoperatively.
4. Totally tubeless PCNL allows shorter hospitalization time.

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Studies on selected molecular factors in endometrial cancers

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1417–1424

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Funding sources
None declared

Conflict of interest
None declared

Received on January 28, 2017
Reviewed on April 10, 2017
Accepted on May 2, 2017

Abstract

Background. Endometrial carcinomas (EC) differ in etiology, clinical course and prognosis.

Objectives. This multi-center study aimed at a closer recognition of molecular factors linked to heterogeneity of EC by evaluating estrogen and progesterone receptors, proteins dependent on MMR genes, proteins linked to poor prognosis and metastases, and mutations in *BRCA1*.

Material and methods. Using sections of paraffin-embedded preparations, in 115 patients with EC type I and 31 with EC type II, expression of ERα, ERβ1, PR, MLH1, and MSH2 proteins, as well as ARID1A, c-MET and BRCA1, was estimated by immunohistochemistry using specific antibodies.

Results. Expression of ERβ1 was augmented in EC type II, in poorly differentiated cancers and with growing clinical advancement. An augmented expression of ERα was noted in well-differentiated EC and at lower clinical stage. An increased expression of PR and decreased of MLH1 were detected in type I EC. The expression of ARID1A and c-MET proteins showed no differences between the types of EC, stages of clinical advancement or grading. In 51.6% patients with type II EC, a loss of BRCA1 expression was disclosed; in this group of cancers a decreased expression of ERα was noted.

Conclusions. An augmented expression of ERβ1 was linked to type II EC. A higher expression of ERα in EC cancers was associated with a lower histopathological grade. A decreased expression of MLH1 protein was estimated in EC type I. Type II EC may be connected to BRCA1 mutation.

Key words: endometrial cancer, BRCA1, estrogen receptors, MMR, ARID1A

DOI
10.17219/acem/70861

Introduction

According to global statistics, endometrial carcinoma (EC) is diagnosed in 4.8% of the female population. In 2012, EC developed in 319,605 women, of which 76,160 cases were fatal. In Europe since 2005 a stable increase has been noted in morbidity and mortality due to EC. In 2012 almost 100,000 women developed EC, which accounts for 6.2% of all morbidities due to malignant tumors in women.[1,2]

Multi-year observations indicate that ECs vary in etiology, clinical course and prognosis. Since Bokhman's hypothesis was 1st proposed, 2 types of EC have been distinguished.[3]

Type I is the most frequently diagnosed in around 80% women: mainly endometrioid, it is linked to an unbalanced estrogen stimulation and metabolic syndrome, manifests a slow course and good prognosis. It carries common receptors for estrogens (ER) and progesterone (P), while their expression depends on the degree of clinical advancement and histological grade. Most of the cancers are sporadic; around 3–5% are linked to mutations in the mismatch repair genes (*MMR*): *MLH1*, *MSH2*, *MSH6* and *PMS2*. In this type of EC, molecular tests demonstrated mutations in *PTEN*, *PIK3CA*, *K-RAS* and β-catenin, as well as microsatellite instability (MSI).[4–9]

Type II is a non-endometrioid cancer that manifests an aggressive biology, encompassing serous, clear-cell and poorly differentiated cancers. Frequent relapses cause an unfavorable course. It is a carrier of *TP53* (p53) and *HER2/neu* mutations. A proportion of the cancers was demonstrated to carry ER hormonal receptors, the effect of which on the clinical course remains controversial.[10–15]

Uterine serous carcinoma (USC) is thought to represent a unique type of EC, which should be treated as a distinct morbid unit.[16]

The results of studies covered by the Human Cancer Genetic Program (543 unselected female endometrial cancers) and focused on MSI demonstrated that, even though MSI was documented in 21.7% of the studied cases, genetic testing for *MMR* (*MLH1*, *MSH2*, *MSH6* and *PMS2*) detected Lynch syndrome in just 1.8% of cases.[17] Another study showed that MSI was more frequent in metastatic than in primary EC, that it appeared late in tumor development and that it might promote progression.[18] Brinton et al., when reporting the results of the Gynecologic Oncology Group (GOG 210 Trial), highlighted the similarity of endometrioid grading 3 (G3) cancers and type II cancers.[19] The authors supported the hypothesis suggesting heterogeneity of EC type II cancers.

As stated above, the mutation of *PIK3CA* is linked to EC type I, although alterations in *PIK3CA* are also present in USC.[20] Studies by Takeda et al. showed that mutations in the *ARID1A* suppressor gene induce an altered expression of many genes, including *MLH1* and genes linked to the *PI3K/AKT* signaling pathway, and are therefore associated with both type I and type II EC.[21] The *PI3K* signaling pathway also involves the *MET* proto-oncogene and its HGF ligand. Studies by Bishop et al. proved that the expression of c-MET occurred likewise in USC.[22]

In recent years USC has been found to be potentially associated with carriership of a mutated *BRCA1* gene. Bruchim et al. subjected women with histologically documented USC to genotyping of 3 main mutations, including *BRCA1* (185delAG and 5382insc) and *BRCA2* (6174delT).[23] They found that over 25% of women with USC carried mutations in *BRCA1/2*. Similar observations were made in English women in 2013; 68% of patients with USC had suffered from breast cancer before being diagnosed with USC. The authors also proposed that at least a subgroup of USC should be recognized as hereditary breast/ovarian cancer, which might carry prophylactic implications (prophylactic adnexectomy) and therapeutic implications (inhibitors of poly adenosine diphosphate ribose polymerase – PARP).[24] The heterogeneity of EC, particular of EC type II, requires further molecular studies.

This study aimed to estimate expression manifested by hormonal receptors (ERα, EBβ1, PR), expression of MSH2 and MLH1 proteins involved in the development of certain endometrial cancers belonging to the Lynch syndrome, expression of proteins linked to poor prognosis and metastases (ARID1A, c-MET), and expression of BRCA1 protein in type II EC, which might indicate that a proportion of the cancers are dependent on mutations in *BRCA1* and represents a proportion of breast/ovarian cancer syndrome.

Material and methods

The study had a multi-center, retrospective character. The research material included archival histopathological preparations of endometrial carcinoma, obtained from 162 patients diagnosed and treated due to EC in 2007–2014 in 8 specialized centers treating the diseases from the oncological gynecology branch. The study group consisted of patients treated primary with surgery. Due to insufficient clinical data in 16 patients, further analyses were conducted on 146 patients. The mean age of the patients was 65.1 years.

In the studied group, EC of endometrioid type was diagnosed in 115 patients (78.8%); 31 patients (21.2%) were diagnosed with EC of non-endometrioid type, including 18 patients with serous cancer (12.3%); 11 patients with clear-cell cancer (7.5%) and 2 patients with mucinous cancer (1.4%).

Seventy-four patients (50.7%) were diagnosed at an early stage of clinical advancement (38 patients manifesting grade IA and 36 patients manifesting grade IB; 26% and 24.7%, respectively). Thirty-seven patients (25.4%) were diagnosed at stage II, 22 patients (15%) at stage III and 13 patients (8.9%) at stage IV, according to International Federation of Gynecology and Obstetrics (FIGO) gynecologic cancer staging system. In 38 patients (25%) endometrial

carcinoma demonstrated a high grade of histological differentiation (G1), 59 patients (38.8%) had an intermediate grade (G2), while 55 patients (36.2%) carried a poorly differentiated tumor (G3) (Table 1).

Tissue material in the form of neoplastic endometrium was fixed in 10% buffered formalin, passed according to classical histopathological techniques and embedded in paraffin blocks. Following the evaluation of hematoxylin and eosin-stained (HE-stained) preparations and diagnosis, further studies were conducted on representative preparations. In order to demonstrate the presence of antigens in the tissue material, antibodies were employed specific for: ARID1A (Novus Biological, Littleton, USA NBP1-88932), ERα (Santa Cruz Biotechnology, Santa Cruz, USA sc-8005, clone D-12), ERβ1 (Zytomed Systems, Berlin, Germany MSK042-05, clone PPG5/10), Met (Santa Cruz Biotechnology, Santa Cruz, USA sc-10, clone C-12), MLH1 (Leica NCL-L, Buffalo Grove, USA NCL-L-MLH1, clone ES05), MSH2 (Invitrogen, Carlsbad, USA 33-7900, clone FE11), PgR (Dako, Santa Clara, USA M3569, clone 636), BRCA1 (Abcam, Cambridge, UK ab16780, clone MS110).

The preparations were incubated in a water bath at 96°C in a citrate buffer, pH 6.0, for 50 min. The activity of endogenous peroxidase was blocked using 3% H_2O_2. The preparations were incubated with the antibody at room temperature for 60 min, followed by 10 min rinsing in tris-buffered saline (TBS). The tissue material was incubated with the EnVision system (DakoCytomation, K5007; Dako, Santa Clara, USA) for 30 min. In all preparations, 3,3'-diaminobenzidine (DAB-3.3) was used to visualize the reaction. Subsequently, the preparations were counterstained with Mayer's hematoxylin, then passed through a row of alcohol to xylene and finally closed under a coverslip.

In immunohistochemical tests the negative control involved a reaction with omission of the primary antibody. Using an Olympus BX 43 light microscope and XC 30 digital camera (Olympus, Shinjuku, Tokyo, Japan), 10 photographs were taken of every stained preparation with the immunohistochemical reaction. The photographs were taken at total magnification of ×400.

In the evaluation of staining intensity reflecting expression of ARID 1A, ERα, ERβ1, Met, MLH1, MSH2 and PR proteins, a 4-degree scale was applied:

- 0 – absence of reaction;
- + – reaction obtained in 1–50 immunopositive cells (cell nuclei or cytoplasm);
- ++ – reaction obtained in 50–75 immunopositive cells;
- +++ – reaction obtained in 75–100 immunopositive cells, in every instance seen in 10 visual fields.

Staining intensity ++ and +++ were considered in further analyses as a positive protein expression.

The expression of BRCA1 protein was evaluated in cancer tissue in patients with non-endometrioid cancer. In the cases where the BRCA1/*MLH1*/*MSH2* expression were evaluated in the studied preparations, it was assumed that the presence of the color reaction indicated an absence of mutations in the *BRCA1*/*MLH1*/*MSH2* gene while the absence of a color reaction indicated the loss of protein expression, which may be the result of the *BRCA1* gene mutation and *MLH1*/*MSH2* mutations or hypermethylation of their promoter in the cancer tissue. The immunohistochemical reaction was detected in both cell nuclei and in the cytoplasm with the use of the ERβ1-specific antibody.

Table 1. Characteristics of patients included in the study

FIGO stage	Grading	Number of patients
Endometrioid adenocarcinoma (n = 115)		
IA	G1	14
	G2	15
	G3	4
IB	G1	9
	G2	15
	G3	8
II	G1	10
	G2	11
	G3	6
IIIA	G2	6
	G3	2
IIIB	G2	2
IIIC1	G1	2
	G2	2
IVA	G2	1
IVB	G1	1
	G2	4
	G3	3
Serous adenocarcinoma (n = 18)		
IA	G3	4
IB	G3	3
II	G3	7
IIIC1	G3	4
Clear cell adenocarcinoma (n = 11)		
IA	G3	1
IB	G3	1
II	G3	2
IIIA	G3	1
IIIC1	G3	3
IVA	G3	2
IVB	G3	1
Mucinous adenocarcinoma (n = 2)		
II	G1	1
IVB	G2	1

FIGO – International Federation of Gynecology and Obstetrics; G1 – endometrial carcinoma demonstrating a high grade of histological differentiation; G2 – endometrial carcinoma demonstrating an intermediate grade of histological differentiation; G3 – poorly differentiated endometrial carcinoma.

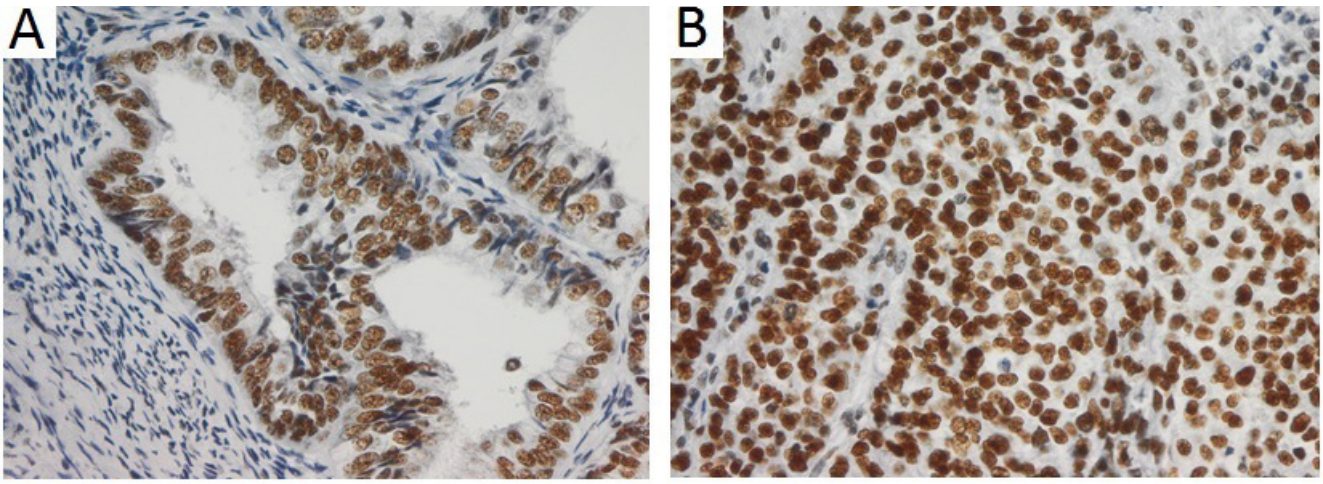

Fig. 1. Nuclear expression of ARID1A: A) in cell lining glands of highly mature adenocarcinoma (immunohistochemical reaction ++); B) in solid tissue (with pronounced reaction +++). Magnification ×400

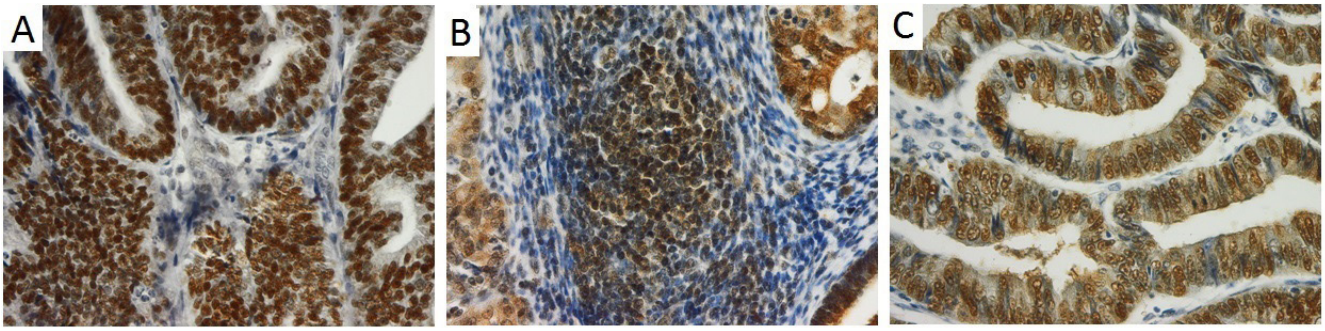

Fig. 2. Nuclear expression of: A) ERα receptor; B) ERβ1 receptor; C) PR receptor in endometrial adenocarcinoma. Magnification ×400

The c-MET protein manifested a cytoplasmic reaction while a nuclear reaction was shown by reactions detecting MLH1, MSH2, BRCA1, PR, ERα and ARID1A (Fig. 1–5).

Statistical calculations were performed with STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The Mann-Whitney, the Kruskal-Wallis and Spearman's tests were used. Statistical significance was set at $p < 0.05$.

Results

Histological type

Based on histopathological diagnosis, the patients were divided into 2 groups: patients with 1) endometrioid type and 2) non-endometrioid type of cancer. The latter group included patients with serous cancer, clear-cell cancer and mucinous cancer. In patients with endometrioid type cancer, a decreased expression of both MLH1 protein and ERβ1 was observed ($p = 0.013$ and $p = 0.035$, respectively) (Fig. 6). A reduced expression of PR receptor was detected (intensity of the reaction of 3–2 vs 1–0) among patients with non-endometrioid EC ($p = 0.041$). The reduced immunohistochemical expression of PR receptor was seen mainly in patients with serous cancer ($p = 0.022$), while intensified immunohistochemical reactions for MLH1 and ERβ1 involved clear-cell cancers ($p = 0.02184$ and $p = 0.00109$). Following the subdivision of non-endometrial cancers to individual subtypes, the subgroup of patients with clear-cell cancers manifested a reduced expression in the immunohistochemical reaction specific for ERα receptors as compared to the expression noted in endometrioid cancers ($p = 0.048$) and a higher expression of immunohistochemical reaction for ERβ1 as compared to expression noted in serous cancers ($p = 0.02658$). No differences were detected in the expression of immunohistochemical reactions specific for the remaining proteins in the studied groups.

Grading

An increase in the expression of ERβ1 receptor was found in parallel to the decrease in histopathological differentiation (G3 vs G1 $p = 0.003$) (Fig. 7). Patients with G1 or G2 endometrial cancer manifested a higher expression of ERα receptors than patients with a G3 cancer (G1 + G2 vs G3) ($p = 0.011$). No differences were detected in the expression of MLH1, MSH2, PR, c-MET and ARID1A dependent on the histopathological grading of the cells.

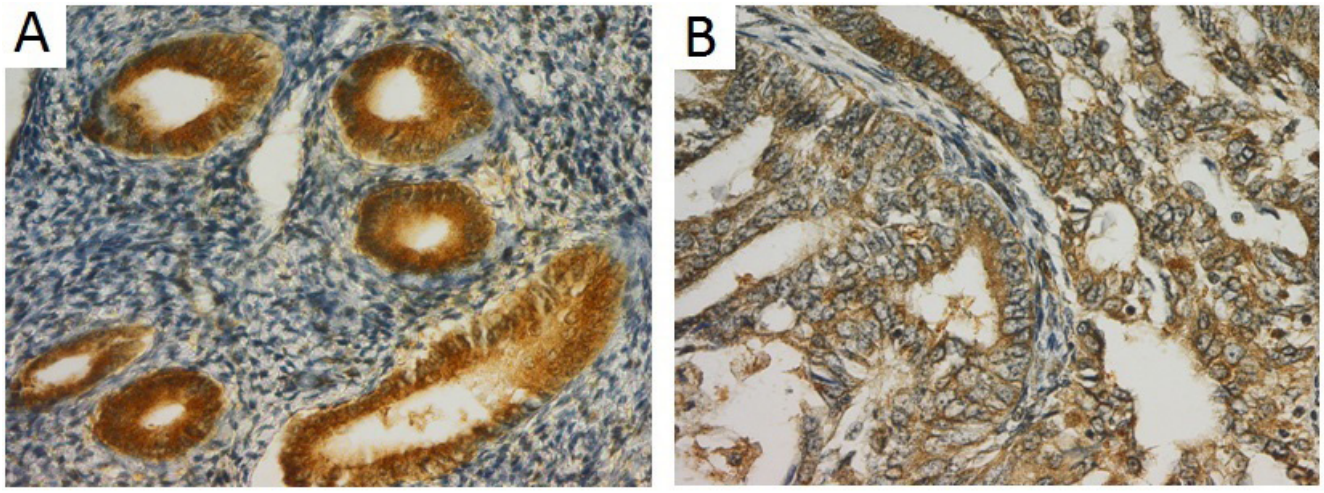

Fig. 3. A pronounced cytoplasmic reaction with c-MET-specific antibody in a section of endometrial adenocarcinoma (A and B). No such reaction in cell nuclei. Magnification ×400

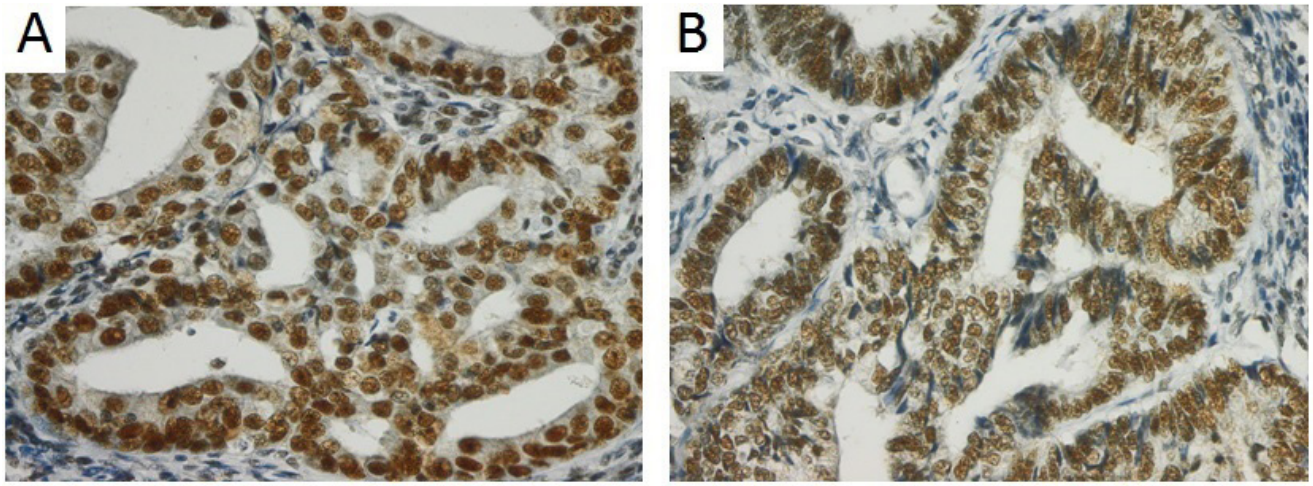

Fig. 4. A pronounced nuclear reaction with A) MLH1-specific antibody and B) MSH2-specific antibody in a section of endometrial adenocarcinoma. Magnification ×400

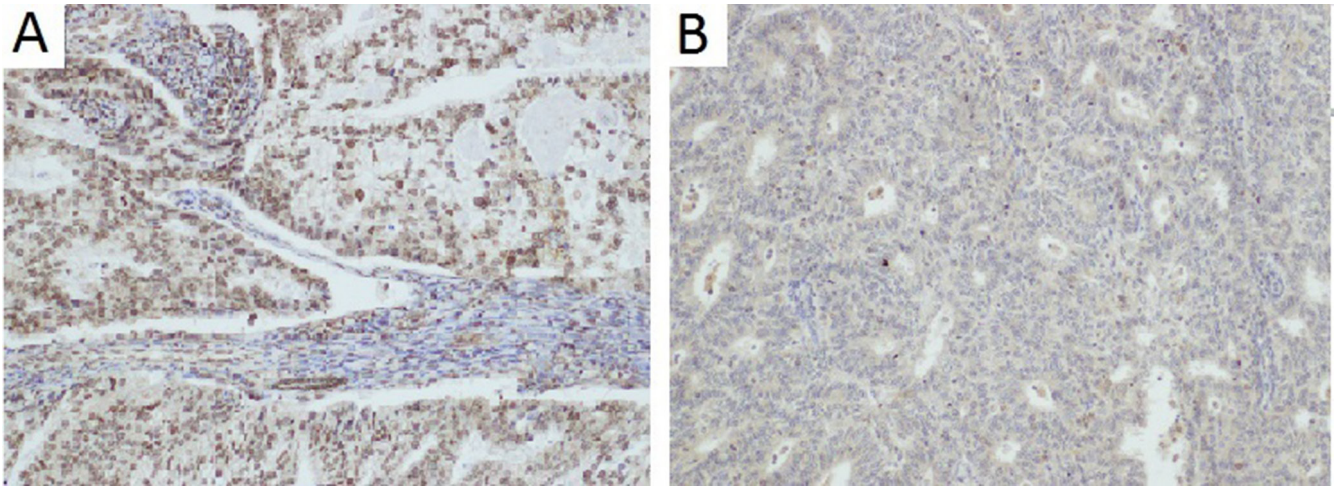

Fig. 5. Positive (A) and negative (B) nuclear reaction with BRCA1 antibody in a section of endometrial adenocarcinoma. Magnification ×10

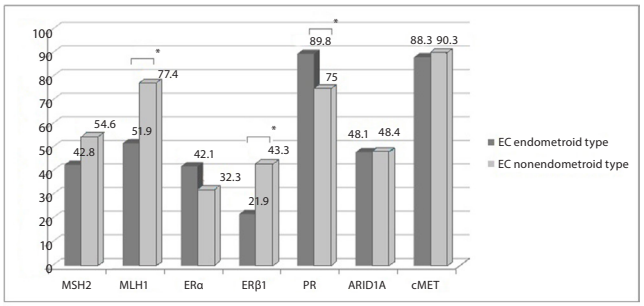

Fig. 6. The values reflect the proportions of endometrial cancer cases manifesting expression of a given protein in cells within the endometrioid and non-endometrioid types (immunohistochemical reaction of +++/++). * p < 0.05

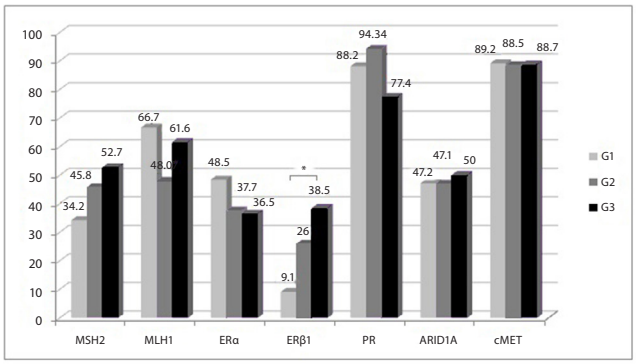

Fig. 7. The values reflect the proportion of endometrial cancer cases manifesting expression of a given protein in cells of a high, moderate or low histopathological grade (immunohistochemical reaction +++/++). * p < 0.05

International Federation of Gynecology and Obstetrics gynecologic cancer staging system

For the analysis, patients with endometrial carcinoma were subdivided depending on the stage of clinical advancement of the disease into early stage of advancement (IA) and the late stage (IB–IV). Women manifesting stage IA showed a statistically significant higher expression of the ERα receptor (p = 0.04). Patients with a more advanced disease manifested an augmented expression of the ERβ1 receptor (p = 0.02) (Fig. 8). No differences were detected in the expression of MLH1, MSH2, PR, c-MET and ARID1A, which would depend on the stage of clinical advancement manifested by the disease.

Mutation in BRCA1

Patients with non-endometrioid cancer were subjected to an evaluation for the expression of BRCA1 protein in cancer tissue. Among 31 patients with type II EC, 16 proved to have negative expression of this protein (Table 2).

Among the patients with non-endometrioid type of EC lack of BRCA1, expression was correlated with reduced expression of ERα receptor (p = 0.02). No other differences in the expression of MLH1, MSH2, PR, ERβ1, c-MET or ARID1A were detected among women with positive or negative BRCA1 staining (Fig. 9).

Discussion

Many endometrioid adenocarcinomas are thought to carry receptors for estrogens and progesterone. According to Reid-Nicholson et al., as many as 84% of endometrioid cancers (type I) manifest G1 and G2 maturity express ER receptors, as compared to ER expression in 9–54% of non-endometrioid (serous and clear-cell cancers).[6] The expression of such receptors manifests a correlation exclusively with histological grading, but not with the clinical stage of the disease.

Estrogen receptors may be present in 2 isoforms: ERα and ERβ, which exhibit distinct functions.[25] Estrogen receptor β is thought to function as a guardian of the endometrium; its disturbed expression has been described in most endometrial cancers.[26] In our patients, the examination of ERβ1 expression demonstrated an increase in non-endometrioid cancer – type II EC (mainly serous carcinoma and clear-cell carcinoma) (Fig. 6) as compared to type I EC: 43.3 vs 21.9, respectively. The expression of ERβ1 increased parallel with histological grading: it was least pronounced in G1 – 9.1, higher in G2 – 26.0 and highest in G3 – 38.5 (Fig. 7). It also increased with the clinical advancement of the cancer: FIGO IA 11.8 vs FIGO IB–IV 31.7. In studies by Chakravarty et al. a decrease was also noted in the expression of ERβ in endometrioid EC cancers, but no differences were detected in the expression as related to grade.[27] On the other hand, on the basis of our studies it may be accepted that an increased expression of ERβ1 in type II EC,

Table 2. BRCA1 protein expression status in studied patients with endometrial carcinoma type II

Histopathological type	FIGO stage	Number of patients with negative BRCA1 expression (n = 16)	Number of patients with positive BRCA1 expression
Serous adenocarcinoma	IA	n = 1	n = 3
	IB	n = 1	n = 2
	II	n = 2	n = 5
	IIIC1	n = 3	n = 1
Clear-cell adenocarcinoma	IA	n = 1	n = 0
	IB	n = 1	n = 0
	II	n = 2	n = 0
	IIIA	n = 1	n = 0
	IIIC1	n = 2	n = 1
	IVA	n = 2	n = 0
	IVB	n = 0	n =1
Mucinous adenocarcinoma	II	n = 0	n =1
	IVB	n = 0	n =1

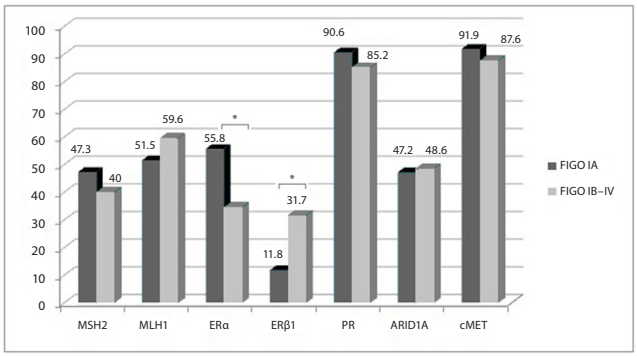

Fig. 8. The values reflect the proportion of endometrial cancer cases manifesting expression of a given protein in cells of early (IA) or more advanced degree (IB–IV) of clinical stage according to FIGO (immunohistochemical reaction +++/++). * $p < 0.05$

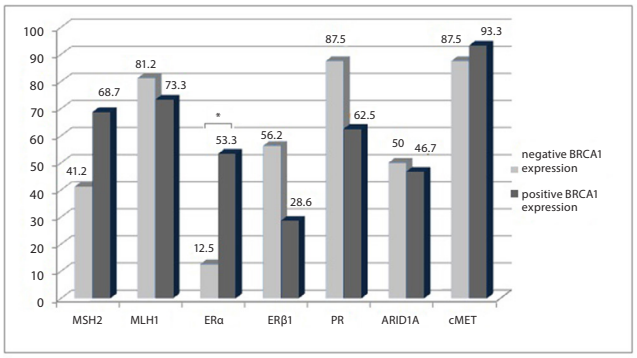

Fig. 9. The values reflect the proportion of endometrial cancer cases of non-endometrioid type manifesting expression of a given protein, as related to the expression of BRCA1 protein (immunohistochemical reaction +++/++). * $p < 0.05$

in the presence of poor differentiation (G3) and a higher stage of clinical advancement, was associated with poor prognosis.

Studies by Kreizman-Shefer et al. demonstrated that early endometrioid cancers preserve their expression of ER and PR, while poorly differentiated and clinically advanced cancers manifested an absence of 1 or both receptors.[12] Similarly, in our studies the expression of ERα was higher in well or moderately differentiated cancers than in G3 cancers (48.5 vs 36.5 – Fig. 7), and it was also significantly higher in IA cancers according to FIGO (Fig. 8, FIGO 1A 55.8 vs FIGO IB–IV 34.6). We could not detect ERα expression in non-endometrioid type II cancers, although Sho et al. described it in 21.2% cases of USC, and this was linked to a poor prognosis.[14]

In studies by Togami et al., ER and PR expression in USC was associated with a good prognosis.[13] In our studies the expression of the progesterone receptor PR was significantly more pronounced in type I endometrioid EC (89.8 in EV type I vs 75 in EC type II), which was consistent with the results of the study by Togami et al.[13] However, we failed to identify differences in PR expression which would depend on histological grading (G), as indicated in the studies by Reid-Nicholson et al. and those by Zhu et al.[6,28]

Since, as mentioned above, around 3–5% of EC are linked to mutations in DNA-repair genes (*MMR*), we estimated the expression of the 2 main relevant proteins, products of *MSH2* and *MLH1* genes, responsible for 85% cases of Lynch syndrome in all our patients.[7,9,29] We detected a lower expression of MLH1 protein in endometrioid cancer (type I 51.9 vs type II 77.4), which may indicate that the *MLH1* gene mutation occurred more frequently in cases of type I EC. Berends et al. noted the loss of MLH1 expression among women with EC connected to Lynch syndrome.[30]

No abnormalities in the expression of ARID1A protein were revealed in our study. Other studies detected a loss of ARID1A expression in around 30% of EEC cancers, in the progression of atypical hyperplasia to cancer as well as in the induction of many genes, including *MLH1*.[21,31]

Furthermore, the expression of the c-MET protein demonstrated no change in any of the parameters we examined, despite the evident association between c-MET and poor prognosis and metastasizing documented in other studies on EC.[32–35]

In 16 of our 31 patients (51.6%) with a diagnosis of EC type II, immunohistochemical tests demonstrated an absent expression of BRCA1 protein, indicating a mutation in *BRCA1*. Such incidence was much higher than described by Bruchim et al.[23] In studies by Raffi et al., 50.5% of USC patients were found to develop breast cancer (17.5% before and 33% following diagnosis of USC), which, according to the authors, suggested that the cases represented a proportion of *BRCA* mutation syndrome.[24] In our studies, the significantly reduced expression of the estrogen receptor α in this group of women might additionally indicate a relationship between some USC and *BRCA* mutation; it has been postulated that certain BRCA1 proteins inhibit ERα activity.[36]

Conclusions

An augmented expression of ERβ1 in EC was linked to type II EC. Higher expression of ERα in EC cancers was associated with a lower histopathological grade. A decreased expression of MLH1 protein was estimated in EC type I, which may indicate a mutation in *MLH1* gene in this type of cancer. Type II EC may be connected to *BRCA1* mutation.

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Evaluation of factors affecting the sense of coherence in women during pregnancy: A prospective pilot study

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

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1425–1430

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Funding sources
The project was funded by the Wroclaw Medical University allocated on the basis of the decision number ST-793.

Conflict of interest
None declared

Received on January 8, 2017
Reviewed on March 16, 2017
Accepted on May 15, 2017

Abstract

Background. Pregnancy is a special time in the life of a woman, which induces many changes not only in the biological, but also in the biopsychosocial dimension.

Objectives. The aim of the study was to evaluate the factors affecting the sense of coherence (SOC) among women during pregnancy. It was hypothesized that a high SOC will depend on a high level of received support, lack of the risk of mental disorders in the perinatal period and physiological course of pregnancy.

Material and methods. Factographic material was collected by a diagnostic survey method. As a 1st research tool, the Polish adaptation of the Sense of Coherence – Orientation to Life Questionnaire (SOC-29) was used. Another research tool was the Edinburgh Postnatal Depression Scale (EPDS), used here for pregnant women and therefore called Edinburgh Depression Scale (EDS).

Results. We analyzed the data of all 200 women with physiological pregnancies and 200 women with complicated pregnancies from whom a complete valid responses were obtained. All women were aged between 18 and 36 years. Significant predictors of low SOC results in the model were: age (odd ratio (OR) = 0.929, 95% confidence interval (CI): 0.870–0.992, p = 0.0280), being multipara (OR = 1.996, 95% CI: 1.271–3.135, p = 0.0027), having never/occasionally husband/partner support (OR = 1.978, 95% CI: 1.070–3.656, p = 0.0295), and EDS results (OR = 1.312, 95% CI: 1.169–1.472, p = 0.0000).

Conclusions. Predisposing factors for the occurrence of a low rate of SOC in pregnant women are: lower age, multiparity, lack of social support, especially from the husband/partner, and the risk of depression during pregnancy. This may result from the fact that a strong SOC develops in the process of socialization, and with age we acquire the ability to accurately assess reality.

Key words: depression, pregnancy, coherence, social support

DOI
10.17219/acem/73802

Introduction

The time directly following birth is not deprived of negative emotions, which can hinder the childcare. This period is usually short-lasting, but when it is prolonged, postpartum depression (PPD) cannot be excluded. Psychosocial factors, in addition to personality, determine the occurrence of this condition to a large extent. Among the 13 potential causes of PPD, Beck indicated low self-esteem, anxiety in the prenatal period and stressful life experiences.[1] Since the effects resulting from the symptoms of the disease can be both short- and long-term, their influence on maternal bonding with the child should be considered.[2]

A tool used to identify the risk of PPD is the Edinburgh Postnatal Depression Scale (EPDS). The authors of EPDS and the British Journal of Psychiatry own the copyright to this tool and they allow its use for scientific purposes.[3] This questionnaire is characterized by high sensitivity and specificity. Validation performed in the group of women outside the period of postpartum allowed us to demonstrate this tool to pregnant women as the Edinburgh Depression Scale (EDP).[4]

In the concept of salutogenesis, Antonowsky emphasizes the importance of global life orientation, known as a sense of coherence (SOC), which allows the individual to function in the bio-psycho-social dimension in an organized manner. Such an individual perceives reality as understandable, manageable and meaningful. These 3 components, according to Antonowsky, determine the optimal mobilization of defense mechanisms in an effort to avoid confrontation with the stressor. The 1st component, the sense of comprehensibility, determines the fact that the stimuli from internal and external environment are organized and can be logically explained. The 2nd component of the SOC, the sense of manageability, allows us to meet the demands which are conditioned by the stimuli. The 3rd component, the sense of meaningfulness, indicates that it is worth getting involved and act to meet the requirements. This ability is related to the essence of the concept of coherence – a SOC.[5–7]

Support is one of the most important elements of human life. During pregnancy the need of support increases to a large extent. Especially important is the social stability based on the emotional and instrumental support, which has significant meaning in the formation of the bond between mother and her unborn child.[8] Psychological comfort of a pregnant woman in the aspect of the development of this relationship is of irreplaceable importance. The lack of support from the husband/partner during the pregnancy as well as after the birth has a negative impact on the self-esteem in women.[9] Emotional support, especially in a situation of high stress levels, can counteract depression. It encourages taking pro-health actions and, therefore, it can eliminate harmful behavior not only in a biological, but also in a psycho-social dimension.[10]

Objectives

The aim of the study was to evaluate the factors affecting the SOC among women during pregnancy. It was hypothesized that a high SOC will depend on a high level of received support, the lack of risk of mental disorders in the perinatal period and physiological course of pregnancy.

Material and methods

Factographic material was collected by a diagnostic survey method. As a 1st research tool, the Polish adaptation of the Sense of Coherence – Orientation to Life Questionnaire (SOC-29) was used to assess the level of the SOC. The 2nd research tool was the EPDS in a version designated as the EDS. The 3rd tool was the personal questionnaire assessing the type and extent of social support received by pregnant women. Questionnaires were distributed to pregnant women during antenatal classes, in primary care units and at the Pathology of Pregnancy Ward of 1st Department and Clinic of Gynecology and Obstetrics at the Wroclaw Medical University, Poland. Women were advised that participation in the study was voluntary and anonymous. The study received a positive opinion of the Bioethics Committee of the Wroclaw Medical University, Poland (KB-655/2014).

The target group consisted of pregnant women. The inclusion criteria involved obtaining an informed consent to participate in the study, the duration of pregnancy equal to or above 30 weeks, physiological or complicated course of pregnancy and age under 40 years. The exclusion criteria included the lack of consent to participate in the study, life-threatening condition to the mother and/or child, gestational age below 30 weeks, age 40 years and above, and chronic illness diagnosed before pregnancy, with particular emphasis on mental disorders.

The SOC-29 questionnaire consists of 29 questions that relate to the 3 components of SOC: comprehensibility (11 questions), manageability (10 questions) and meaningfulness (8 questions). The respondent answers each question by marking the answer on a scale from 1 to 7, where 7 is the maximum intensity of the characteristics associated with 1 of the 3 dimensions of SOC. The results are calculated by summing the obtained points. The higher the score, the higher the SOC. A score over 156 points indicates a high SOC, a result below 156 points indicates a low SOC.

The EDS scale consists of 10 questions. Each woman chooses 1 of 4 possible answers corresponding to her feelings in the last 7 days. A score of 12 points and above (out of a max. 30 points possible) may suggest the probability of depressive disorders of varying severity.

The personal questionnaire also consisted of 10 questions. The questionnaire covered basic information about the participant of the study (the woman's age, duration

of pregnancy, type of pregnancy complications, and the type and extent of social support received by the pregnant woman).

Statistical analysis

All analyses were performed using STATISTICA v. 12.4 software (StatSoft Inc., Tulsa, USA). Results are presented as mean, median, minimum, and maximum results and standard deviation (SD) or percentages. Participants were divided into subgroups according to Sense of Coherence Scale (low SOC vs high SOC). Normality of distribution was assessed using the Shapiro-Wilk test. The χ^2 test or the Mann-Whitney U test were used (where appropriate) to compare subgroups. Multivariate forward logistic regression analysis using the stepwise method included factors potentially favoring the occurrence of a low SOC: age, pregnancy (physiological vs complicated), parity (primipara/multipara), husband/partner support (never/occasionally vs often/always), family support (never/occasionally vs often/always), medical staff support (never/occasionally vs often/always), and EDS (in points). Odds ratios (OR) are presented with 95% confidence intervals (CI). The fit of the regression model was assessed with the Hosmer-Lemeshow test. The results were considered significant with a p-value <0.05.

Results

Characteristics of the group

We analyzed the data of all 400 respondents (200 women with physiological pregnancy and 200 women with complicated pregnancy) from whom complete and valid responses were obtained. The characteristics of the participants are displayed in Table 1. It shows that 50% of the participants were primipara, 84.8% had often/always husband/partner support, 52% had often/always family support and 80.2% had often/always medical staff support. All women were aged between 18 and 36 years.

Results of the logistic regression are shown in Table 2. They showed that parity was associated with SOC (OR = 1.67 (1.12–2.49)), husband/partner support (OR = 2.56 (1.46–4.48)) and EDS (OR = 1.35 (1.21–1.51)).

Results of the multiple logistic regression of Sense of Coherence Scale are shown in Table 3. The goodness of fit statistics (Hosmer-Lemeshow test, p = 0.3661) indicated a satisfactory fit for the model. Significant predictors of low SOC results in the model were: age (OR = 0.929, 95% CI: 0.870 – 0.992, p = 0.0280), being multipara (OR = 1.996, 95% CI: 1.271–3.135, p = 0.0027), having never/occasionally husband/partner support (OR = 1.978, 95% CI: 1.070–3.656, p = 0.0295), and EDS results (OR = 1.312, 95% CI: 1.169–1.472, p = 0.0000).

Discussion

Pregnancy is a special time in the life of woman, which induces many changes not only in the biological, but also in the biopsychosocial dimension. Psychologists classify it as a critical life event. In the Social Readjustment Rating Scale (SRRS) created by Holmes and Rahe, pregnancy occurs between other stressors with 40 out of 100 possible points.[11]

Although studies indicate that up to 80% of women experience mood swings during pregnancy and after childbirth, only a few researchers undertook the problem.[12] The emotions experienced by a pregnant woman result from conscious or unconscious evaluation of a new life situation. The symptoms of depression are diagnosed in about 15% of pregnant women and 13% of mothers within the 1st 6 months after the birth.[13–17] The impact of the emotional state of a woman, not only on the fetus but also on the infant, is an important issue. Identification of emotional disorders during pregnancy is particularly relevant in terms of health and safety of the newborn.[18,19] McFarland et al. showed that clinically diagnosed depression during pregnancy affects the "maternal-fetal attachment".[20] The authors indicate that this condition causes the abnormal relations between the mother and child and may affect its development.[18–20]

The predictors of PPD are presented by many authors. In particular, they emphasize the occurrence of depression in a previous pregnancy, low self-esteem, stress during pregnancy, lack of social support, unplanned/unwanted pregnancy, and low socioeconomic status.[16,21–25]

Considering the fact that threatened pregnancy is a significant source of emotional strain, it is likely that the stress will be at a considerably higher level. Lewicka et al.,

Table 1. Characteristic of the group

Characteristic of the group (n = 400)		
Age [years]		mean = 27.4 median = 27.0 min–max = 18.0–36.0 SD = 3.5
Pregnancy	physiological	n = 200 (50%)
	complicated	n = 200 (50%)
Parity	primipara	n = 200 (50%)
	multipara	n = 200 (50%)
Husband/partner support	never/occasionally	n = 61 (15.3%)
	often/always	n = 339 (84.8%)
Family support	never/occasionally	n = 192 (48%)
	often/always	n = 208 (52%)
Medical staff support	never/occasionally	n = 79 (19.8%)
	often/always	n = 321 (80.2%)
Edinburgh Depression Scale [points]		mean = 6.5 median = 6.0 min–max = 4.0–14.0 SD = 2.1

SD – standard deviation.

evaluating the intensity of fear in a group of pregnant women hospitalized in the department of pathology of pregnancy, showed that it was at the secondary level. Only 0.98% of the women experienced a serious exacerbation of anxiety.[26]

In this study, a comparative analysis between 2 groups of pregnant women (physiological and complicated course of pregnancy, n = 200 vs n = 200) showed no statistically significant differences between the EDS results ($p > 0.4678$). In the study groups the quality of pregnancy is, therefore, not a risk exponent of depressive disorders in perinatal period. Both groups also showed no statistically significant difference in SOC ($p > 0.0546$). Nevertheless, the group of pregnant women with a low SOC achieved higher EDS score (OR = 1.312). Other studies demonstrated that the SOC is a strong predictor of well-being both in pregnancy and in the postnatal period, and that it can make a meaningful difference in the clinical, holistic women's health care.

The study also emphasizes the value of, subjective evaluation of pregnant women in terms of needed support, conducted by the midwife.[27] The deficit of social support, especially the lack of support from the husband/partner in studied group of women, was noticeably correlated with a low SOC. Two times more women who reported the lack of husband's/partner's support had a low SOC than women who reported obtained support (OR = 1.978); the result was statistically significant ($p < 0.0295$). Psychological comfort of the pregnant woman in the aspect of the development of partner bond is an undeniable fact. The lack of support from a partner during pregnancy and after childbirth has a negative impact on self-esteem in women.[9]

An important factor influencing the positive emotions in pregnant women is the support offered to the woman by the partner before the birth and a declaration of support after the birth, especially in the participation in childcare.[28] The lack of support, especially the partner support,

Table 2. Results of the logistic regression of Sense of Coherence Scale (SOC)

Variable		Sense of Coherence Scale (SOC)		p-value	OR (95% CI)
		low SOC (n = 171)	high SOC (n = 229)		
Age [years]		mean = 27.1 median = 27.0 min–max = 18.0–36.0 SD = 3.6	mean = 27.6 median = 27.0 min–max = 19.0–34.0 SD = 3.3	0.1285	0.96 (0.90–1.01)
Pregnancy	complicated	n = 84 (49.1%)	n = 116 (50.7%)	0.7617	0.94 (0.63–1.41)
	physiological	n = 87 (50.9%)	n = 113 (49.3%)		1.000
Parity	multipara	n = 98 (57.3%)	n = 102 (44.5%)	0.0118	1.67 (1.12–2.49)
	primipara	n = 73 (42.7%)	n = 127 (55.5%)		1.000
Husband/partner support	never/occasionally	n = 38 (22.2%)	n = 23 (10.0%)	0.0010	2.56 (1.46–4.48)
	often/always	n = 133 (77.8%)	n = 206 (90%)		1.000
Family support	never/occasionally	n = 82 (48%)	n = 110 (48%)	0.9871	0.99 (0.67–1.48)
	often/always	n = 89 (52%)	n = 119 (52%)		1.000
Medical staff support	never/occasionally	n = 34 (19.9%)	n = 45 (19.7%)	0.9539	1.02 (0.62–1.67)
	often/always	n = 137 (80.1%)	n = 184 (80.3%)		1.000
Edinburgh Depression Scale [points]		mean = 7.2 median = 7.0 min–max = 4.0–14.0 SD = 2.6	mean = 6.0 median = 6.0 min–max = 4.0–11.0 SD = 1.4	0.0000	1.35 (1.21–1.51)

SD – standard deviation; OR – odds ratio; CI – confidence interval.

Table 3. Results of the multiple logistic regression of Sense of Coherence Scale (SOC)

Sense of Coherence Scale (low SOC)							
Variables*		regression coefficients (B)	SE	OR	95% CI lower	95% CI upper	p-value
Age		–0.073	0.033	0.929	0.870	0.992	0.0280
Parity	multipara	0.691	0.230	1.996	1.271	3.135	0.0027
Husband/partner support	never/occasionally	0.682	0.313	1.978	1.070	3.656	0.0295
EDS		0.272	0.059	1.312	1.169	1.473	0.0000

OR – odds ratio, CI – confidence interval; SE – standard error; EDS – Edinburgh Depression Scale.
* Only significant variables are shown from the variables entered: age [years], pregnancy (physiological vs complicated), parity, husband/partner support (never/occasionally vs often/always), family support (never/occasionally vs often/always), medical staff support (never/occasionally vs often/always), Edinburgh Depression Scale (EDS) [points].

is of particular importance in the case of an unwanted pregnancy. In addition, these women may be exposed to psychosocial stressors, which may increase the level of depressive symptoms and also reduce general life satisfaction.[29–31] The relationship between the social support and the risk of PPD has been studied by many researchers. Morikawa et al. in cohort studies showed that postpartum women with a low level of social support had a significantly higher risk of depression than those who received support in line with their expectations.[32] These results suggest a protective effect of social support, particularly from the husband/partner, as a prevention of depressive disorders during pregnancy and the postpartum period. Thus, the need to identify mothers with a low level of support, as they are at increased risk of PPD, gains special importance. Understanding the relationship and the role of social support during pregnancy and after birth also becomes crucial.[33]

Taking into account the fact that the presented study showed the correlation of a low SOC with the risk of depression, the age of women was also considered in this group. We observed that the lower age of women conduces the lower result of SOC ($p < 0.0280$). This is confirmed by the results of other authors. Especially teenage mothers compared with older women are more prone to mood disorders, including depression during the postpartum period. This problem affects 26% of young mothers, which is 2 times more often compared to the result for the general population of 13%. It should be mentioned that possible emotional immaturity to motherhood can significantly affect the pregnant adolescent.[34,35]

The lack of support from the partner is not only expressed by his absence or lack of interest. Ludermir et al. proved that psychological abuse against a woman strongly correlates with PPD, both in cases of physical and sexual abuse.[36] They studied 1,045 women using the EPDS. The risk of depression was identified in 26% of the respondents, of which 28% reported being abused by their partner. Also, the study showed that women experiencing psychological violence were 2 times more often exposed to the occurrence of PPD. About 10% of all cases of PPD are related to women who have experienced mental abuse from their partners.[36]

In the group with a low SOC, we also analyzed the women's parity. Multiparous women 2 times more often obtained a low SOC score compared to the primiparous (OR = 1.996); the result was statistically significant ($p < 0.0027$). The researchers are not unanimous regarding the influence of parity on the SOC or depression. Particularly, the parity is not an unambiguous risk factor for depression, since both primiparity and multiparity are indicated as risk factors for this disorder.[37–40] Therefore, a correlation with different factors seems to have a higher predictive value. It should be assumed that the low rate of SOC may be an important predictor in a multifaceted analysis of the risk of emotional disorders in women, not only in the postpartum period, but also during the pregnancy.

Conclusions

Predisposing factors for the occurrence of a low rate of SOC in pregnant women are: lower age, multiparity, lack of social support (especially from the husband/partner) and the risk of depression during pregnancy. It may result from the fact that a strong SOC develops in the process of socialization, and with age we acquire the ability to accurately assess reality. Social support is one of the most important aspects of human life. As social beings, in order to achieve an optimal biopsychosocial development, which is the paradigm of health, we need the help of others, especially loved ones.

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Immunopathogenesis of ophthalmological paraneoplastic syndromes: Recent findings

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

Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1431–1439

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Funding sources
Pbmn-168 (grant for Young Researchers funded by Wroclaw Medical University), Poland

Conflict of interest
None declared

Received on November 24, 2016
Reviewed on April 8, 2017
Accepted on May 18, 2017

Abstract

The aim of this study was to summarize the current knowledge of paraneoplastic syndromes involving eyes. The main interest was the immunopathogenesis of the abovementioned entities. A web search was conducted using Medline, Web of Science and Scopus engines. Key words concerning ocular paraneoplastic syndromes (OPS) such as: "ocular paraneoplastic syndrome", "cancer-associated retinopathy", "cancer-associated cone dysfunction", "melanoma-associated retinopathy", "bilateral diffuse uveal melanocytic proliferation", and "paraneoplastic optic neuritis" were combined with "immunology", "immune response", "antibodies", or "autoantibodies". Numerous papers were found as a result of "ocular paraneoplastic syndrome" search and many of them matched the chosen criteria. We focused on the most recent papers – published in the last 5 years – and eventually, 92 items were found. Only several papers from each detailed category fulfilled both OPS and immunologic criteria. Site-specific paraneoplastic syndromes still remain a difficult clinical challenge. The immunopathogenesis of some of them seems to be more complex than previously thought.

Key words: ophthalmologic findings, optic neuropathy, immunopathogenesis, paraneoplastic syndrome, paraneoplastic retinopathy

DOI
10.17219/acem/73860

Introduction

The incidence of paraneoplastic syndromes is estimated at 7–15% in patients suffering from malignant neoplasms, varying in different papers.[1] However, the frequency of the eye site-specific manifestation is unknown. This disorder is caused by chemical substances (produced by neoplasm) that have an impact on distant tissues and organs, not simply by mass effect or metastatic infiltration. Sawyer et al. in 1976 were the first to describe the visual acuity loss caused by retinal degeneration connected with systemic cancer. Since that report, many patients have been described suffering from ocular paraneoplastic syndromes (OPS). The usual presented classification of OPS distinguishes paraneoplastic retinopathies, paraneoplastic optic neuropathy and paraneoplastic tonic pupils. Other authors divide OPS into those with visual loss and with abnormal eye movements, incorporating the last mentioned group of diseases as only ophthalmological.

The tumor during its neoplastic transformation can develop some antigens similar to those naturally occurring in different organs. Human immune system tries to protect the organism via humoral or cellular response against the tumor. This immunologic response plays an important role or is the main cause for the paraneoplastic syndromes occurring in patients suffering from neoplasms. Moreover, in organs and systems where the specific blood-tissue barriers can be found, the neoplastic transformation can lead to expressing proteins similar to those that can be found in restricted sites (Fig. 1). The described situation promotes an aggressive response of the patient's own immune system towards healthy tissues. In previous papers, the autoantibody against eye-specific proteins as well as rare infiltrates of T cells, plasma cells and macrophages in both retinal and cancer tissues were described.

The aim of this paper is to summarize the available knowledge about immunopathogenesis of OPS. The resources were searched using Medline, Web of Science, Scopus, PubMed, and worldwide websites. Searched terms included: "ocular paraneoplastic syndrome", "cancer-associated retinopathy", "cancer-associated cone dysfunction", "melanoma-associated retinopathy", "bilateral diffuse uveal melanocytic proliferation", and "paraneoplastic optic neuritis", combined with "immunology", "immune response", "T cell", "B cell", "innate lymphoid cell", "antibodies", and "autoantibodies".

Numerous papers were found for "ocular paraneoplastic syndrome" – 299 items. We focused on the most recent papers, published within the last 5 years – in this case 92 items were found. Only a few papers from each category fitted both OPS and immunologic criteria.

Cancer-associated retinopathy

There is a theory that cancer-associated retinopathy (CAR) is primarily caused by *p53* tumor suppressor gene mutation in tumor cells, which express highly immunogenic recoverin protein. In response, the patient's immune system produces a high level of anti-recoverin

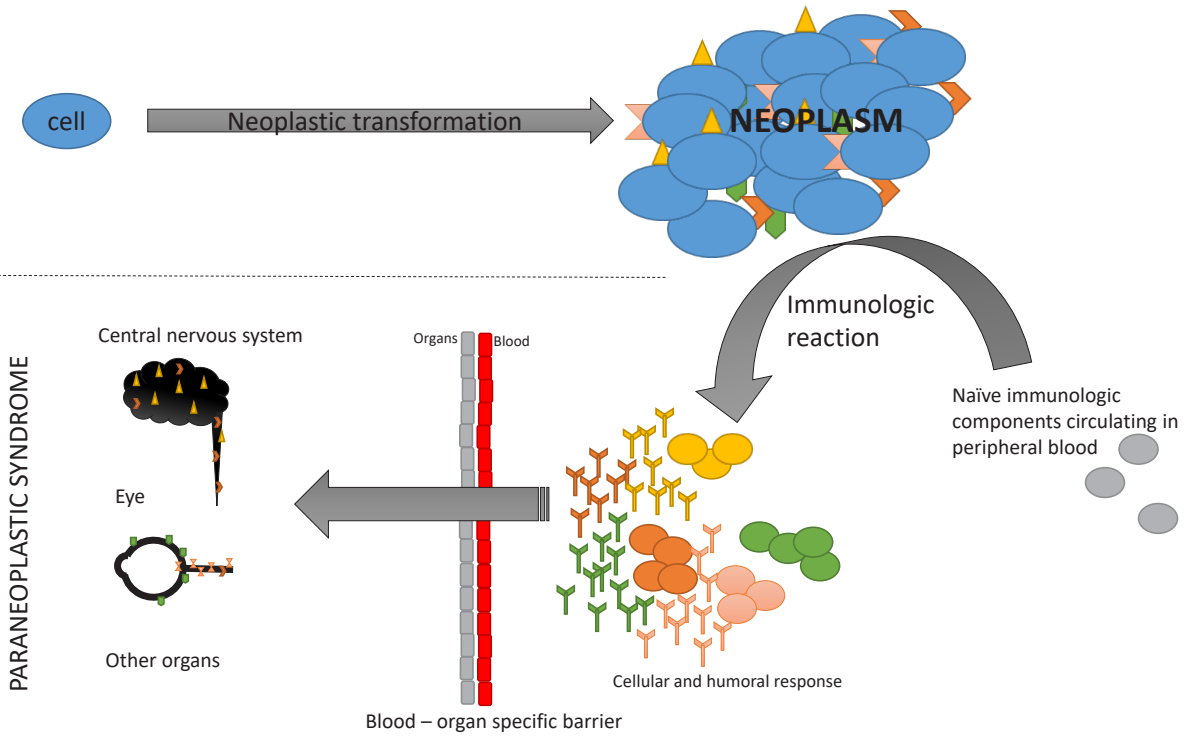

Fig. 1. Development of site-specific paraneoplastic syndrome. The importance of immunologic reactions in its pathogenesis

IgG. It penetrates through the blood-retinal barrier and by binding to recoverin molecules on the photoreceptors cells, activates caspase-dependent apoptotic pathways. Immunological reaction is conducted via cytotoxic T-lymphocytes antigen 4 (*CTLA-4*) and vascular endothelial growth factor (*VEGF*) receptor-1. In such cases, the recoverin-specific T cells including CD8+ lymphocytes were found in the peripheral blood.[1] All the abovementioned changes lead to photoreceptor cell death. Rods and cones are equally affected; as a consequence, flattening of the alpha wave in electroretinogram (ERG) is observed.[2,3]

After the first description of recoverin, many other autoantigens that generate a clinical presentation similar to recoverin were discovered.[1,2,4] According to some authors, different molecules transmitted via different pathways cause slightly different clinical symptoms. However, nowadays CAR is thought to be a group of clinically similar paraneoplastic retinopathies with a different molecular background that leads to photoreceptor death.[1] Other antigens associated with CAR are: retinal enolase, Tubby-like protein 1 (*TULP1*), aryl hydrocarbon receptor interacting protein-like 1 (*AIPL1*), interphotoreceptor retinoid binding protein (IRBP), photoreceptor cell-specific nuclear receptor (PNR), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), aldolase, transducing-α, as well as heat shock cognate protein 70 (hsc-70) and 60 (hsc-60).[5–9] Additionally, some authors indicate other possible antigens that can be associated with CAR, including: guanylyl cyclase-activating proteins (*GCAP*s), heat shock protein 27 (HSP27), Rab6A GTPase (*Rab6A*), carbonic anhydrase II (CA II), anti-CV2/collapsin response mediator protein (CRMP5), and anti-Hu antibodies.[7,10–13] Furthermore, Adamus et al. showed that CA II unique epitopes can be used to differentiate autoimmune retinopathies (AR) from CAR. Autoimmune retinopathies sera predominantly reacted with the N-terminal epitope 85–90 and CAR sera have been found to be reactive with the peptide 218–222 clustered within the α-helix.[10] Turaka et al. described a case of CAR with positive antiretinal autoantibodies against 48-kDa (arrestin), 64-kDa and 94-kDa, probably in the course of ovarian mass, in 14-year-old girl of African origin.[3]

Common clinical manifestations of the first ophthalmological symptoms include both cone dysfunction (photosensitivity, prolonged glare, decreased best-corrected visual acuity – BCVA, color discrimination, and central scotomas) and rode dysfunction (night blindness, prolonged adaptation to darkness and peripheral or ring scotomas). Other clinical symptoms have been described as arteriolar narrowing, retinal pigment epithelial thinning and motting, vitritis, cells in anterior chamber, sheathing of retinal arterioles, periphlebitis, and pallor of the optic disc. In ERG rods- and cones-mediated responses are not recordable or significantly decreased.

Neoplasms causing CAR can be as follows: small-cell carcinoma of the lung, other neoplasm of the lung, breast cancer, osteosarcoma, cancers of the cervix, ovary, uterus and thymus, Warthin tumor of parotid gland, as well as carcinoma of unknown origin.[1–3,5–7,11,12] There are single reports of patients with prostate, pancreatic neurocrine and small intestine tumors.[14,15] In the past CAR has been reported in bladder and laryngeal neoplasms as well as in lymphomas (systemic follicular cell lymphoma) or colon adenoma – only as case reports or case series.[4,8]

Cancer-associated cone dysfunction

Dysfunction of the cone system alone without any symptoms or electrophysiological signs of rods system failure has rarely been reported.[16] Clinical symptoms include: mild to moderate BCVA loss, sudden photosensitivity, and total or subtotal loss of color perception. In addition, patients usually notice improvement of the visual acuity while wearing sunglasses. The ERG shows the suppression of cones response.[17,18]

Cancer-associated cone-specific dystrophy involves an abnormal amount of immunologic activity with 2 retinal antigens that are possibly isoforms of the same 40-kD photoreceptor protein.[19] This protein may be expressed only in cones, and perhaps more in L and M than S cones.[17] The circulating antibodies in cancer-associated cone dysfunction (CACD) include the CAR antigen/recoverin and protein whose molecular weight is 50 and 40 kDa.[19]

Finger et al. described serum derived 42-kDa retinal antigen in a patient suffering from CACD and paraneoplastic optic neuropathy (PON).[16]

Paraneoplastic retinopathies also include the anti-enolase retinopathy that characteristically occurs together with cone dysfunction. In enolase-associated autoimmune retinopathy, visual impairment and the course of the disease may vary from relative stability to slow progression with loss of central vision.[19]

Reported cases of the abovedescribed paraneoplastic syndrome were associated with small-cell endometrial cancer, primary cervical intraepithelial neoplasia and laryngeal carcinoma.[18,19]

Melanoma-associated retinopathy

Patients suffering from melanoma can develop paraneoplastic retinopathy caused by pure dysfunction of rods without any cone involvement. Clinical symptoms may include: sudden shimmering, flickering, difficulty with the night vision, and photopsias (pulsating, continuous or intermittent) with occasional hyperphotosensitivity and floaters. Symptoms usually are observed in patients already treated with melanoma and the development of melanoma-associated retinopathy (MAR) is considered

to be a bad prognosis marker. Lesions are usually bilateral with the time-shift between the eyes from weeks to months. Typically unaffected, BCVA, visual field and color vision can, however, be deteriorated in rare cases (e.g., generalized depression in flicker perimetry and red-green axis or blue-yellow axis demonstrated with Farnsworth-Munsell 100 Hue Color Vision Test). In the fundus examination, of the disc pallor, non-specific retinal pigment changes or the attenuated retinal vessels can be found. The ERG findings are similar to those associated with congenital stationary night blindness: markedly reduced or absent dark-adapted b-wave with normal a-wave ("a negative-appearing waveform"), preserved or slightly reduced amplitude of light-adapted response, as well as prolonged implicit time and amplitude reduction of oscillatory potentials. It has been proven that some changes can be restricted only to the magnocellular pathway.[20]

Recently, MAR-like retinopathies have been described.[21–23] Aronow et al. in 2012 described paraneoplastic vitelliform retinopathy (PVtR).[23] In reported cases of PVtR, the average age of patients was 56 years at the moment of onset, equally distributed between males and females. The majority had metastatic cutaneous melanoma. Paraneoplastic vitelliform retinopathy is characterized by subjective visual loss with no BCVA reduction, nyctalopia and photopsias. Funduscopic findings vary among reporting authors from bilateral serous macular detachments, "vitelliformpseudohypopyon macular appearance" to multiple yellow and white lesions scattered throughout the posterior pole and mid-periphery.[22] Aronow et al. described nummular, flat and well-demarcated lesions that clinically appear to be located within the deep retinal layers.[23] However, ERG recordings showed reduction in the b-wave amplitude, which implies that there is selective destruction of bipolar cells. In the case presented by Aronow et al., ERG revealed a mild reduction in both the a-wave and the b-wave amplitudes for both scotopic and photopic waveforms – it shows involvement of both rods and cones. Electrooculography (EOG) demonstrated a pathologically reduced Arden ratio (reflecting retinal pigment epithelium (RPE) dysfunction) only in 40% of the patients that have been examined (5 cases evaluated with EOG).[23] Lincoff et al. described an exudative polymorphous vitelliform retinopathy case of 65-year-old male suffering from metastatic melanoma with little subjective ocular-related complaints.[21] This patient has been treated successfully with ipilimumab, an anti-cytotoxic T lymphocyte-associated antigen 4 antibody. Metastatic lesions, as well as the exudative macular lesions, improved with the administered treatment within 1 year of follow-up.[21]

In the sera of patients suffering from MAR and MAR-like retinopathies, specific autoantibodies against human rod bipolar cells can be found.[24] Wang et al. presented postmortem paraneoplastic retinopathy as a cross-reaction of cutaneous metastatic melanoma autoantibodies.[22,24] Authors reported local retinal thinning with the damaged inner nuclear layer and outer plexiform layer. Those changes resulted in focal retinal degeneration, edema and atrophy. There were no signs of infiltration of immune-specific or melanoma cells. Immunohistochemical reactions showed evidence of melanoma-associated autoantibodies directed against transient receptor potential M1 channels that target the ON-bipolar cell structures in the inner nuclear and outer plexiform layers in PVtR.[22] Several cases of melanoma-associated retinopathy have been reported with serum autoantibodies to transient receptor potential cation channel, subfamily M, member 1 (*TRPM1*) labeled on ON-bipolar cells.[20,25,26]

Other autoantibodies similar to those associated with CAR can be found in MAR and MAR-like retinopathies, such as: autoantibodies directed against α-enolase, recoverin or hsc-70; autoantibodies specific for bipolar cells and non-specific for bipolar cells; carbonic anhydrase II (CA II), interphotoreceptor retinoid binding protein (IRBP), bestrophin, myelin basic protein, and rod outer segment proteins have been reported.[23]

Munk et al. described the case of a reversible nyctalopia in a 70-year-old patient with lung adenocarcinoma diagnosed 8 months previously, who has been enrolled in a phase I trial with erlotinib hydrochloride combined with AUY922. The extensive loss of the ellipsoid zone as well as external limiting membrane was shown in spectral domain optical coherence tomography (SD-OCT) with the intact central zone (4 regular layers). The ERG findings included extinguished responses to blue scotopic light and significantly decreased responses in different light conditions. The anti-α-enolase was found in the serum. Seventeen months after the cessation of AUY922 treatment the symptoms significantly improved, but there were still detectable irregularities within the ellipsoid zone and external limiting membrane in SD-OCT.[27]

Audemard et al. described a treatment option in the patients not responding to classical surgery and chemotherapy with metastatic melanoma presenting MAR.[24] The ipilimumab has been reported previously to improve survival in previously treated patients with metastatic melanoma and with MAR-like PVtR.[21] On the other hand, ipilimumab has not been recommended in the patients suffering from MAR at this same time due to the ability of the drug to increase autoimmunity. In the presented case of 70-year-old woman, the ipilimumab treatment enabled to free patient from the metastases (2-year observation) but exacerbated her vitiligo as well as MAR.[24]

Serous retinopathy-associated mitogen-activated protein kinase inhibition (binimetinib) for metastatic cutaneous and uveal melanoma has been reported recently by van Dijk et al.[28] It is a time-dependent, reversible retinopathy with mild and transient visual symptoms. Treatment with binimetinib may be a cause of the described serous retinopathy; however, authors consider autoantibodies role in the pathogenesis of this entity.

Table 1. Ocular paraneoplastic syndromes – summary

Paraneoplastic syndrome	Underlying neoplasm	Circulating antibodies in peripheral blood	Clinical findings	Histopathological findings	Genetic findings	Source
CAR	small-cell carcinoma of the lung, other neoplasm of the lung, breast cancer, cancers of the cervix, ovary, uterus and thymus, osteosarcoma, Warthin tumor of parotid gland, prostate, pancreatic neuroendocrine, small bowel, bladder and laryngeal neoplasms, lymphomas (systemic follicular cell lymphoma) and colon adenomas	Recoverin (23-kDa), retinal enolase (46-kDa), TULP1, hsc-70 and 60, AIPL1, IRBP, PNR, GAPDH (36-kDa), aldolase C (40-kDa), transducin-α possibly: GCAPs, HSP27 and Rab6A, CA II (30-kDa), CRMP5 and anti-Hu, antiretinal autoantibodies against 48-kDa (arrestin) and 64-kDa and 94-kDa	cone dysfunction: photosensitivity, prolonged glare, decreased BCVA, color discrimination and central scotomas; rode dysfunction: night blindness, prolonged adaptation to darkness and peripheral or ring scotomas arteriolar narrowing, retinal pigment epithelial thinning and motting, vitritis, cells in anterior chamber, sheathing of retinal arterioles, periphlebitis and pallor of the optic disc	relatively small cells with little cytoplasm intensively positive for recoverin as well as positive for *NSE*, *Ki-67* and *tp53*	no additional data available apart from data that confirms the circulating antigens involvement	Weixler, Oertli and Nebiker 2016; Dalin et al. 2016; S. Yang et al. 2016; Adamus, Yang and Weleber 2016; Bhavsar et al. 2015; Adamus 2015; M. Morita et al. 2014; Turaka et al. 2014; Machida et al. 2014; Adamus, Choi, et al. 2013; M. Saito et al. 2014; Adamus, Bonnah, et al. 2013; Makiyama et al. 2013; W. Saito et al. 2013
CACD	small-cell endometrial cancer, primary cervical intraepithelial neoplasia, occult small cell lung carcinoma and laryngeal carcinoma	recoverin and protein whose molecular weight is 50 and 40 kDa	mild to moderate best-corrected visual acuity loss, sudden photosensitivity, total or subtotal loss of color perception, visual acuity improvement while wearing sunglasses	no data	no data	Finger, Thirkill and Borruat 2012; Javaid et al. 2015; Parc et al. 2006; Hargitai et al. 2004
MAR and MAR-like retinopathies	cutaneous melanoma	TRPM1, α-enolase, recoverin or hsc-70, CA II, IRBP, Bestrophin, myelin basic protein, mitofilin, titin, and rod outer segment proteins	sudden shimmering, flickering, difficulty with night vision and photopsias (pulsating continuous or intermittent) with occasional hyperphotosensitivity and floaters	local retinal thinning with the damaged inner nuclear layer and outer plexiform layer positive melanoma-associated autoantibodies directly against transient receptor potential M1 channels that target the ON-bipolar cell structures in the inner nuclear and outer plexiform layers in PVtR	no additional data available apart from data that confirms the circulating antigens involvement	Lincoff et al. 2016; Wang et al. 2012; Dalal et al. 2013; Xiong et al. 2013; Y. Morita et al. 2014; Dhingra et al. 2011; Aronow et al. 2012

CAR – cancer-associated retinopathy; CACD – cancer-associated cone dysfunction; MAR – melanoma-associated retinopathy; BDUMP – bilateral diffuse uveal melanocytic proliferation; BCVA – best-corrected visual acuity; RPE – retinal pigment epithelium; PON – paraneoplastic optic neuritis; PVtR – paraneoplastic vitelliform retinopathy; *TULP1* – Tubby-like protein 1; hsc-70 – heat shock cognate protein 70; *TRPM1* – transient receptor potential cation channel; subfamily M member 1 (that is labeled on ON-bipolar cells); CA II – carbonic anhydrase II; IRBP – interphotoreceptor retinoid binding protein; CMEP factor – cultured melanocyte elongation and proliferation factor; CRMP5 – collapsin response mediator protein 5; MBP – myelin binding protein; ANNA-1 – type 1 antineuronal nuclear antibody; *GCAPs* – guanylyl cyclase-activating proteins; HSP27 – heat shock protein 27; *Rab6A* – Rab6A GTPase; PNR – photoreceptor cell-specific nuclear receptor; *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase; *AIPL1* – aryl hydrocarbon receptor interacting protein-like 1.

Table 1. Ocular paraneoplastic syndromes – summary (cont.)

Paraneoplastic syndrome	Underlying neoplasm	Circulating antibodies in peripheral blood	Clinical findings	Histopathological findings	Genetic findings	Source
BDUMP	ovarian, cervix, uterus, colon and rectum cancer, gallbladder cancer, neoplasm of the retroperitoneal space, and a variety of lung cancers	CMEP factor, AAbs against 35-kDa, 46-kDa, 30-kDa, 50-kDa, and 70-kDa proteins	classical findings: slow, painless, bilateral (usually asymmetric), progressive loss of vision for several months, subretinal infiltration and exudative retinal detachment that result as outer retinal damage development of cataract, iridocyclitis or glaucoma additional clinical findings: iris nodules, pigmented keratic precipitates, anterior chamber and vitreous cells, peripheral retinal arterial areas of non-perfusion, loss of RPE in a pattern described as nummular or dermal, conjunctival melanocytic proliferation	infiltration consisted of more benign-appearing melanocytes – stained positively with melan-A	deletions in chromosome 19, gain in chromosomes 5, 6, 8q and X lack of mutations in *GNAQ*, *GNA11* and $BRAF^{V600F}$	Mittal et al. 2015; Mudhar et al. 2012; Rahimy, Coffee and McCannel 2015; Lin and Mruthyunjaya 2012; Navajas et al. 2011; Yonekawa, Shildkrot and Eliott 2013; Pulido et al. 2013; Jansen et al. 2015; Adamus et al. 2013; Miles et al. 2012
PON	adenocarcinoma and small cell carcinoma of the lung, prostate carcinoma, stomach carcinoid tumor, colon adenocarcinoma, cutaneous melanoma, occult pancreatic non-secretory neuroendocrine tumor, thymoma	CRMP5, aquaporin 4, MBP, ANNA-1, recoverin, enolase	neuroretinitis and positive autoantigens against neuronal part of retina and the optic nerve	no histological data available in recent papers due to tissue sampling consequences concerning visual function	no additional data available apart from data that confirms the circulating antigens involvement	Verschuur, Kooi and Troost 2015; M. Saito et al. 2014; Adamus et al. 2011; Finger, Thirkill and Borruat 2012; Carboni et al. 2012; Al-Harbi et al. 2014; Chao et al. 2013; Slamovits et al. 2013; Schoenberger, Kim and Lavin 2012; H. K. Yang et al. 2014; Iyer et al. 2014

CAR – cancer-associated retinopathy; CACD – cancer-associated cone dysfunction; MAR – melanoma-associated retinopathy; BDUMP – bilateral diffuse uveal melanocytic proliferation; BCVA – best-corrected visual acuity; RPE – retinal pigment epithelium; PON – paraneoplastic optic neuritis; PVtR – paraneoplastic vitelliform retinopathy; *TULP1* – Tubby-like protein 1; hsc-70 – heat shock cognate protein 70; *TRPM1* – transient receptor potential cation channel; subfamily M member 1 (that is labeled on ON-bipolar cells); CA II – carbonic anhydrase II; IRBP – interphotoreceptor retinoid binding protein; CMEP factor – cultured melanocyte elongation and proliferation factor; CRMP5 – collapsin response mediator protein 5; MBP – myelin binding protein; ANNA-1 – type 1 antineuronal nuclear antibody; *GCAPs* – guanylyl cyclase-activating proteins; HSP27 – heat shock protein 27; *Rab6A* – Rab6A GTPase; PNR – photoreceptor cell-specific nuclear receptor; *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase; *AIPL1* – aryl hydrocarbon receptor interacting protein-like 1.

Bilateral diffuse uveal melanocytic proliferation

In the first reports describing diffuse uveal melanocytic proliferation (noted in the 1960s by Machemer and named in the 1980s by Barr et al.), the findings were thought to be a result of infiltration of the choroid by malignant ocular melanoma or choroidal metastases from cutaneous malignant melanoma. The later histopathologic findings revealed that the infiltration is composed by more benign-appearing melanocytes. The cellular infiltration, not the degeneration as in previously described conditions (CAR, MAR and cancer-associated cone dysfunction), is responsible for symptoms and findings that characterize the bilateral diffuse uveal melanocytic proliferation (BDUMP).[29]

The pathogenesis of the BDUMP is unknown due to its rare presentation and there is no consensus of whether it is a neoplastic, hyperplastic or paraneoplastic process. Three main mechanisms have been proposed: 1. unknown carcinogenic factor that simultaneously inducts primary neoplasm and melanocytic proliferation development; 2. BDUMP is secondary to the secreted by cancer (primary cause) hormone or factor; 3. genetic predisposition that causes coincidental development of the BDUMP and extraocular neoplasm. Interestingly, the melanocytic proliferation can be induced ex vivo in cultured melanocytes by adding the IgG fraction of human serum (by some authors named as cultured melanocyte elongation and proliferation factor – CMEP factor) from patients with BDUMP and the proliferation of melanocytes usually reveals regression after total excision of primary neoplasm.[30] In a clinical diagnosis of BDUMP, the elongation and selective proliferation of cultured human melanocytes, if cultured with patients serum, has been confirmed.[30,31] Positive visual as well as ophthalmoscopic response to plasmapheresis have been described.[31–34] Recently, Jansen et al. demonstrated a relation between the level of serum CEMP factor (during treatment with plasmapheresis in conjunction with radiation and chemotherapy) and the proliferation of cultured human melanocytes.[31] Adamus et al. described 2 patients presented with BDUMP in the course of gynecological cancers that had autoantibodies against 35-kDa, 46-kDa, 30-kDa, 50-kDa and 70-kDa proteins in serum.[12] In addition, the lack of the mutation in genes *GNAQ*, *GNA11* and $BRAF^{V600F}$ supports the thesis of BDUMP being a part of the neoplastic process and probably different oncogenic pathway to uveal nevi and the majority of uveal melanoma.[29,35,36]

Patients suffering from BDUMP experience slow, painless, bilateral (usually asymmetric), progressive loss of vision for several months. The melanocytic proliferation leads to subretinal infiltration and exudative retinal detachment as a result of outer retinal damage. Visual acuity can also deteriorate due to the development of cataract, iridocyclitis or glaucoma.[29] Gass et al. described the main features of the BDUMP: multiple round or oval red spots of patches at the level of retinal pigment epithelium (found in the posterior pole), multifocal hyperfluorescence during fluorescein angiography corresponding to the lesions, diffuse thickening of the uveal tract with multiple elevated pigmented and non-pigmented uveal melanocytic tumors, exudative retinal detachment (a late finding), and rapidly progressive cataract formation.[37] In recent years, the increasing number of described cases adds to our knowledge of the BDUMP. Additional clinical findings are: iris nodules, pigmented keratic precipitates, anterior chamber and vitreous cells, peripheral retinal arterial nonperfusion, loss of RPE in a pattern described as nummular or dermal, and conjunctival melanocytic proliferation.[34,38–40] Electrophysiological findings are non-specific to the syndrome and may involve reduction of amplitude of scotopic or photopic response. The systemic findings (apart those associated to the neoplasm itself) usually do not occur; however, in rare cases there have been described hyperpigmented lesions of the oral mucosa, penis and skin.

The pathologic spectrum of the BDUMP varies between patients. The infiltrating cells can be small and spindle-shaped as well as more rounded or even epithelioid with homogenous dark-staining pattern. The described cells that infiltrate RPE, stain with S-100 protein and they contain pigment granules of various density, with rare mitotic figures. In rare cases, the cells can develop more malignant-appearing epithelioid cells that suggest the malignant melanocytic transformation. Often the melanophages are seen in the area of necrosis and the photoreceptors with retinal pigment epithelium show signs of degeneration.

The BDUMP is associated with a number of carcinomas: cervix, uterus, clear-cell carcinoma of the endometrium, colon and rectum cancer, gallbladder cancer, malignancy of the retroperitoneal space, as well as a variety of lung cancers.[29,37,41–43] The described ocular paraneoplastic syndrome precedes identification of the malignancy in 1/2 or 2/3 of cases (even by a few years). In patients with diagnosed malignancy, the BDUMP usually develops within 1 year.

Paraneoplastic optic neuropathy

Paraneoplastic retinopathy including CAR and PON are autoimmune diseases in which the host response to tumor antigens triggers cross-reactions to an overlapping epitope in the retina and/or the optic nerve. Depending on whether the syndrome is targeting the retina, optic nerve or both, the pathogenesis of these syndromes is poorly elucidated and may be characterized by progressive loss of visual acuity and/or visual field sensitivity, loss photoreceptors and/or retinal ganglion cells (RGCs) and their axons, and optic nerve head changes, such as pallor, hyperemia, edema, swelling, or dropout of the retinal nerve fiber layer (RNFL) by optic coherence tomography (OCT) imaging.

Patients diagnosed with paraneoplastic or non-paraneoplastic retinopathy often present with optic nerve problems not related to glaucoma. The perceived pathology

of autoimmune optic neuropathy and glaucoma is different, although both might have a similar underlying autoimmune cause.

Paraneoplastic optic neuropathy – called by some authors "optic neuritis associated with neoplasm" – can be characterized by funduscopic features of neuroretinitis and positive autoantigens to neuronal part of retina and optic nerve.[13] As for autoantigens causing PON, collapsin response mediator protein (CRMP5) has been most frequently reported.[44] Patients with anti-CRMP5 antibody-positive PON generally develop funduscopic features of neuroretinitis.[44] The pathogenesis of neuroretinitis is regarded as vasculitis at the optic disc.[45]

Autoimmunity might significantly influence the outcome of optic nerve degenerative process but the pathogenic process is not fully elucidated. For example, autoantibodies (AAbs) can exert their pathogenic effects if they gain access from the periphery to the central nervous system (CNS) or the eye when the integrity of the blood barriers is compromised.

Taking into account a large variety of possibly involved cells, such as RGCs, Muller cells, bipolar cells, amacrine cells, and, on the other hand optic nerve head as a part of neuronal system, this results in large variety of autoantigens.

The major optic nerve antigenic targets for patient's auto-antibodies can be divided into 4 categories as follows: (1) classical glycolytic enzymes involved in energy production, including α and γ enolases, glyceraldehyde 3-phosphate dehydrogenase, which also reacted with retinal antigens; (2) neuronal-specific myelin proteins (myelin basic protein – MBP, myelin oligodendrocyte glycoprotein – MOG) or type 1 antineuronal nuclear antibody (ANNA-1)[46]; (3) collapsin response mediator protein 5 (CRMP5)[47]; and (4) aquaporin-4 (AQP4).[46,48,49]

Also, other antibodies labeled as optic nerve components, such as axons, astrocytes and oligodendrocytes are present, but their role in the pathogenicity of the optic neuropathy needs to be evaluated further. The identity of cellular targets is important in better understanding the etiology of autoimmune retinopathy associated with optic neuropathies, either with or without retinopathy, and for developing better treatments.

Paraneoplastic optic neuropathy is associated with number of carcinomas such as adenocarcinoma and small cell of the lung, prostate carcinoma, stomach carcinoid tumor, colon adenocarcinoma, cutaneous melanoma, occult pancreatic non-secretory neuroendocrine tumor, and thymoma.[13,16,44,46,50–55]

Conclusions

Ophthalmological neoplastic syndromes can be associated with a wide range of neoplasms. Large variety of clinical findings as well as different autoantibody targets in each condition makes the differential diagnosis more difficult. The recent advancements in this field should be widely used to improve the patient-centered health care. However, basing only on the retinal autoantibody results without proper history-taking procedure and clinical evaluation is inappropriate.

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Elastic dental prostheses – alternative solutions for patients using acrylic prostheses: Literature review

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1441–1445

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Funding sources
None declared

Conflict of interest
None declared

Received on January 26, 2017
Reviewed on March 9, 2017
Accepted on March 30, 2017

Abstract

Elastic dentures are prostheses made of thermoplastic material. This category includes: nylons, acetals, acrylic polymers, and acrons. Elastic prostheses have been recognized for several years; however, their properties are constantly being modified. In the opinion of the majority of patients, elastic prostheses are comfortable, handy and long-lasting. Elastic dentures are a good choice for esthetic reasons. They may be recommended for patients who do not accept clasps in framework dentures, but cannot afford dentures supported with precise elements or implant-based fixed appliances. Such dentures can be applied in masticatory organ rehabilitation in patients with increased absolute reflexes, especially retching. Furthermore, such features like size, construction weight or material plasticity and smoothness are considered to be advantages of thermoplastic materials. Elastic dentures are the only removable appliances for patients allergic to metal or acrylic. They are better tolerated by patients with an uneasy prosthetic base or with systemic diseases, e.g., diabetes, who are more susceptible to the traumatic activity of the hard plate of traditional dentures made of acrylic material. Adaptation time is shortened and the number of necessary corrections is reduced. Hygiene rules as well as follow-up visits terms must be strictly obeyed.

Key words: thermoplastic materials, elastic dental prostheses, antiallergic dentures

DOI
10.17219/acem/70044

Elastic dentures are prostheses made of thermoplastic material.[1] This category includes: nylons, acetals, acrylic polymers, and acrons. Elastic prostheses have been recognized for several years and their properties are constantly being modified. In the opinion of the majority of patients, elastic prostheses are comfortable, handy, esthetic, and long-lasting.[2] This type of appliance is an alternative method in the rehabilitation of partially edentulous patients allergic to metals such as chrome, cobalt or nickel.[3,4] They allow constructing elements supported on teeth with periodontal diseases and designing the plate in the case of inclined teeth requiring a deep approach. Elastic dentures are also less burdensome for patients with a gagging sensation or a retching problem.[3] They are alternative solutions in the case of both small and more extensive gaps in patients with contraindications for acrylic dentures or in those who are allergic to acrylic.[3–7] The application of thermoplastic materials in dentistry is very widespread. Elastic materials in dental prosthetics should have some very specific mechanical properties.[2]

Nylons are used mainly in partial dentures. Nylon is a synthetic polyamide resulting from the polycondensation of adipic acid and hexamethylenediamine. It is formed of fibers which can be divided into aliphatic, cycloaliphatic and aromatic ones.[1,8,9] It undergoes condensation polymerization, and methacrylates undergo additional polymerization.[10–13] Nylon is characteristic for its high elasticity, inflexibility, hardness, durability as well as mechanical strength. In comparison to metals, its mechanical strength and hardness are smaller. However, shape stability and fatigue strength as well as sliding properties, optimum abrasion resistance, strong suppression of vibrations, high impact resistance, good chemical resistance, low thermal expansion, good optic properties, coloration option, and the possibility to produce transparent objects are the main advantages of nylon.

In nylons, some organic solvents may provoke strain micro-cracks and the material has some confined compatibility when combined with acetals. It is relatively easy to treat mechanically (cutting, punching, milling).[2] Dura-Flex (Myerson LLC, Chicago, USA) is one of the most popular elastic materials. It is a highly esthetic polyamide which gives the possibility to form the maximally thin construction at adequately preserved stability.[14–16] Elastic materials used in prosthetic appliances are constantly being improved.

More advanced materials are characteristic for definitely smaller water absorbency, considerable resistance to color change, and bigger and stable stiffness, which enables the production of increasingly thinner appliances. They are also less translucent. Quite recently, Flex Star nylon has been introduced (Nobilium CMP Industries LLC, New York, USA), replacing the older generation nylon Valplast N1 10 (Valplast International Corp., New York, USA).[14] However, Valplast and Flex Star have one common feature: their elasticity is enhanced at the increased temperature of the oral cavity, which distinguishes them among other nylon materials. This quality largely influences their use, denture construction design, the thickness of elements and plate, appliance range, and storage method (moisty environment is recommended).[14,15]

Acetals are used in supported dentures, elastic clasps and construction elements of other prosthetic devices. Acetal is a crystalline structure polymer with reduced residual monomer content and is a product of formaldehyde polymerization.[10] It exhibits high resistance to wear, mechanical strength, perfect flexibility and elasticity, low thermal conductivity as well as adequate stiffness of the construction cores, and it can be sterilized at a temperature of 120°C.[10–13,17] The material is available in many colors and shades; it does not form air bubbles even during the fabrication of very tiny elements.[3] It is resistant to water, weak and strong alkalis, organic solvents such as paraffin hydrocarbons, alcohols, ethers, ketones, fuels, diesel oil, and fats. It has limited compatibility with nylon, high fatigue resistance, environment humidity resistance, size and shape stability at high temperatures, low friction coefficient in typical material combinations, high bacterial and fungal resistance, good sliding properties as well as very high rebound resilience.[2] Acetals are divided into homo- and copolymers. Dental D (3M ESPE AG, Seefeld, Germany) is one of homopolymers which are products of worse physical and chemical properties in comparison with copolymers, e.g., DurAcetal (Myerson LLC, Chicago, USA).[14] DurAcetal is used in the production of a variety of appliances, such as overdentures and snap-on overlays. In order to produce them, injection or scanning technologies are incorporated. Snap-on type overlays prove to be an expensive appliance used in the region of the maxilla and the mandible as well for pterygoid deficits in the mandible. They may be in the form of gaps, so they do not change the occlusal relations between the jaws.[14–16] Acetal is also used in the production of stabilizing splints and space retainers as well as in the fabrication of protector pads for sportsmen.[3,5]

Acrylic polymers are a good material for complete dentures, crowns and temporary bridges. Polyvinyl resin (vinyl polymer) is a multi-particle substance, a mixture of vinyl acetate copolymers, which is elicited by vinyl monomers polymerization. Acrylic polymers are processed with the use of the injection method or hot stamping. Materials can be either translucent or in many colors. They are characteristic for their very low water absorption and the stability of color.[10,12,18]

Acrons are used in the production of complete or partial dentures. Acron is a thermoplastic material combining acrylic advantages (high luster) and polyamide excellence (fracture resistance and elasticity). Its melting temperature of 270°C assures that it is non-yielding in the oral cavity.[19]

All the above-mentioned materials are characteristic for their thick, non-porous internal structure, which prevents saliva penetration and the development of microorganisms, as well as for the manufacture technique based on plasticized material injection, and final extreme elasticity.[1,2,8,9,20]

A typical elastic denture is fabricated like an acrylic denture in respect to the initial clinical and laboratory stages. It is very important to qualify patients correctly for the appropriate method of denture fabrication. Acetal or nylon dentures cannot be applied in edentulous patients in whom adhesion force is mainly responsible for the denture support.[10,11,18] There is no adhesion force between the bedding elastic dentures. In order to stabilize these dentures, thickening of the filling construction is necessary. In acetal dentures, modeled clasps should be thicker than in a traditional denture. Short and thick clasps have stronger fixation, whereas long and thin ones are more flexible. In turn, long clasps are recommended in very deep flanges, where traditional metal clasps are impracticable to use. In a traditional supported denture, construction adequate durability is attained at the plate thickness of 0.3–0.5 mm. In turn, acetal or nylon denture optimum thickness amounts to 1.5 mm.[1,9] Also, the fact that after years the structure of dentures remains resilient is considered an advantage of elastic materials.[4] Elastic nylon dentures are also settling dentures. The nylon clasp arm contacts linearly with the tooth crown at the site of its most prominent bulge. With time, periodontal structures as well as abutment teeth are overpressed. In the case of elastic appliances construction, teeth flanges as well as alveolar process flanges can be either blocked or used in denture retention increase.[10,13,18] Conventional teeth-supported dentures are always shifted from the periodontium, whereas in the case of elastic ones it does not always happen. Sometimes, retention areas are incorporated to the denture fixation. Denture plate elasticity makes the appliance more prone to strain. Mastication forces do not strike denture balance, as there is no leverage force between the appliance sides. Mechanical damage like fractures or cracks is very rare.

However, the most important advantage of elastic appliances is no release of harmful monomer. In acrylic-based conventional appliances, mistakes that occur at the polymerization stage result in residual monomer release (1–2%).[1,6,8,9,21] Elastic materials are chemically inert and they do not provoke the formation of galvanic couples as it happens in the case of appliances based on metal construction. They do not provoke electro-metalloses, either. Apart from many other advantages, elastic materials are very esthetic. The colors of clasps made of these materials are similar to those of oral cavity tissues.

Thermoplastic materials, however, are not free from defects. Microbiological surveys show that *Candida albicans* and *Staphylococcus aureus* strains exhibit bigger affinity to nylon than to acrylic. The cases of slight inflammatory reactions in the areas of contact with mucous membrane have been reported. Besides, thermoplastic materials are more brittle due to a smaller tendency to deformations.[1,4,10,17,20] Some disadvantages of thermoplastic materials may be reduced by the introduction of glass fibers.[22] Glass fibers increase the strength of elastic materials, improve their bending strength and resistance to congelation. The content of >50% of fibers in the mass base significantly influences the above-mentioned characteristics.[22] The presence of <10% of fibers in the denture composition does not change the physical properties. However, besides glass fibers content, their diameter is very important as well. Glass fibers elasticity modulus is close to this of the dentine. Acrylic polymers dentures reinforced with glass fibers prove stronger than prostheses devoid of them.[22] Sometimes, unfavorable esthetic effects occur: nylon pellots visually shorten dental crowns and acetal clasps unnaturally thicken teeth.

The patient's correct classification and qualification for the particular type of appliance is very significant, and among many criteria there are: adequate prosthetic basis (it cannot be loose), dental gaps arrangement (partial gaps are mostly favorable).[14–16] The treatment should always be preceded by compiling the medical history and performing a clinical investigation of the patient. Also, diagnostic impressions can be taken or, on the basis of elicited information, conservative, surgical and periodontology treatment may be instituted.[14] The recognition of the physical and chemical parameters of the materials is very important as well.

The difference between conventional and elastic dentures concerns mainly the laboratory stage of fabrication. After taking individual impressions, a provisional cast is made of plaster class IV and occlusal patterns are performed in it. It is very important to duplicate the models (with the use of silicone and plaster class IV and of 1.5% expansion).[14,19] Avoiding this procedure may result in inexact adhesion or excessive activity of retention elements pressure, which gives the sensation of a "too tight" denture and requires numerous corrections. In the case of a complete denture made of acron, flanges are not blocked in the model. Elastic dentures are modeled on a duplicated mould.

First, the technician patterns the framework of the elastic denture of the mould wax. In the case of acron dentures, the plate should reach the tooth preparation margin.[19] In the case of partial, elastic supported dentures, the model paralelometric evaluation should be performed, flanges should be partially blocked and crevices relieved. The model is placed in a special container and injections canals are formed. Unskillful formation, avoiding the use of the protection mass for thermoplastic materials as well as inobservance of material hardening times cause many imprecisions.[14] The material is introduced to the container using the thermal injection method. It is melted and subsequently pressurized; the manufacturer's recommendations are absolutely important while the melting temperature, injection time and pressure are defined on the injection moulding machine panel. This technique is difficult and requires maximum accuracy. The procedures of material heating, pressure increase, injection force control, and cooling time keeping should be watched. Disobedience of the above-mentioned procedures results in air bubbles trapped in thermoplastic material, teeth loss, teeth occlusion shift, plate adhesion incorrectness or too small amount of injected material

in peripheral regions.[14] After releasing denture medium from the container, it is modeled and adjusted to the mould so that it has appropriate thickness and shape. On the prepared framework of the elastic denture, teeth are positioned. Teeth are positioned in a laboratory on the basis of the information registered by the dentist after elastic prosthesis framework elaboration. Acron does not bond chemically with acrylic teeth, so each tooth must be prepared individually, crow step milled and openings at the tooth basis drilled. Correct preparation is a solid warrant of the tooth stability in an acron plate.[19] It is important to position teeth of good quality and low abrasiveness, avoid flat-cuspid ones used in the Wrocław method of fabrication of extensive dentures. At subsequent stages, the technician carries out canning and polymerization, perfects and polishes the ready denture, and controls adhesion accuracy on the diagnostic mould. Thermoplastic materials should not be cut and heated again, as it causes denture fragility, water increased absorption and material staining.[14] Final refinement of elastic dentures constitutes a big problem. It requires a special technique based on the abrasion materials which are not cutting. The use of inappropriate instruments decreases the quality of appliances quality by the formation of micro scratches. Polishing at an increased temperature causes melting and softening of the material, especially nylon. All the above-mentioned errors cause a decrease in esthetics and staining. The correction of elastic dentures requires the "short-term goals" rule. Polymers and nylons dentures are corrected using a low speed. For this purpose, the stones are white or green with a diamond coating.[14–16] Polishing should be carried out at low rotation with the use of hard rubber tips and Scotch-Brite micro discs or Habra's abrasion discs as well as Marvel and Flexi-Brites type preparations.[14]

Acetal dentures are corrected with sharp mills designed for acrylic. In the case of vast corrections, plaster mill provides good results, whereas small errors can be corrected with diamond-coated stones, hard rubber tips and sometimes carborundum stones. Polishing is achieved with the use of hard rubber tips and polishing systems (e.g., Marvel, Scratch remover CDM Dental). Their disadvantage is a limited possibility of modification after denture completion, e.g., widening the prosthetic field after teeth extraction. A new tooth can be positioned only close to the acrylate plate.[3,14] Furthermore, improper preparation of teeth while settling them in the denture saddle during the laboratory process may make the retention areas susceptible to dental plaque. Humid environment of the oral cavity favors fungal colonization, mainly *Candida albicans*, which occur in the normal flora of the mouth, but they remain in the biological equilibrium. Any imbalance of this condition triggered by improper hygiene or material porosity favors the development and spread of bacteria and fungi.[4,23] In conventional dentures, after acrylic surface silanizing, *Candida albicans* – appliance adhesion drops significantly. Also, material to water affinity decreases and the mechanical strength of dentures grows.[4,23] Hygiene is especially important in using elastic dentures. As in the case of conventional appliances, special means for cleaning elastic dentures have been elaborated and they are manufactured by pharmaceutical laboratories.[14] Preservatives ProTech and Smile Again (Boynton Beach, USA) are recommended. They also prove applicable in acrylic appliances, especially those in which soft materials are incorporated as liners, retainers or hard-soft materials in splints.

Apart from their long-term application, thermoplastic materials have auxiliary employment in temporary appliances, both permanent and removable.

FJP (Pressing Dental, San Marino) is one of the representatives of copolymers. Its resilience degree positions this material between acetals and nylons. It can be used for the fabrication of partial dentures, along with retention elements, esthetic pellots or clasps and occlusal support. It is free from many disadvantages of thermoplastic materials, especially their unfavorable influence on the prosthetic base, and it is easier in mechanical treatment. FJP appliances may be repaired and subsequent teeth may be added to the denture, as this material combines with acrylic. It is mainly incorporated in temporary appliances or provisional dentures. Due to its hypoallergenic properties, it may replace acrylic in framework dentures in patients sensitive to acrylic material.[7,24,25]

Luxaform (DMG Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany) is another thermoplastic material applied in fixed temporary prosthetic appliances. It is an alternative solution in provisional tasks performed directly after impression making.[26] It reduces the time of prosthetic appliance fabrication, as the dentist, ex tempore, makes the matrix of Luxaform before the tooth preparation. This polymer (polycaprolactone), at room temperature is found in the form of blue non-translucent tablets. They are plasticized while heating in water at a temperature of 70°C for 1–2 min or in a microwave oven in 1/4 glass of water for 1 min.

When the material is already plastic, it should be spread over the tooth waiting to be prepared as well as over the adjacent teeth from the mesial and distal sides, and pressed firmly. Then, the whole area should be dried with air until there is opacity and color. Further steps in the procedure of the fabrication of temporary fixed appliances are similar to those of the impression taking method. However, acrylic should not be applied to the matrix, as it bonds both thermally and chemically with Luxaform. For this reason, the following composite materials are recommended: Luxatemp (DMG Chemisch-Pharmazeutische Fabrik GmbH), Protemp 3 Garant (3M ESPE AG, Seefeld, Germany), Provitemp K (Bisico Bielefelder Dentalsilicone GmbH & Co. KG, Bielefeld, Germany).

Luxaform (DMG Chemisch-Pharmazeutische Fabrik GmbH) is also very useful while constructing the splint, which follows the positioning of an implant during the implantation procedure in accordance with the "simple in practice" routine.[26]

It may be an alternative if there is no laboratory splint at disposal or in emergency cases during implantological treatment.

Elastic thermoplastic materials are increasingly common in dentistry. However, their disadvantages should always be recognized and kept in mind. In planning complex treatment, all available prosthetic methods should be considered, including elastic dentures, which ought to be confronted with conventional appliances.

Elastic dentures are a good choice for esthetic reasons. They may be recommended for patients who do not accept clasps in framework dentures, but cannot afford dentures supported with precise elements or implant-based fixed appliances. Such dentures can be applied in masticatory organ rehabilitation in patients with increased absolute reflexes, especially retching.

Moreover, such features as size, construction weight, material plasticity and smoothness are considered the advantages of thermoplastic materials. Elastic dentures are the only removable appliances for patients allergic to metal or acrylic. They are better tolerated by patients with an uneasy prosthetic base or with systemic diseases, e.g., diabetes, i.e., more susceptible to the traumatic activity of the hard plate of traditional dentures made of acrylic material. The adaptation time is shortened and the number of necessary corrections is reduced. Hygiene rules as well as follow-up visit terms must be strictly obeyed.

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Review

The pathogenesis and available prevention options in patients with diabetic thrombophilia

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1447–1452

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Funding sources
None declared

Conflict of interest
None declared

Received on April 23, 2017
Reviewed on April 27, 2017
Accepted on May 5, 2017

Abstract

Diabetes mellitus (DM), a growing health problem itself, is accompanied by an increased risk of cardiovascular and thrombotic complications. The imbalance between coagulation and fibrinolysis processes observed in patients with diabetes may be defined as diabetic thrombophilia. Several mechanisms are involved in the hypercoagulability state in diabetics, including endothelial cell damage, altered platelet structure and function, increased microparticle formation, different structure of fibrin clots, disturbances in the activity of coagulation factors, fluctuations in the concentrations of fibrinolysis activators and inhibitors, and qualitative changes of proteins due to glycation and oxidation processes. These all are the reasons why DM is the most common cause of acquired thrombophilia. Moreover, diabetes changes the efficacy of certain medications. Results of various trials seem to suggest that thrombolytic drugs are less effective in patients suffering from this disease. The impact of DM on the effectiveness of treatment with acetylsalicylic acid (ASA) remains unclear. Awareness of thrombotic complications in diabetic patients may enable earlier diagnosis and proper therapy.

Key words: diabetic thrombophilia, diabetes mellitus, thrombosis, coagulation factors, hypercoagulability

DOI
10.17219/acem/71054

Introduction

Thrombophilia (hypercoagulability) is a congenital or acquired condition caused by the predominance of coagulation processes over fibrinolysis and resulting in thrombosis. The most common congenital causes are factor V Leiden mutation, prothrombin G20210A mutation, protein S deficiency, protein C deficiency, antithrombin deficiency, and *MTHFR* gene mutations. Acquired thrombophilia can be caused by antiphospholipid syndrome or drugs, such as oral and transdermal contraceptives or chemotherapeutic agents.[1]

Diabetes is a group of metabolic diseases characterized by high blood sugar levels over a long period. It is associated with an increased risk of thrombotic and cardiovascular complications, such as myocardial infarction and ischemic stroke, which are the most common causes of death among diabetics.[2]

The imbalance between the clotting system coagulation and fibrinolysis (favoring coagulation) in patients with diabetes is described as diabetic thrombophilia.[3] Diabetes mellitus (DM) is considered to be a leading cause of acquired thrombophilia.[4] The aim of our paper was to investigate the pathogenesis of diabetic thrombophilia, and to discuss primary and secondary prevention strategies.

Endothelial dysfunction

Endothelium is the lining in all the blood vessels of the human body and it plays an important role in the regulation of the hemostatic processes.[5] In physiologic conditions, due to its antithrombotic activity, the endothelium maintains continuous blood flow through the inhibition of platelet aggregation and of the inflammatory activation of leukocytes and coagulation factors. Endothelial function is already impaired in patients with insulin resistance, diabetes or in individuals at a high risk for developing type 2 diabetes (T2D), which is further worsened by hypertension, dyslipidemia and nicotinism.[6,7] The biochemical and cellular factors behind endothelial dysfunction in diabetes include increased activation of protein kinase C, elevated levels of growth factors (endothelin, angiotensin II and/or cytokines), nonenzymatic glycation of proteins and other molecules, oxidative stress, and impaired insulin activation of phosphatidylinositol (3,4,5)-triphosphate (PIP-3) kinase, but normal response of mitogen-activated protein kinase (MAP).[7] It is worth mentioning that in T2D, endothelial progenitor cells (EPCs) count is low and their functionality is impaired. Endothelial progenitor cells are responsible for blood vessel formation and they support the repair of damaged endothelium. Although the mechanism of EPCs dysfunction is not fully understood, it is suggested that hyperglycemia-induced increased oxidative stress is an important factor in this process.[8]

The well-known mechanisms contributing to endothelial dysfunction phenotype in T2D are impaired glucose metabolism, altered insulin signaling and low-grade inflammatory state.[9] Many important relationships between insulin resistance and endothelial dysfunction have been discussed in the literature. In insulin resistance, the phosphatidylinositol 3-kinase-dependent signaling is altered, causing an imbalance between the production of nitric oxide (NO) and the secretion of endothelin-1. This leads to a decrease in blood flow, which exacerbates insulin resistance. Besides the regulation of endothelial NO and endothelin-1 synthesis, insulin also stimulates the expression of vascular cell adhesion molecule (VCAM)-1 and E-selectin on endothelium.[10] Moreover, by acting through monocyte receptors, insulin may produce anti-inflammatory effects.[11]

The consequences of endothelial dysfunction include increased vascular permeability, decrease in heparin sulphate concentration, reduced production of NO, reduced release of microparticles, and the expression of adhesion molecules, which allow leukocyte migration during inflammation. Damage of endothelial cells leads to the exposition of subendothelial matrix, which has prothrombic properties.[12] In recent years, much attention has been paid to the procoagulant activity of microparticles, which are small fragments of cell membrane vesicles, released mainly from the platelets. They play a significant role in diabetic atherogenesis as a reservoir of bioactive substances involved in inflammatory and thrombotic processes. The concentration of microparticles is dependent on the degree of glycemic control and in the future it may become a clinically relevant biomarker of diabetic vascular dysfunction.[13]

Additionally, hyperhomocysteinemia and hyperuricemia, through their direct cytotoxic effect on endothelium, promote thrombosis and formation of atherosclerotic lesions in obese patients.[14] The degree of endothelial cell injury is correlated with increased concentrations of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and C-reactive protein (CRP).[15] Hemostasis and inflammation are closely interrelated processes, responsible for the restoration of normal tissue function after injury. The cooperation between the immune and hemostatic systems takes place on multiple levels and involves many hemostatic components, such as vascular EPCs, platelets, plasma coagulation cascade, anticoagulant pathways, and fibrinolytic activity. The main proinflammatory cytokines linked to the activation of thrombotic process are TNF-α, IL-1 and IL-6.[16] A recent report presented a novel inflammatory pathway, in which Wnt5a signaling and c-Jun N-terminal kinase (JNK) activation mediate impaired endothelial function in diabetes.[17]

It should be noted that endothelial dysfunction may precede the development of T2D. Meigs et al. showed that plasma markers of endothelial dysfunction (plasminogen activator inhibitor-1 antigen and von Willebrand factor antigen) increased the risk of incident diabetes independently of other diabetes risk factors such as obesity, insulin resistance and inflammation.[18] In a population-based study, biomarkers of oxidative stress, inflammation and

endothelial dysfunction predicted type 2 diabetes.[19] Biomarkers of oxidative stress, F2-isoprostanes and oxidized low-density lipoprotein (LDL) were positively associated with incident T2D; however, the associations were attenuated by the adjustment for body mass index (BMI).[20]

Altered platelet function in patients with diabetes

Platelet involvement in the pathogenesis and development of diabetic macro- and microangiopathy has been proven in many studies.[21,22] Platelets, due to their ability to interact with endothelial cells, are the key elements of thrombotic and embolic complications. Recently, many disturbances concerning platelet structure and function in patients with diabetes have been described. They include increased platelet volume, altered plasma membrane fluidity, elevated concentration of cell calcium, increased P-selectin expression, reduced production of NO, diminished sensitivity to antiaggregating agents (such as NO and prostacyclin – PGI 2), and enhanced activation of the arachidonic acid pathway, resulting in increased thromboxane A_2 synthesis.[23–25] Another finding concerning the function of platelets in diabetics was that they produce more tissue factor (TF) than platelets in control subjects due to the loss of insulin inhibition.[26] These changes cause firm adhesion of platelets and their aggregation, as well as their interaction with monocytes, granulocytes, erythrocytes, and endothelial cells.[27] Active platelets are also the source of aforementioned microparticles.[28]

Soma et al. showed that platelet surface markers and the percentage of activated platelets were elevated in diabetics with and without cardiovascular manifestation compared to healthy individuals. The highest percentage of activated platelets and increased microparticle formation were observed in the group of patients with diabetes and cardiovascular complications.[29]

Increased platelet reactivity in diabetics is caused by many factors, e.g., hyperglycemia, hypertriglyceridemia, oxidative stress, inflammation, and absolute or relative insulin deficiency, which further favor the development of vascular complications.[30] Hyperglycemia following disturbances in insulin secretion, insulin action or both may be a cause of platelet hyperactivity in subjects with diabetes. It is established that hyperglycemia disrupts Ca^{2+} homeostasis in platelets by mobilizing Ca^{2+} from intracellular storage pools, which leads to an increase in intracellular Ca^{2+} levels. Moreover, hyperglycemia may promote nonenzymatic glycation of platelet membrane proteins with changes in protein structure and conformation, as well as alterations of membrane lipid dynamics.

The other mechanism is impaired platelet response to high insulin levels (platelet insulin resistance), associated with a change in intracellular signal transduction and the suppression of the action of inhibiting factors (fibrin degradation products – FDP).[31] Insulin regulates platelet function by cell surface receptors, counteracting the activation and aggregation of platelets. It also sensitizes platelets to PGI 2 and NO actions, inhibiting their aggregation, and reduces the prothrombotic effects of a number of agonists (adenosine diphosphate (ADP), collagen, thrombin, and epinephrine).[30] It has been shown that insulin maintains platelet sensitivity to PGI 2 by increasing surface expression of PGI 2 receptors.[9] Moreover, insulin increases platelet concentrations of both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which are the main inhibitory second messengers for platelet activation. Another study, conducted by Ferreira et al., revealed that insulin attenuates platelet functions by interfering with cAMP suppression through insulin receptor substrate-1 (IRS-1) and G-protein (Gi). The inhibition of Gi activity by insulin is associated with a decrease in Ca^{2+} mobilization and the reduction of adhesion and aggregation. Thus, the authors speculated that the hyperresponsiveness of platelets in diabetes explains the absence of platelet inhibition by insulin.[25] It has been shown that platelet hyperactivity in T2D is probably caused by a defect in the mechanisms through which insulin interferes with signaling by the P2Y12 receptor.[32] The other mentioned cause of high platelet activity is decreased number and affinity of platelet insulin receptor (IR).[9] In addition, Zhang et al. demonstrated that genetic background might play a role in diminished antiplatelet efficacy of dual antiplatelet therapy. The authors presented an association between IRS-1 polymorphisms and high platelet reactivity in subjects with T2D and coronary artery disease (CAD).[32]

Several biomarkers of diabetic thrombocytopathy have been proposed to be used in clinical practice. Since platelet volume indices (PVI), including mean platelet volume (MPV), platelet distribution width (PDW) and platelet-large cell ratio (P-LCR) are considered indicators of platelet hyperactivity, they can be utilized as potential biomarkers for diabetic complications. Recently, Buch et al. determined MPV and PDW as predictive biomarkers of diabetic vascular complications (their prognostic value is more significant for microvascular than macrovascular complications). Moreover, it was found that improved glycemic control decreases MPV, which indicates that diminished platelet activity achieved by proper glycemic control may delay or even prevent vascular complications. In a cross-sectional study, a group of patients with glycated hemoglobin (HbA1c) >7.0% had higher MPV than a group with HbA1c ≤7.0%. The authors found a positive correlation between MPV and both short-term and long-term glycemic markers in subjects with T2D. It has been hypothesized that an increase in MPV can result from hyperglycemia, which exerts direct osmotic effects on platelets, causing osmotic swelling. In addition, increased MPV may be associated with a higher platelet turnover rate.[4,33]

Coagulation factors and fibrinolysis

Changes in the concentration and activity of coagulation factors in patients with diabetes lead to the dominance of thrombogenesis over fibrinolysis. The common feature of this phenomenon is the influence of chronic hyperglycemia on the increase of TF expression and the activity of factor VII (responsible for the activation of coagulation in the extrinsic pathway), II, V, VIII, and X. Moreover, the concentration of C- and S-protein (coagulation cascade inhibitors) is decreased. The changes mentioned above contribute to hypercoagulability by enhanced thrombin generation.[34,35] Von Willebrand factor (vWF) mediates the protection of factor VIII from proteolysis and is prerequisite for platelets adhesion to subepithelium.[36] Its elevated plasma levels have been observed in patients with poor-controlled DM and with cardiovascular disease.[37] Tissue factor pathway inhibitor (TFPI) concentration is conversely correlated to fasting glucose and HbA1c.[38,39] An essential reaction in the fibrinolysis process is the conversion of plasminogen to plasmin, which causes the proteolysis of fibrin clots. This reaction is controlled by activators (tissue plasminogen activators – t-PA) and inhibitors (α2-antiplasmin, α2-macroglobulin, plasminogen activator inhibitor – PAI).[40] Apart from quantitative changes in coagulation factors and fibrinolysis, in chronic hyperglycemia there are also qualitative changes due to glycation and oxidation processes. Protein glycation is covalent bonding (post translational modification) of a simple sugar molecule to free amino groups of proteins, without the controlling action of an enzyme. The amino groups of lysine in fibrinogen could be bonded with the carbonyl groups of glucose. Hyperglycemia increases plasma protein glycation. The absence of free amino groups of lysine, which are a binding site for fibrinolytic proteins (t-PA and plasminogen) to fibrin, impairs clot lysis. Additionally, the glycation of plasminogen decreases the specific activity of plasmin and reduces its profibrinolytic properties.[41] There is a noticeable difference in the structure of fibrin clots, which are more compact and, therefore, less susceptible to fibrinolysis. The observed increased fibrinogen levels, an independent risk factor for atherosclerosis and cardiovascular disease, may be a predisposing factor to the deposition of fibrin clots (a potential role in the development of diabetic nephropathy) and increased platelet aggregation.[42] Hess et al. demonstrated that incorporating complement component 3 into the fibrin clots of patients suffering from diabetes had a greater effect on the prolongation of clot lysis compared to the clots from healthy controls.[43] Fibrinolysis impairment in diabetic patients depends primarily on the increased concentration of PAI, which is embedded in fibrin clots by activated factor XIII. Moreover, Hori et al. noticed that diabetes patients had an elevated level of thrombin activatable fibrinolysis inhibitor (TAFI), which protects fibrin clots against lysis.[44]

Treatment and prophylaxis

Both prophylaxis and treatment of thrombophilia associated with diabetes should be based primarily on the well-controlled disease, close to normoglycemia, measured by glycated hemoglobin.[45] It is noticeable that hypoglycemia can also lead to hypercoagulability due to elevated fibrinogen and PAI levels.[46] Another very important issue concerns the prevention of atherosclerosis, which can be achieved by a change of dietary habits, enhancement of physical activity (up to moderate-intensity exercise for 30 min, 5 times a week) and smoking cessation. Decreased concentration of LDL, stabilization of atherosclerotic plaque and anti-inflammatory effect are benefits of therapy with 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins). According to the observations of Kim et al., diabetic patients treated with statins or angiotensin receptor blockers showed a decreased endogenous thrombin potential ratio and increased protein C levels.[34] Because of a high risk of myocardial infarction and ischemic stroke, it is advisable to use acetylsalicylic acid (ASA) at a prophylactic dose. However, diabetic patients have a high prevalence of suboptimal effect or even aspirin resistance.[47] Depending on the cause, various groups of drugs are being used to treat the hypercoagulation complications of diabetic thrombophilia.

Antiplatelet drugs decrease platelet aggregation and inhibit thrombus formation. They are effective in the treatment and prophylaxis of arterial thrombosis. The class of antiplatelet drugs includes, among others, irreversible cyclooxygenase inhibitors (ASA), adenosine diphosphate receptor inhibitors (clopidogrel, prasugrel) and glycoprotein IIB/IIIA inhibitors (abciximab, tirofiban). Bhatt et al. noticed that clopidogrel is superior to ASA in reducing recurrent ischemic events in patients with diabetes, thereby causing fewer bleeding complications.[48] According to Wiviott et al., oral antiplatelet therapy provided with prasugrel is of particular benefit to patients with diabetes. They compared prasugrel to clopidogrel in the secondary prevention of major cardiovascular events.[49] In the observation by Perkan et al., abciximab treatment was associated with a lower in-hospital rate and 30-day mortality rate, and a lower incidence of death and reinfarction at 30 days in diabetic patients undergoing primary percutaneous coronary intervention due to acute myocardial infarction.[50]

Anticoagulants inhibit the coagulation cascade that happens after the initial platelet aggregation. They are used in the treatment and prophylaxis of venous thrombosis. Anticoagulants are divided into: vitamin K antagonists (warfarin, acenocoumarol), direct factor Xa inhibitors (rivaroxaban, apixaban), direct thrombin inhibitors (bivalirudin, dabigatran), and unfractionated heparin (UFH), as well as low-molecular-weight heparin (enoxaparin, nadroparin, dalteparin). Bansilal et al. aimed to examine the safety and efficacy of rivaroxaban vs warfarin in patients with non-valvular atrial fibrillation and diabetes. The relative

efficacy and safety of both drugs was similar.[51] According to the observations of Morrow et al., in high-risk ST elevation myocardial infarction (STEMI) patients with diabetes undergoing fibrinolysis, a reperfusion strategy with the use of enoxaparin significantly improved the outcomes compared to UFH.[52]

The last group of drugs includes thrombolytic drugs, which are responsible for dissolving blood clots. These include streptokinase, urokinase and recombinant t-PA (alteplase, reteplase). Masoomi et al. noticed that complete ST-resolution after streptokinase infusion occurred in 31.6% of diabetic and 51.0% of non-diabetic patients, respectively.[53] The failure of ST-segment resolution 180 min after drug application was notably higher in diabetic than nondiabetic patients. Another observation by Strbian et al. was that poor outcome of thrombolysis after ischemic stroke patients was associated with diabetes and elevated admission blood glucose.[54] The results of trials seem to suggest that thrombolytic drugs are less effective in the case of patients suffering from diabetes.

Conclusions

The pathogenesis of diabetic thrombophilia is not fully understood. Despite a number of studies available on this issue, potential factors and mechanisms that may exacerbate hypercoagulability in diabetic patients are constantly being sought. A better understanding of the causes of hemostatic disorders in these patients makes it possible to provide adequate primary prevention and provides an opportunity to increase the effectiveness of sufficient therapy. Because of the large and still growing population of diabetics, diabetic thrombophilia is a crucial issue because of its complications, such as cardiovascular disease.

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Challenges in diagnosis and treatment of sporadic inclusion-body myositis

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1453–1457

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Funding sources
None declared

Conflict of interest
None declared

Received on May 21, 2016
Reviewed on June 30, 2016
Accepted on March 27, 2017

Abstract

Sporadic inclusion body myositis (sIBM) is a rare yet increasingly prevalent disease and the most common cause of inflammatory myopathy in people over the age of 50. The exact cause of the disorder is unknown. In sIBM 2 processes, first autoimmune and the other degenerative, parallelly occur in the muscle cells. The inflammation aspect is characterized by the cloning of T cells that appear to be driven by specific antigens to invade muscle fibers. The degeneration aspect is characterized by the appearance of holes in the muscle cell vacuoles, deposits of abnormal proteins within the cells and in filamentous inclusions. The disease has a major impact on patients' motor functionality and their quality of life. The treatment of sIBM still remains a major challenge. Early diagnosis of sIBM (already at the histopathology stage), when one still cannot observe fully developed clinical symptoms, may stop help to the progression of the disease.

Key words: biomarker, early diagnosis, sporadic inclusion body myositis

DOI
10.17219/acem/69855

Table 1. Sporadic inclusion-body myositis (sIBM) diagnostic criteria from 2011

Classification	Clinical features	Pathological features
Clinicopathologically defined sIBM	1. duration of weakness >12 months 2. creatine kinase ≤15 × ULN 3. age at onset >45 years 4. finger flexion weakness > shoulder abduction weakness, and/or 5. knee extension weakness > hip flexor weakness	All of the following: 1. endomysial inflammatory infiltrate 2. rimmed vacuoles 3. protein accumulation or 15–18 nm tubulofilaments
Clinically defined sIBM	1. duration of weakness >12 months 2. creatine kinase ≤15 × ULN 3. age at onset >45 years 4. finger flexion weakness > shoulder abduction weakness 5. knee extension weakness > hip flexor weakness	One or more: 1. endomysial inflammatory infiltrate 2. upregulation of MHC class I 3. rimmed vacuoles 4. protein accumulation or 15–18 nm tubulofilaments
Probable sIBM	1. duration of weakness >12 months 2. creatine kinase ≤15 × ULN 3. age at onset >45 years 4. finger flexion weakness > shoulder abduction weakness, or 5. knee extension weakness > hip flexor weakness	One or more: 1. endomysial inflammatory infiltrate 2. upregulation of MHC class I 3. rimmed vacuoles 4. protein accumulation or 15–18 nm tubulofilaments

sIBM – sporadic inclusion body myositis; MHC class I – major histocompatibility complex class I; ULN – upper limit of normal.

Sporadic inclusion-body myositis (sIBM) is one of the causes of myopathy that should be considered in rheumatological and neurological diagnosis. The onset of the disease is usually concealed and clinical symptoms develop slowly (over the years), leading to weakness and atrophy of both proximal and distal muscles.[1] The term sIBM was introduced in 1971 by Yunis and Samaha to describe patients with polymyositis, for whom the histopathological examination revealed the presence of rimmed vacuoles and specific intercalations localized in the cytoplasm and nuclei of muscle fibers.[1,2] Generally, sIBM is taken into consideration in the diagnosis of myopathy when a patient does not respond to the treatment with glucocorticosteroids, or when the clinical symptoms include muscle involvement seen in distal muscles, particularly in the foot extensors or deep fingers flexors.[1–4] Moreover, the facial and swallowing muscles are affected; hence, one of the clinical symptoms of sIBM could be difficulty in swallowing.[5] The disease usually manifests itself after the age of 50 and is more common among males (2:1). Its steady progress leads to significant motor impairment. The muscle involvement can be asymmetric and can occur selectively in the quadriceps (muscles of the thighs), iliopsoas muscle, triceps, biceps, and forearm flexor muscles.[5] Laboratory tests may show elevated or normal levels of creatine kinase (CK). Asymptomatic presentation of sIBM is rare.[6]

Electromyography (EMG) can be used to exclude typical causes of neurogenic disorders and to classify the disease into the group of muscle damage; however, it cannot accurately extract its cause (myositis, toxic or dystrophic myopathic processes).[5] Therefore, muscle biopsy with histopathologic, immunohistochemical and microscopical examination remains the best method for diagnosing sIBM. In the analysis of the muscle biopsy, sIBM can be signalized by: inflammatory infiltrations, infiltration of inflammatory cells intact muscle fibers (partial infiltration), rimmed vacuoles, deposits of amyloid, and 15–18 nm tubulofilaments seen in electron microscopy. Moreover, it should be noted that not all of these changes can be observed in every patient simultaneously; this fact is included in the diagnostic criteria from 2011 (Table 1).[7] So far, the existing diagnostic criteria for sIBM have been mainly based on histopathological changes (Table 2).[7] In definite IBM, patients must exhibit all muscle biopsy features, including invasion of non-necrotic fibers by mononuclear cells, vacuolated muscle fibers and intracellular (within muscle fibers) amyloid deposits or 15–18 nm tubulofilaments. If the muscle biopsy shows only inflammation (invasion of non-necrotic muscle fibers by mononuclear cells) without other pathological features of IBM, then a diagnosis of possible IBM can be given if the patient exhibits the characteristic clinical and laboratory features.

Table 2. Sporadic inclusion-body myositis (sIBM) diagnostic criteria from 1995

Clinical features	1. duration of illness >6 months 2. age of onset >30 years old 3. muscle weakness affecting proximal and distal muscles of arms and legs and patient must exhibit at least 1 of the following features: a. finger flexion weakness b. wrist flexion weakness > wrist extension weakness c. quadriceps muscle weakness (≤grade 4 MRC)
Laboratory features	4. serum creatine kinase <12 times normal 5. muscle biopsy a. inflammatory myopathy characterized by mononuclear cell invasion of non-necrotic muscle fibers b. vacuolated muscle fibers c. either intracellular amyloid or 15–18 nm tubulofilaments 6. electromyography – inflammatory myopathy

MRC – Medical Research Council scale.

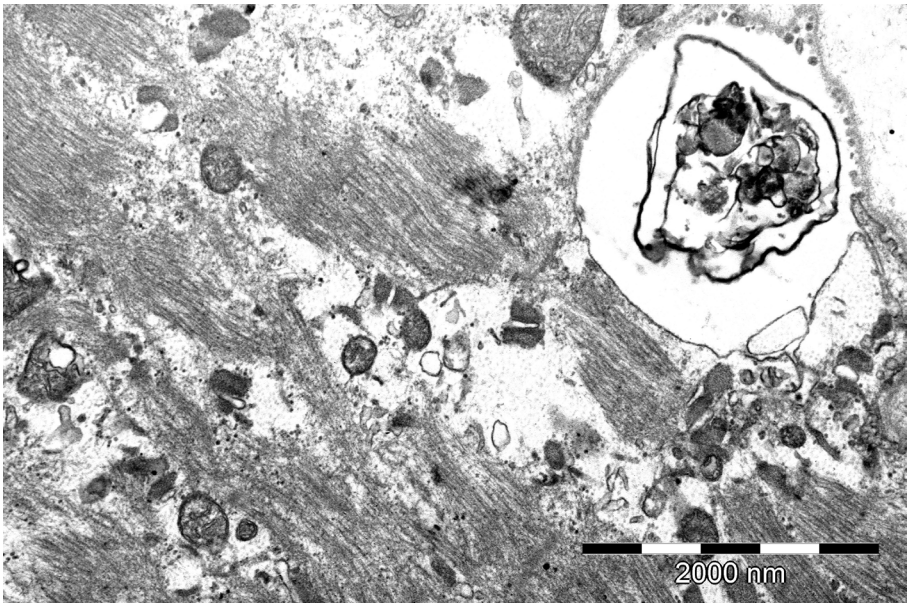

Fig. 1A. Myelin structure, transmission electron microscopy (TEM)

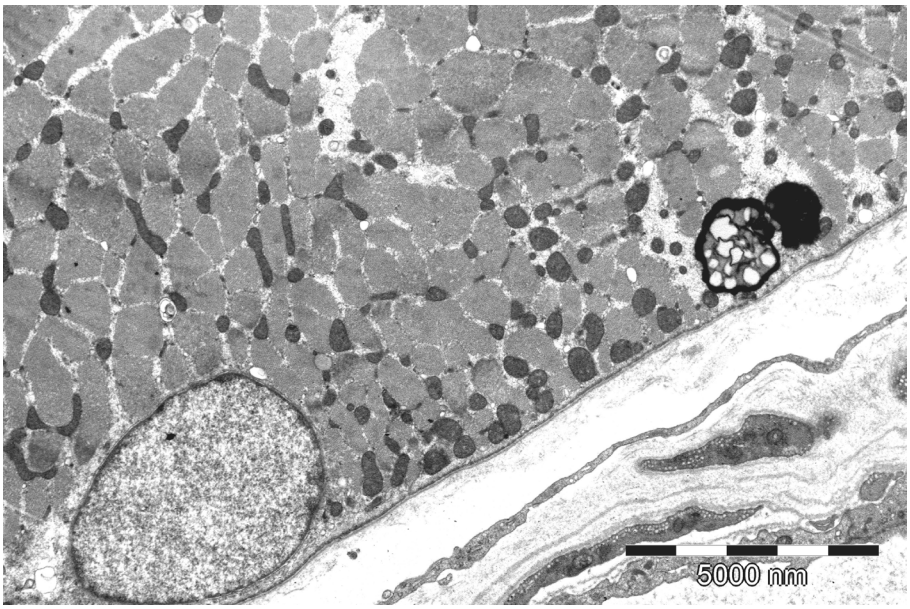

Fig. 1B. The muscle ultrastructure. Rimmed vacuole is recognized by a lining of granules at the margins of the vacuole, transmission electron microscopy (TEM)

Prognostic factors in sporadic inclusion body myositis

The disease has a major impact on the patients' motor functionality and their quality of life. As the time of disease development is slow, the survival rate of patients with sIBM is probably not comparable to the life expectancy of the general population.[8] Causes of death associated with IBM mainly include malnutrition, wasting (cachexia), aspiration, respiratory infections as a result of dysphagia, and decreased respiratory muscle function in advanced cases of the disease.[7] The increased cancer risk was not proven like in other inflammatory myositis.[9] According to Benveniste et al., a few factors can contribute to the progression of the disease towards disability and they include: male gender, older age (>60 years) and immunosuppressive therapy.[10] Cortese et al. also suggested that advanced age (>55 years) is associated with an unfavorable prognosis.[11] Furthermore, the time to stand up and start the movement was the prognostic factor in patients with sIBM.[12]

The cause of the disease is still not fully understood. So far, the proposed theories suggest a role in the etiology of disease of: viral infection, accumulation of toxic proteins, the autoimmune attack, degeneration of muscle cells (rimmed vacuoles filled with nuclear and lysosomal proteins, and inclusions in the cytoplasm of muscle cells composed of the TDP-43 (TAR DNA-binding protein 43) (Fig. 1A,B); the role of lysosomal membrane protein LAMP1 and LAMP2 in the degeneration of the muscle cells' proteins and the formation of typical vacuole), and impaired proteasome and autophagocytosis function (accumulation of P62 (a protein associated with the nuclear pores-lamin involved in the transportation between the nucleus and cytoplasm), LC3 (microtubule-associated proteins 1A/1B

light chain 3-autophagy membranes) and NBR1 (neighbor of the *BRCA1* gene 1 protein, receptor associated with P62)).[5,13–15] New insights into the role of p62 in sIBM pathogenesis indicate that p62 join to perform selective autophagy.[16] Furthermore, p62 could be induced by some cellular stresses. Nakano et al. recently published the theory that in sIBM, compromised binding of the p62-ubiquitinated protein complex to LC3 could stop the autophagy process in its initial stages, which causes the formation of aggregates of p62-oligomers with Lys63-ubiquitinated proteins.[16] Also, the upregulation of TGF-β (transforming growth factor-β) signaling may have an important influence on sIBM pathogenesis.[17]

Among patients with sIBM, the histopathological muscle biopsy finds: atrophied fibers along with the normal and overgrown fibers with properly preserved differentiation in the types of metabolic, infiltration with mononuclear cells (mainly lymphocytes around blood vessels and muscle fibers that do not show the features of necrosis), presence of rimmed vacuoles in the fibers and eosinophils inclusions in the cytoplasm of muscle fibers, as well as vacuoles comprising basophilic granularities.[7,18]

The fact that sIBM belongs to the group of autoimmune diseases is indicated by the presence of infiltrates composed of cytotoxic lymphocytes T $CD8^+$ that form an immunological connection with major histocompatibility complex class I (MHC-I). This leads to a failure of muscle fibers through the mechanism of special cytolytic proteins-perforins, which provides the reason for the use of immunosuppressive drugs.[19–25] Additionally, apart from the inflammatory process, in sIBM coexist features of the degeneration of muscle fibers in the form of rimmed vacuoles, and the depositions of congophilic amyloids and other specific proteins.[21] Rare missense variants in FYCO1 (a protein accumulated at rimmed vacuoles) may impair autophagic function, leading to rimmed vacuoles formation.[26] In sIBM there were also observed activated lymphocytes of B type, dendritic cells of plasmacytoid origin in the area of perivascular and perimysium, as well as increased expression of adhesion molecules and significant amount of cytokines and chemokines such as, for example: γ-interferon, interleukin (IL)-1b, tumor necrosis factor alpha (TNF-α), CXCL3-CXCL10, and deregulated expression of mitochondrial proteins in oxidative phosphorylation.[13,27] Still, there are no established biomarkers for sIBM that could be used in everyday clinical practice.[28] The proposed biomarkers of the disease are presented in Table 3. The determination of biomarkers of sIBM is limited by the lack of specific standards for the use of specific biomarkers (medium cut, accountability), the lack of comparable sensitivity and specificity with other than sIBM muscle diseases, and the need to perform invasive muscle biopsy. Nowadays, the most suitable determination method appears to be the use of antigens anti-cN1A (cytosolic 5'-nucleotidase 1A antibodies), described for the first time by Larman et al.[29] The antigen localizes itself predominantly in the perinuclear and rimmed vacuoles region. Their advantage is the determination of blood serum taken from patients. Their sensitivity was between 60% and 70% and specificity was enclosed in the range of 83–92% at low titer and correspondingly 33–34% and 96–98% at high titer.[29–32] Probably, this antibody could be connected with a more severe phenotype and a higher adjusted mortality risk.[33]

Table 3. The biomarkers of sporadic inclusion-body myositis (sIBM)

Biomarker	Mechanism of action
Rimmed vacuoles (1.3–16% of muscle fibers in IBM)	degeneration of muscle cells
Tubulofilaments	theory of viral infection – unconfirmed
Congo red-stained material	protein accumulation
Beta-amyloid accumulation (6–25% of patients with IBM)	theory of molecular toxicity
TDP-43 accumulation (inside and around rimmed vacuoles, sensitivity – 91% and specificity – 77% vs 0% and 8% in polymiosyositis)	metabolism of nucleic acids, degeneration of muscle cells
Immunoreactive SMI-31 accumulations (initially named phosphorylated tau protein)	unknown
p62 accumulation	protein accumulation, autophagocytosis
cN1A antibodies	metabolism of nucleic acids
Invasion of nonnecrotic fibers	autoimmune etiology
BACE-1 PS-1 sAPPβ	amyloidogenic-related molecules

BACE-1 – beta-secretase-1; PS-1 – presenilin-1; sAPPβ – soluble Aβ precursor protein.

Treatment of sIBM still remains a major challenge due to the coexistence of autoinflammatory and degenerative processes. So far, there has not been enough evidence in favor of using glucocorticosteroids, methotrexate, cyclosporine, azathioprine, mycophenolate mofetil, or intravenous immunoglobulin. Even though up to 30% of patients during the initial period of treatment may show some response, the disease is still slowly progressing.[21,34,35] Recently, it was suggested that the aggressive anti-inflammatory treatment in sIBM might stop the degenerative process. Therefore, a few studies were carried out with the use of alemtuzumab (humanized monoclonal IgG1 kappa causing long-term reduction in lymphocytes). Indeed, the results showed that this antibody significantly reduces the number of T cells in the muscles and inhibits certain molecules associated with degenerative process, which slowed down the progression of the disease in the 6-month period under consideration. However, there was no improvement in muscle strength.[36,37] Other substance, arimoclomol, has been already used in neurology as it affects the expression of the heat shock proteins (HSP). In the course of therapy, slow improvement was observed in patients' exercise capacity, assessment of manual muscle (MMT) and grip strength in the right upper extremity. However, no effect was found

on the quantitative assessment of muscle tissue with dual-energy X-ray absorptiometry (DEXA) and the level of HSP70 in the muscle biopsy taken at the beginning and at the end of the treatment. The study in question has yet to be fully published.[38] Currently, we are expecting the results from 2 other studies that were carried out with the administration of rapamycin.[21,39,40] The research that was carried out with the use of immunosuppressive drugs represents an introduction to new treatment options and a better understanding of sIBM's pathomechanism. Early diagnosis of sIBM (already at the histopathological stage), when one still cannot observe fully developed clinical symptoms, may help to stop the progression of the disease.

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The importance of the polymorphisms of the *ABCB1* gene in disease susceptibility, behavior and response to treatment in inflammatory bowel disease: A literature review

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2018;27(10):1459–1463

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Funding sources
None declared

Conflict of interest
None declared

Received on April 24, 2016
Reviewed on July 20, 2018
Accepted on July 5, 2018

Abstract

Crohn's disease (CD) and ulcerative colitis (UD) are the 2 common clinical subtypes of idiopathic inflammatory bowel disease (IBD) characterized by chronic inflammation of the gastrointestinal tract. The multifactorial etiology and pathogenesis of IBD is still unknown; however, the interaction between genetic, environmental and immunological factors seems to be crucial. A member of the adenosine triphosphate (ATP)-binding cassette family, P-glycoprotein, encoded by the human *ABCB1* gene, is among the most extensively studied transporters involved in drug disposition and effects. Single nucleotide polymorphisms (SNPs) located in exons 21, 26 and 12, i.e., G2677T/A, C3435T and C1236T, are of the greatest clinical importance. Functional defects of the intestinal epithelial barrier due to the lack of P-glycoprotein expression may constitute possible reasons for the development of colitis. Given that several drugs central to the therapy of IBD are also P-glycoprotein substrates, it has been hypothesized that its altered expression in IBD patients could modify the response to medical treatment. Nevertheless, there are conflicting reports of an association between these 3 SNPs and IBD. This article aims to review all relevant studies investigating the role of the polymorphisms of the *ABCB1* gene in disease susceptibility, behavior and response to treatment in IBD.

Key words: inflammatory bowel disease, MDR, P-glycoprotein

DOI
10.17219/acem/92936

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the 2 common clinical subtypes of idiopathic inflammatory bowel disease (IBD) characterized by chronic inflammation of the gastrointestinal tract. They may occur in various geographic regions, however, with different frequency. They affect mainly citizens of developed countries from Europe and North America. A large multicenter study conducted in 1991–1993 in 12 European countries estimated the incidence rate of UC and CD to be 10.4 and 5.6 per 100,000 person-years, respectively.[1] The incidence of IBD may have been changing over time, while the incidence of UC appears to remain stable, and a rise in incidence of CD has been observed.[2,3] The peak incidence of IBD occurs between 15 and 40 years of age, with a possible 2nd peak between 50 and 80 years of age.[4]

Ulcerative colitis is characterized by recurring episodes of inflammation limited to the mucosal layer of the colon. It involves the rectum and may spread proximally in a continuous fashion. Crohn's disease may involve the entire gastrointestinal tract from mouth to perianal area and is characterized by transmural inflammation and skip lesions, i.e., lesions appearing in a place surrounded by healthy mucosa. The transmural inflammation may lead to fibrosis and strictures as well as to microperforations and fistulae. Patients with UC usually present with bloody diarrhea. Associated symptoms include colicky abdominal pain, urgency, tenesmus, and fecal incontinence. Crampy abdominal pain, prolonged diarrhea and weight loss are the hallmarks of CD. Gross bleeding is less frequent than in UC.[5]

The underlying pathogenesis remains unclear but may involve persistent bacterial infection, defective mucosal barrier and imbalance in the regulation of the immune system. Epidemiological and family studies suggest that genetic factors play a significant role in determining susceptibility to IBD. Approximately 10–25% of individuals with IBD have a first degree relative with either CD or UC.[6] In a study conducted among Danish twins with IBD, the concordance rate among monozygotic pairs was 58.3% for CD and 18.2% for UC.[7] Inflammatory bowel disease appears to follow a non-Mendelian pattern of inheritance. It is likely that the aggregate effect at several loci contributes to the IBD phenotype. The first identified gene associated with CD was NOD2/CARD15 gene (nucleotide-binding oligomerization domain, caspase recruitment domain), localized on chromosome 16. Three mutations in the sequence of the *NOD2*/*CARD15* gene, Arg702Trp, Gly908Arg and Leu1007fsinsC, appeared to be the factors strongly related to CD. Wild-type NOD2 protein activates nuclear factor kappa B, making it responsive to bacterial lipopolysaccharides; this induction is deficient in patients with the mutant form of *NOD2*. Patients with CD, who are the carriers of at least 1 of the 3 *NOD2*/*CARD15* gene mutations, are at a higher risk for early onset and development of stenosis and small intestine involvement.[8] The functional properties of the proteins encoded by the genes implicated in the susceptibility to IBD have enabled the identification of specific pathways important in the pathogenesis of IBD: ATG16L1 (the autophagy pathway), interleukin (IL)-17 and IL-23 receptor genes (pathways regulating adaptive immunity), and OCTN2 (the pathway regulating the epithelial function).[9–11]

P-glycoprotein has been the most extensively studied member of the adenosine triphosphate (ATP)-binding cassette superfamily encoded by the human *ABCB1* gene (previously known as *MDR1*). P-glycoprotein is a phosphorylated and glycosylated protein that consists of 1280 amino acids and 2 homologous and symmetric sequences, each containing 6 transmembrane domains (TMDs), and a nucleotide-binding domain (NBD). P-glycoprotein functions as a transmembrane efflux pump, thereby moving drugs from the intracellular to the extracellular domain. Adenosine triphosphate hydrolysis provides the energy for active drug transport against steep concentration gradient.[12] P-glycoprotein is expressed in several human tissues, including peripheral blood lymphocytes, epithelial cells in the small and large bowel, hepatocytes, pancreatic ductile cells, kidneys, adrenal glands, the epithelium of the brain choroids plexus, the capillaries of the brain, placenta, etc. Thus, it plays an important role in the excretion of xenobiotics and endogenous substrates via the canalicular membrane of hepatocytes into the bile, via the brush border membrane of enterocytes into the gut lumen and via the brush border membrane of proximal tubules into the urine. In the endothelial cells of the blood-brain barrier, P-glycoprotein prevents the entry of substrates into the central nervous system.[13,14] P-glycoprotein transports a wide range of substances with diverse chemical structures, among them anticancer agents, cardiac drugs (e.g., digoxin, quinidine), HIV protease inhibitors, immunosuppressants (e.g., cyclosporine), and ß-blockers. Localized in gastrointestinal tract, it decreases their absorption and bioavailability.[15] Interestingly, most substrates of P-glycoprotein are also metabolized by the isoenzyme CYP3A4. This is of particular importance, because P-glycoprotein and CYP3A4 are co-localized in the small intestine and liver, organs crucial for absorption, distribution and excretion of drugs.[16]

ABCB1 gene polymorphisms and IBD

The *ABCB1* (*MDR1*) gene is located on chromosomal region 7q21 and consists of 28 exons encoding a protein composed of 1280 amino acids. It has been shown to be highly polymorphic, with 50 single nucleotide polymorphisms (SNPs) and 3 insertion/deletion polymorphisms reported so far. Three SNPs are currently considered to be the most clinically relevant, i.e., in exons 21 (G2677T/A), 26 (C3435T) and 12 (C1236T).[17] Hoffmeyer et al. were the first to demonstrate a 2-fold reduction in P-glycoprotein

expression in duodenal biopsy samples among healthy Caucasian subjects homozygous for the mutant 3435T allele in comparison to subjects homozygous for the C3435 allele (wild-type). TT genotype was also shown to be associated with higher digoxin plasma concentrations after oral administration, suggesting greater drug absorption in individuals with low intestinal P-glycoprotein levels.[18] These observations were only in apparent contradiction with the fact that 3435C→T mutation in exon 26 is a synonymous single-nucleotide polymorphism (i.e., does not alter the amino acid encoded). There is a linkage disequilibrium between SNPs in exon 26 (C3435T) and exon 21 (G2677T/A), suggesting that the observed differences in P-glycoprotein, initially attributed to the exon 26 SNP, may be the result of the associated tri-allelic polymorphism in exon 21. The latter is a nonsynonymous single nucleotide polymorphism (i.e., one causing an amino acid change, Ala893Ser/Thr).[19] It has also been recently shown that a synonymous SNP in exon 12 (C1236T) is linked to the C3435T and G2677T/A SNPs.[20]

However, there are conflicting reports on an association between these 3 SNPs and IBD. The *ABCB1* gene is an attractive candidate for the pathogenesis of IBD and, perhaps, it may also condition response to therapy. Firstly, it is located in a region of the human genome (7q21) that was found to possibly harbor a disease gene involved in susceptibility to IBD.[21] Its role in IBD was then investigated in a mouse knockout model (mdr1a–/–) in which developmentally normal mice spontaneously developed a colitis resembling UC in humans. They also developed a spontaneous colitis when maintained under specific pathogen-free conditions. The colitis was prevented and reversed with the administration of antibiotics, suggesting that the intestinal flora is necessary to initiate and perpetuate the inflammation.[22] All this suggests that functional defects of the intestinal epithelial barrier (in terms of both loss of the xenobiotic efflux mechanism and host–bacteria interaction) due to the lack of P-glycoprotein expression are possible reasons for the pathogenesis of colitis.

C3435T polymorphism

In the initial case-control study conducted in Germany by Schwab et al., investigating the C3435T polymorphism, an increase of the T allele and TT genotype frequencies was identified in 149 patients with UC, but not with CD, compared with controls ($p = 0.049$, odds ratio (OR) = 1.4; $p = 0.005$, OR = 2.1, respectively).[23] This finding has been replicated in several studies but in a handful of other works such observation was not evident. Ho et al. in a study conducted in Scotland reported a statistically significant association between UC and a higher frequency of the mutant T allele ($p = 0.02$, OR = 1.28) and of TT genotype ($p = 0.04$, OR = 1.60).[24] Similarly, Farnood et al. observed in the Iranian population a higher risk of UC development for the 3435T allele carriers and 3435TT homozygotes.[25] In contrast, negative findings have been reported in large studies from Germany and the UK,[26] North America,[27] Slovenia,[28] and Italy.[29] Paradoxically, Urcelay et al. found a significant association between the wild CC3435 genotype and CD in Spanish patients ($p = 0.007$), recognizing the 3435T allele as a risk factor for UC, and the 3435C allele as a risk factor for CD.[30] The meta-analysis of 9 studies (1743 UC cases, 2311 CD cases and 2931 controls in total) has confirmed an association between C3435T and UC, with an OR = 1.12 (95% confidence interval (CI): 1.02–1.23). For CD, the pooled ORs were not significant for either the fixed-effect or the random-effects model.[31] Similar results, i.e., a significant association of UC with T allele (OR = 1.17, 95% CI: 1.06–1.31) and TT genotype (OR = 1.36, 95% CI: 1.05–1.76), were obtained in another meta-analysis.[32]

It is worth mentioning that Ho et al. undertook a genotype–phenotype analysis and estimated the risk rate of UC with the proximally spreading lesions (i.e., extensive colitis) as 1.70 ($p = 0.009$) for the 3435T allele carriers and as 2.64 ($p = 0.003$) for the 3435TT homozygotes.[24] Likewise, Ardizzone et al. found Italians carrying the mutant 3435T allele to present a 3-fold increased risk for developing CD with ileocolonic localization as compared to individuals with the wild-type allele.[33]

G2677T/A polymorphism

Investigating another polymorphism of *ABCB1* gene, the tri-allelic *G2677T/A* SNP (Ala893Ser/Thr) in a multicenter North American cohort study, Brant et al. found a significant association of the G2677 allele (Ala893), known to decrease P-glycoprotein function, with IBD.[27] An association with another allele of this SNP, i.e., 2677T (893Ser), was identified in UC patients ($p = 0.029$) by Potočnik et al. in Slovenia.[28] Conversely, Ho et al. did not find any relation between this polymorphism and IBD.[24] In the study by Onnie et al., the 2677T allele was significantly increased in British UC cases compared with controls (45.2% vs 39.6%; $p = 0.034$). In particular, the TT genotype was significantly associated with severe UC (OR = 1.90; 95% CI: 1.01–3.55) and the use of steroids in UC (OR = 1.77; 95% CI: 1.08–2.88).[31] After pooling data from the available studies, the meta-analysis performed by Annese et al. found no significant relation between allele and genotype frequencies of the G2677T/A SNP and UC, as well as between CD and IBD as a whole.[32]

Linkage disequilibrium and heterogeneity considerations

As significant linkage disequilibrium has been observed between 3 SNPs (G2677T/A, C3435T and C1236T) in different populations, a haplotype analysis seems to be

justified in IBD patients. In the study by Ho et al., a 2-locus haplotype (3435T/G2677) was significantly associated with UC (p = 0.03).[24] On the other hand, Urcelay et al. found a trend towards an increased frequency of 2-locus 2677T/C3435 haplotype in CD patients.[30] Onnie et al. did not discover any significant C3435T-G2677T/A haplotype association with either CD or UC.[31] The advantage of haplotype analysis in comparison to analysis of single polymorphisms in complex diseases has been confirmed in the study by Potočnik et al. They found haplotype defined by T–T–T (1236T–2677T–3435T) alleles to be significantly associated with higher risk for refractory CD (p = 0.044, OR = 3.1) and UC (p = 0.026, OR = 1.6), although using each of these SNPs separately resulted in association only at the border of significance.[28]

Reasons for the discrepancy among the abovementioned studies may lie in population heterogeneity, sample size, selection of control population, incomplete phenotype description, and the lack of statistical power to detect the moderate effect size. It has been proven that there is a significant heterogeneity of the allele frequencies in various populations. For example, the frequency of the C3435 allele has been reported as 43–54% in Caucasians, 34–63% in Asians and 73–90% in Africans. The incidence of C/T and C/C3435 genotypes in Africans is much higher than in other racial populations.[34]

Response to pharmacotherapy

Of interest, several drugs central to the therapy of IBD are also P-glycoprotein substrates like glucocorticoids, cyclosporine and methotrexate.[32] The hypothesis that altered P-glycoprotein expression in IBD patients could modify the response to medical therapy was verified by Farrell et al. Compared with controls, the expression of P-glycoprotein in peripheral blood lymphocytes was significantly elevated in patients with CD who required bowel resection and patients with UC who required proctocolectomy for failed medical therapy. However, Farrell et al. did not investigate the role of genetic variants of the *ABCB1* gene.[35] Conversely, Palmieri et al., who compared allele and genotype frequencies in 594 patients using systemic steroids and 297 patients taking immunosuppressive drugs, did not find any influence of both C3435T and G2677T/A polymorphisms on the response to therapy.[29]

Polish population

Two studies have recently been published investigating the importance of C3435T and C1236T polymorphisms in determining IBD susceptibility in a population from central Poland. The 1st, evaluating the C3435T polymorphism, comprised 108 patients with IBD (61 with UC and 47 with CD) and 137 healthy volunteers.[36] The 2nd, evaluating the C1236T polymorphism, comprised 85 patients with IBD (45 with UC and 40 with CD) and 70 healthy volunteers.[37] The identification of the polymorphisms in the *ABCB1* gene was carried out using the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method. In both studies, the observed differences in genotype and allele frequencies were not significant. However, 3435CC genotype and 3435C allele carriers were present more frequently among IBD patients than in controls (OR = 1.72, 95% CI: 0.95–3.12 and OR = 1.35, 95% CI: 0.94–1.93, respectively).[36] In parallel, 1236CT genotype and 1236T allele carriers were more frequent in IBD patients compared to controls (OR = 1.26, 95% CI: 0.66–2.42 and OR = 1.08, 95% CI: 0.41–2.14, respectively).[37] Neither G2677T/A polymorphism nor the impact of G2677T/A, C3435T and C1236T polymorphisms on disease behavior and response to therapy in IBD have been explored so far in Poland. It is worth noting that even in our country a substantial heterogeneity of the allele frequencies in relation to the *ABCB1* gene has been observed. For example, the frequency of the 3435CC, 3435CT and 3435TT genotype was 23.3%, 56.3% and 20.4%, and 42.0%, and 41.0% and 17.0%, respectively, in subjects originating from Western Pomerania and Łódź region.[38,39] There has been so far no study determining the importance of the polymorphisms of the *ABCB1* gene in pediatric onset IBD. It seems equally challenging to evaluate the *ABCB1* gene expression in the tissues of the gastrointestinal tract, because, as was stated by Yacyshyn et al., intra-epithelial, lamina propria and peripheral blood lymphocytes may demonstrate different gene expression and activity of its product, P-glycoprotein.[40]

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