Advances in Clinical and Experimental Medicine

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

2019, Vol. 28, No. 3 (March)

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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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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"Enhancing the scientific standards and internationalization level of published scientific journals, and improving the level of information dissemination on scientific research outcomes or development work results" – project financed based on agreement No. 915/P-DUN/2019 from the resources of Ministry of Science and Higher Education allocated to science dissemination activities.

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Ministerstwo Nauki i Szkolnictwa Wyższego

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
Cover: Monika Kolęda
Printing and binding: EXDRUK

Advances
in Clinical and Experimental Medicine

MONTHLY 2019, Vol. 28, No. 3 (March)

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

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Early propranolol treatment ameliorates endothelial dysfunction in experimental septic lung

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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*. 2019;28(3):291–297

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Funding sources
This study was funded by the Scientific Research Project Unit of Çukurova University, Balcalı, Turkey.

Conflict of interest
None declared

Received on July 7, 2017
Reviewed on July 31, 2017
Accepted on October 4, 2017

Published online on July 3, 2018

Cite as
Özyılmaz E, Büyüknacar HSG, Bağır EK, et al. Early propranolol treatment ameliorates endothelial dysfunction in experimental septic lung. *Adv Clin Exp Med*. 2019;28(3):291–297. doi:10.17219/acem/78248

DOI
10.17219/acem/78248

Abstract

Background. Recent reports have indicated an improved prognosis in sepsis with β-blocker agents; however, the underlying action mechanism is still under debate.

Objectives. The aim of this study was to investigate the potential effect of propranolol on endothelial dysfunction in septic rats.

Material and methods. The cecal ligation and puncture model (CLP) was used to generate sepsis. Adult male Wistar-Albino rats were divided into 4 groups: group 1 was a sham group, group 2 received sterile saline, group 3 received 10 mg/kg of propranolol 3 days before the intervention, and group 4 received 10 mg/kg of propranolol 30 min after CLP. Six rats from each group were sacrificed 24 h postoperatively. The remaining rats were followed for survival. We have also evaluated the effects on systemic inflammation, coagulation and the lung tissue with immunohistochemical and electron microscopic evaluation.

Results. Serum tumor necrosis factor alpha (TNF-α) and plasminogen activator inhibitor-1 (PAI-1) levels, as well as tissue TNF-α scores were elevated in septic rats. Electron microscopic examination of the lung tissue showed endothelial dysfunction in the sepsis group. Pretreatment significantly improved survival. Moreover, pre-treatment altered serum vascular endothelial growth factor receptor-1 (VEGFR-1) levels and post-treatment reduced serum PAI-1 and VEGFR-1 levels. In both the pre- and post-treatment groups, electron microscopic examination revealed improvement of the destroyed lung endothelium and showed only mild alterations in the cytoplasmic organelles, especially in the mitochondria of the endothelial cells.

Conclusions. These results suggest that the improved outcome with β-blockers in sepsis may be due to the ameliorated endothelial dysfunction. Further studies focusing on the potential effect of β-blockers on the endothelium may lead to a better understanding of sepsis.

Key words: sepsis, endothelial dysfunction, experimental, β-blockers

Introduction

Sepsis is a leading cause of mortality and morbidity.[1] Although its precise pathogenetic mechanisms are not fully understood, it is now clear that endothelial activation and dysfunction lead to persistent microvascular dysfunction and related multi-organ failure.[2] However, the underlying mechanism of endothelial dysfunction in sepsis is still unknown.

The adrenergic system is not only a key modulator of organ function and cardiovascular homeostasis, but also a powerful regulator of the immune system. Both the lymphoid organs and the vast majority of lymphoid cells express β-adrenergic receptors on their surfaces.[3] Sympathetic activation is a well-known feature of sepsis, essential for cardiovascular and metabolic compensation. In addition, sympathetic activation may mediate immune modulation in sepsis.[4] Adrenergic activation induces monocyte production and monocyte-mature macrophage differentiation in bone marrow via β2 receptors that are functionally different in cytokine response.[5,6] Although plasma catecholamine levels are increased during inflammation and sepsis, it has been shown that persistent elevations due to excessive stimulation lead to β-adrenoceptor downregulation and immune suppression through the modulation of cell-mediated immunity.[7,8] In a clinical study, Boomer et al. showed clear immune suppression in patients who died following sepsis.[9] Today, β-blockers are one of the mainstay of treatments of coronary artery disease and chronic heart failure. Also, recent studies have demonstrated that β-blockers might have a novel therapeutic potential against several hypermetabolic states, such as sepsis or burn injury, by attenuating catecholamine-induced metabolic alterations and excessive inflammatory responses.[10] However, the underlying mechanism of action is still under debate.

The aim of this study was to investigate the potential effect of propranolol, a non-selective β-blocker, on endothelial dysfunction in septic rats. Toward the realization of this goal, we treated rats with propranolol before and after lethal sepsis. We evaluated the effects of this treatment strategy on systemic inflammation, coagulation and the lung tissue with immunohistochemical and electron microscopic evaluation, and on survival.

Material and methods

The study protocol was approved by the animal research ethical committee of the institute and all experiments were performed according to the rules of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996).

Drugs and the experimental model

The propranolol was obtained from Sigma Laboratories (P-0884) (Sigma-Aldrich, St. Louis, USA). Experimental sepsis was induced with cecal ligation and puncture (CLP). After 80 mg/kg of ketamine + 5 mg/kg of xylazine anesthesia, a midline laparotomy was performed, and the cecum was ligated at the 75% proximal part of the ileocecal valve and thoroughly perforated with an 18-gauge needle. The cecum was then relocated into the abdominal cavity, and the peritoneum, fasciae and abdominal muscles were closed by simple interrupted sutures.[11]

Animals and the experimental schedule

Forty-two adult male Wistar-Albino rats weighing 300 ±25 g were procured from the experimental animal laboratory of our institute. They were maintained on a standard diet and given water ad libitum under appropriate conditions. The animals were randomly divided into 4 groups as follows: group 1 was the sham group (n = 6), in which laparotomy was performed without the induction of sepsis with CLP; group 2 was the septic control group (n=12), which was twice injected intraperitoneally (i.p.) sterile saline (the same volume as propranolol) for 3 consequtive days before CLP; group 3 was the pre-propranolol group (n = 12), which was given an i.p. dose of 10 mg/kg of propranolol for 3 consecutive days before CLP; and group 4 was the post-propranolol group (n = 12), which was administered i.p. a dose of 10 mg/kg of propranolol 30 min following CLP. The abovementioned doses of either propranolol or saline were selected according to a previous study and were administered every 12 h for both the pre-and post-propranolol groups.[12] Cecal ligation and puncture was performed in all groups as previously described, except group 1 (sham), and on the 3rd day of the experiment and 1 h after the last injection, all animals were given i.p. 2 ml/100 g of pre-warmed (37°C) sterile saline. After CLP, survival analysis was initiated and the animals were observed each hour over the subsequent 120 h for mortality, and the survival time was recorded. On the 4th day, 24 h after the last CLP, 6 animals from each group were re-anesthetized and blood samples were obtained; the animals were sacrificed with cardiac puncture, and the lung tissue was immediately excised and divided in half for immunohistochemical analysis and electron microscopic evaluation. The samples were kept at –80°C under appropriate conditions until analyzed. The remaining animals underwent survival follow-up.

Biochemical assay

The plasma levels of rat tumor necrosis factor alpha (TNF-α) (Ref. No. BMS622; e-Bioscience, Vienna, Austria), plasminogen activator inhibitor-1 (PAI-1) (Ref. No. DZE201110637; Sunred, Shanghai, China), vascular

endothelial growth factor (VEGF) (Ref. No. DZE201110660; Sunred), and VEGF receptor-1 (VEGFR-1) (Ref. No. E20150604052; Hangzhou, China) were measured with standard sandwich enzyme-linked immunosorbent assay (ELISA) methods, and the results were presented in pg/mL for TNF-α, AU/mL for PAI-1, ng/L for VEGF, and ng/mL for VEGFR-1.

Light microscopy and immunohistochemical examination

In order to investigate lung tissue inflammation due to sepsis, immunohistochemical staining was performed on 5 µm sections of formalin-fixed, paraffin-embedded tissue, using antibody to TNF-α (ab6671; Abcam, Cambridge, UK) and interleukin (IL)-6) (ab6672; Abcam, Cambridge, UK). The visualization system used was BenchMark XT with heat-induced epitope retrieval (CC1 solution; Ventana Medical Systems, Inc., Tucson, USA) and the iView DAB detection kit (Ventana). Slides stained with anti-TNF-α and anti-IL-6 were examined in 5 high-power microscopic fields (×400 magnification; Nikon-ECLIPSE 80I; Nikon, Tokyo, Japan). Immunohistochemical evaluation was performed by the histological score (HSCORE) system. The HSCORE was calculated using the following equation: HSCORE = _Pi (i+1), where i is the intensity of labeling with a value of 1,2 or 3, and Pi is the percentage of the labeled cells, varying from 0 to 100%.

Electron microscopy

For electron microscopic examination, the lung tissue samples were fixed in 5% glutaraldehyde solution for 4 h. The tissue samples were then washed twice in Millonig phosphate buffer for 10 min, then postfixed in 1% osmium tetraoxide (OsO_4) for 2 h and washed in a buffer solution for 10 min to remove the OsO_4. Then, the tissue samples were dehydrated in graded ethanols. For clearing, the tissue samples were put in propylene oxide, embedded in araldite and polymerized at 64°C for 48 h. Tissue sections 50 nm in thickness were obtained using Reichert Ultracut-S ultramicrotome (Leica, Wetzlar, Germany), and the sections were stained with uranyl acetate and lead citrate. After staining, they were examined with a Jeol-JEM 1400 transmission electron microscope (Jeol, Ltd., Tokyo, Japan).

Statistical analysis

For each continuous variable, normality was checked by the Kolmogorov-Smirnov and Shapiro-Wilk tests, and by histograms. The comparisons between groups were conducted using Student's t-test or the analysis of variance (ANOVA) for normally distributed data, and the Mann-Whitney U test and Kruskal-Wallis test were used for the data not normally distributed. Bonferroni correction was applied for multiple comparisons. The rates of mean or median survival were estimated with the use of the Kaplan-Meier method and the curves were compared with the use of the log-rank test. Data was expressed as mean ± standard deviation (SD) and as median (min–max); p-value <0.05 was considered statistically significant. All reported p-values are 2-tailed. Statistical analysis was performed using the statistical package SPSS v. 20.0 (IBM Corp., Armonk, USA).

Results

Pre-propranolol treatment improved the overall survival in septic rats

Pretreatment of septic rats with propranolol significantly increased survival hours (120 h) compared to the septic control group (mean: 28 h; median: 14 h) and post-propranolol group (mean: 38.9 h; median: 29.5 h) (log-rank test; p = 0.003) (Fig. 1). The mortality rate reached 83.7% at the 17th h in the septic controls (5 dead) and it reached the same rate (83.7%) later (at the 62nd h) in the post-propranolol group (6 dead). The mortality rate was prominently lower (16.3% at the 120th h) in the rats which received propranolol before the induction of sepsis (only 1 dead). None of the animals in the sham group died.

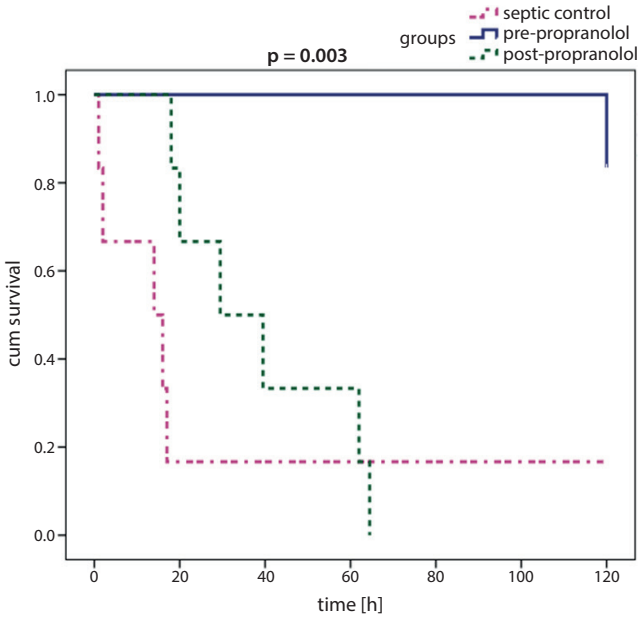

Fig. 1. Cumulative survival curves according to study groups

Pre-treatment of septic rats with propranolol significantly increased survival hours (120 h) compared to the septic control and post-propranolol groups.

Propranolol improved serum inflammatory and endothelial markers

Serum TNF-α and PAI-1 levels were elevated in septic rats in comparison to the sham group ($p = 0.036$ and $p = 0.006$, respectively). Pre- or post-propranolol treatment failed to decrease serum TNF-α levels; pretreatment altered serum VEGFR-1 levels ($p = 0.037$) and post-treatment reduced serum PAI-1 levels ($p = 0.013$) and serum VEGFR-1 levels ($p = 0.016$) (Table 1).

Light microscopy and immunohistochemical evaluation

The TNF-α score of the lung tissue was significantly higher in septic rats compared to the sham group ($p < 0.05$). Both pre- and post-propranolol treatment failed to improve lung immunohistochemical results (Table 2, Fig. 2).

Electron microscopy

Electron microscopic examination of the lung tissue of the sham group revealed a normal histological appearance in which the nucleus, cytoplasmic organelles and the underlying basal lamina of the capillary endothelial cells preserved their structure. In contrast, the septic control group showed an increased amount of heterochromatin in the nuclei of the capillary endothelial cells, which were located in the interalveolar septum. Furthermore, there was increased vacuolization in the cytoplasm of the endothelial cells. This group also displayed enlarged mitochondria and distractions in the crista of the mitochondria. There were surfactant-like structures and phagocytic bodies in the cytoplasm of the macrophages. It is noteworthy that the amounts of collagen fibers were increased, along with the interalveolar septum. Moreover, it was observed that the interalveolar septum became thickened in this group.

In the pre-propranolol treatment group, it was observed that the capillary endothelial cells preserved their fine structures in many areas. However, it was seen in some areas that the amount of heterochromatin increased in the nuclei of the endothelial cells and there were mild alterations in the mitochondria. The macrophages were noticeably higher in number in the interstitium and they had phagocyte surfactant-like structures.

In the post-propranolol treatment group, electron microscopic examinations showed that the capillary endothelial cells and the underlying basal lamina preserved their normal structures in many areas, as in the pre-propranolol group. However, in some areas there were mild alterations in the cytoplasmic organelles, especially in the mitochondria of the endothelial cells. Enlarged mitochondria and fragmentation were found in the crista of the mitochondria (Fig. 3).

Discussion

The present study showed that systemic administration of the non-selective β-adrenergic blocker propranolol

Table 1. The distribution of the TNF-α, PAI-1, VEGF, and VEGFR-1 levels between the groups

Biomarker	Group 1 sham	Group 2 saline	Group 3 pre-propranolol	Group 4 post-propranolol	p-value§
Serum TNF-α [pg/mL]	70.8 ±6.8 71.8 (58–77)	193 ±276* 82 (75–757)	76.7 ±1.9 76.2 (75–81)	100.5 ±3.4 99 (97–106)	**0.030**
Serum PAI-1 [AU/mL]	14.5 ±1.6 14.1 (13–18)	18.9 ±2.3* 18 (16–23)	19.8 ±3.2 19.2 (16–26)	15 ±2.5*** 15.8 (10–18)	**0.021**
Serum VEGF [ng/L]	631 ±56.9 612 (564–707)	652 ±212 697 (290–920)	742 ±114 758.9 (568–884)	865 ±336 783.7 (401–1376)	0.147
Serum VEGFR-1 [ng/mL]	15.7 ±2.1 16.4 (11.7–17.8)	17.9 ±1.6 18.2 (15.1–19.4)	14.4 ±3.0** 13.7 (11.6–19.1)	13.9 ±3.0*** 14.1 (9.8–17.3)	0.053

PAI-1 – plasminogen activator inhibitor-1; TNF-α – tumor necrosis factor alpha; VEGF – vascular endothelial growth factor; VEGFR-1 – vascular endothelial growth factor receptor-1; § the Kruskal-Wallis test between 4 groups; * $p < 0.01$ between sham and sepsis; ** $p < 0.01$ between sepsis and pre-propranolol; *** $p < 0.01$ between sepsis and post-propranolol; the values are expressed as mean ± standard deviation (SD) and as median (min–max); values in bold are statistically significant.

Table 2. Immunocytochemical HSCORE staining among the groups

Immunocytochemical parameters	Group 1 sham	Group 2 saline	Group 3 pre-propranolol	Group 4 post-propranolol	p-value§
TNF-α score median (min–max)	22 (2–39)	130* (119–192)	205** (180–245)	139 (112–261)	**0.002**
IL-6 score median (min–max)	163 (125–205)	185 (145–235)	218 (190–255)	218 (155– 260)	0.069

IL-6 – interleukin-6; HSCORE – histological score; TNF-α – tumor necrosis factor alpha; § the Kruskal-Wallis test between 4 groups; * $p < 0.05$ between sham and sepsis; ** $p < 0.05$ between sepsis and pre-propranolol; values in bold are statistically significant.

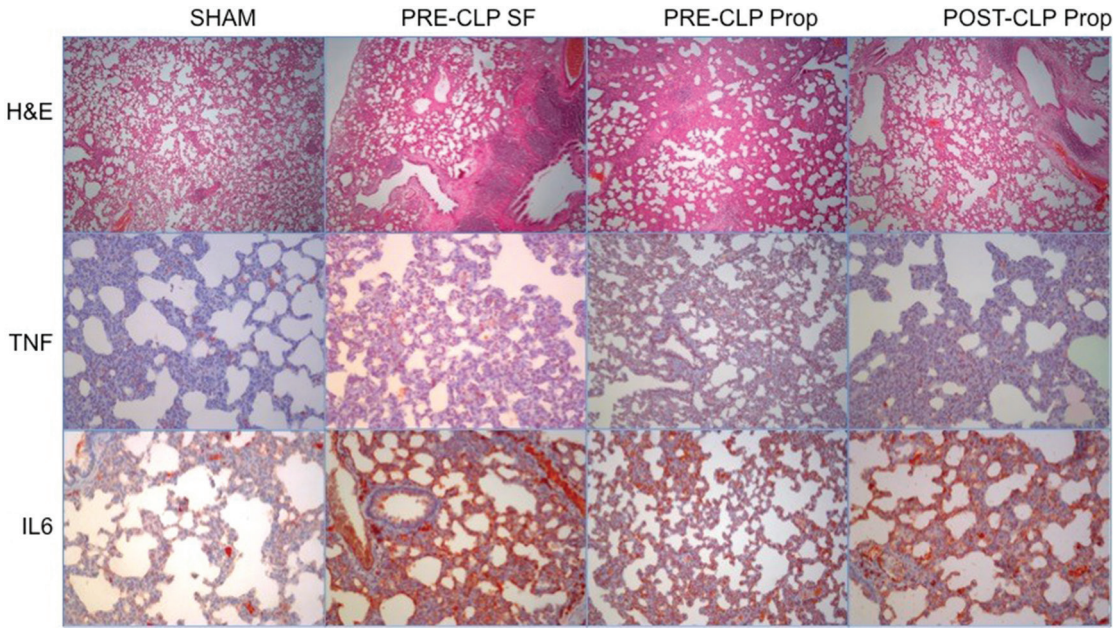

Fig. 2. Immunohistochemical staining of IL-6, TNF-α, and H&E sections of the lung tissue from each group

SHAM – sham group; PRE-CLP SF – saline group; PRE-CLP Prop – pre-propranolol group; POST-CLP Prop – post-propranolol group; CLP – cecal ligation and puncture; H&E – hematoxylin and eosin; IL-6 – interleukin-6; TNF-α – tumor necrosis factor alpha; Prop – propranolol; SF – saline.

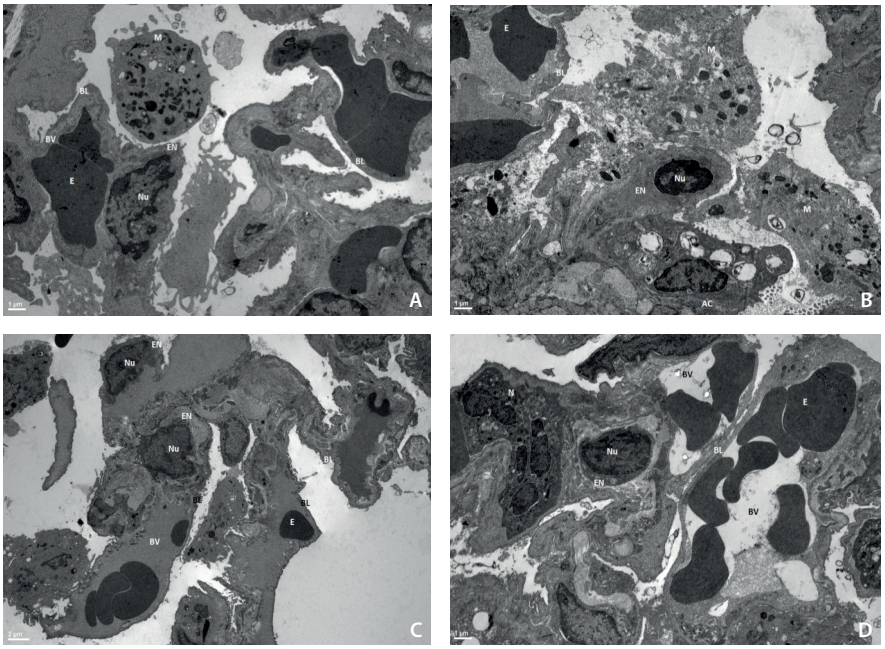

Fig. 3. Electron microscopic examination of the lung tissue of the animals

A – electron microscopic view from the sham group. The capillary endothelial cells preserved their normal structure in the nuclei and cytoplasmic organelles. Bar: 1 µm; B – electron microscopic view from the septic control group. There was an increase in the amount of heterochromatin in the nucleus of the endothelial cells. There were phagocytic bodies in the cytoplasm of the macrophages. Bar: 1 µm; C – electron microscopic view from the pre-propranol group. The capillary endothelial cells and basal lamina preserved their ultrastructure in many areas. However, in some areas there was an increase in the amount of heterochromatin in the nuclei of the endothelial cells. Bar: 2 µm; D – electron microscopic view from the post-propranol group. The capillary endothelial cells and basal lamina preserved their normal structure in many areas. Bar: 1 µm.

AC – type 2 alveolar cell; BL – basal lamina of capillary endothelial cell; BV – blood vessel; E – erythrocyte; EN – endothelial cells; M – macrophage; N – neutrophil; Nu – nuclei.

exerts a protective effect on systemic inflammation, coagulation and survival when applied before the septic insult. To the best of our knowledge, we showed for the first time that the underlying mechanism of the action of propranolol in sepsis may be the improvement of endothelial function as well.

The idea of administering β-blockers in sepsis is not novel. In the first study, which was performed in the late 1960s, Berk et al. documented a favorable survival rate with no pathological changes of pulmonary congestion in septic dogs with the use of propranolol.[13] Since then, there has been growing interest in the use of β-blockers in sepsis, with a number of mostly experimental studies being published; however, the mortality data is contradictory.[10,12,14–16] This is possibly due to the inconsistency in animal sepsis models, the receptor specificity of the selected agents, the dosage, and the timing of treatment.[17] In a previous report, Ackland et al. showed that metoprolol and atenolol markedly improved survival and reduced inflammation when initiated 48 h before sepsis. However, the favorable survival effect disappeared in this study when the β-blockage was implemented 6 h after the induction of sepsis.[14] In another report, Wilson et al. verified a similar effect with propranolol when administered

30 min prior to and 24 h after sepsis.[12] The results of the present study are in accordance with the previous experimental reports, which showed enhanced survival with the prior administration of β-blockers in sepsis. This endpoint is also supported by 2 clinical studies. In the first report, Macchia et al. studied the database of 9,465 patients who were hospitalized in critical care units for sepsis, and despite a higher risk profile, the patients previously prescribed β-blockers had a lower mortality rate after 28 days (odds ratio (OR): 0.78; 95% confidence interval (CI): 0.66–0.93; p = 0.005).[18] The only randomized clinical trial which was performed with esmolol also confirmed these results, although its findings have been criticized, particularly due to the unexplicably high mortality in the control group.[19] Currently, clinical trials are ongoing to elucidate this inconsistent clinical data.

The physiological rationale behind the clinical application of β-blockers in sepsis was firstly based on the cardiovascular modulation effect.[13,20] Later, various mechanisms of action, including inflammatory, metabolic and coagulation effects, have been proposed.[3] Another notably logical reason for the use of β-blockers in sepsis is catecholamine-derived endothelial dysfunction.[21] A growing body of evidence showed that β-blockers counteract the adrenergic storm in sepsis by modulating cytokine profile production and modulate the coagulation system.[3–5,22] Specifically, propranolol was reported to suppress the catecholamine-driven increase in natural killer cells and result in a marked decrease in the production of chemokine (C-C motif) ligand 3 (CCL3), which is a macrophage-derived inflammatory protein that facilitates the clearance of the pathogen.[23,24] Herein, we report that β-blocker treatment, either administered prior to or after the induction of sepsis, significantly suppressed serum levels of VEGFR-1, which plays a key role in activating monocytes/macrophages. Besides, our results demonstrate that propranolol treatment, when administered after septic insult, alleviates the PAI-1 levels. Plasminogen activator inhibitor-1 is a well-known crucial glycoprotein of hemostasis synthesized by the endothelium and the liver, and is incrementally released during inflammation. Further evaluation of the potential effect of β-blockers on inflammation and the coagulation system might help to find a potential pharmacological strategy for the treatment of sepsis.

Interestingly, a huge amount of previous evidence has yielded potential favorable effects of β-blockers on the endothelium in non-septic conditions.[25–28] In a previous report, Jawa et al. showed that β-blockers had a beneficial effect on endothelial function in patients with diabetes and hypertension.[25] Also, Joseph and Levine reported that β-blockers induced anti-hyperalgesia via the endothelial cells, which has been implicated in diverse pain syndromes, such as migraines.[29] Later, Perros et al. indicated that β-blockage improves endothelial function in pulmonary arterial hypertension.[30] Additionally, it is an effective treatment modality in infantile hemangioma, whose potential mechanism of action has been suggested to be a combination effect on the endothelium.[31] Recently, a meta-analysis which included 16 clinical studies with 1,273 patients with cardiovascular disease clearly confirmed a potential ameliorating effect of β-blockers on endothelial dysfunction.[32]

On the other hand, data which focuses on the effect of β-blockers on the endothelium in sepsis is extremely limited. In a recent experimental study, Jacquet-Lagrèze et al. investigated the effect of esmolol on gut and sublingual microcirculation after the septic insult, and they reported that esmolol allowed a better maintenance of gut microcirculation despite a reduction in stroke index scores.[33] In the first pilot clinical study to specifically investigate the microcirculatory effects in septic shock patients with side stream dark field imaging, Morelli et al. demonstrated the preserving influence of esmolol on the microvascular flow.[34] However, various β-blockers may have different effects due to fitful receptor specificity, and to the best of our knowledge, no previous report has focused on the effect of propranolol on endothelial dysfunction. Our results are principally in agreement with previous reports, which demonstrated that propranolol treatment may alleviate endothelial dysfunction.

Several limitations of this study must be taken into account. First of all, one can propose that prior administration of propranolol may not be feasible due to the inconsistency of developing sepsis in clinical practice. However, this may be essential in high-risk groups, such as surgery or burn patients, in whom sepsis development risk and its related mortality are expected to be high. Secondly, we did not perform hemodynamic monitoring. Although propranolol treatment is expected to reduce the heart rate or cardiac output, we selected a dosage which is 30% less than that applied in a previous study, which showed no hemodynamic compromise.[16] Moreover, we had formerly performed a pilot study to test the potential fatal effect of the propranolol administration itself and we did not observe any mortality. Owing to the fact that we succeeded in showing the improved endothelial function and survival, we think that the lack of hemodynamic monitoring did not significantly affect our results. However, further studies with higher doses may better show the survival advantage in post-propranolol groups. Although we evaluated endothelial function in terms of barrier function, inflammation and hemostasis, we could not evaluate glycocalyx due to the collapse of the tissue during processing.

Conclusions

This study documented a significant survival advantage for septic rats who were given a non-selective β-blocker prior to the septic insult. This outcome benefit may be due to the ameliorated endothelial dysfunction or to previously described effects. Further experimental and clinical studies, which may lead to a full understanding of the

underlying mechanisms by which propranolol exerts its effects on sepsis, should not only allow for new treatment options, but may also improve survival.

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The effect of silver nanoparticles on the reproductive system of adult male rats: A morphological, histological and DNA integrity study

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):299–305

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Funding sources
The study was funded by vice-chancellor for Research and Technology, Hamadan University of Medical Sciences, Iran (No. 9205011568).

Conflict of interest
None declared

Acknowledgements
This study has been adapted from an MSc thesis at Hamadan University of Medical Sciences, Iran. The authors would like to thank Dr. Hajilui for his technical assistance.

Received on September 1, 2016
Reviewed on March 25, 2017
Accepted on December 28, 2017

Published online on August 29, 2018

Cite as
Fathi N, Hoseinipanah SM, Alizadeh Z, et al. The effect of silver nanoparticles on the reproductive system of adult male rats: A morphological, histological and DNA integrity study. *Adv Clin Exp Med*. 2019;28(3):299–305. doi:10.17219/acem/81607

DOI
10.17219/acem/81607

Abstract

Background. Silver nanoparticles (AgNPs) are more often used in various products, and consequently the potential deleterious effects associated with exposure to them are of concern. Several lines of evidence have demonstrated that the toxicity of AgNPs affects different organs and leads to some side effects, including weight loss, inflammation and cell death.

Objectives. The aim of this study was to evaluate the effect of different concentrations of AgNPs on sperm parameters and testicular histology.

Material and methods. In the present study, 28 male adult Wistar rats were categorized into a control group and 3 experimental groups (AgNP-1, AgNP-2 and AgNP-3), intraperitoneally (i.p.) receiving 30, 125 and 300 mg/kg of AgNPs, respectively. Twenty-eight days after injection the epididymes and the testes of each rat were dissected in order to evaluate sperm parameters, sperm chromatin integrity and histomorphometric changes in the testicular tissue.

Results. The results showed a significant decrease in sperm count ($p < 0.0001$), vitality ($p < 0.05$) and morphology changes ($p < 0.001$) in the group receiving 300 mg/kg of AgNPs compared to the control group. A significant decrease was also observed in the number of spermatogonia, Sertoli and Leydig cells in the AgNP-2 and AgNP-3 groups ($p < 0.05$). The evaluation of sperm chromatin did not show any significant differences among the experimental groups ($p > 0.05$).

Conclusions. The data showed some dose-dependent adverse effects of AgNPs on sperm and seminiferous tubules. More experimental investigations are necessary to draw better conclusions regarding the safety of nanoparticles (NPs) on the male reproduction system.

Key words: testis, sperm chromatin, sperm parameters, histomorphometry, silver nanoparticles

Introduction

Silver nanoparticles (AgNPs) are clusters of silver atoms (varying from 1 to 100 nm in diameter) that are used as antibacterial and antimicrobial agents in clinical medicine. Silver nanoparticles are integrated into various food-contact materials, such as plastics used to manufacture food containers, refrigerator surfaces, storage bags, and chopping boards, and are used to preserve foods by inhibiting microorganism growth.[1,2] Evidence has shown that AgNPs have a potent anti-inflammatory effect.[3] Silver nanoparticles are of great importance in the treatment of diseases.[4]

Regardless of their widespread utility, the influence of AgNPs on human health and the mechanisms of their action are not fully understood. Therefore, it is important to survey their potential toxicity in living organisms, especially in mammals, in order to provide a reliable human risk assessment.[4] Nanoparticles (NPs) like silver can induce reactive oxygen species (ROS) production, which causes oxidative stress and toxicity in various cell types.[5,6] Silver nanoparticles can pass through cell membranes, the blood-brain barrier and the blood-testis barrier.[7] The main target organs for AgNP deposition after systemic availability are the spleen, liver and kidneys, while there is less distribution to other organs. Additionally, high levels of silver have sometimes been found in the testes.[5,6]

The effects of AgNPs on testis and sperm function have been reported. Pothuraju and Kaul reported the effects of these NPs on buffalo sperm parameters, which showed a dose-dependent decrease in sperm viability without a change in sperm motility at a concentration of 50 mg/kg.[8]

However, NPs must be investigated on a case-by-case basis to determine whether an NP will have a positive or negative effect on spermatogenesis, because it is dependent on the chemical composition, size, treatment period, dose level, route, duration of exposure, and recovery time.[9,10]

Therefore, the main purpose of the present study was to evaluate the effect of different concentrations of AgNPs on sperm parameters and chromatin, and testicular histomorphometry in rats.

Material and methods

Nanoparticle preparation for analysis

We prepared AgNPs as described previously.[11] Silver nanoparticles were purchased from US Research Nanomaterials, Inc. (Houston, USA). Based on the specifications provided by the manufacturer, the AgNPs were of 99% purity as a nanopowder, with a nominal diameter of 60–80 nm. The AgNPs were suspended by sonication in deionized water (TKA Fisher Scientific Smart2Pure 30123; Thermo Fisher Scientific, Burladingen, Germany), and then diluted to concentrations of 30, 125 and 300 mg/kg at the time of injection.

Nanoparticle characterization

The size distribution of AgNPs was measured by dynamic light scattering (DLS) (Zetasizer Nano ZS apparatus ZEN 3600; Malvern Panalytical, Malvern, UK) at 25°C and was started 2 min after the cuvette was placed in the DLS apparatus to reach thermal equilibrium. Measurements were conducted 24 h after the suspensions were prepared.[11,12] A Philips-EM 208 transmission electron microscopy (TEM) and a scanning electron microscope (SEM) (Philips, Amsterdam, the Netherlands) were also employed to monitor the size and morphological changes in AgNPs.

Animals

Twenty-eight adult male Wistar rats weighing 200–250 g were kept under standard conditions (12-hour light/dark cycle at 22–24°C, with free access to water and food). All of the procedures were carried out with minimal stress and discomfort in accordance with national guidelines and protocols approved by the Institutional Animal Ethics Committee of Hamadan University of Medical Sciences, Iran.

Experimental design

The animals were weighed and randomly divided into 4 groups (n = 7 per group): a control group and 3 experimental groups (AgNP-1, AgNP-2 and AgNP-3), receiving a single intraperitoneal (i.p.) dose of AgNPs at 30, 125 and 300 mg/kg concentrations, respectively. The animals in the control group were injected with distilled water. The animals were sacrificed 4 weeks after the injection. In rats, the duration of the spermatogenesis cycle is approx. 48–56 days. A half of this cycle and 2 cycles of the seminiferous epithelium takes 28 days.[10,13]

Sperm collection and count

After 28 days, the left cauda epididymis of each animal was dissected and placed in a Petri dish containing Ham's F10 medium (Sigma-Aldrich, St. Louis, USA). The dishes were placed in the incubator for 15 min. Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of a hemocytometer and allowed to stand for 5 min. The cells which settled during this time were counted by a light microscope (Zeiss, Munich, Germany) at ×200 magnification.[13]

Sperm morphology

The sperm morphology was also determined as described previously.[14] Morphological abnormalities were classified as headless sperm, a flattened head, a reduced hook or banana head, a pin or nail head, a bent neck, a bent tail, a kinked tail, and multiple abnormalities. The percentage

of normal morphology of 100 spermatozoa per rat was assessed by light microscopy.

Sperm motility

Aliquots of the sperm suspension prepared for analysis were placed on a slide. The spermatozoa were classified as motile or immotile. The percentage of motility was evaluated for each of the animals used.[15]

Sperm viability

The sperm viability was also determined using eosin stain as described before.[14] Eosin penetrated non-viable, dead spermatozoa with disrupted membranes, which appeared stained in red.

Histological evaluation

The left testes were removed and fixed in a 10% buffered formalin solution for 2 weeks, and were then embedded in paraffin. The paraffin blocks were cut into 5-micrometer slices. The sections (3 per animal) were stained with hematoxylin and eosin (H&E).[10] Twenty-five seminiferous tubules from each testis were randomly evaluated on circular cross-sections, and the spermatogonia, spermatocyte, spermatid, and Leydig and Sertoli cells were counted.

Parameters of seminiferous tubules

For each set of 25 seminiferous tubules from each testis, the parameters, including area, circumference and diameter, were determined under a light microscope equipped with Motic Moticam 2000 2.0M Pixel camera (Motic, Kowloon, Hong Kong).[10]

Evaluation of sperm nuclear chromatin

For the evaluation of sperm nuclear chromatin, 3 different techniques were applied.

Aniline blue staining

Aniline blue (AB) selectively stains lysine-rich histones and has been used for distinguishing sperm chromatin condensation anomalies. For this purpose, air-dried smears were prepared from fresh sperm samples of each rat and fixed in 3% buffered glutaraldehyde in a 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min. For light microscopy evaluation, 100 spermatozoa were counted in each slide and pale-blue spermatozoa were considered normal, while dark-blue-stained ones were treated as abnormal spermatozoa.[13]

Toluidine blue staining

Toluidine blue (TB) is a metachromatic dye which measures the rate of sperm nuclear chromatin condensation via binding to phosphate groups of DNA strands. For this staining, air-dried sperm smears were fixed in fresh 96% ethanol:acetone (1:1) at 4°C for 30 min, and then hydrolyzed in 0.1 N hydrochloric acid (HCl) at 4°C for 5 min. Next, the slides were rinsed thrice in distilled water for 2 min, and finally stained with 0.05% TB for 10 min. The component of staining buffer was 50% citrate phosphate (pH 3.5).[16] For light microscopic assessment, using ×100 magnification, the chromatin quality of the spermatozoa was determined according to metachromatic staining of sperm heads in light blue (TB–) and purple (TB+).

Acridine orange staining

Acridine orange (AO) is a fluorescence probe for measuring the susceptibility of sperm nuclear DNA to in situ acid-induced denaturation. Sperm DNA integrity was determined by AO staining. For this purpose, the smears were first air-dried and then fixed overnight in Carnoy's solution (methanol:glacial acetic acid, 3:1). Each sample was stained for 10 min in freshly prepared AO (0.19 mg/mL) in citrate phosphate buffer (pH 2.5) for 10 min. The smears were evaluated on the same day with an Olympus fluorescence microscope (Zeiss) with a 460-nanometer filter. The duration of illumination was limited to 40 s per field. The percentage of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample was calculated.[16]

Statistical analysis

Statistical analysis was performed using SPSS v. 16 (SPSS Inc., Chicago, USA) and the variables were analyzed by one-way analysis of variance (ANOVA), the Kruskal-Wallis test and the Mann-Whitney U test. The Tukey method was used for the following multiple comparison tests. All data was expressed as mean ± standard deviation (SD). The statistical level of significance was set at $p < 0.05$.

Results

Characterization of silver nanoparticles

As previously mentioned, the DLS analysis demonstrated a broad hydrodynamic diameter peak with an average size of 250 nm (nominally <100 nm in diameter, but actually ~250 nm) (Fig. 1).[11] The diameters of NPs observed under TEM were in agreement with the DLS results (Fig. 2).[17] According to the SEM micrographs, the AgNPs were almost spherical (Fig. 3), but had a tendency to agglomerate or aggregate in the solution.

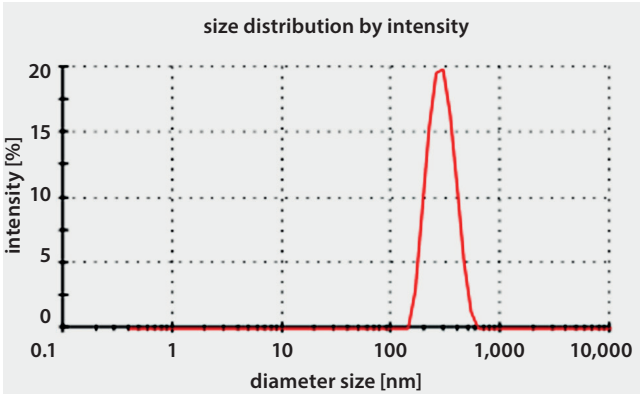

Fig. 1. Dynamic light scattering results of the characterization of nanosilver used in the study

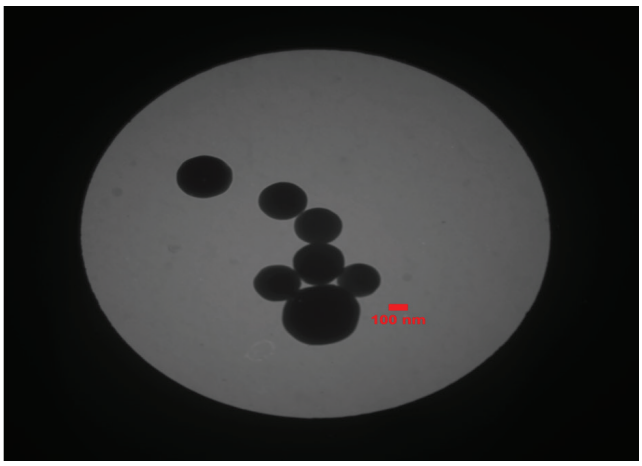

Fig. 2. Transmission electron microscopy (TEM) image of silver nanoparticles (AgNPs); scale bars indicate 10 nm

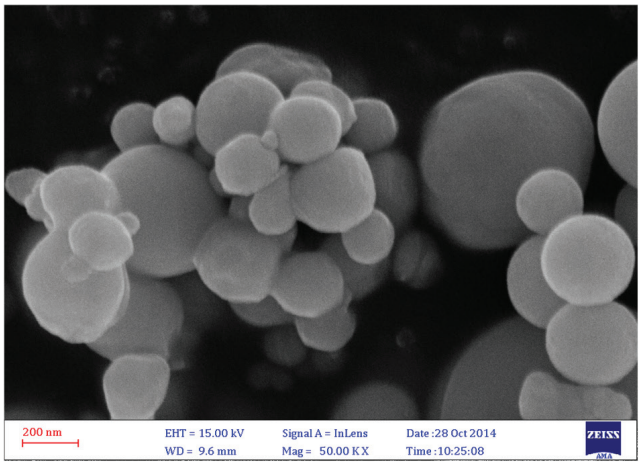

Fig. 3. Scanning electron microscopy (SEM) image of silver nanoparticles (AgNPs); bars indicate 200 nm

Body weight

There were no significant differences ($p > 0.05$) in mean body weight between the treated groups and the control group (data not shown).

Sperm parameters

Table 1 shows the effect of AgNPs on sperm parameters. A significant reduction in sperm vitality ($p < 0.05$), normal sperm morphology ($p < 0.001$) and sperm count ($p < 0.0001$) was observed in the AgNP-3 group, while there were no significant differences in sperm motility in the AgNP-injected groups compared to the control rats.

Testicular histomorphometry

The data showed a significant reduction in the number of spermatogonia, Sertoli and Leydig cells in the AgNP-2 and AgNP-3 groups ($p < 0.05$), whereas there were no significant differences in the number of spermatocytes and spermatid cells among the 4 study groups (Fig. 4).

Parameters of seminiferous tubules

We observed a significant reduction in the diameter, circumference and mean area of seminiferous tubules in the AgNP-2 group compared to the control group ($p < 0.001$) (Table 2).

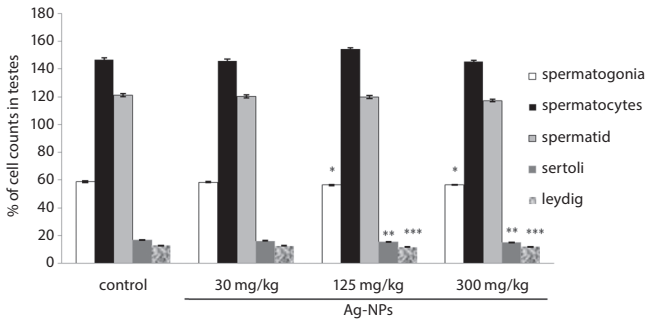

Fig. 4. The effect of silver nanoparticles (AgNPs) on testicular histomorphometry (mean ±SD)

*$p < 0.01$, **$p < 0.001$, *** $p < 0.0001$: (compared to controls); results presented as mean ± standard deviation (SD).

Assessment of sperm DNA and chromatin

Sperm DNA and chromatin assay is shown in Fig. 5. As can be seen in Fig. 6, there were no significant differences between the experimental and control groups regarding AB, TB and AO staining ($p > 0.05$).

Discussion

The main purpose of the present study was to evaluate the effect of different concentrations of AgNPs on sperm parameters and chromatin, and testicular histomorphometry. Our in vivo data revealed that AgNPs could reduce sperm parameters, such as normal sperm morphology, sperm vitality and sperm count. Silver nanoparticles caused a decrease in Sertoli and Leydig cell number. To better understand the role of AgNPs, we observed the

Table 1. The effect of AgNPs on sperm parameters

Parameters	Control	30 mg/kg	125 mg/kg	300 mg/kg
Motility [%]	62.8 ±3.03	44.4 ±8.41	53.8 ±4.18	42 ±5.8
Vitality [%]	96.7 ±0.56	94.5 ±0.71	97 ±0.72	93.1 ±1.37
Normal morphology [%]	96.7 ±0.56	92.5 ±0.75	93.5 ±0.57	89.5 ±1.87**
Sperm count [$\times 10^6$/mL]	53.1 ±1.30	51.6 ±1.48	49.0 ±1.6	36.0 ±1.7***

AgNPs – silver nanoparticles; * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ (compared to controls); results presented as mean ± standard deviation (SD).

Table 2. The effect of AgNPs on seminiferous tubule parameters in testes

Feature	Control	30 mg/kg	125 mg/kg	300 mg/kg
Area [$\times 10^{-8}$ m^2]	11.6 ±0.19	11.27 ±0.17	10.44 ±0.18*	11.62 ±0.19
Circumference [$\times 10^{-3}$ m]	1.20 ±0.01	1.18 ±0.00	1.18 ±0.04*	1.20 ±0.00
Mean diameter [$\times 10^{-3}$ m]	0.36 ±0.00	0.36 ±0.00	0.34 ±0.00*	0.36 ±0.00

AgNPs – silver nanoparticles; * $p < 0.001$ (compared to controls); results presented as mean ± standard deviation (SD).

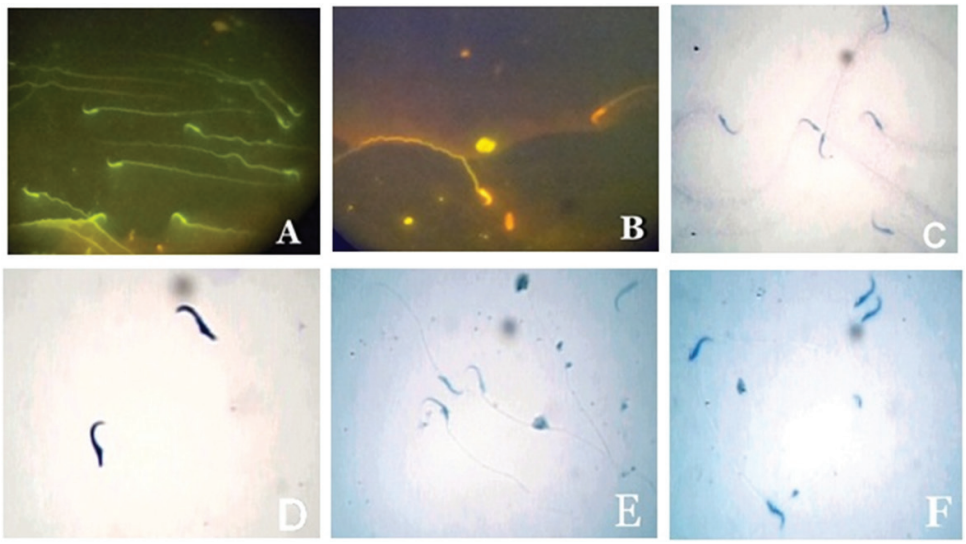

Fig. 5. Sperm chromatin integrity assay

A – acridine orange (AO), green-stained spermatozoa were considered to be AO-negative (non-denatured DNA); B – AO, orange-red-stained spermatozoa were considered abnormal (denatured DNA); C – toluidine blue (TB), unstained or pale blue-stained spermatozoa were considered normal; D – TB, dark blue-stained spermatozoa were considered abnormal; E – anilin blue (AB), unstained or pale blue-stained spermatozoa were considered normal; F – AB, dark blue-stained spermatozoa were considered abnormal.

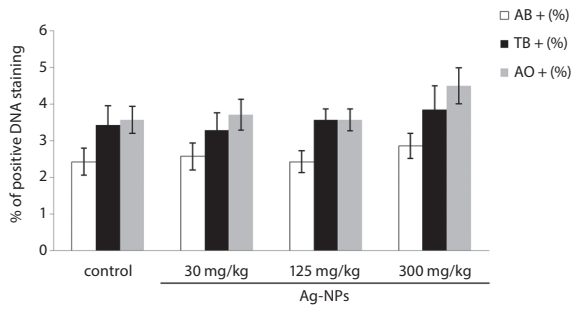

Fig. 6. The effect of AgNPs on sperm nuclear chromatin

AgNPs – silver nanoparticles; AB – anilin blue; TB – toluidine blue; AO – acridine orange; results presented as mean ± standard deviation (SD).

sperm chromatin packaging by AB, TB and AO staining. Silver nanoparticles did not destroy sperm DNA or chromatin integrity in any of the 3 concentration levels (30, 125 and 300 mg/kg).

In the past decade, with the development of nanotechnology, there has been a remarkable increase in application of NPs and nanomaterials in everyday products in industrial and medical sciences.[18] Despite the beneficial effects of NPs on improving the quality of life, there is in turn an increase in the risk of human as well as animal exposure to these nanomaterials through various routes. The toxic role of NPs, especially AgNPs, has been under investigation by researchers. In vitro studies have shown that NPs induce necrosis, apoptosis and mitochondrial dysfunction in mouse spermatogonia stem cells.[19] Similarly, in vivo studies have confirmed the in vitro observations and have suggested that the deleterious effects of NPs are related to their chemical composition, size and dosage.[20]

The i.p. route is one of the most neglected routes for testing AgNPs toxicity. The i.p. route delivers substances into circulation faster than the oral route. In this study, rats were treated with AgNPs at concentrations of 30, 125 and 300 mg/kg. These dosage levels were selected based on a previous 28-day oral toxicity study by Kim et al.[21] The pharmacokinetics of substances administered i.p. is more similar to this seen after oral administration.[22]

Consistent with previous reports, our results showed a significant reduction in sperm function in a dose-dependent manner. A high dose of AgNPs could affect conventional sperm characteristics, such as normal morphology and viability. Sleiman et al. indicated that AgNPs decreased sperm parameters in the pre-pubertal period of rats.[23] Sperm count is one of the most sensitive tests for spermatogenesis, since it gives the cumulative result of all stages of sperm production and is highly correlated with fertility.

Silver nanoparticles could damage sperm membranes and/or penetrate the cells, subsequently increasing free radicals, including ROS, that cause membrane lipid peroxidation and, in consequence, loss of motility and viability, and can injure the sperm membrane and flagellum structure, eventually leading to sperm motility and morphology perturbation.[6,19,24]

Importantly, based on our data, AgNPs caused a significant reduction in testicular morphological characteristics, such as the diameter, area and circumference of seminiferous tubules, at a concentration of 125 mg/kg, compared to the control group. In contrast to our result, Gromadzka-Ostrowska et al. showed a significant increase in the testicular morphology of animals treated with 200 nm of AgNPs at a dose of 5 mg/kg 28 days after intravenous injection.[10] Furthermore, Miresmaeili et al. showed that there were no significant changes in seminiferous tubule diameter in animals treated with oral AgNP administration at doses of 25, 50, 100, and 200 mg/kg after 48 days (the time period of spermatogenesis in rats).[25] Many in vivo studies have shown that chemicals, hypoxia or metals such as chromium, cadmium or lead can decrease the diameter of seminiferous tubule epithelial cells.[10] Accordingly, it seems that AgNPs have a dual effect on testicular morphological characteristics: at higher concentrations they reduce these features, and at lower doses increase them. Intraperitoneal injection of AgNPs might influence the endocrine system, and, consequently, may manipulate endogenous testosterone and estradiol levels; hence, these endocrine alterations might protect seminiferous tubules at high concentrations.[23]

Moreover, a histological evaluation of the testis tissue indicated a significant decrease in the mean number of spermatogonia, Leydig and Sertoli cells at doses of 125 and 300 mg/kg compared to controls. In support of our finding, Miresmaeili et al. showed a significant decrease in the number of primary spermatocyte and spermatid cells at doses of 50, 100 and 200 mg/kg.[25] It has been shown that AgNPs markedly decreased spermatogonial stem cell proliferation by some intracellular pathways.[26]

Silver nanoparticles, like other NPs, can damage the DNA structure of Leydig cells, leading to apoptosis. During spermatogenesis, histones are replaced by protamines in the nuclear DNA. Anilin blue can determine the protamines deficiency in sperm chromatin. The presence of disulfide bonds in the chromatin of mature sperm cells can prevent the denaturation of DNA, and TB can measure the rate of sperm nuclear chromatin condensation and DNA fragmentation via binding to DNA phosphate. Purple sperm heads showed DNA damage. Acridine orange is a fluorescence probe for the detection of denatured or single-stranded DNA in spermatozoa, and red fluorescence in sperm heads showed DNA damage or altered chromatin structure in sperm cells.[16] Our study also showed no significant changes in chromatin integrity.

Our results were in agreement with the findings of various studies which reported no significant correlation between sperm chromatin condensation and sperm parameters, including motility, vitality, normal morphology, and sperm count.[27,28] In contrast, other studies reported significant correlations between sperm DNA fragmentation and poor sperm morphology.[29,30]

Conclusions

Collectively, these findings indicate the effect of AgNPs on the male reproductive system. Normal sperm morphology and viability was disrupted by AgNPs. Silver nanoparticles at a high concentration (300 mg/kg) also reduced the number of testicular tubules, and spermatogonia, Sertoli and Leydig cells. Spermatozoa DNA was not affected by AgNPs, which needs more investigation on an intracellular level. The in vitro results indicated that the harmful effect of AgNPs is related to their chemical composition, size, dosage, means of administration, and duration of exposure.[31] Further investigation is necessary to clarify the mechanisms of NP-induced male reproductive dysfunction.

Given these findings, we can conclude that AgNPs pose a potential risk to male fertility, depending on their dose, and taking into consideration the protective role of AgNPs in relation to some parameters, a study over a long period of time should be carried out in our future work.

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Do CTRC mutations affect the development of alcoholic chronic pancreatitis and its course among Poles: Preliminary study

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):307–312

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Funding sources
None declared

Conflict of interest
None declared

Received on March 19, 2017
Reviewed on May 2, 2017
Accepted on July 28, 2017

Published online on October 2, 2018

Abstract

Background. Genetic mutations are one of the etiological factors that predispose people to develop chronic pancreatitis.

Objectives. The aim of our study was to examine the effect of p.Trp55*, p.Arg254Trp and c.738_761del mutations in the chemotrypsin gene (*CTRC*) on the development of alcoholic chronic pancreatitis (ACP) in order to answer the questions whether these mutations vary between gender groups, whether they were related to the age when ACP was first diagnosed, and whether they affected the morphological changes in the pancreas and the course of ACP.

Material and methods. The study included 124 patients with ACP, 52 with nonalcoholic pancreatitis and 52 controls. The p.Trp55*, c.738_761del and p.Arg254Trp mutations in the *CTRC* gene were tested by the polymerase chain reaction (PCR).

Results. The c.738_761del and p.Arg254Trp mutations occurred in 3.07% and 1.31% of cases, respectively. None of the examined patients were found to have the p.Trp55* mutation. The frequency of detected mutations did not significantly differ between the study groups. The c.738_761del mutation was detected more frequently in women than in men. No significant differences were found in the age at ACP onset, morphological changes affecting the pancreas, or in the course of ACP between the patients with and without the 2 examined mutations. The c.738_761del mutation was significantly more frequent in the diabetic patients than in the non-diabetics. The patients with this mutation more frequently required surgery than those without the c.738_761del mutation.

Conclusions. No relationship between the c.738_761del and p.Arg254Trp mutations and the development of APC was found. The c.738_761del mutation was more frequent in females than in males. Neither mutation affected the patient's age at ACP onset or its course. In contrast to p.Arg254Trp, the c.738_761del mutation correlated with diabetes development and the need for surgery in the course of ACP.

Key words: alcohol, alcoholic chronic pancreatitis, *CTRC* mutations

Cite as
Cichoż-Lach H, Michalak-Wojnowska M, Lis-Janczarek E, et al. Do CTRC mutations affect the development of alcoholic chronic pancreatitis and its course among Poles: Preliminary study. *Adv Clin Exp Med*. 2019;28(3):307–312. doi:10.17219/acem/76130

DOI
10.17219/acem/76130

Introduction

Chronic pancreatitis (CP) can present as recurrent episodes of acute inflammation or as progressive inflammatory conditions that result in fibrosis, calcification or altered morphology of the pancreas, the consequence of which is endocrine and exocrine failure of the organ.[1,2] Global annual incidence rates of CP range from 5 to 12 cases per 100,000 people; the prevalence of CP is about 50 cases per 100,000 people.[3] The disease occurs with a varied geographic distribution. In developed and western European countries, CP is generally due to alcohol consumption (38% of men and 11% of women). In the USA, Italy and Denmark, more than 50% of cases are alcohol-related, but in Brazil, the proportion reaches 89.6%.[4,5] Alcohol consumption has been increasing in developing countries (China and India) due to rapid urbanization and increased affluence; therefore, this rise is expected to increase the burden of alcohol-related pancreatitis in these countries.[6]

Among many etiological factors, the role of genetic predisposition to CP is clearly highlighted. The genetic factors of CP include a mutation/polymorphism of the cationic trypsinogen gene (*PRSS1*), the serine protease inhibitor Kazal type 1 gene (*SPINK1*), the chemotrypsin gene (*CTRC*), the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), and the calcium sensing receptor gene *(CASR). CASR* is responsible for calcium homeostasis and some patients with hypercalcemia develop CP. This observation may encourage one to postulate that a mutation in the *CASR* gene may favor CP. Polymorphisms of interleukin-encoding genes, such as tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta-1 (TGF-β1), interferon-gamma (INF-γ), vascular endothelial growth factor (VEGF), and intercellular adhesion molecule 1 (ICAM-1), are also major genetic contributors to the development of CP.[7,8] In 2013, the carboxypeptidase A1 *(CPA1)* gene was identified as a novel gene of pancreatitis susceptibility.[7,9,10] According to studies carried out on Polish individuals, the NS34 mutation of the *SPINK1* gene seems to be significantly correlated with alcoholic chronic pancreatitis (ACP).[11]

The *CTRC* gene encodes chemotrypsin C, a digestive enzyme produced by lobular pancreatic cells. Prematurely activated trypsin is destroyed by *CTRC*, which acts on the molecule within the calcium-binding loop in the absence of calcium, and therefore is a crucial candidate gene in the pathogenesis of CP. Since trypsin degradation serves as a protective mechanism against pancreatitis, it may be hypothesized that a loss of function in trypsin-degrading enzymes increases the risk for pancreatitis.[12,13] Numerous *CTRC* mutations and polymorphisms have been presented so far. However, the p.Trp55*, p.Arg254Trp and c.738_761del mutations at exon 7 have not been studied among Poles with ACP, which poses a serious clinical challenge.

The aim of this study was to examine the effect that the p.Trp55*(W55X), p.Arg254Trp (R254W) and c.738_761del (del24) mutations in the *CTRC* gene have on the development of ACP, and to answer the question whether these mutations vary between gender groups. Moreover, we wanted to learn whether the mutations were related to the age when ACP was first diagnosed, and whether they affected the morphological changes in the pancreas and the course of ACP. The focus was on the 3 above-mentioned mutations of *CTRC*, because it seems that they have the greatest impact on the development of CP and are the most frequently observed mutations in exon 7.[7]

Material and methods

Material

The study group was comprised of a homogenous Caucasian ethnic group of 228 patients, including 124 with ACP, 52 with nonalcoholic CP (NCP), and 52 healthy volunteers who made up the control group.

Chronic pancreatitis diagnosis was based on the standard criteria: a clinically confirmed history of recurrent episodes of acute pancreatitis and the results of imaging tests on the pancreatic structure (ultrasonography – USG, computed tomography – CT, endoscopy, or endoscopic retrograde cholangiopancreatography), such as calcifications, fibrosis of the pancreatic parenchyma, intraductal calcifications, and widened or irregular pancreatic ducts. Histological tests confirmed the diagnosis in 3 patients with ACP; in other cases, a biopsy was not performed. Alcoholic etiology was established on the basis of a medical history, i.e., the consumption of 80 g of pure ethanol in a 24-h period (males) or >40 g of pure ethanol in a 24-h period (females) in the previous 2 years or more. In addition, diabetes was diagnosed in 53 patients with ACP and insulin-dependent diabetes was diagnosed in 27 patients. Patients with a biliary, toxic, metabolic, or family history of CP were excluded from this group.

The group of patients with NCP included idiopathic CP cases. Chronic pancreatitis was diagnosed on the basis of the criteria presented above. The patients with an alcoholic, toxic, metabolic, or family history of CP were excluded from the study group. Four patients suffered from type 2 diabetes, treated with oral medication.

The control group was comprised of healthy volunteers with no history of alcohol consumption. They did not present any clinical symptoms or abnormalities on abdominal USG. There were no episodes of acute pancreatitis in this study group. Table 1 presents the characteristics of the study groups.

To examine whether there is an association between the p.Trp55*, p.Arg254Trp and c.738_761del mutations in the *CTRC* gene and the development of ACP, we compared the frequencies of the p.Trp55*, p.Arg254Trp and c.738_761del mutations in the *CTRC* gene in our 3 groups of patients: with ACP, with NCP, and in healthy controls.

Table 1. Characteristics of the study groups

Patient group	Number of patients	Gender		Age [years]	Presence of diabetes [n]	Mean BMI [kg/m^2] ±SD	Mean age at CP onset [years] ±SD
		F	M	mean ±SD			
ACP	124	34	90	43.07 ±9.04	53	24.32 ±4.35	38.4 ±8.17
NCP	52	28	24	39.97 ±11.03	4	25.45 ±5.97	36.93 ±9.04
Controls	52	24	28	40.38 ±7.88	0	24.67 ±3.47	–

CP – chronic pancreatitis; ACP – alcoholic chronic pancreatits; NCP – nonalcoholic chronic pancreatitis; F – female, M – male; BMI – body mass index; SD –standard deviation.

The study protocol was approved by the local Ethical Committee (No. 131/2013) (Medical University of Lublin, Poland), and all participants gave written informed consent to participate in the study.

Methods

The p.Trp55*, c.738_761del and p.Arg254Trp mutations in the *CTRC* gene were tested in all patients.

DNA isolation

DNA was isolated from peripheral blood leukocytes, using a Blood DNA Purification Kit (EURx, Gdańsk, Poland) according to the instructions of the manufacturer. The lymphocytes were separated by the Ficoll gradient technique.

Determination of the p.Trp55* mutation in the *CTRC* gene

Polymerase chain reactions (PCRs) were performed with 100 ng of genomic DNA in a total volume of 20 µL, using a Biometra T Personal thermal cycler (Biometra, Gottingen, Germany). DNA was amplified with the primers named p.Trp55*_F and p.Trp55*_R (Table 2), designed by the Primer3 application in the Genetic Testing Laboratory in Lublin, Poland, and with a Taq PCR Master Mix kit (EURx), according to the manufacturers' instructions. The setting parameters are listed in Table 2.

The PCR products were digested overnight at 37°C in a CLN 15 STD INOX/G incubator by the restriction enzyme PmlI (New England Biolabs, Ipswich, USA). The composition of the 20.4 µL restriction mix was 12 µL of the PCR product, 5 U of PmlI, 2 µL of CutSmart buffer, and 6 µL of water. Fifteen microliters of the digestion reaction products were used for electrophoresis. The products of digestion were separated in 3% agarose gel (Sigma Aldrich, St. Louis, USA), stained with Simply Safe (EURx), and visualized on a transilluminator (JW Electronic, Warszawa, Poland). The wild alleles were digested into fragments of 202 bp and 262 bp; the mutated allele did not have a restriction site and was identified as a band of 462 bp. To confirm the results, the analyzed samples underwent sequencing in both directions by means of primers 3R and 3F (Table 2), using a BigDye Terminator 3.1 kit (Life Technologies, Carlsbad, USA). The products of sequencing were electrophoretically separated on an ABI3100 sequencer, on a 50-centimeter capillary in the POP6 polymer, and analyzed by the Sequencing Analysis (Applied Biosystems, Foster City, USA).

Determination of the c.738_761del and p.Arg254Trp mutations in the *CTRC* gene

Both mutations were determined in 1 assay by using the same primer pairs, named c.738_761del_F and c.738_761del_R (Table 2), designed by Primer3 in the Genetic Testing Laboratory in Lublin, Poland. The PCR amplification was performed using a Biometra T Personal thermal cycler (Biometra) in a volume of 20 µL with 100 ng of genomic DNA, 2 primers and a Taq PCR Master Mix kit (EURx), according to the manufacturers' instructions. The setting parameters are listed in Table 2. Following that, the PCR products were digested overnight at 37°C

Table 2. Primers used to determine the p.Trp55*, p.Arg254Trp and c.738_761del mutations in the *CTRC* gene

Application	Primer name	Primer sequence	Annealing temperature	Product length
PCR-RFLP	p.Trp55*_F	5′ AGCCCTATTCACTGGTTCTTCTG 3′	59°C	464 bp
	p.Trp55*_R	5′CAACTGAGTTACTGGGTGTGAGTAG 3′		
	c.738_761del_F	5′ TGGTGGCTTATGCCCTCCCG 3′	59°C	209 bp
	c.738_761del_R	5′ GGACAGCTGTGGAGGCAGCAC 3′		
Sequencing	*CTRC*_3F	5′ ACCTGCAGGCTGACACACA 3′	59°C	325 bp
	*CTRC*_3R	5′ GCTGGTTCCTGGCACATAAT 3′		
	*CTRC*_7F	5′ GCAGGCTGAGGCCAAAT 3′	58°C	467 bp
	*CTRC*_7R	5′ TGAATGAGTGACTGAATAAGTG 3′		

PCR-RFLP – polymerase chain reaction-restriction fragments length polymorphism; both mutations, c.738_761del and p.Arg254Trp, were determined by using the same primers (c.738_761del_F and c.738_761del_R).

in a CLN 15 STD INOX/G incubator by the restriction enzyme SmaI (New England Biolabs). The composition of the 20.36 µL restriction mix was 12 µL of the PCR product, 10 U of SmaI, 1.6 µL of CutSmart buffer, 0.16 µL of bovine serum albumin (BSA), and 6 µL of water. Fifteen microliters of the digestion reaction products were used for electrophoresis. The products of digestion were separated in 3% agarose gel (Sigma Aldrich), stained with Simply Safe (EURx) and visualized on a transilluminator (JW Electronic). The wild allele was digested into fragments of 152 bp and 57 bp; the mutated allele (p.Arg254Trp) did not have restriction sites and appeared as a band of 209 bp. In the case of the c.738_761del mutation, the wild allele was seen as a band of 209 bp, but a product of 185 bp in length corresponded to the allele with a 24-bp deletion. To confirm the results, the analyzed samples underwent sequencing in both directions by means of primers 7F and 7R (Table 2) (the same primers for both mutations), using a BigDye Terminator 3.1 kit (Life Technologies) according to the manufacturer's instructions. The products of sequencing were electrophoretically separated on an ABI3100 sequencer, on a 50-centimeter capillary in the POP6 polymer, and analyzed by the Sequencing Analysis (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis

To describe the quantitative characteristics, the average values with standard deviations (SDs) were used. The comparison of the age at ACP onset was analyzed by Student's t-test for independent samples. The qualitative data was described as numbers. To compare the frequency of the p.Trp55*, c.738_761del and p.Arg254Trp mutations in the *CRTC* gene between the study groups, the ANOVA test was performed. To compare the frequency of the examined mutations in the *CRTC* gene between women and men, the χ^2 test with Yates's correction or Fisher's exact test was used, as appropriate. Statistical significance between the differences was assumed at $p < 0.05$. All calculations were done by means of STATISTICA PL software (StatSoft, Kraków, Poland).

Results

The frequency of the examined mutations in the study groups is presented in Table 3. In the group of 228 patients, the c.738_761del mutation occurred in 3.07% of cases and the p.Arg254Trp mutation was found in 1.31% of patients (Fig. 1,2). In none of the examined patients was the p.Trp55* mutation detected. All mutations were heterozygotic. The c.738_761del mutation was detected only in the group of patients with ACP (4.35%); the p.Arg254Trp mutation was found in 1.45% of patients with ACP and in 1.92%

Table 3. The frequency of the examined mutations in the *CRTC* gene in the study groups

Mutation	ACP	NCP	Controls	p-value
p.Trp55*	0/124	0/52	0/52	NS
c.738_761del	7/124	0/52	0/52	NS
p.Arg254Trp	2/124	0/52	1/52	NS

ACP – alcoholic chronic pancreatitis; NCP – nonalcoholic chronic pancreatitis; NS – no statistically significant differences.

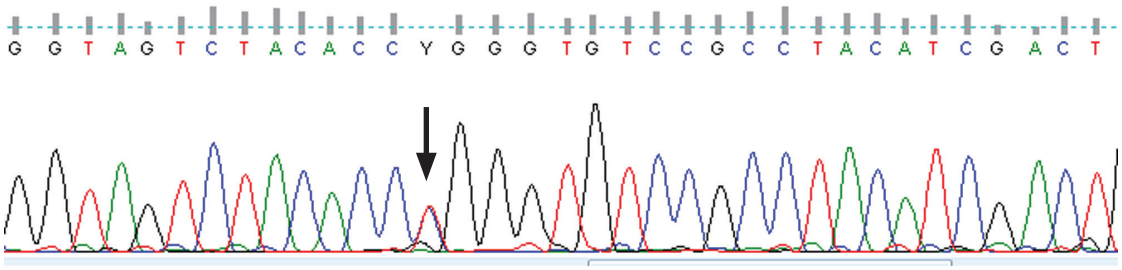

Fig. 1. Electropherogram of F strand of showing the p.Arg254Trp mutation in exon 7 of the *CTRC* gene. Cytosine was replaced by thymine. The affected individual is a heterozygote

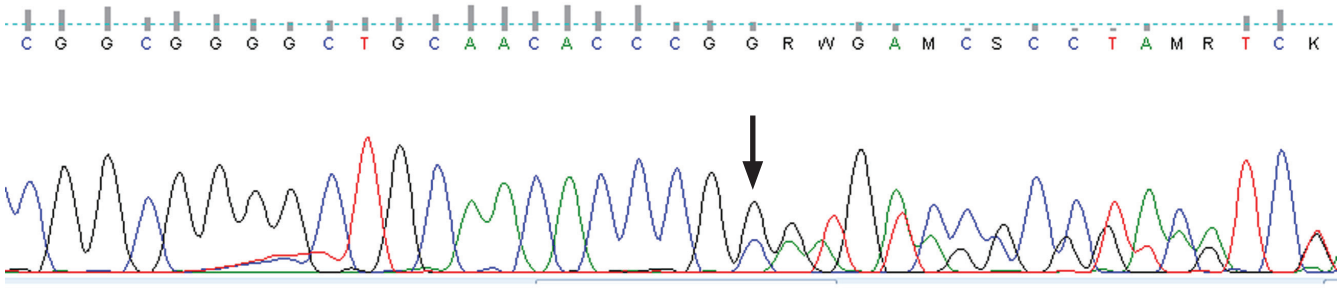

Fig. 2. Electropherogram of F strand showing the c.738_761del mutation in exon 7 of the *CTRC* gene. 24 bp are missing. The affected individual is a heterozygote

Table 4. Morphological changes in the pancreas and the clinical course of ACP in the patients with and without the c.738_761del and p.Arg254Trp mutations

Course of ACP	c.738_761del			p.Arg254Trp		
	mutation (n)	no mutation (n)	p-value	mutation (n)	no mutation (n)	p-value
Calcifications (n = 124)	7	117	NS	3	121	NS
Cysts (n = 54)	5	49	NS	1	53	NS
Widened Wirsung duct >3 mm (n = 36)	4	32	NS	1	37	NS
Stones in Wirsung duct (n = 21)	3	18	NS	2	19	NS
Operation (n = 14)	5	9	0.0002	0	14	NS
Diabetes (n = 53)	6	47	0.0484	2	51	NS

ACP – alcoholic chronic pancreatitis; n – number of patients; NS – no statistically significant differences.

of controls (Table 3). There were no statistically significant differences between the groups.

The c.738_761del mutation was detected in 6.98% of women and was statistically significantly more frequent in women than in men (0.70%) (χ^2 = 5.130; p = 0.0235). The p.Arg254Trp mutation was detected in 1.16% of women and in 1.41% of men, and was not statistically significantly different (χ^2 = 0.025; p = 0.8746).

The mean age at ACP onset in patients with the c.738_761del mutation was 39.87 ±5.56 years and without this mutation it was 38.22 ±8.15 years; with and without the p.Arg254Trp mutation, the mean age at ACP onset was 36.67 ±6.85 years and 38.94 ±7.87 years, respectively. The statistical analysis showed no statistically significant differences in the age at ACP onset between the patients with the c.738_761del and p.Arg254Trp mutations.

The relationship between the examined mutations and the course of ACP were analyzed with reference to morphological changes detected in the pancreas (calcifications, cysts, widening of the Wirsung duct by >3 mm, stones in the Wirsung duct), the occurrence of diabetes and the need for surgery. The patients with and without the c.738_761del and p.Arg254Trp mutations did not differ statistically in terms of morphological changes affecting the pancreas in the course of ACP. Fourteen patients were operated on: 2 had distal pancreatic resection, 7 had pancreaticoduodenectomy and 7 had cystojejunostomy. There were no statistical differences in the frequency of the examined mutations between patients operated on and not operated on in the course of ACP. However, the c.738_761del mutation was statistically significantly more frequent in patients with diabetes (13.21%) in the course of ACP compared to the examined non-diabetic patients (0%) (p = 0.0204); no differences were observed for the p.Arg254Trp mutation (Table 4).

Discussion

Our results revealed a frequency of 5.65% and 2.42% for the c.738_761del and p.Arg254Trp mutations in the *CTRC* gene, respectively, in the group of patients with ACP compared to 0% and 1.92%, respectively, in the control group.

Rosendahl et al. studied the frequency of the del24 (c.738_761del) mutation in a group of German patients with ACP, and they found a 0.6% frequency of the del24 (c.738_761del) mutation in that group compared to 0.2% with alcoholic liver disease without pancreatitis.[13] According to these authors, the frequency of the R2 54W (p.Arg254Trp) mutation in other types of CP was as follows: 1.7% in idiopathic CP, 4.2% in hereditary CP and 2.1% in healthy controls. However, the del24 (c.738_761del) mutation in the German patients occurred in 1.5% of idiopathic CP cases, and in 1.2% of controls; it was not detected in the hereditary CP group.[13] Chemotrypsin C *(CTRC)* variants that diminish activity or secretion are associated with chronic pancreatitis. According to Felderbauer et al., the R2 54W (p.Arg254Trp) mutation occurred in 6.5% of the group of German patients with CP and hyperthyroidism, but was not detected in the group with hyperthyroidism without CP. The authors emphasized the correlation of that mutation type and the course of CP. However, they found no other mutation types in the *CTRC* gene.[14] A study carried out in a group of patients with primary CP found a frequency of 1.7% for the R2 54W (p.Arg254Trp) mutation, 0.7% for the del24 (c.738_761del) mutation in a group of patients with idiopathic pancreatitis and 0.3% in healthy controls.[15]

The research results of studies on *CTRC* mutations in CP etiology are controversial. Some researchers believe that *CTRC* mutations contribute to the so-called secondary CP (including this of alcoholic origin), while other genetic risk factors are either absent (e.g., *PRSS1* mutations) or they suggest a considerably lesser influence in comparison to primary CP (e.g., *SPINK1* mutations). Therefore, those genes should be considered important contributing factors rather than causative factors.[16,17] The studies in non-European populations found a higher frequency of the R2 54W (p.Arg254Trp) mutation in the group of patients with tropical pancreatitis (2.8%) compared to healthy volunteers (1.2%) of Indian origin. However, the del24 (c.738_761del) mutation, a considerably frequent type among German patients, was not found among Indian patients at all. Nonetheless, the interpretation of these results cannot be conclusive, because the Indian cohort was much smaller than the German group.[13] The studies

carried out in the Asian Pacific region found a significant correlation between the *CTRC* gene mutation and tropical calcific pancreatitis.[18] Considering the biochemical activity of *CTRC* and the functional properties of the mutation, 3 mechanisms seem to contribute to the risk of developing CP: 1. a weakened degradation of trypsinogen and/or trypsin; 2. an impaired activation of A-carbopeptidase; and 3. the induction of endoplasmic reticulum (ER) stress. Hence, the carriers of a *CTRC*-mutated gene are more likely to be exposed to ER stress in the exogenic region of the pancreas, which may contribute to apoptotic damage to the lobular pancreatic tissue.[12,16,19]

The frequency of the c.738_761del and p.Arg254Trp mutations in the *CRTC* gene among females and males has not been investigated so far. Our study seems to be the first in that respect. Our results found a statistically significantly higher frequency of the c.738_761del mutation in females than in males. However, no such correlation was found for the p.Arg254Trp mutation.

Most research focuses on different roles of various mutations in the etiology of pancreatitis. When a mutation is confirmed, the course of CP is rarely examined in terms of environmental and demographic factors. It seems quite interesting to learn whether a *CTRC* mutation quickens and affects the course of CP, e.g., by developing diabetes. Our study found that a *CTRC* mutation did not affect the age at ACP onset or the course of the disease.

Moreover, diabetes correlated with the c.738_761del mutation. There has been no research on *CTRC* gene mutations and CP course so far. However, the researchers have found that the N34S mutation in the *SPINK1* gene favors ACP development and predisposes patients to developing diabetes at a younger age than patients without a mutated gene.[5,11] We also found that patients with the c.738_761del mutation more often required surgery in the course of APC than patients without this mutation.

To our knowledge, this is the first study on the effect of the p.Trp55*, p.Arg254Trp and c.738_761del mutations in the *CTRC* gene on the development of ACP in Polish patients. Our results are preliminary and further large-population studies should be conducted to confirm these findings.

Conclusions

Our results lead to following conclusions:

1. No relationship between the c.738_761del or p.Arg254Trp mutations in the *CTRC* gene and the development of APC or the course of APC was found in the examined group of Polish patients.

2. The mutation c.738_761del occurred statistically significantly more frequently in women than in men; however, the p.Arg254Trp mutation was not gender-dependent.

3. Neither the c.738_761del mutation nor the p.Arg254Trp mutation affected the patient's age at ACP onset or the course of the disease.

4. Contrary to p.Arg254Trp, the c.738_761del mutation in the *CTRC* gene correlated with diabetes development and with the need for surgery in the course of ACP.

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Hepatitis C infection among pregnant women in central Poland: Significance of epidemiological anamnesis and impact of screening tests to detect infection

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):313–318

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Funding sources
None declared

Conflict of interest
None declared

Received on November 14, 2016
Reviewed on December 26, 2016
Accepted on August 31, 2017

Published online on January 17, 2019

Abstract

Background. Mother-to-child transmission is one of the main sources of hepatitis C virus (HCV) infection in children. However, because of the asymptomatic course of the illness, certain women may not be aware of their infection.

Objectives. The aim of this study was to estimate the significance of epidemiological anamnesis in diagnoses of HCV infection in women of reproductive age and to evaluate how screening among pregnant women impacts the detection of HCV infection.

Material and methods. Epidemiological interviews of 432 mothers infected with HCV (but free of human immunodeficiency virus (HIV)) were conducted in the Warsaw Hospital for Infectious Diseases (Poland) from 1998 to 2012.

Results. Complaints or abnormalities in laboratory tests were the reasons for anti-HCV antibody testing in 28.2% of mothers, whereas specific interview responses or occupational health care services group affiliation were the reasons for testing in 35.6%. However, in a large group of women, infection was only detected because of screening examinations. The introduction of routine screening for pregnant women (since 2010 in Poland) has led to the increased detection of HCV infection in women who did not present with infection risk factors (9.9% before 2010 vs 46.1% after 2010). This practice has also led to an increase in the percentage of women diagnosed during pregnancy (21.5% before 2010 vs 30.8% after 2010).

Conclusions. Establishing HCV infection risk factors during the interview process is the most common indicator for serological testing; however, not all infected cases can be diagnosed in this manner. Screening for anti-HCV antibodies in pregnant women increases the detection of HCV infection in this group.

Key words: pregnancy, HCV, screening, anti-HCV

Cite as
Aniszewska M, Pokorska-Śpiewak M, Kowalik-Mikołajewska B, Pluta M, Marczyńska M. Hepatitis C infection among pregnant women in central Poland: Significance of epidemiological anamnesis and impact of screening tests to detect infection. *Adv Clin Exp Med*. 2019;28(3):313–318. doi:10.17219/acem/76739

DOI
10.17219/acem/76739

Introduction

The hepatitis C virus (HCV) causes an inflammation of the liver that leads to chronic disease in 50–85% of cases. Hepatitis C virus infection is usually asymptomatic or subclinical for many years; however, after 20–30 years, it can lead to cirrhosis (in 5–25% of patients) and an increased risk of hepatocellular carcinoma. Transmission of the virus can occur during the transfusion of HCV-infected blood, the performance of medical and non-medical procedures (piercings or tattoos) using equipment contaminated with infectious material, and intravenous drug use (IVDU) with needles shared by an infected person. Infection through sexual contact with HCV-infected persons is possible, although the risk is low.[1–4] Currently, one of the main routes for HCV infection in children in developed countries is vertical transmission from infected mothers. Anti-HCV antibodies (anti-HCV) in pregnant women in Europe are detected at a prevalence of 0.1–1.7%,[5,6] and the risk of vertical HCV infection is estimated to be 3–10%.[7] No specific methods of prevention (vaccination or specific immunoglobulin) are available to reduce the risk of transmission in children after birth. The main factor affecting vertical transmission is maternal viral load. The use of antiviral therapy before pregnancy can lead to the reduction or elimination of HCV vireamia. However, because of the asymptomatic nature of HCV, women may not know they are infected. The diagnosis of HCV involves testing for anti-HCV (ELISA method), and a positive result is followed by an evaluation of viral replication using a reverse transcription polymerase chain reaction assay (RT-PCR).

The aims of the study were as follows: to estimate the significance of epidemiological anamnesis in diagnoses of HCV infection in women of reproductive age and to evaluate how screening among pregnant women impacts HCV infection detection.

Material and methods

The study group consisted of women who attended the Department of Children's Infectious Diseases (Medical University of Warsaw, Hospital for Infectious Diseases, Warszawa, Poland) in order to test for HCV infection in their infants. Epidemiological information was collected via questionnaires distributed to 432 mothers living in the Warsaw agglomeration area from 1998 to 2012. The Warsaw agglomeration is located in central Poland and it has an area of 6,200 km^2 and a population of 3 million, with 80% living in the cities and 20% living in the countryside. The average prevalence in this area is 10/1,000 live births per year. The Hospital for Infectious Diseases is the primary center for diagnosis and treatment of HCV-infected children in the agglomeration of Warszawa. The sample group of HCV-infected women were not selected using a screening population. The reasons provided for undergoing the HCV diagnostic tests were the subject of the survey. The respondent group was not homogeneous, and all women who visited the clinic to have diagnostic HCV tests performed on their infants were qualified for the study.

Child qualification criteria for the vertical infection tests changed over the course of this study. Between 1998 and 2009, children born to mothers with detectable anti-HCV antibodies (anti-HCV+) were tested regardless of HCV-RNA status. However, from 2010 to 2012, the tests were only performed on children born to anti-HCV(+) mothers that were positive for HCV-RNA – HCV-RNA(+), which resulted in a change in the recruitment of respondent mothers.

The survey questions were related to the putative source of infection, infection risk factors, and reasons for performing the diagnostic tests.

The data obtained was summarized into 3 time intervals according to the following 2 events that could have had a significant effect on the epidemiology of hepatitis C and the detection of HCV infection: the introduction of the RT-PCR HCV-RNA test for blood donation in Poland in 2002 and the introduction of the recommended anti-HCV test for pregnant women in 2010. In the 1st period (1998–2002), data was obtained from 54 women; in the 2nd period (2003–2009), data was obtained from 300 women; and in the 3rd period (2010–2012), data was obtained from 78 women. The putative sources of infection and the reasons for undergoing HCV infection testing were compared with respect to the women's age (<24, 25–35, >35 years) and place of residence (urban and rural). Women living in towns with more than 10,000 residents or fewer than 10,000 residents but with non-agricultural labor forces that commuted to work in larger cities were considered residents of urban areas.

The Local Ethics Committee of the Medical University of Warsaw gave its approval for the study. Each patient and/or parents/guardians gave written informed consent for the study. The study complies with the principles outlined in the Declaration of Helsinki.

Statistical analysis

Categorical variables were compared using either the χ^2 test or Fisher's exact test as appropriate. A p-value of <0.05 was considered statistically significant. All of the statistical analyses were performed using MedCalc Statistical Software v. 12.1.4.0 trial software (MedCalc, Mariakerke, Belgium).

Results

The questionnaire data on 432 HCV-infected mothers was analyzed. From 1998 to 2009, the analysis involved 354 anti-HCV(+) mothers: 182 (51.4%) were HCV-RNA(+), 59 (16.7%) were HCV-RNA(–), and 113 (31.9%) had an unknown HCV-RNA status. From 2010 to 2012,

epidemiological information was gathered from 78 women: 59 (75.6%) were HCV-RNA(+) and 19 (24.4%) had an unknown HCV-RNA status (children who were adopted and in foster families (biological mother unavailable)). Epidemiological data on the mothers of adopted children was obtained from the children's medical records. Almost all of the women (431/432) were Caucasian (427 of Polish nationality and 4 of Ukrainian nationality), while 1 woman was Asian. The average age of the mothers was 29.85 years (range: 18–50), including 37 women younger than 24 years, 346 women between 24–35 years of age, and 49 women older than 35 years. In addition, 367 women (85%) lived in urban areas and 65 women (15%) lived in rural areas.

Events in the women's history that might have increased the risk of HCV infection are presented in Table 1.

Table 1. Events in the women's history that could increase the risk of HCV infection and characteristics of the group

Risk event in the history	Number of women	%
Surgical procedure	106	24.5
Blood product transfusion	94	21.8
IVDU age of women <24 years 24–35 years >35 years	74 (8/37) (63/346) (3/49)	17.1 (21.6) (18.2) (6.1)
Multiple hospitalizations	39	9.0
Health care workers with needlestick/scarification in history	24	5.6
Infected sexual partner	11	2.5
Infected other family member	5	1.2
Tattoo or piercing	5	1.2
Mother infected with HCV	3	0.7
Unknown source of infection	71	16.4
Total	432	100

IVDU – intravenous drug use.

Based on the place of residence, we found a higher incidence of IVDU among urban residents compared with rural residents (70/367 (19.1%) vs 4/61 (6.1%); p = 0.01). Information on the type, duration, and number of the doses of intravenous drugs was not collected, recognizing that even a single incidence of drug use with HCV-contaminated equipment carries a risk of HCV infection. Among the children of mothers with a history of IVDU, 25/74 (33.8%) remained in foster care (adoption or orphanages), 49/74 (66.2%) were in the care of their mothers, 28/74 of the mothers (37.8%) declared abstinence, and 21/74 of the mothers (28.4%) participated in methadone programs.

The characteristics of anti-HCV-positive mothers stratified by intravenous drug use status are presented in Table 2.

In addition, the history of HCV-infected women was analyzed for changes in risk events in the periods: 1998–2002, 2003–2009 and 2010–2012. We observed an increasing incidence of IVDU (3.7%, 17.7% and 24.4%, respectively; p = 0.007) and decreasing history of blood transfusion among HCV infected women (31.5%, 22.3% and 12.8% in the subsequent periods; p = 0.03). All of the blood transfusions leading to HCV infection in this study were performed prior to 1992.

An event with the probability of increasing the risk of HCV infection was not determined in 71/432 women (16.4%), including 35/354 (9.9%) from 1998 to 2009 and 36/78 (46.1%) between 2010 and 2012 (p < 0.001).

The reasons for undergoing diagnostic tests are presented in Table 3.

In 77/432 cases (17.8%), tests were performed according to a planned gynecological recommendation before or during pregnancy. Changes in the responses in subsequent years are presented in Fig. 1.

The women were divided into 3 groups according to the time of HCV infection diagnosis: 287/432 (66.4%) were

Table 2. Characteristics of anti-HCV-positive mothers stratified by intravenous drug-use status

Characteristics of mothers	Mothers without IV drug use; n = 358 n (%)	Mothers with IV drug use; n = 74 n (%)	In total; n = 432 n (%)
Maternal hepatitis C RNA status* HCV-RNA positive HCV-RNA negative HCV-RNA not done	 191 (53.4) 57 (15.9) 110 (30.7)	 50 (67.6) 2 (2.7) 22 (29.7)	 241 (55.8) 59 (13.7) 132 (30.5)
Place of living urban area rural area	 297 (83.0) 61 (17.0)	 70 (94.6) 4 (5.4)	 367 (85.0) 65 (15.0)
Age of women <24 years 24–35 years >35 years	 29 (8.1) 283 (79.1) 46 (12.8)	 8 (10.8) 63 (85.1) 3 (4.1)	 37 (8.6) 346 (80.1) 49 (11.3)
Time of HCV infection diagnosis before pregnancy during pregnancy after parturition	 238 (66.5) 89 (24.9) 31 (8.6)	 49 (66.2) 11 (14.9) 14 (18.9)	 287 (66.4) 100 (23.2) 45 (10.4)

*HCV-RNA status tested with RT PCR; P – NS for all comparisons; IV – intravenous; HCV-RNA – hepatitis C virus ribonucleic acid; RT-PCR – reverse transcription polymerase chain reaction.

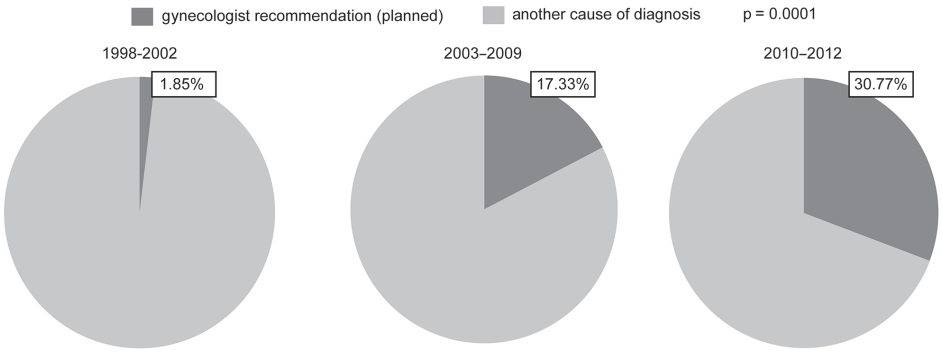

Fig. 1. Percentage contribution of gynecologists and obstetricians in HCV infection detection in women

Table 3. Reasons for performing HCV diagnostics in women

Reason for HCV testing	Number of women	%
Interview response (risk factor for HCV infection in anamnesis)	125	28.9
Gynecologists' recommendation (planned, without recognized risk factors in history)	77	17.8
Acute hepatitis C or other complaints	48	11.1
Blood donor examination	45	10.4
Increased aminotransferases activity	44	10.2
Obstetric cholestasis	30	7.0
Periodical examination of health care worker or after occupational exposure	29	6.7
Detection of anti-HCV antibodies in child or HCV-RNA in umbilical cord blood or placenta	19	4.4
During other examination	11	2.6
Examination at the patient's initiative	4	0.9
In total	432	100

HCV-RNA – hepatitis C virus ribonucleic acid.

diagnosed before pregnancy, 100/432 (23.1%) were diagnosed during pregnancy, and 45/432 (10.4%) were diagnosed after parturition. A comparison of the periods 1998–2009 and 2010–2012 showed that the proportion of women with diagnosed infection during pregnancy increased from 76/354 (21.5%) to 24/78 (30.8%), respectively.

Discussion

Complaints or abnormalities in laboratory tests (symptoms suggesting acute hepatitis, cholestasis during pregnancy, elevated aminotransferases, etc.) were the reasons for anti-HCV testing in 28.2% of the participants.

Specific interview responses or employment in health care services was the reason for testing in 35.6% of the cases (154/432) (Table 3). Extended and complementary interviews conducted during the survey were used to determine risk events in the women's histories in an additional 48% of cases (207/432). Knowledge of the epidemiology of HCV infection specific to the given population and appropriately conducted interviews by the general practitioner were likely to increase the detection of HCV infection.[8,9] Events that could carry a risk of HCV infection in the respondents' histories are presented in Table 1.

The most important changes in HCV sources in recent years are related to blood donation centers, with the safety of transfused blood products increasing significantly because of the introduction of HCV testing in blood donation centers in Poland (anti-HCV since 1992 and HCV-RNA RT-PCR since 2001/2002).[10,11] It is estimated that 1.4% of blood donors in Poland were anti-HCV(+) before the introduction of routine serological tests.[12] All blood transfusions resulting in HCV infection in this study were performed prior to 1992. It is likely that a history of blood transfusion will not be a risk factor for HCV infection in women of reproductive age within 25 years.

Intravenous drug users represent a significant group among the HCV-infected population, and multiple exposures to HCV and a lower incidence of spontaneous HCV-RNA clearance are found among intravenous drug users.[13–15] The prevalence of IVDU among the interviews of HCV-infected women increased from 3.7% (1998–2002) to 17.7% (2003–2009) and to 24.4% (2010–2012) ($p = 0.0075$). Young women (under 24 years old) living in cities accounted for the majority of IVDU ($p = 0.01$). From a reproductive health perspective, an increase in the percentage of teenagers reporting at least 1 contact with an intravenous drug is a worrisome problem; according to Sieroslawski, this percentage was 0.3–0.6% in 2003 and 0.6–1.2% in 2011 (higher in the group of 15- and 16-year-olds than in those aged 17–18 years).[16]

A mother actively using drugs is not able to care for her child responsibly. Among children born to IVDU mothers, 33.8% remained in foster care and 66.2% remained in the care of mothers who reported abstinence or participation in a methadone program.

Determining a direct relationship between surgical procedures or multiple hospitalizations and HCV infection is difficult unless the patient presented with symptoms of acute hepatitis after a period corresponding to HCV incubation.[4] Such time-dependence was not demonstrated in any of the women; thus, it is likely that hospitalizations in most of these cases had no effect on HCV infection. However, the data for these women should be analyzed against a control group with no risk factors.

Health care workers may be a group with an increased risk for HCV infection.[4,17–20] The risk of infection after incidental occupational exposure is less than 2% on average (range 0–10%). Flisiak et al. analyzed the presence of anti-HCV among health care workers in Poland and found significant differences depending on the workplace, with anti-HCV detected in 0.98% of basic health care employees, in 2% of employees in multi-profile hospitals, and in 3.28% of employees in specialist centers.[21] All of the HCV-infected health care workers in our study confirmed that they had been stabbed with a needle or sharp instrument while dealing with a patient.

Postgraduate training in HCV epidemiology may improve the effectiveness of HCV detection, and such courses for gynecologists and obstetricians led to the following results: among women examined from 1998 to 2002, HCV infection was diagnosed based on gynecologists' recommendations in 1.8% of cases, whereas in 2003–2009 HCV infection was diagnosed based on gynecologists' recommendations in 17.3% of cases (Fig. 1). A further statistically significant ($p < 0.001$) increase in gynecologist involvement in HCV diagnosis after the year 2010 (30.77%) was related to the implementation of the Act on Pregnant Woman Care, which recommends anti-HCV examinations for all pregnant women in Poland.[22] This act also led to an increase in the percentage of women diagnosed during pregnancy after 2010.

There is a large group of women for whom infection was only detectable because of screening examinations. The introduction of screening recommendations for pregnant women in 2010 led to an increase in the percentage of HCV infections detected in women who did not disclose risk factors in an interview (9.9% before 2010 vs 46.1% after 2010; ($p < 0.001$). Certain women may not disclose risk factors in their history for reasons of moral and social censure. A study by Lamber et al. determined that infection risk factors were not observed for 27% of Irish women infected with HCV, over half of whom were immigrants from Eastern Europe.[6] In an examination of pregnant women in Austria, Diab-Elschahawi found that 21% of them learned about their infection from a screening examination, despite a lack of risk factors.[23]

A significant number of women of reproductive age who are infected with HCV are unaware of their infection and will not qualify for examination because of a lack of disclosed risk factors or clinical abnormalities. This group may only be detected through screening examinations.[24,25] In our study, 36.1% of women were diagnosed without presenting risk factors in the interview, physical examination, or laboratory results.

Because of its cost-effectiveness, universal/antenatal screening is primarily considered in populations in which the prevalence of anti-HCV is higher than 1–2%.[24,26,27] Such rates of infection significantly increase the risk of contact with HCV through casual sexual contact and non-sterile medical and non-medical procedures. Currently, the United States has implemented a routine examination for anti-HCV in people born 1945–1965, regardless of other risk factors. Anti-HCV antibodies are detected in 3.5% of people in this group age, with the estimated average for the whole population at 1–1.5%.[28] The prevalence of anti-HCV in Europe is in the range of 0.4–5.2% and Flisiak estimated the prevalence in Poland at 1.9%.[5,21]

The most beneficial method of reducing the risk of vertical infection is to detect infection in the pre-reproduction period and to use antiviral therapy to eliminate or reduce HCV viraemia.[29–31] Because of toxicity, drug therapies that are applicable during pregnancy are not available.[4]

Among the women included in the study, 287/432 (66.4%) knew of their infection prior to pregnancy, whereas the remaining participants (145/432 (33.6%)) did not. The results of this study confirm the low awareness of HCV infection, which is consistent with findings from studies conducted among various European populations.[23,32] The data presented here suggests the benefits of performing screening examinations for HCV infection in young women of reproductive age, although detecting infection during pregnancy also has benefits. For example, therapy is more effective when performed shortly after birth because of changes in the immune system,[33,34] effective therapy provides benefits for subsequent pregnancies, and maternal infection awareness programs provide recommendations on slowing the course of the disease, such as avoiding alcohol and receiving vaccinations against other hepatotropic viruses (HAV, HBV). Detecting HCV infection in mothers will promote diagnostic testing in their children, and children infected with HCV will benefit from early treatment.[24]

The authors are aware of the following study limitations: 1. the group of women infected with HCV was not selected from the population based on screening examinations, and 2. the study group was not uniform and included respondents who were anti-HCV+, though HCV replication was not confirmed in all cases. (It is estimated that an active replication of the virus occurs in 31% of Polish anti-HCV(+) individuals on average, with 44% of cases occurring in patients <25 years old and 27% of cases occurring in patients >40 years old).[21]

Although the authors have acknowledged these limitations, they believe that the study presents new information on HCV infection among women of reproductive age in Poland. To our knowledge, this is the only work of this type concerning women in Poland.

Conclusions

Establishing HCV infection risk factors by analyzing interview responses is the most common indicator for serological testing. However, performing examinations on women according to their interview responses alone will not lead to a diagnosis of all infected cases. Screening for anti-HCV in pregnant women increases the detection of HCV infection in this group. Universal screening of women of reproductive age should be considered.

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Comorbidities in chronic obstructive pulmonary disease: Results of a national multicenter research project

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):319–324

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Funding sources
The study was funded by National Center for Research and Development, Warszawa, Poland; project "Chronic obstructive pulmonary disease (COPD) – systemic disease, the biggest threat of the 21st century" (13 0034 06/2009).

Conflict of interest
None declared

Acknowledgements
We would like to thank Monika Kosacka MD, PhD, Barbara Mackiewicz MD, PhD, Grzegorz Gąsior MD, PhD, and Krzysztof Kędziora MD, PhD for their help in data collection.

Received on March 28, 2017
Reviewed on July 28, 2017
Accepted on September 26, 2017

Published online on August 29, 2018

Cite as
Rubinsztajn R, Przybyłowski T, Grabicki M, et al. Comorbidities in chronic obstructive pulmonary disease: Results of a national multicenter research project. *Adv Clin Exp Med*. 2019;28(3):319–324. doi:10.17219/acem/78024

DOI
10.17219/acem/78024

Abstract

Background. Chronic obstructive pulmonary disease (COPD) is associated with various comorbidities, which influence the course of COPD and worsen prognosis.

Objectives. The aim of this study was to analyze the comorbidities in a cohort of COPD patients in Poland during 12 months of observation.

Material and methods. A total of 444 COPD patients (median age: 66.1 years) in all stages of airflow limitation severity were enrolled. Medical histories and a questionnaire concerning comorbidities were analyzed at baseline and after 12 months (data of 267 patients available). Anthropometric data, pulmonary function, and body mass index, airflow obstruction, dyspnea, and exercise capacity (BODE index) were assessed.

Results. No comorbidities were reported in 9 patients (2.0%), 101 patients (22.7%) had 1–2 comorbidities, 243 (54.7%) had 3–5, and 91 (20.6%) had more than 5 comorbidities. Cardiovascular diseases (CVDs) were the most frequent ones, followed by peptic ulcer, obstructive sleep apnea (OSA), diabetes, gastroesophageal reflux disease (GERD), and osteoporosis; 11 patients had a history of lung cancer. Cachexia was observed in 11 cases, overweight in 136 cases and obesity in 139 cases. The incidence of CVDs increased with time. The number of comorbidities correlated with the body mass index (BMI) and the number of hospitalizations for extra-pulmonary causes, but not with airflow limitation. The BODE index score increased with the number of comorbidities.

Conclusions. In a cohort of Polish COPD patients, the most frequent comorbidities were CVDs. The number of comorbidities affected the BODE index, but not airflow limitation. The BODE index is better than forced expiratory volume in 1 s (FEV_1) in the rating of COPD patients' condition. The BMI correlated with the number of comorbidities as well as the number of hospitalizations for extra-pulmonary causes.

Key words: comorbidity, chronic obstructive pulmonary disease, cardiovascular disease, BODE index

Introduction

Chronic obstructive pulmonary disease (COPD) is currently recognized as a systemic disease and, as such, it is associated with many systemic disorders and comorbidities. According to the recommendations of the Global Initiative for Obstructive Lung Disease (GOLD) guidelines, the most frequent disorders are cardiovascular diseases (CVDs), body composition disorders, sarcopenia, osteoporosis, depression, and lung cancer.[1] Additionally, some other concomitant disorders, like asthma-COPD overlap[2] or obstructive sleep apnea (OSA)-COPD overlap syndrome,[3] are also listed. Mapel et al. showed that only 6% of the studied patients with COPD had no other chronic medical conditions. The average number of diseases in the COPD cohort was 3.7 vs 1.8 in the control group.[4] Van Manen et al. reported that over 50% of 1,145 patients with COPD had more than 1 comorbidity, 15.8% had between 3 and 4 comorbidities, and 6.8% had 5 or more comorbid conditions.[5] The comorbidities influence the frequency and duration of hospitalization[6,7] and contribute to COPD-related mortality.[7] In COPD patients, cardiovascular disorders, malignancies and other pulmonary diseases (acute or chronic respiratory failure, cor pulmonale with edema, or pulmonary infections) were responsible for deaths of 14–40%, 7–60%, and 4–26% of the patients, respectively.[8] This finding was validated by our previous study, in which approx. 12% of deaths were associated with CVDs.[9] Comorbidities also affect quality of life,[10] as well as COPD severity.[11] Depression, osteoporosis and musculoskeletal alterations are frequently diagnosed in patients with severe COPD.[11] Frei et al. found that depression, anxiety, peripheral artery disease, cerebrovascular disease, and symptomatic heart disease most strongly affected the patients' health status, and they proposed the use of an index that reflects the impact of comorbidities on the health status of patients with COPD – Comorbidities in Chronic Obstructive Lung Disease (COMCOLD).[12] It is known that a common risk factor for COPD and some comorbidities is smoking. In the Polish part of the Burden of Obstructive Lung Disease (BOLD) study, the prevalence of COPD increased with exposure to tobacco smoke.[13]

The main aim of our study was to evaluate the prevalence of comorbidities in a cohort of patients with COPD in Poland during 1 year of observation, and to estimate the relationship between the presence of comorbidities and airflow limitation severity and the body mass index, airflow obstruction, dyspnea, and exercise capacity (BODE) index.

Material and methods

The study is a part of a National Center for Research and Development project called "Chronic obstructive pulmonary disease (COPD) – a systemic disease, the biggest threat of the 21st century" (13 0034 06/2009). Chronic obstructive pulmonary disease was diagnosed according to the GOLD definition.[1] The study group consisted of 444 consecutive COPD patients (69% males) at a median age of 66.1 years, with an interquartile range (IQR) of 59.0–74.0, in all stages of disease severity, recruited from the outpatient clinics of the Department of Internal Medicine, Pulmonary Diseases and Allergy, Medical University of Warsaw, Poland, and our partners from Poznań, Gdańsk, Lublin, Wrocław, and Katowice. The median COPD duration was 6.0 years (IQR: 3.0–10.0) and the median number of pack-years was 37.8 (IQR: 25.0–50.0). The inclusion criteria were COPD diagnosis and written informed consent. The patients were evaluated at baseline (V1) and 267 patients were reassessed during a follow-up examination after 12 months (V2). The reasons for the discrepancies between the numbers of subjects in V1 and V2 were death of the patients (n = 40), withdrawal of consent (n = 34) and being lost to follow-up (n = 103).

The basic characteristics of the investigated group are presented in Table 1.

Chronic obstructive pulmonary disease comorbidities were defined as other diseases coexisting with COPD and affecting the natural history of COPD.[1] The list of comorbidities for each patient was established during the 1st consultation (V1) based on the detailed analysis of medical documentation, medications used, clinical examination, and laboratory results. Data on the presence of the following comorbidities was analyzed: CVDs (arterial hypertension, coronary heart disease, myocardial infarction, and arrhythmia), cerebrovascular diseases, diabetes, peptic

Table 1. Basic anthropometric data of the investigated group at the 1st (V1) and 2nd (V2) examination

Variables	V1 (n = 444)	V2 (n = 267)	p-value
BMI [kg/m^2]	27.1 (23.4–31.2)	27.6 (24.0–31.8)	0.46
mMRC	2.0 (1.0–3.0)	2.0 (1.0–3.0)	0.43
FEV_1 [% of predicted value]	50.0 (37.4–64.8)	52 (39–63)	0.31
FEV_1 ≥50% of predicted value FEV_1 <50% of predicted value	221 (49.8) 223 (50.2)	142 (53.2) 125 (46.8)	0.36
Number of hospitalizations due to pulmonary and extra-pulmonary causes	1.0 (0–1.0)	0.0 (0–1.0)	0.99

Data is presented as median (interquartile range – IQR) or as n (%); BMI – body mass index; mMRC – modified Medical Research Council scale; FEV_1 – forced expiratory volume in 1 s; COPD – chronic obstructive pulmonary disease; V1 – the 1st consultation; V2 – the follow-up consultation.

ulcers, gastroesophageal reflux disease (GERD), anemia, obstructive sleep apnea (OSA), malignancy (lung and other cancers), bone density abnormalities (osteoporosis and osteopenia), impaired nutritional status, chronic renal disease, and hypothyroidism.

The patients were subjected to the following procedures:

– spirometry with bronchial reversibility testing (Lungtest 1000; MES sp. z o.o., Skawina, Poland) in accordance with the recommendations of the American Thoracic Society/European Respiratory Society (ATS/ERS)[15];

– a 6-minute walk test (6MWT) in accordance with the ATS recommendations[15];

– an assessment of dyspnea with the modified Medical Research Council scale (mMRC)[16];

– the BMI evaluation, calculated as body mass divided by the square of the height (underweight was defined as BMI < 18.5 kg/m^2, healthy weight as 18.5 ≤ BMI < 25 kg/m^2, overweight as 25 ≤ BMI < 30 kg/m^2, and obesity as BM ≥ 30 kg/m^2);

– the bone mineral density (BMD) assessment within the posterior–anterior lumbar spine (L1–L4) and the femoral neck, performed with dual-energy X-ray absorptiometry (DEXA), using a Discovery Densitometer (Hologic, Waltham, USA) according to the manufacturer's recommended standard procedures (osteopenia was diagnosed for a T-score between –1.0 and –2.5, osteoporosis for a T-score ≤–2.5 SD);

– laboratory tests (blood count, thyroid stimulating hormone (TSH), serum creatinine concentration, glomerular filtration rate (GFR), and glucose), all performed in the morning after fasting in the central hospital laboratory, using routine methods;

– the BODE index calculation[17];

– monitoring the number of COPD exacerbations and hospitalizations due to pulmonary and extra-pulmonary causes.

The same procedures were performed at V1 and V2.

For further analyses, the patients were divided into:

– group I (without comorbidities);
– group II (1–2 comorbidities);
– group III (3–5 comorbidities);
– group IV (more than 5 comorbidities).

According to the degree of airflow limitation, measured by forced respiratory volume in 1 s (FEV_1), the patients were divided into:

– group 1 (patients with mild and moderate airflow limitation, FEV_1 ≥ 50% of predicted value);

– group 2 (patients with severe and very severe airflow limitation, FEV_1 < 50% of predicted value).

According to GOLD 2017 guidelines, the patients were divided into:

– group A;
– group B;
– group C;
– group D.

Statistical analysis

Statistical analysis was performed using STATISTICA v. 10 (StatSoft Inc., Tulsa, USA). The Shapiro-Wilk test was used to estimate the normality of the data distribution. The data was presented as mean ± standard deviation (SD), or as median and interquartile range (IQR), when appropriate. As the majority of the analyzed variables demonstrated a non-normal distribution, the Mann-Whitney U test and the analysis of variance (ANOVA) Kruskal-Wallis rank test were applied for group comparisons; the correlations were analyzed by the Spearman's rank correlation test. Categorical data was compared by the χ^2 analyses. A p-value <0.05 was regarded as statistically significant.

The study was approved by the Bioethical Committee of the Medical University of Warsaw, Poland (KB 207/2008), and all patients gave written informed consent before enrollment in the study.

Results

No comorbidities were found in 9 patients (2.0%, group I), 101 patients (22.7%, group II) had 1–2 comorbidities, 243 (54.7%, group III) had 3–5 comorbidities, and 91 (20.6%, group IV) had more than 5 comorbidities. Patients with more comorbidities were older and had higher BMI and

Table 2. Patient characteristics in relation to the number of comorbidities

Variables	Group I n = 9	Group II n = 110	Group III n = 243	Group IV n = 91
Age [years]	59 (56–68)$^{\#}$	62 (57–70)$^{@,**}$	67 (59–75)$^{@,\&}$	72 (63–76)$^{\#,**,\&}$
BMI [kg/m^2]	24.6 (21.2–28.7)	25.1 (21.8–29.4)$^{@,**}$	27.1 (23.7–31.5)$^{@}$	28.7 (24.8–32.4)**
mMRC	2.0 (2.0–2.0)	2.0 (1.0–2.0)$^{@,*}$	2.0 (1.0–3.0)$^{@}$	2.0 (2.0–3.0)*
FEV_1 [% of predicted value]	44.9 (26.4–58.2)	53.3 (41.7–68.3)$^{@}$	46.6 (35.6–60.4)$^{@,\&}$	56.0 (46.0–68.4)$^{\&}$
Pack-years of tobacco smoking	30.0 (28.2–40.0)	40.0 (29.0–48.0)	36.0 (22.0–50.0)	37.5 (26.5–50.0)
COPD duration [years]	4.0 (3.0–10.0)	5.0 (2.0–10.0)$^{@,*}$	7.0 (3.0–11.0)$^{@}$	6.5 (3.0–11.0)*

BMI – body mass index; mMRC – modified Medical Research Council scale; FEV_1 – forced expiratory volume in 1 s; COPD – chronic obstructive pulmonary disease; group I – no comorbidities; group II – 1–2 comorbidities; group III – 3–5 comorbidities; group IV – more than 5 comorbidities; I vs IV: $^{\#}$ p < 0.05; II vs III: $^{@}$ p < 0.05; II vs IV: * p < 0.05; ** p < 0.001; III vs IV: $^{\&}$ p < 0.05.

mMRC scores. We did not observe a correlation between the number of comorbidities and COPD airflow limitation severity (Table 2).

The number of comorbidities was related to the BODE index. The BODE index scores for group I vs group II vs group III vs group IV were 1.5 ±0.7 vs 2.6 ±2.6 vs 3.5 ±2.5 vs 2.8 ±2.5, respectively. Differences in the BODE index between groups II and III as well as groups III and IV were statistically significant ($p = 0.002$ and $p = 0.02$, respectively).

Cardiovascular diseases were the most frequent comorbidities. Forty-five patients underwent percutaneous transluminal coronary angioplasty (PTCA) and 9 patients had coronary artery bypass grafting (CABG). We did not find any difference in the number of comorbidities in patients stratified according to the degree of airflow limitation (Table 3).

During V1 we observed significant differences between groups 1 and 2 in the nutritional status. Patients classified as group 1 had higher BMI than patients from group 2 (29.0 ±6.1 kg/m^2 vs 26.7 ±6.1 kg/m^2). There were 145 patients (33%) with normal body weight: 28% of patients in group 1 and 37% in group 2. Overweight was diagnosed in 32% of the whole group (32% and 29% of patients in groups 1 and 2, respectively) and obesity was diagnosed in 32% of the whole group (37% and 25% of patients in groups 1 and 2, respectively). The distribution of nutritional categories in patients with different degrees of bronchial obstruction was significant ($p < 0.001$). We also found some significant correlations between the number of comorbidities and BMI ($r = 0.25$; $p < 0.0001$), and the number of hospitalizations due to extra-pulmonary causes ($r = 0.27$; $p < 0.0001$).

Bone densitometry was performed in 144 subjects during V1 and revealed osteopenia and osteoporosis in 66 patients (45.8%) and 41 patients (28.5%), respectively. No differences in the frequency of bone metabolism abnormalities were found between groups 1 and 2.

We analyzed the number of comorbidities according the GOLD 2017 groups for 373 patients and found differences only for the total number of comorbidities between groups A and D (3.7 ±1.9 vs 4.4 ±2.0; $p = 0.017$).

Two hundred and sixty-seven patients attended the 2nd consultation after 12 months. The most frequently reported new incidents were CVDs: coronary heart disease (n = 12), myocardial infarction (n = 6), arterial hypertension (n = 7), arrhythmia (n = 13), cerebral stroke (n = 1), and transient ischemic attack (n = 2). Two new cases of diabetes and 5 new cases of lung cancer were diagnosed.

Discussion

The present study showed that in a cohort of Polish COPD patients, there was a significant rate of comorbidity. Only 2% of patients did not report any comorbidities. The greatest was the incidence of CVDs. Cardiovascular diseases were also the most frequently reported newly diagnosed diseases during the 12-month observation. The prevalence of the individual comorbidities did not differ significantly in relation to the degree of airflow limitation, with the exception of cachexia, which was present only in patients with severe and very severe airway obstruction. The correlations between the number of comorbidities and the BODE index suggest that the presence of comorbidities affects the natural history of COPD. Our study contributes to the evaluation of the global burden of COPD and offers an insight into the characteristics of COPD patients in the Polish population.

Table 3. The prevalence of comorbidities in patients with $FEV_1 \geq 50\%$ and $FEV_1 < 50\%$ of the predicted value (groups 1 and 2, respectively)

Comorbidities	Whole group (n = 444) n (%)	$FEV_1 \geq 50\%$ (n = 221) n (%)	$FEV_1 < 50\%$ (n = 223) n (%)	p-value
Arterial hypertension	273 (61.5)	144 (65.2)	129 (57.8)	0.31
Coronary heart disease/ myocardial infarction	134 (30.1) 48 (10.8)	72 (32.6) 23 (10.4)	62 (27.8) 25 (11.2)	0.53 0.93
Arrhythmia	112 (25.2)	54 (24.4)	58 (26.0)	0.92
Diabetes	85 (19.1)	41 (18.6)	44 (19.7)	0.62
Cerebrovascular diseases: cerebral stroke transient ischemic attacks	 16 (3.6) 6 (1.3)	 9 (4.1) 4 (1.8)	 7 (3.1) 2 (0.9)	 0.79 0.27
Peptic ulcers	96 (21.6)	55 (24.9)	41 (18.4)	0.29
GERD	81 (18.2)	44 (19.9)	37 (16.6)	0.16
Anemia	62 (14.0)	31 (14.0)	31 (13.9)	0.34
OSA	96 (21.6)	51(23.1)	45 (20.2)	0.64
Malignancy: lung cancer other cancer locations	 11 (2.5) 32 (7.2)	 5 (2.3) 19 (8.6)	 6 (2.7) 13 (5.8)	 0.81 0.15
Chronic renal disease	361 (81.3)	172 (77.8)	189 (84.8)	0.048
Hypothyroidism	36 (8.1)	18 (8.1)	18 (8.1)	0.63

FEV_1 – forced expiratory volume in 1 s; GERD – gastroesophageal reflux disease; OSA – obstructive sleep apnea.

Our results confirm the observations of other authors. The Rotterdam study showed that the risk of sudden cardiac death is higher in COPD patients.[18] According to Chatila et al., the frequency of CVDs in COPD patients ranged from 13 to 56%; the frequency of arterial hypertension was as high as 15–82%.[8] In our study, arterial hypertension was also the most prevalent CVD. The common risk factors for COPD and CVDs are smoking and systemic inflammation.[8] However, other researchers do not share this opinion. Sin and Man found that a concomitance of COPD and ischemic heart disease was independent of smoking history, BMI, arterial hypertension, and serum cholesterol concentration; however, these authors found that a decrease of FEV_1 by 10% was associated with a 14% increase in all-cause mortality, a 28% increase in mortality due to CVDs and an increase of nonfatal coronary events by almost 20%. The authors concluded that COPD is a powerful, independent risk factor for cardiovascular morbidity and mortality.[19] The Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) study showed that COPD patients with CVDs reported a worse quality of life, a shorter walking distance in the 6MWT, a higher MRC score, and a higher value of the BODE index.[20] Our study confirmed that the BODE index values increased with the total number of comorbidities. Nevertheless, the incidence of CVDs did not increase with COPD airflow limitation severity. This finding is in agreement with the report by Lange et al.[21] However, these authors reported a higher incidence of cerebral stroke in patients at the GOLD 2 stage of severity. In our cohort, the incidence of cerebral stroke was similar in all groups.

We found a relatively high incidence of gastrointestinal diseases in our patients. In the study by Chatila et al., the frequency of gastrointestinal disorders was 15–62%.[8] Martinez et al. demonstrated that in patients with COPD, GERD had a negative influence on dyspnea severity, quality of life, the incidence of cardiovascular diseases, and the number of COPD exacerbations.[22] The relationship between GERD and COPD exacerbations has also been reported by other authors.Liang et al. demonstrated that GERD was associated with lung hyperinflation.[23] However, we could not evaluate this association, as our study protocol did not involve body plethysmography or computed tomography of the chest.

The relationship of diabetes and respiratory diseases has been the subject of research for many years. Hyperglycemia, systemic inflammation and bacterial infection may affect the lung function in patients with diabetes.[24,25] Literature data shows that COPD is a significant risk factor for type 2 diabetes and that COPD patients with diabetes have a more rapid disease progression and a worse prognosis.[26] We did not find a relationship between the prevalence of diabetes and COPD airflow limitation severity. McGhan et al. demonstrated that diabetes increased the risk of re-hospitalization after exacerbation in patients with severe COPD.[27]

The majority of the patients in our cohort were overweight or obese. This could explain the relatively high proportion of patients with OSA (22.3%). In the study by Greenberg-Dotan et al., the prevalence of COPD among patients with and without OSA was 7.6 and 3.7%, respectively.[28] Shiina et al. reported a prevalence of COPD in 12% of patients with OSA.[29] This overlap syndrome increased arterial stiffness and cardiovascular risk.[30] However, we cannot exclude that the high incidence of OSA in our group may be related to a selection bias. All centers that recruited patients have sleep laboratories and outpatient clinics dedicated to the treatment of OSA, to which a larger proportion of patients with OSA/COPD overlap syndrome may be referred.

More than 25% of the studied subjects reported symptoms from the motor organs; osteoporosis was diagnosed in 16% of the patients in whom bone densitometry was performed. Chen et al. provided evidence that COPD is an independent risk factor for osteoporosis.[30] According to the results of McGarvey et al., osteoporosis, along with older age, severely impaired the lung function and a high mMRC score increased the risk of severe exacerbations, requiring hospitalization.[31]

Lung cancer is one of the leading causes of mortality in patients with COPD.[9] There is evidence that COPD and emphysema are important risk factors for lung cancer.[32] In our study, 2.6% of the patients had a history of lung cancer; moreover, 5 new cases were diagnosed over the 12-month observation period. Of note, none of these 5 patients presented with new symptoms and were referred for further diagnostic work-ups upon the detection of focal lesions in a routine chest radiogram.

The incidence of anemia in our patients did not differ significantly from the incidence reported previously by other authors. In a systematic review on the impact of anemia on the course of COPD by Yohannes and Ershler, the incidence of anemia in COPD patients ranged from 7.5 to 34%.[33]

Chronic renal disease (CRD) is not listed as a typical COPD comorbidity. In our study group, the prevalence of CRD was very high. Our observation confirmed the results of the meta-analysis by Gaddam et al., who demonstrated the increased prevalence of CRD in patients with COPD.[34]

Eight percent of our patients were diagnosed with hypothyroidism. Terzano et al. showed that patients with overt hypothyroidism had lower PaO2, and maximal inspiratory and expiratory pressure compared with subjects with subclinical hypothyroidism and the control group.[35]

The data on comorbidities in our patients came mainly from a detailed questionnaire and medical documentation, but were not verified with diagnostic tests. This is the major limitation of our study. However, we made every effort to exclude a potential overdiagnosis and we carefully analyzed the available medical documentation of the patients. Another limitation of our study is the number of patients who attended the follow-up consultation: they

accounted for only 60% of the initial study group. Finally, as the patients were recruited only in university hospitals, we cannot exclude a selection bias, which may have influenced the results of the study.

We conclude that in a cohort of Polish COPD patients, the presence of comorbidities is frequent in all stages of the disease. The most frequent comorbidities were CVDs. This is in agreement with other studies from different regions. The correlation between the number of comorbidities and the BODE index confirms the negative impact of the comorbidities on the course of COPD and is irrespective of airflow limitation severity.

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Clinical-pathological characteristics of patients treated for cancers of the eyelid skin and periocular areas

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):325–330

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
We would like to thank Dr. David Aebisher for linguistic editing.

Received on May 26, 2017
Reviewed on July 18, 2017
Accepted on September 26, 2017

Published online on October 2, 2018

Abstract

Background. Malignant cancers of the eyelid skin and the periocular area comprise 5–10% of facial skin malignant cancers. Basal cell carcinomas (BCCs) dominate, comprising approx. 80–90% of cancers of this area of the face. Considerably less often, in 9–14% of cases, squamous cell carcinomas (SCCs) are observed.

Objectives. The aim of this study is to provide an epidemiological-clinical assessment of patients treated over the period 2006–2015 for primary malignant cancers of the eyelid skin considering histological diagnosis and clinical staging.

Material and methods. This study was based on an analysis of the medical records of 262 patients. Demographic data, age, sex, size and location of lesions, histological types, and clinical staging were assessed.

Results. In the analyzed group of 262 patients, most were over the age of 60 years. Primary cancers were most frequently located in the area of the medial canthus of the eye and in the lower eyelid skin. They were mainly lesions of 6–10 mm and 16–20 mm with histologically diagnosed basal cell carcinoma of nodular and cystic types.

Conclusions. The most represented group was that of patients aged 60–70 years with primary cancers located in the medial canthus of the eye (47.3%) and in the lower eyelid. Basal cell carcinoma was diagnosed, mainly nodular and cystic types.

Key words: skin, malignant tumor, eyelid, epidemiological studies

Cite as
Brodowski R, Pakla P, Dymek M, et al. Clinical-pathological characteristics of patients treated for cancers of the eyelid skin and periocular areas. *Adv Clin Exp Med*. 2019;28(3):325–330. doi:10.17219/acem/78023

DOI
10.17219/acem/78023

Eyelids constitute a facial aesthetic unit that has a complex anatomical structure which, apart from the function of protecting the eyeball, is of high importance for expressing emotions, feelings and moods. Accordingly, the diagnostics and treatment of cancers located in this part of the face cause difficulties in surgical practice. Epidemiological data shows that 5–10% of malignant cancers of the facial skin affect eyelids and the periocular area.[1–4]

Environmental factors, among others, play a significant role in the etiopathogenesis of malignant skin cancers of the periocular tissues and face. Facial skin, including the periocular area, as an exposed part of body, is vulnerable to the intense effects of UVB radiation, mainly from sunlight. Other factors predisposing to facial skin cancer development are potentially malignant lesions, i.e., actinic keratosis, sunburn and pigment changes.[5–8]

Among the histological types of malignant eyelid cancers, the most common is basal cell carcinoma (BCC), which comprises approx. 80–90% of all cancers of this area of the face. Considerably less often, in 9–14% of cases, squamous cell carcinoma (SCC) is observed. Adenocarcinomas are least often diagnosed.[9–12]

Objectives

The aim of this study was to provide an epidemiological-clinical assessment of patients treated at the Department of Maxillofacial Surgery of the Frederic Chopin Clinical Regional Hospital in Rzeszów, Poland, over the period 2006–2015 for primary malignant cancers of the eyelid skin. Moreover, the study was aimed at histological diagnosis and the clinical staging of particular cancer types located in the periocular area.

Material and methods

This study was based on an analysis of the medical records of 262 patients treated at the Department of Maxillofacial Surgery of the Frederic Chopin Clinical Regional Hospital in Rzeszów over the period 2006–2015 for primary malignant cancers of the eyelid skin and periocular tissues. Demographic data, age, sex, size and location of lesions, histological types, and clinical staging were assessed. The collected data was subjected to statistical analysis using STATISTICA v. 10.0 system (StatSoft Inc., Tulsa, USA). Two tests were used for the analysis: Pearson's χ^2 test, which assessed variable dependence in which the qualitative (not quantitative) data was taken into consideration, and the unilateral structure indicators significance test, which was used for comparing the amount and the percentage of a given option in order to determine if any of the indicated options occurs considerably more often than the others. The tests used in the analysis were non-parametric. Statistical significance was taken as $p < 0.05$.

Results

The results obtained from the conducted test material assessment are presented in the form of tables and descriptions. Between 2006 and 2015, a total of 2,062 patients were hospitalized at the department and treated on an outpatient basis for malignant head and neck skin cancers. This study solely concerns the 262 patients operated on due to malignant cancers of the eyelid skin and the periocular area on an inpatient basis, which constituted 12.7% of the total number of patients treated for malignant head and neck skin cancers. Table 1 shows the number of patients treated during particular years for skin cancers located in the periorbital area and demonstrates that in the last decade, annually 20–30 patients were treated for cancer located in this area. A slight increase in the number of patients with eyelid skin and periocular area cancer occurred year by year, yet at that time, this was not statistically significant $p < 0.05$.

Among the patients, there were 139 (53%) women and 123 (47%) men in the 11–94 age group. Data concerning the patients' sex and age is presented in Table 2.

In the analyzed material, the majority, i.e., 62.9% of patients are over 60 years of age. Among the patients, there were 6 people (2.3%) under the age of 30 who were admitted to the hospital in order to excise minor skin lesions

Table 1. Number of patients treated for malignant cancers between 2006 and 2015

Year	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	Total
Number (%)	20 (7.6)	28 (10.7)	19 (7.2)	30 (11.4)	32 (12.2)	23 (8.8)	26 (9.9)	22 (8.4)	30 (11.4)	32 (12.2)	262 (100.0)

Table 2. Sex and age of the treated patients

Sex	Age [years]							
	<30	31–40	41–50	51–60	61–70	71–80	≥81	Total
Female; n (%)	3 (2.2)	3 (2.2)	16 (11.5)	32 (23.0)	31 (22.3)	28 (20.1)	26 (18.7)	139
Male; n (%)	3 (2.4)	1 (0.8)	13 (10.6)	26 (21.1)	26 (21.1)	34 (27.6)	20 (16.3)	123
Total; n (%)	6 (2.3)	4 (1.5)	29 (11.1)	58 (22.1)	57 (21.8)	62 (23.7)	46 (17.6)	262
p-value	$\chi^2(6) = 2.77$; $p = 0.8376$							

(<5 mm) of the benign lump type in cases where histopathological examination of the material suggested the presence of basal cell carcinoma. Over time, an increase in the number of patients with eyelid skin cancer was observed. The statistical analysis of the material did not confirm a statistically significant relationship between the increase in the number of patients treated in particular 10-year age brackets and their sex. The difference between the number of patients in subsequent age brackets and the previous age bracket was statistically insignificant. Patients were referred for surgical treatment mostly by dermatologists, ophthalmologists, oncologists, general practitioners, and maxillofacial surgeons.

Patients admitted to the Clinic did not report any complaints, including pain, in most cases. The reason for being referred for treatment were mostly non-healing skin ulcerations and periocular tissue wounds, visual impairment caused by a growing tumor mass obscuring the field of vision, bleeding, and, rarely, aesthetic reasons.

In the case of 124 patients, who constituted 47.3% of patients, primary cancers were located in the area of the medial canthus of the eye, while in the case of 83 patients (31.7%) they concerned the lower eyelid. In 26 patients (9.9%), it was the upper eyelid, while in 29 patients (11.1%), lesions were located in the area of the lateral canthus of the eye.

The statistical analysis of the data shows that the occurrence of cancers in the area of the medial canthus of the eye was considerably more frequent than in the remaining areas, which was statistically confirmed ($p < 0.05$).

The sizes of lesions in several cases varied between less than 5 mm, found in 22 patients (8.4%), to more than 20 mm. Cancers ranging in size between 6 and 10 mm were treated most often (in 113 patients; 43.1%). Lesions between 11 and 15 mm were present in 40 patients (15.3%), whereas those between 16 and 20 mm occurred in 67 cases (25.5%). Lesions larger than 20 mm which spread into the surrounding tissues were found in 7 patients (2.8%). The statistical analysis shows that the differences in the frequency of eyelid primary cancers between 6 and 10 mm in size were statistically significant ($p < 0.05$) in comparison with the remaining eyelid and periocular area cancerous lesion sizes.

On the basis of clinical examination and histopathological verification, BCCs were observed most frequently, namely, in 213 patients (81.4%), while SCCs were found in 38 patients (14.5%). In 8 cases, i.e., 3.0%, eyelid adenocarcinomas were present, whereas 3 patients (1.1%) were diagnosed with eyelid skin malignant melanomas. The occurrence of BCC cancer was 4 times higher than the remaining types of malignant periocular area skin cancers.

An assessment of the patients with regard to the relationship between the type of malignant cancer and its location was conducted. Basal cell carcinomas were most frequently located in the medial canthus of the eye (in 102 patients; 47.9%), while in 68 cases (31.9%), it was found in the lower eyelid. The outbreak of BCCs in the upper eyelid affected 23 patients (10.8%), while 20 patients (9.4%) had it in the lateral canthus of the eye. Squamos cell carcinomas were also most often located in the medial canthus of the eye (in 17 patients; 44.7%), while in 11 patients (28.9%), it was located in the lower eyelid. Table 3 presents data regarding the relationship between the type of malignant cancer and its location with respect to the histopathological diagnosis, which shows that both BCCs and SCCs were mostly located in the medial canthus of the eye and concerned the lower eyelid.

The statistical analysis of the data regarding location shows that there are no statistically significant differences between the type of malignant cancer confirmed by histopathological examination and its location in periocular tissues ($p = 0.6134$).

Over the period 2006–2015, a total of 213 patients were treated at the clinic for different types and forms of eyelid and periocular area BCC. Data regarding the particular forms of BCC is presented in Table 4.

Table 3. Location and histopathological type of malignant cancer

Location	SCCs	BCCs	Adenocarcinoma	Melanoma malignum	Total
Lower eyelid; n (%)	11 (28.9)	68 (31.9)	2 (25.0)	2 (66.7)	83 (31.7)
Upper eyelid; n (%)	2 (5.3)	23 (10.8)	1 (12.5)	0	26 (9.9)
Medial canthus; n (%)	17 (44.7)	102 (47.9)	4 (50)	1 (33.1)	124 (47.3)
Lateral canthus; n (%)	8 (21.1)	20 (9.4)	1 (12.5)	0	29 (11.1)
Total; n (%)	38 (100)	213 (100)	8 (100)	3 (100)	262 (100)
p-value	$\chi^2(9) = 7.23$; $p = 0.6134$				

SCCs – squamous cell carcinomas; BCCs – basal cell carcinomas.

Table 4. Occurrence of particular BCC forms in the group of treated patients

Number	Morphological types of BCCs				
	nodular	ulcerative	cystic	cicatricial	pigmented
213	90 (42.2%)	21 (9.8%)	81 (38.0%)	13 (6.1%)	8 (3.9%)

BCCs – basal cell carcinomas.

Table 5. Type of BCCs depending on location

Location	Morphological types of BCCs				
	nodular n (%)	ulcerative n (%)	cystic n (%)	cicatricial n (%)	pigmented n (%)
Lower eyelid	23 (33.8)	18 (26.5)	18 (26.5)	7 (10.3)	2 (2.9)
Upper eyelid	11 (47.8)	3 (13.0)	7 (30.4)	2 (8.7)	0
Medial canthus	51 (50)	0	46 (45.1)	3 (2.9)	2 (2.0)
Lateral canthus	5 (25.0)	0	10 (50.0)	1 (5.0)	4 (20.0)
Total	90 (42.3)	21 (9.9)	81 (38.0)	13 (6.1)	8 (3.8)
p-value	$\chi^2(12) = 60.13$; p = 0.0000				

BCCs – basal cell carcinomas.

Most often, in a total of 90 patients (42.2%), the nodular form of BCC was diagnosed, while there were 81 cases (38.0%) with the cystic form of BCC. The pigmented form of BCC found in 3.9% of patients was the least frequently represented. The primary cancers of BCC in an ulcerative form were present in 21 patients (9.8%), while 13 patients (6.1%) suffered from the cicatricial type of BCC. The frequency of occurrence of the nodular and cystic forms in the analyzed material was similar. There were no statistically significant differences between the occurrence of these 2 forms (p = 0.2880). The nodular and cystic forms occurred statistically more frequently than the remaining variants of BCC. With respect to the further ulcerative form, in terms of occurrence, the difference was statistically significant for both the nodular type (p = 0.0027) and the cystic type (p = 0.0069). The statistical analysis shows that the nodular and cystic forms of BCC occurred considerably more frequently in the periocular area than did the remaining variants of BCC.

A detailed assessment of the particular morphological forms of BCC shows that the lower eyelid was the location of mainly nodular and cystic lesions which, together with the cicatricial forms, accounted for more than 80% of cases. The lesions located in the upper eyelid were the most common nodular variations (in 47.8% of cases). In the medial canthus of the eye area, the nodular form also occurred most frequently (in 51 patients), while the cystic form was diagnosed in 46 patients (45.1%). The cicatricial and the pigmented forms were mostly located in the lateral canthus area of the eye. The material presented indicates that the differences in the occurrence of the types and forms of BCC cancer were significantly related to their location (p = 0.0000). The abovementioned data is presented in Table 5.

Over the period 2006–2015, 38 patients with SCCs of the periocular area skin were treated. The degree of histopathological differentiation according to the Broder's scale regarding the percentage of mature, differentiated cells and atypical cells is shown in Table 6. Most frequently, in 19 patients, forms of well-differentiated cancer cells (G1) were observed; 13 patients (34.9%) were diagnosed with cancer of type G2 (moderately differentiated) and 6 patients (15.8%) had a poorly differentiated type of cancer (G3). There were no anaplastic SCCs (type G4) in the discussed material.

The statistical analysis demonstrates that differences in the occurrence of particular stages of histologically mature SCCs located in the periocular area were not statistically significant (p >0.05).

On the basis of the simplified TNM classification (feature T), developed by the Union of International Cancer Control (UICC), the clinical staging of eyelid skin cancer was assessed.[2] In the analyzed group, the most frequent were outbreaks of cancer in stage $T_2M_0N_0$ (43.1%). Tumors in stage $T_3M_0N_0$ were operated on in 40.8% of patients. Stage $T_1M_0N_0$ was treated in 35 patients (13.3%). The analysis confirmed that the differences in the occurrence of eyelid skin cancers in clinical stages ranging from T_1 and T_2, T_1 and T_3, T_1 and T_4, T_2 and T_4, T_3 and T_4 were statistically significant. A detailed overview of the clinical staging of eyelid cancers is included in Table 7.

It seemed of interest whether there is a relationship between the clinical staging of malignant skin cancers (TNM) and their location in the periocular area. In the analyzed group of patients, the malignant cancers located in the medial canthus area of the eye were the most frequently operated on (in the cases of 124 patients [47.3%]). They were mostly in the T_3 and T_4 clinical stages. In this location, there were also 3 patients in the T_4 stage who required a wide-margin excision of tissues surrounding the cancer. Detailed data regarding the relationship between

Table 6. Broder's eyelid SCC differentiation stages

Number	Differentiation stages of SCCs		
	G_1	G_2	G_3
38 (100.0%)	19 (50.0%)	13 (34.2%)	6 (15.8%)
p-value	$pG_1–G_2 > 0.05$; $pG_1–G_3 > 0.05$; $pG_2–G_3 > 0.05$		

SCCs – squamous cell carcinomas.

Table 7. Clinical staging of eyelid cancers according to the TNM classification

Number	$T_1N_0M_0$	$T_2M_0N_0$	$T_3M_0N_0$	$T_4M_0N_0$
262 (100%)	35 (13.3%)	113 (43.1%)	107 (40.8%)	7 (2.8%)
p-value	$pT_1–T_2 < 0.05$; $pT_1–T_3 < 0.05$; $pT_1–T_4 < 0.05$; $pT_2–T_4 < 0.05$; $pT_3–T_4 < 0.05$; $pT_2–T_3 > 0.05\%$			

Table 8. Clinical stage depending on primary outbreak location

Location	Clinical stage according to feature T				
	T_1	T_2	T_3	T_4	total
Medial canthus; n (%)	10 (28.6)	52 (46.1)	59 (55.3)	3 (42.8)	124 (47.3)
Lateral canthus; n (%)	0	17 (15.1)	11 (10.2)	1 (14.3)	29 (11.1)
Lower eyelid; n (%)	9 (25.7)	35 (31.0)	37 (34.5)	2 (28.6)	83 (31.7)
Upper eyelid; n (%)	16 (45.7)	9 (7.8)	0	1 (14.3)	26 (9.9)
Total; n (%)	35 (100.0)	113 (100.0)	107 (100.0)	7 (100.0)	262 (100.0)
p-value	($p > 0.05$)				

the clinical staging and the location of the cancers is shown in Table 8.

The differences in the occurrence of the particular clinical stages of cancers and their location were not statistically significant ($p > 0.05$).

Discussion

In the analyzed group of patients operated on at the Department of Maxillofacial Surgery of the Frederic Chopin Clinical Regional Hospital in Rzeszów, the dominant group were patients aged over 60 years, which is in accordance with the epidemiological data published by other authors and regarding not only primary cancers, but also the recurrence of facial skin malignant cancers.[13,14]

The most frequently histologically diagnosed eyelid skin cancer in the examined group was BCC, which occurred in 81.4% of patients. A similar frequency of occurrence was noted by Spiteri and McCormick, as well as Allali et al.[1,15] Among the excised BCCs, the most often diagnosed form were the nodular and cystic variations, which were found in 38.0% of patients. This was confirmed by statistical analysis. Similar results were obtained by Ben Simon et al.[8,14]

The nodular and cystic types of BCC occurred considerably more often in the medial canthus area of the eye in comparison to the remaining periocular areas. The location was the medial canthus of the eye in the examined group in 47.3% of the overall number of periocular area tumors operated on. The results of these examinations are compliant with the observations published by Ilijn et al.[6,10,13]

An analysis of our own material shows that the largest group were the cancers with diameters ranging from 10 to 20 mm and classified as T_2 or T_3 according to the UICC classification. This may result from the characteristics of the patients who qualified for surgical treatment at the Department. They were patients aged over 60 years, mostly afflicted with ailments and diseases of other systems. Minor lesions are mostly operated on at dermatological, laryngological and general surgery clinics. Histopathological verification of the excised eyelid tumors allowed for a diagnosis of SCC in 38 patients (14.5%) with dominant, well-differentiated G_1 cancer constituting 50% of histological diagnoses. There was no statistically significant correlation between the clinical stage of the SCC and the location of the tumor. The results obtained from these observations correspond with the publication published by Nemet's and Donaldson's teams.[9,10] On the basis of the retrospectively studied characteristics of the treated patients, the following conclusions are presented.

Conclusions

Between 2006 and 2015, 262 patients were treated for periocular area skin cancers at the Department of Maxillofacial Surgery of the Frederic Chopin Clinical Regional Hospital in Rzeszów. They represent 12.7% of all patients hospitalized due to head and neck skin cancers at that time.

The most represented group (45.3%) was that of patients aged between 60 and 70 years.

Primary cancers were mostly located in the medial canthus of the eye (47.3%) and in the lower eyelid (31.7%).

In 81.4% of treated patients, BCCs of mainly nodular and cystic types were diagnosed, while 14.5% of patients had SCCs.

The majority of the treated patients were diagnosed with cancer outbreaks in the T_3 (16–20 mm) and T_2 (6–10 mm) clinical stages.

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The impact of low alcohol consumption on the liver and inflammatory cytokines in diabetic rats

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):331–337

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
The authors gratefully thank the College of Pharmacy, Babylon University, Al-Hilla, Iraq, for the availability of the laboratory and technical assistance.

Received on February 16, 2017
Reviewed on March 6, 2017
Accepted on September 19, 2017

Published online on August 29, 2018

Abstract

Background. Diabetes mellitus (DM) and alcohol consumption is still one of the important research models that simulate variable clinical conditions and metabolic diseases, such as alcoholic liver diseases.

Objectives. The aim of this study was to evaluate the long-term cumulative effects of low alcohol consumption on the liver tissue, biochemical assays and some inflammatory cytokines in experimentally-induced DM rats.

Material and methods. Ethanol was administered in the drinking water (3% v/v) for 30 days to adult male Sprague-Dawley rats, with or without DM induced by streptozocin injection. Histological and biochemical parameters as well as some inflammatory cytokines – interleukin (IL)-4, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) – were measured.

Results. A significant increase in blood glucose level in the combination group was accompanied by a significant decrease in plasma insulin ($p < 0.001$ vs controls). Hepatic histopathology of the combination group revealed steatosis and fibrosis in addition to a significant increase in the gamma-glutamyltransferase (γ-GT) and alkaline phosphatase (ALP) levels ($p < 0.05$ and $p < 0.001$, respectively). A non-high-density lipoprotein (HDL) lipid profile (total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL)) revealed a significant increase in comparison to controls ($p < 0.05$), while HDL showed no significant change. The IL-4 and IL-6 levels were significantly higher ($p < 0.05$), while IL-10 and TNF-α revealed non-significant changes.

Conclusions. Depletion of the hyperglycemic response in the case of low alcohol consumption in DM rats was associated with elevated plasma cytokines, especially IL-6 and IL-4, which could be a part of a host defense mechanism to repair the hepatic and pancreatic damage through this inflammatory process. The severe liver damage under insult of low alcohol consumption and DM could serve as inhibitory factors in gluconeogenesis and glycogenolysis, with little or no impact on insulin levels.

Key words: alcohol, cytokines, diabetes, liver, rat

Cite as
Al-Humadi HW, Al-Saigh R, Sahib A. The impact of low alcohol consumption on the liver and inflammatory cytokines in diabetic rats. *Adv Clin Exp Med*. 2019;28(3):331–337. doi:10.17219/acem/77085

DOI
10.17219/acem/77085

Introduction

Diabetes mellitus (DM) is a public health problem which has reached epidemic proportions worldwide.[1] Insulin-deficient DM is associated with severe complications.[2,3]

Therefore, inflammatory cytokines provide important signals in the pathophysiology and complications of DM, being markedly elevated, and are thought to contribute to several complications (neuropathy, nephropathy, retinopathy, and periodontal lesions).[4–7] The relationships between plasma inflammatory markers and an increased risk of clinical DM are inconsistent, just like the relationships between these cytokines and other metabolic risk factors, such as dyslipidemia and hepatotoxicity, although hepatic damage is probably a major contributor to dyslipidemia in insulin-deficient DM.[3,8,9]

Recent studies have shown that moderate alcohol intake is associated with changes in the levels of several cytokines, especially pro-inflammatory ones – interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) – in different physiological and pathological states.[10]

Interleukin-6 protects against alcoholic liver injury via the activation of the signal transducer and activator of transcription 3 (STAT3), regulating liver fibrosis and inflammation, and promoting liver regeneration,while IL-10 inhibits alcoholic liver inflammation via the activation of STAT3 in Kupffer cells/macrophages (targeting immune cells) and the subsequent inhibition of liver inflammation.[11,12] Recent studies have suggested that IL-10 may play a dual role in controlling ethanol-induced steatosis and liver injury via the inhibition of pro-inflammatory cytokines, such as TNF-α, thereby decreasing alcoholic liver injury, or via the inhibition of the hepatoprotective cytokine IL-6, thereby potentiating alcoholic liver injury.[13]

Furthermore, it has been suggested that ethanol might modulate the production of these cytokines and their clearance at several sites, including adipose tissue.[14] Nevertheless, data on the relationship between alcohol intake and circulating cytokine levels is scant.

The precise role of inflammatory cytokines in the initiation and progression of pathological processes in DM, and its relationship with low alcohol consumption are not fully clear. However, our study contributed to evaluating the changes of the liver (histologically and biochemically) in addition to evaluating the inflammatory response by measuring proinflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-4 and IL-10) cytokines under insult of low alcohol consumption in streptozotocin (STZ)-induced DM in rats.

Material and methods

Animals

Twenty-four male Sprague-Dawley rats (8–10 weeks old, weighing 221 ±39 g) were used in the experiments. The rats were purchased from the National Center for Drug Research and Quality Control, Baghdad, Iraq. They were kept in the animal house of the Department of Pharmacology and Toxicology, Pharmacy College, University of Babylon, Al-Hilla, Iraq. They were housed 6 per cage, at a constant room temperature (22 ±1°C) and relative humidity (60–70%), and under a 12-hour light/12-hour dark cycle (light 8:00 am–8:00 pm). Standard food and water (treated or not) were provided ad libitum. All experiments were performed according to the international guidelines of laboratory animal care and the ethical guidelines for investigations on experimental animals (International Society for Applied Ethology).[15]

Induction of diabetes and alcohol consumption

The rats were divided into 4 main groups (n = 6 per group) as follows: control (C), alcohol consumption (ACH), diabetic (DM), and alcohol consumption with diabetic (ACH+DM). Diabetes mellitus was induced with a single intraperitoneal injection of STZ (65 mg/kg body weigh (b.w.)), diluted in a 0.1 mol/L citrate solution, pH 4.5 (Sigma-Aldrich), while low alcohol administration was performed ad libitum through drinking water consumption with 3% v/v ethanol (99% pure EtOH) (Sigma-Aldrich, St. Louis, USA) to simulate a dosage ranging from 4.0 to 5.0 g/kg b.w.[16] This dose might be enough to induce peripheral insulin sensitization.[17] The body weight of rats was measured once every 5 days. The rats were sacrificed after 30 days of the experiment.

Blood sample collection

On the last day of the experiment, blood was collected from the inferior vena cava and the serum was used to estimate the examined biochemical parameters and inflammatory cytokines.

Histological examination

The liver specimens were excised and fixed in 4% formalin, then embedded in paraffin wax as per conventional techniques. The sections cut (4 µm) were stained with hematoxylin and eosin (H&E). The specimens were examined by a double-blinded examiner. The morphological changes produced in the liver were histopathologically estimated under a high power field (×400) microscope.[18]

Biochemical assays

In order to assess blood glucose, hepatic functional integrity – serum alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyltransferase (γ-GT), and alkaline phosphatase (ALP), as well as lipid profile – total cholesterol (TG), triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL), a biochemical automatic analyzer was used (Hitachi, Roche, USA).

Plasma insulin and cytokines

Plasma insulin was measured by an enzyme-linked immunosorbent assay (ELISA) kit (EZRMI-13K; Linco Research, St. Charles, USA) and the quantity was recorded in ng/mL. Inflammatory cytokines were also measured by an ELISA kit (R&D Systems, Minneapolis, USA). Interleukin-6 was determined by an R6 000B Quantikine kit, IL-4 by an R4 000 Quantikine kit, IL-10 by an R1 000 Quantikine kit, and TNF-α by an RTA00 kit according to the manufacturer's protocol (R&D Systems); each assay was run with known standards (provided with the kit) to determine the quantity of cytokines in each sample in pg/mL.

Statistical analysis

The data is expressed as means ± standard deviation (SD) and was analyzed using a one-way analysis of variance (ANOVA), followed by multiple comparisons with Bonferroni's method and Tukey's honestly significant difference method. The significance level for all analyses was set at a probability $p < 0.05$. All analyses were performed by GraphPad Prism v. 5.3 for Windows (GraphPad Software, San Diego, USA).

Results

Body weight

The body weight of adult rats was not significantly different in the ACH group compared to the controls, but a marked and significant decrease in weight gain appeared in both the DM and ACH+DM groups between 10 and 30 days of the experimental period ($p < 0.05$ and $p < 0.01$ for DM, $p < 0.001$ for ACH+DM vs controls) (Fig. 1), in addition to a significant increase in the liver weight/body weight ratio in the ACH and ACH+DM groups ($p < 0.001$ for both groups vs controls) (Fig. 2).

Blood glucose and plasma insulin levels

Blood glucose level was significantly higher in the DM and ACH+DM groups ($p < 0.001$) in comparison to the controls, while non-significant changes were reported in the ACH group compared to the controls (Fig. 3), in contrast to plasma insulin level, which was significantly lower in both the DM and ACH+DM groups compared to the controls ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 4).

Liver enzymes

In the ACH, DM and ACH+DM groups, there is a non-significant increase in the enzymes that used for assessment of liver parenhymal inflammation and/or injury (ALT and AST) in comparison with the control with different proportions, while the response is different in the enzymes used for the assessment of cholestatic liver inflammation and/or injury (ALP and γ-GT); the ALP level in the ACH+DM group revealed a statistically significant increase in comparison to all groups (control ($p<0.001$), ACH and DM ($p<0.05$)). The γ-GT levels were statistically significantly higher in the ACH+DM group only compared to the controls ($p < 0.05$) (Table 1).

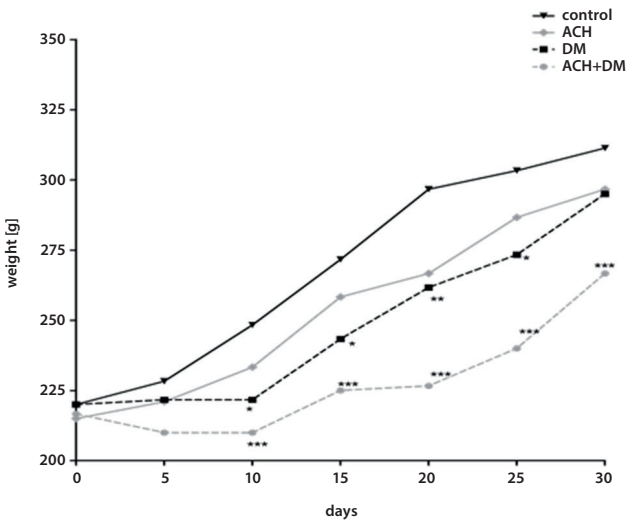

Fig. 1. Body weight in diabetic and non-diabetic rats exposed to 30 days of low-dose alcohol consumption

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; * $p < 0.05$ and ** $p < 0.01$ (DM vs controls); *** $p < 0.001$ (ACH+DM vs controls). All values refer to mean ± standard deviation (SD).

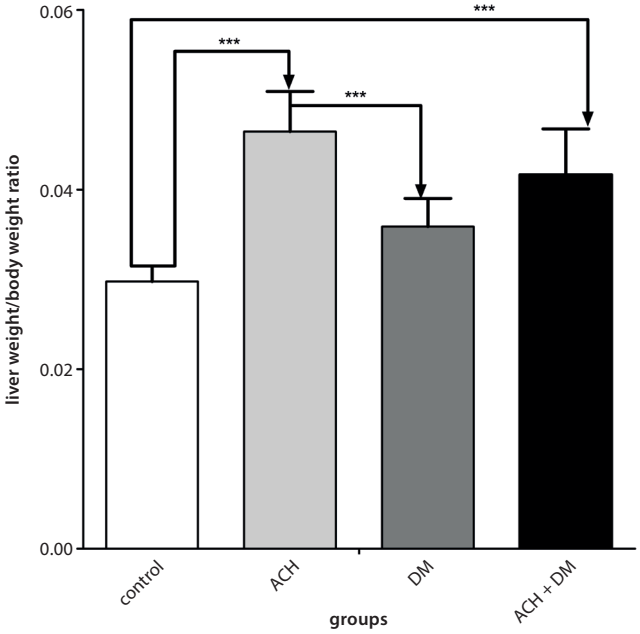

Fig. 2. The liver weight/body weight ratio in diabetic and non-diabetic rats exposed to 30 days of low-dose alcohol consumption

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; *** $p < 0.001$. All values refer to mean ± standard deviation (SD).

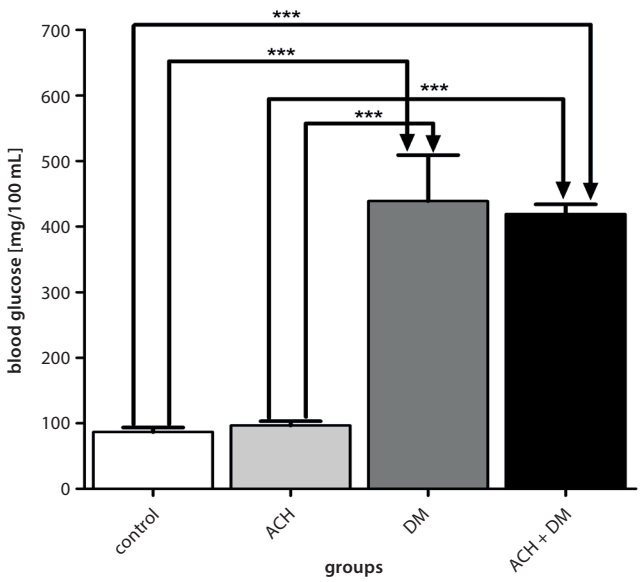

Fig. 3. Blood glucose levels in diabetic and non-diabetic rats exposed to 30 days of low-dose alcohol consumption

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; the number of asterisks (*) corresponds to the level of statistical significance (*** $p < 0.001$). All values refer to mean ± standard deviation (SD).

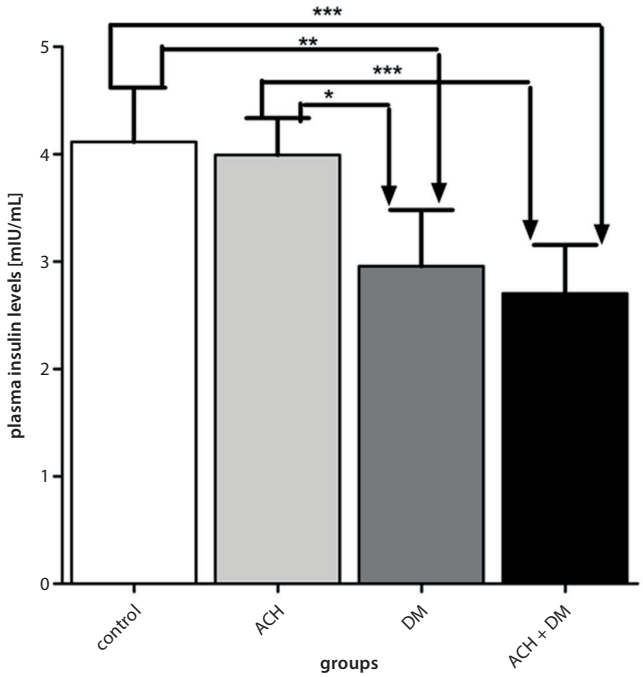

Fig. 4. Plasma insulin levels in diabetic and non-diabetic rats exposed to 30 days of low-dose alcohol consumption

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; the number of asterisks (*) corresponds to the level of statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All values refer to mean ± standard deviation (SD).

Determination of the lipid profile

The TC and TG levels revealed a significant increase in the ACH+DM group compared to the control group and the ACH group ($p < 0.05$) (Table 2). Low-density lipoprotein level was significantly higher in the ACH+DM group compared to the controls ($p < 0.05$), while in the ACH and DM groups, there was no significant difference compared to the control group. High-density lipoprotein measurement revealed no significant differences in the ACH, DM and ACH+DM groups compared to the control group (Table 2).

Histopathology

The histological examination of the ACH group revealed a mild inflammatory reaction with progressive micro- to macrovesicular steatosis with no fibrosis, while the DM group showed microvesicular steatosis that became marked with some fibrosis (Fig. 5). The livers obtained from rats exposed to STZ and alcohol (ACH+DM group) were enlarged, pale in color, soft, and greasy compared to the livers obtained from rats belonging to the control group. The histological examination of the ACH+DM liver sections revealed micro- and macrovesicular steatosis with degeneration and progressive development of lesions due to the effects of alcohol and STZ. Most liver sections showed an increase in fibrosis with plasma cell infiltrate, causing distortion of the usual concentric arrangement of hepatocytes. Moreover, there was congestion of the portal vessels and sinusoids, and the veins were dilated (Fig. 5).

Inflammatory cytokines

The IL-4 and IL-6 levels were significantly higher in the DM and ACH+DM groups compared to the control group ($p < 0.05$ and $p < 0.01$, respectively), while non-significant changes were found in the ACH group in comparison to the controls (Fig. 6A,6B, respectively). Furthermore, the IL-6 level was significantly higher in the DM and ACH+DM groups than in the ACH group ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 6B). Moreover, the IL-10 level was non-significantly higher in all experimental groups than in the control group (Fig. 6C), while the TNF-α level was non-significantly lower in the DM and ACH+DM groups than in the control group (Fig. 6D).

Discussion

Diabetes mellitus is still one of the important chronic diseases that have a significant impact on different inflammatory and metabolic markers. Our study focused on some of these markers under insult of low alcohol consumption over 30 days. Liver histological examination in the ACH+DM group after 30 days revealed progressive

Table 1. The levels of serum liver enzymes indicative of liver functional integrity in diabetic and non-diabetic rats exposed to low-dose alcohol consumption

Experimental group	ALT	AST	ALP	γ-GT
Control	351.2 ±26.3	74.3 ±16.1	162.2 ±13.4	0.33 ±0.15
ACH	373.6 ±37.1	109.8 ±38.6	383.4 ±73.2	1.6 ±0.24
DM	392.5 ±29.9	128.1 ±25.4	409.1 ±161.8	2.5 ±1.07
ACH+DM	388.1 ±82.1	128.2 ±13.9	699.2 ±365.0***###	3.2 ±2.50*

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; ALT – alanine aminotransferase; AST – aspartate aminotransferase; ALP – alkaline phosphatase; γ-GT – gamma-glutamyltransferase; the number of asterisks (*) or hashes (#) corresponds to the level of statistical significance (*** $p < 0.001$ combination vs control; # $p < 0.05$ combination vs ACH; ## $p < 0.05$ combination vs DM). All values refer to mean ± standard deviation (SD) serum levels, expressed in IU/L.

Table 2. The lipid profile in diabetic and non-diabetic rats exposed to low-dose alcohol consumption

Experimental group	TC	TG	HDL	LDL
Control	51.1 ±15.2	131.2 ±58.9	34.1 ±3.4	17.3 ±10.5
ACH	48.6 ±13.1	132.1 ±71.3	31.1 ±2.8	20.7 ±6.6
DM	59.3 ±16.4	165.5 ±65.1	29.5 ±3.2	22.6 ±11.7
ACH+DM	80.5 ±24.6*#	299.4 ±192.5*#	24.5 ±12.6	38.8 ±16.3*

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; TC – total cholestrol; TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; the number of asterisks (*) or hashes (#) corresponds to the level of statistical significance (* $p < 0.05$ combination vs control; # $p < 0.05$ combination vs ACH). All values refer to mean ± standard deviation (SD) serum levels, expressed in mmol/L.

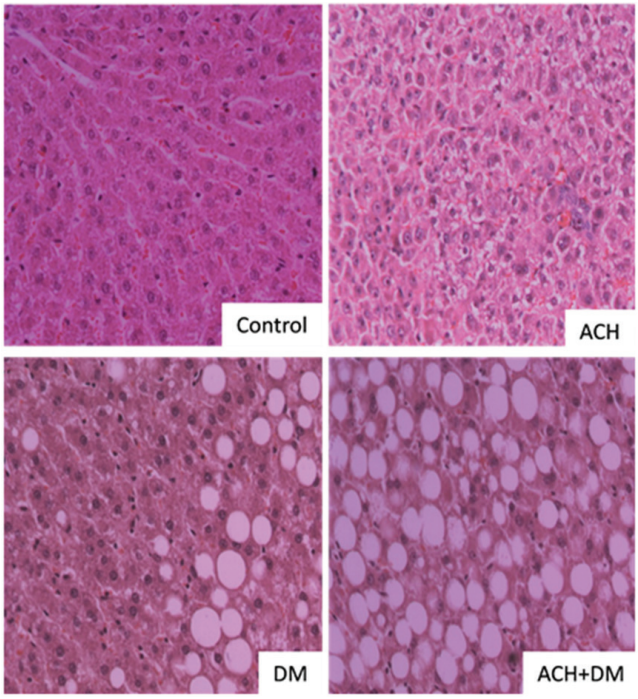

Fig. 5. Overview of the hematoxylin and eosin (H&E) staining of liver sections obtained from diabetic and non-diabetic rats exposed to 30 days of low-dose alcohol consumption

Liver sections representing the control group show normal liver morphology; the ACH group liver sections present with mild micro- and macrovesicular steatosis; liver sections obtained from the DM group show mild steatosis; the ACH+DM group sections present with micro- and macrovesicular steatosis with degeneration; control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin (STZ)-treated rats; ACH+DM – rats exposed to low doses of ethanol and STZ.

micro- and macrovesicular steatosis with degenerative changes; these hepatic fatty deposits were probably due to the inability of the liver to export triglycerides, which had been previously rationalized by the effects of hypoinsulinemia induced by STZ.[19] Moreover, mild fibrosis with plasma cell infiltrate and congestion of the portal vessels and sinusoids were found.[20,21] On the other hand, significant weight loss was observed in the combination group, in spite of the fatty liver, due to progressive and severe liver damage, which affected its function and disturbed the normal metabolism by augmenting the catabolic state. This result is in contrast to the weight gain in the DM-only group that was shown by Chowdhury et al., who detected the changes in weight after the induction by STZ with hyperphagia.[22] Moreover, there was a significant increase in the liver weight/body weight ratio, which is in accordance with previous studies, confirming a decrease in body weight gain in diabetic rats without the influence of food consumption.[16] Furthermore, this combination group (our model) led to a leakage of liver enzymes (ALT, AST, ALP, and γ-GT), which became significant only in the case of ALP and γ-GT. This may be related to the duration of the experiment, resulting in a fluctuation in the levels of enzymes in addition to the hepatotoxic effect of both alcohol and DM.[23,24] Additionally, inflammatory cytokines variably reacted to the pro- or anti-inflammatory mediators at the end of day 30 in the combination group. Tumor necrosis factor alpha as a pro-inflammatory cytokine showed a non-significant decrease, which might enhance the repair processes of liver damage, while IL-6 showed

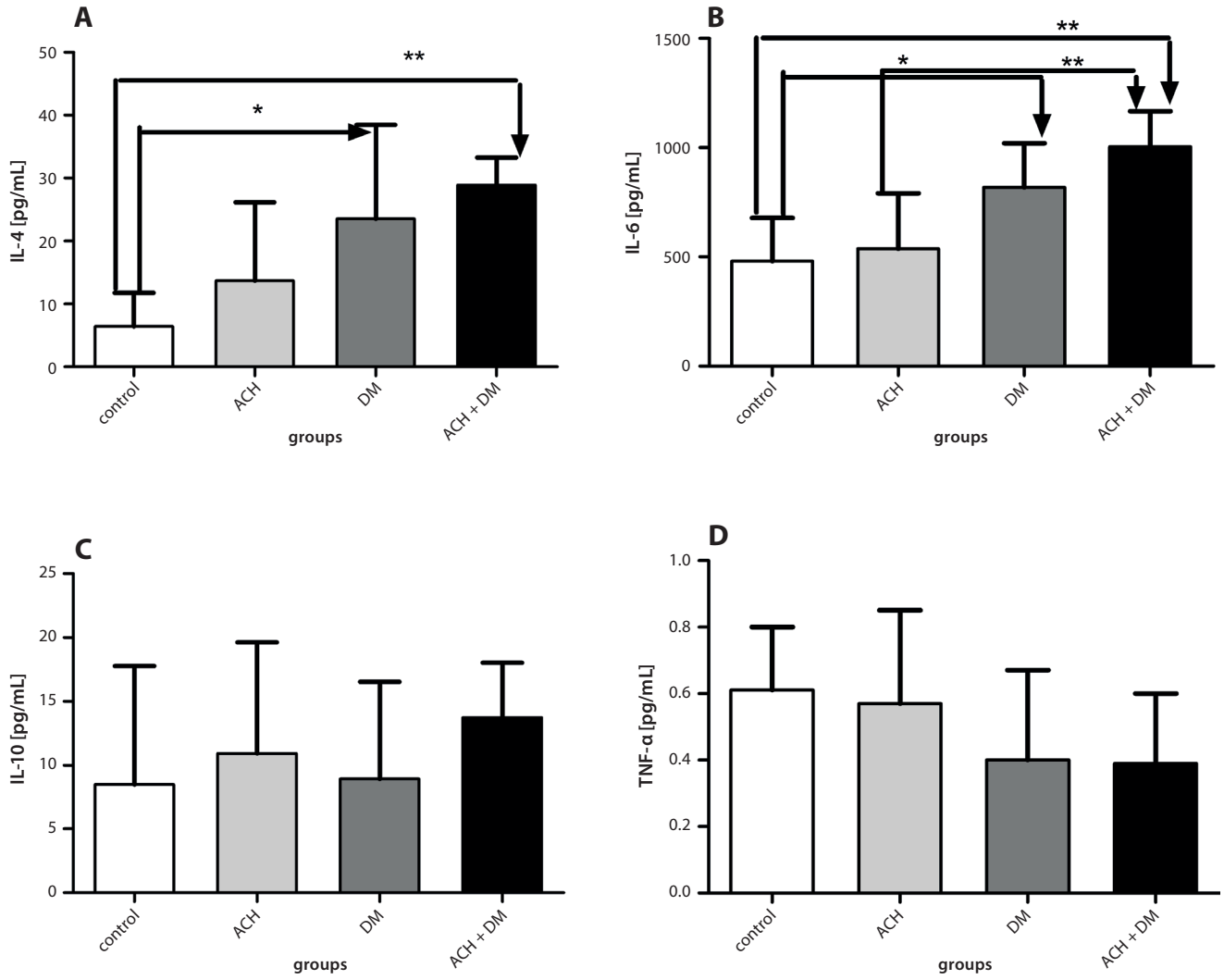

Fig. 6. Plasma inflammatory cytokine levels (pg/mL) in diabetic and non-diabetic rats exposed to 30 days of low-dose alcohol consumption

Control – rats receiving a normal diet and water; ACH – rats receiving low doses of ethanol; DM – streptozocin-treated rats; ACH+DM – rats exposed to low doses of ethanol and streptozocin; IL-4 – interleukin-4; IL-6 – interleukin-6; IL-10 – interleukin-10; TNF-α – tumor necrosis factor alpha; the number of asterisks (*) corresponds to the level of statistical significance (* $p < 0.05$; ** $p < 0.01$). All values refer to mean ± standard deviation.

a significant increase in all groups, with high levels in the combination group as an important pro-inflammatory mediator that acts as a protective marker in liver pathologies.[13,25] On the other hand, some anti-inflammatory cytokines (IL-4 and IL-10) reacted positively. Interleukin-4 had an important role in protection against type 1 DM during pancreatic cell destruction and IL-10 level was non-significantly increased in response to Kupffer cell mediators with the inhibition of TNF-α.[26,27]

The non-HDL lipid profile generally showed a significant increase by augmenting the effects of hypoinsulinemia and the lipolytic response with chronic low consumption of alcohol.[28] High inflammatory cytokines also play an important role in lipid metabolism, stimulating the production of triglycerides in the liver.[29–31] Hyperglycemia was still progressive, but to a lesser extent than in the DM group. This might be related to the hepatic insulin sensitizing effect of low doses of alcohol, which added to the low level of glucagon and decreased gluconeogenesis caused by alcohol in spite of the hyperglycemic effect of DM.[17,32]

Therefore, a combined state of both low alcohol consumption and DM is associated with a depletion of the hyperglycemic response with elevated plasma cytokines, especially IL-6 and IL-4, which could be a part of a host defense mechanism to repair the hepatic and pancreatic damage through this inflammatory process. The severe liver damage under insult of low alcohol consumption and DM could serve as inhibitory factors in gluconeogenesis and glycogenolysis, with little or no impact on insulin levels.

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Potential plasma biomarkers of bladder cancer identified by proteomic analysis: 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*. 2019;28(3):339–346

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Funding sources
None declared

Conflict of interest
None declared

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Received on April 13, 2017
Reviewed on July 18, 2017
Accepted on October 27, 2017

Published online on July 2, 2018

Abstract

Background. Bladder cancer diagnosis and surveillance includes cystoscopy and cytology. New methods for the detection of bladder cancer are needed, because cystoscopy is invasive and expensive, and because urine cytology is not sensitive enough.

Objectives. The aim of the study was to select potential plasma protein markers for bladder cancer which could be useful in developing a specific laboratory test to improve diagnosis and to establish treatment strategies in order to prevent the recurrence of the disease.

Material and methods. Plasma proteome maps were prepared based on 2-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), combined with image gel analysis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of plasma samples from patients with urothelial bladder cancer, and they were compared to normal samples.

Results. The analyses of bladder cancer plasma samples allowed us to distinguish 3 groups of proteins whose relative abundance differed from that in normal samples. The 1st one comprised modified forms of plasma transferrin, fibrinogen gamma and complement C3b, which were absent in normal plasma. The 2nd group comprised haptoglobin, alpha-2-macroglobulin, vitamin D-binding protein, and pigment epithelium-derived factor, which occurred in the cancerous samples in large quantities. The 3rd group consisted of 3 molecular forms of immunoglobulin M (IgM), the relative abundance of which was significantly lower in the cancerous plasma samples.

Conclusions. The data indicated potential plasma biomarkers associated with inflammation, immunity and coagulation processes accompanying bladder cancer. They could be used for the development of a laboratory test(s) useful in clinical practice.

Key words: bladder cancer, biomarkers, proteomics, blood plasma glycoproteins, 2-dimensional gel electrophoresis

Cite as
Lemańska-Perek A, Lis-Kuberka J, Lepczyński A, et al. Potential plasma biomarkers of bladder cancer identified by proteomic analysis: A pilot study. *Adv Clin Exp Med*. 2019;28(3):339–346. doi:10.17219/acem/79296

DOI
10.17219/acem/79296

Introduction

Bladder cancer is often symptomless or may produce symptoms similar to a urinary tract infection. The tumors are routinely evaluated by cystoscopy, an invasive endoscopic procedure in order to aid diagnosis and post-treatment surveillance; intensive efforts have been undertaken to research and develop specific, sensitive and non-invasive tests based on a marker or group of markers.[1] The aim is the early detection of primary or recurrent bladder cancer, which might help in establishing new treatment strategies, monitoring treatment and preventing recurrence.[2,3]

Biomarkers described to date are tumor-associated molecules that degrade the components of the extracellular matrix and the urothelium. The US Food and Drug Administration has approved only a few of them, i.e., bladder tumor antigen (BTA), nuclear matrix protein 22 (NMP22) and fibrinogen degradation products.[4–6] Moreover, the following molecules that are still under investigation have been reported to be associated with urinary carcinogenesis: BLCA-4, cytokeratin, fibronectin degradation products, uroplakins and their glycovariants, psoriasin, zinc-alpha-2-glycoprotein, pro-matrix metalloproteinase-2 (proMMP-2), matrix metalloproteinase-7 (MMP-7), and endostatin.[7–13] It has also been reported that the serum levels of CYFRA 21-1 and soluble E-cadherin are associated with advanced and high-grade urothelial bladder carcinoma.[14,15] In the opinion of Goodison et al., most of these biomarkers may be more useful as prognostic factors aiding therapeutic decisions. So far, none of the recommended urinary cancer biomarkers have been found to be sufficiently sensitive and specific to detect the entire spectrum of bladder cancers and to be used in routine clinical practice.[3]

A new powerful and complex tool – proteomics, using 2-dimensional gel electrophoresis (2DE) to separate complete proteins in a sample, and immunoblotting or mass spectroscopy (MS) techniques, integrated with advanced computer software, make it possible to precisely identify, characterize and quantify the data. It is usually used to select potential marker proteins from complex protein mixtures for further analysis in larger groups. So far, a 2-dimensional gel-based proteomic approach coupled with MS and database searching has been used in order to explore the biomarkers of bladder cancer in urinary samples, tissue samples derived from biopsies from the transurethral resections of the bladder and patients' plasma samples.[16–18] Despite the abovementioned pioneering studies which have shed some light on the molecular mechanisms of bladder transitional cell carcinoma, there is still a great need to identify novel proteins or a group of bladder cancer biomarkers.

The aim of our study was to analyze differentially expressed levels of proteins in the plasma samples of patients with urothelial bladder cancer and acute cystitis by 2-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), combined with image gel analysis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. Furthermore, the goal was to select a group of potential protein markers of bladder cancer which might be used in the development of a laboratory test(s) useful in clinical practice.

Material and methods

Blood plasma sample collection

Blood samples were obtained from 6 patients (54–91 years old) from the Department of Urology and Oncologic Urology of Wroclaw Medical University, Poland, who were being treated for bladder cancer. They were included in the study after providing written informed consent. The study was approved by the local Bioethical Committee (KB-406/2014).

The tumor was staged according to the TNM Classification of Malignant Tumors (TNM) and graded according to World Health Organization/International Society of Urologic Pathology (WHO/ISUP) 2004 criteria.[19,20] The bladder cancer patients' samples were divided into 2 groups according to the disease stage. The 1st group (C) comprised samples derived from 4 patients (2 women and 2 men, aged 70.8 ±17 years) with bladder tumors. The 2nd group (S), suspected of having cancer, consisted of 2 samples of patients (2 men, aged 62.5 ±0.7 years) who had undergone the transurethral resection of urothelial bladder cancer about 2 years earlier and were recurrence-free, but suffered from acute cystitis, lasting approx. 2 weeks.

The normal group (H) consisted of healthy volunteers aged 48.7 ±4 years (3 women).

Blood plasma sample preparation

The blood was drawn into plastic test tubes containing sodium citrate as an anticoagulant (1 part of 3.8% (w/v) citrate to 9 parts of the sample) and the plasma was separated from the blood cells by centrifugation at 2,000 × g for 15 min. The samples were stored at –76°C until used.

Blood plasma samples were depleted of the 2 most abundant proteins, using a ProteoExtract Albumin/IgG Removal Kit, Maxi (Calbiochem Merck, Geneva, Switzerland) to enrich the content of medium- and low-abundant protein in this medium. Depleted samples were subsequently precipitated with 4 volumes of cold acetone (–20°C) for 2 h and then centrifuged (20,800 × g, 4°C, 30 min) to obtain protein pellets. The protein pellets were dissolved in lysis buffer, containing 7 M of urea, 2 M of thiourea, 4% w/v of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% w/v of DL-dithiothreitol (DTT), 0.2% w/v of 3–10 carrier ampholytes, and 2 mM of tributylphosphine (TBP). The protein concentration in the samples was determined by a modified Bradford assay (Bio-Rad6 Protein Assay; Bio-Rad, Hercules, USA), according to the manufacturer's instructions.

Two-dimensional electrophoresis

Isoelectric focusing (IEF) was performed after loading blood plasma proteins (800 µg) on 5–8 24 cm nonlinear (NL) ReadyStrip IPG Strips (Bio-Rad). The proteins were separated for a total of 90,000 Vh, using a Protean IEF Cell (Bio-Rad). After IEF, the immobilized pH gradient (IPG) strips gained equilibrium at room temperature in a buffer solution containing 6 M of urea, 0.5 M of Tris/hydrochloric acid (pH 6.8), 2% w/v of sodium dodecyl sulfate (SDS), 30% w/v of glycerol, and additional 1% w/v of DTT to reduce the separated polypeptides (15 min) or 2.5% w/v of iodoacetamide to alkylate the proteins (20 min). Subsequently, the proteins were separated according to molecular range (SDS-PAGE) in 12% polyacrylamide gels (20 × 25 cm) at 40 V for 2.5 h, and then at 100 V for 16 h at 10°C, using a Protean Plus Dodeca Cell electrophoretic chamber (Bio-Rad). The proteins were visualized using Coomassie brilliant blue G-250 according to the protocol described by Lepczyński et al.[21]

Image analysis

The acquisition of gel images was performed using a GS-800 Calibrated Densitometer (Bio-Rad). To align and quantify the protein spots, the PDQuest Advanced 2D-Gel Analysis Software v. 8.0.1 (Bio-Rad) was used. PDQuest enables the user to calculate the value of relative protein expression based on several measurements of the optical density of spot coloration intensity, and to quantify the protein spot area in order to represent it as a dimensionless quantity. The normalization of the experimental data was performed using a local regression model (LOESS). The significance of the differences in protein spot abundances was confirmed using Student's t-test within the PDQuest software. The significance of the differences was set at $p \leq 0.05$. To evaluate the intragroup variability, the coefficients of variation (CV) for each group were calculated based on the information about the location and relative expression for each protein spot within the groups. The experimental molecular masses of the identified protein spots were computed using Precision Plus Protein Kaleidoscope Standard for SDS-PAGE (Bio-Rad) as the reference.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry protein identification

Following 2DE, the protein spots that showed significantly differentiated expression were identified by MALDI-TOF MS as previously described by Ożgo et al.[22] Briefly, the protein spots were excised from the gels, decolorized (washed in a buffer solution containing 25 mM of ammonium bicarbonate [NH_4HCO_3] in 5% v/v of acetonitrile [ACN]), then washed twice in a solution of 25 mM of NH_4HCO_3 in 50% v/v of ACN, dehydrated (100% ACN), vacuum-dried, and incubated overnight with trypsin (8 µL/spot of 12.5 µg of trypsin/mL in 40 mM of NH_4HCO_3) (Promega, Madison, USA) at 37°C. After extraction with 100% ACN, combined with an equal volume of matrix solution (2.5 mg/mL of α-cyano-4-hydroxycinnamic acid [CHCA], 0.1% v/v of trifluoroacetic acid [TFA], 50% v/v of ACN), the obtained peptides were loaded onto a MALDI-MSP AnchorChip 600/96 plate (Bruker Daltonics, Bremen, Germany). Peptide Mass Standard II with a mass range of 700–3200 Da (Bruker Daltonics) was used to calibrate the mass scale.

The protein identification was done in the positive-ion reflector mode of a Microflex MALDI-TOF MS (Bruker Daltonics). The peptide mass fingerprinting (PMF) data were compared with the SwissProt/NCBI databases. The parameters used for the database search were: monoisotopic mass, 150 ppm mass accuracy, trypsin as an enzyme with 1 missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. The results were further validated by the Mascot score and sequence coverage.

Heat map generation

PermutMatrix software v. 1.9.3 (The free access to the software is available on-line http://www.atgc-montpellier.fr/permutmatrix/) was used to create a heat map based on the data of the expression of the proteins in the healthy individuals, and in the cancer-suspected and cancerous patients.[23] The data on protein expression level for each spot was normalized within rows, i.e., the mean expression level for each sample gel was calculated for each statistically differentially altered protein spot and the expression level of this protein for each gel was divided by the arithmetic mean. After division, it was subjected to base 2 logarithmic transformation. Thus, a negative value of the logarithm indicated a decrease in the expression, whereas a positive value showed an increase. In addition, the mean values were calculated based on the resulting values, but when the spot was absent on the gel (0 abundance), the 0 value was substituted by 1.

Results

Analysis of plasma proteome differences

The analysis revealed from 361 to 387 protein spots per analyzed 2D gel, representing the protein profiles of the human plasma. Of them, 327 spots matched to each analyzed gel were subjected to statistical analysis between the cancer groups C and S, and group H. The CV was estimated to be 51.5%, 46.6% and 53.3% for groups H, C and S, respectively.

The significant quantitative differences in protein profiles revealed 15 differentially expressed protein spots (Fig. 1) in the plasma samples of patients with urothelial

Table 1. Characterization of the plasma protein spots revealed by proteomic analyses of the bladder cancer plasma samples

Spot No.	Database protein name/ identified blood plasma protein	Accession No.	Calculated Mr [kDa]	Theoretical pI/Mr [kDa]	Mean relative abundance in the groups			MALDI-TOF MS		
					H	C	S	Mascot score	Sequence coverage (%)	Mass values matched
1	haptoglobin/haptoglobin β chain	Q9UC67	43.1	6.13/45.86	29,968.2	78,764.0	93,212.1	81	18	10
2	haptoglobin/haptoglobin β chain	Q9UC67	42.9	6.13/45.86	2,616.9	12,149.3	14,759.0	115	29	13
3	vitamin D-binding protein	P02774	59.2	5.40/54.53	5,771.8	47,832.1	47,612.2	228	59	25
4	alpha-2-macroglobulin	1009174A	201.6	5.95/162.07	234.8	731.1	940.5	90	13	13
5	fibrinogen gamma	0602239A	54.8	5.54/46.82	4,206.8	8,799.9	6,449.0	206	59	22
6	fibrinogen gamma	0602239A	54.1	5.54/46.82	137,003.0	246,912.8	279,217.8	146	51	14
7	fibrinogen gamma	0602239A	51.7	5.54/46.82	N/D	2,274.0	1,510.3	133	53	15
8	serotransferrin	P02787	54.9	6.81/79.29	N/D	1,626.1	1,617.6	107	22	17
9	human complement component C3b, chain B	2I07_B	125.4	5.16/104.91	N/D	645.7	980.2	71	18	15
10	human complement component C3b, chain B	2I07_B	80.0	5.16/104.91	3,139.7	7,066.4	5,018.1	70	23	19
11	pigment epithelium-derived factor	AAA84914	51.0	6.56/40.22	1,270.5	2,831.3	2,127.8	78	34	11
12	insulin-like growth factor binding protein, acid labile subunit	AAH25681	89.3	6.33/66.77	1,367.2	870.4	1,065.8	111	27	13
13	Ig mu chain C region/IgM heavy chain	P01871	83.7	6.35/49.96	15,616.3	3,876.4	1,819.6	113	36	14
14	Ig mu chain C region/IgM heavy chain	P01871	83.0	6.35/49.96	35,847.6	7,263.2	4,501.8	129	36	14
15	Ig mu chain C region/IgM heavy chain	P01871	82.2	6.35/49.96	51,699.2	16,930.2	15,115.7	86	42	16

Table 1 lists the differentially expressed plasma proteins abundantly occurring in the plasma samples of patients with bladder cancer and of healthy controls. The proteins were revealed (see labeled spots in Fig. 1) and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS), following 2-dimensional electrophoresis (2DE) analysis. Protein names and accession numbers are given as found in the SwissProt/NCBI databases. C – patients with bladder tumors; H – healthy controls; IgM – immunoglobulin M; Mr – molecular mass; N/D – not detected in the normal plasma samples; pI – isoelectric point; S – patients suspected of having cancer.

bladder cancer and acute cystitis spots. The matched protein names identified by SwissProt/NCBI databases are given in Table 1. Fifteen selected spots were identified as 9 proteins (Table 1). They include the following proteins: 2 molecular forms of haptoglobin β chain (spot 1: 43.1 kDa and spot 2: 42.9 kDa), vitamin D-binding protein (spot 3: 59.2 kDa), alpha-2-macroglobulin (spot 4: 201.6 kDa), 3 forms of fibrinogen gamma (spots 5–7: 54.8 kDa, 54.1 kDa and 51.7 kDa, respectively), serotransferrin (spot 8: 54.9 kDa), 2 forms of human complement component C3b (spot 9: 125.4 kDa and spot 10: 80.0 kDa), pigment epithelium-derived factor (spot 11: 51.0 kDa), insulin-like growth factor binding protein (spot 12: 89.3 kDa), and 3 forms of immunoglobulin M (IgM) heavy chain (spots 13–15: 83.7 kDa, 83.0 kDa and 82.2 kDa, respectively).

Figure 2 presents a heat map which shows a comparative visualization of the individual levels of the differentially expressed protein spots in the control samples (H1–H3), the cancer-suspected samples (S1 and S2) and the bladder cancer patients' plasma samples (C1–C4). The observed trends of the up- and downregulation of protein abundances in the cancerous and cancer-suspected samples analyzed in respect to those in the samples of healthy individuals are shown in Tables 1 and 2.

The protein spots identified as transferrin (54.9 kDa), fibrinogen gamma (51.7 kDa) and complement C3b (125.4 kDa) occurred abundantly in both groups C and S, but were absent in the normal plasma samples. The remaining identified proteins, apart from a low relative abundance of IgM, showed a high abundance in groups C and S compared with group H.

The ratio values, which compare the relative abundance of the respective proteins between groups C and H, S and H, and C and S, are given in Table 2. The highest ratio values of C/H and S/H were found for vitamin D-binding protein (8.29 and 8.25, respectively). This indicates that the relative abundance of vitamin D-binding protein in both groups C and S was more than 8 times higher than that of the normal (H) group. The ratio values of C/H and S/H were 2.0 or higher for 42.9 kDa haptoglobin (4.64 and 5.64,

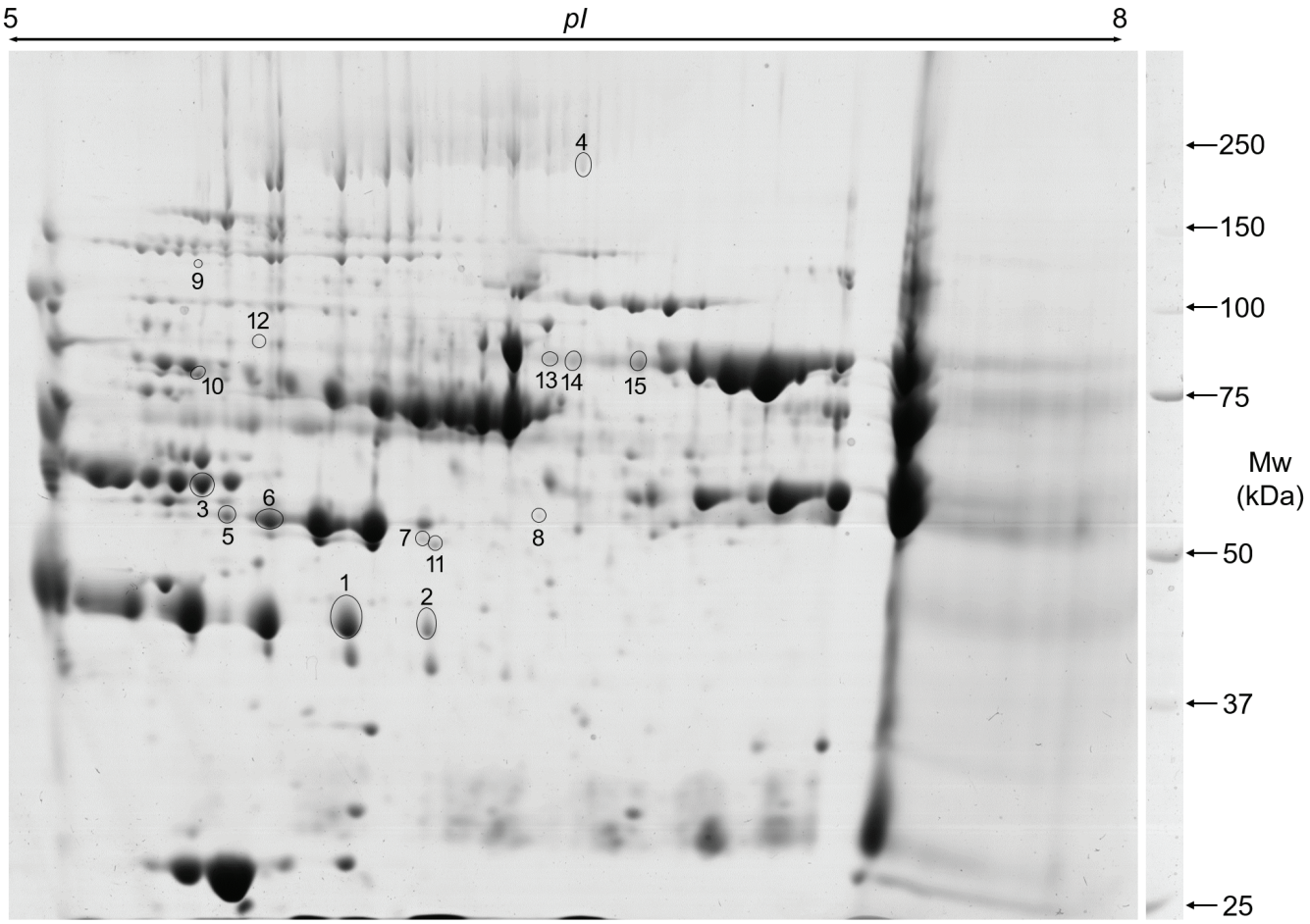

Fig. 1. Two-dimensional electrophoresis (2DE) map of the bladder cancer plasma proteins

Spots with significantly variable expression are identified. The differentially expressed protein spots are numbered and their characterization parameters are given in Table 1.

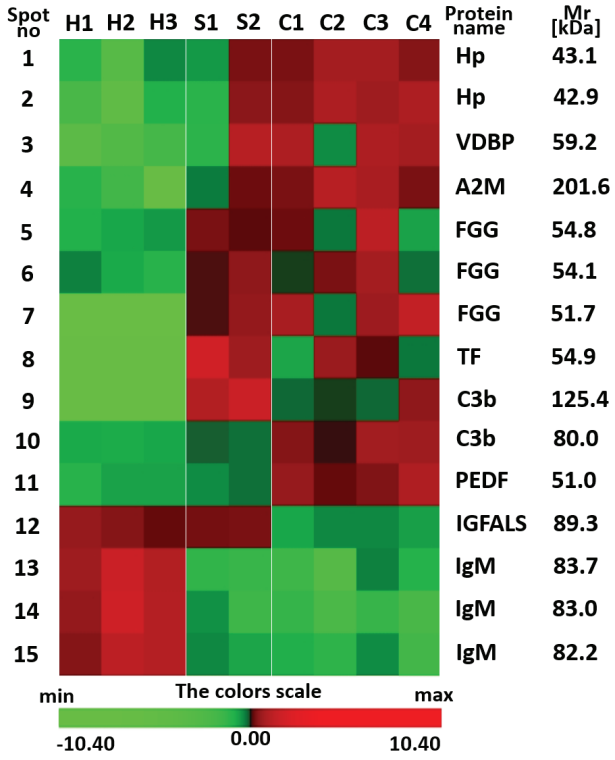

Fig. 2. A heat map describing the differences in protein expression patterns in the plasma of bladder cancer patients and healthy individuals

The comparative visualization was done for the differentially expressed proteins. The spot numbers correspond to those in Tables 1 and 2.

respectively), 43.1 kDa haptoglobin (2.63 and 3.11, respectively), and alpha-2-macroglobulin (3.11 and 4.00, respectively). The ratio values of C/H and S/H for 54.8 kDa and 54.1 kDa fibrinogen forms (2.09 and 1.53, respectively, and 1.8 and 2.04, respectively) and 80.0 kDa complement C3b (2.25 and 1.6, respectively), and pigment epithelium-derived factor (2.23 and 1.67, respectively) ranged from 1.5 to 2.0. In contrast, low values of the ratio were found for IgM molecular forms 83.7 kDa (0.25 and 0.12, respectively), 83.0 kDa (0.20 and 0.13, respectively) and 82.2 kDa (0.33 and 0.29, respectively), when groups C and S were compared to group H. Finally, the ratio value was nearly 1 for insulin-like growth factor binding protein when comparing C and H (0.64), and S and H (0.78) groups. On the other hand, similar ratio values were observed when comparing protein abundances in groups C and S.

Table 2. Relative abundance of the potential plasma biomarkers of bladder cancer identified by proteomic analyses

Spot No.	Identified protein	Function	Mr [kDa] of a protein or its subunit	Ratio of the relative protein abundance (Table 1) between the groups		
				C/H	S/H	C/S
1	haptoglobin	positive acute phase protein, inflammatory response[24]	43.1	2.63	3.11	0.84
2	haptoglobin		42.9	4.64	5.64	0.82
3	vitamin D-binding protein	transport of vitamin D metabolites, immune functions, chemotactic cofactor[25]	59.2	8.29	8.25	1.0
4	alpha-2-macroglobulin	protease inhibitor, binding various cytokines and growth factors[26]	201.6	3.11	4.00	0.78
5	fibrinogen gamma	blood coagulation[27]	54.8	2.09	1.53	1.36
6	fibrinogen gamma		54.1	1.8	2.04	0.88
7	fibrinogen gamma		51.7	2,274.0*	1,510.3*	1.51
8	transferrin	iron homeostasis, erythropoiesis, innate immune system, negative acute phase protein[28]	54.9	1,626.1*	1,617.6*	1.01
9	complement C3b	complement pathway activation and regulation[29]	125.4	645.7*	980.2*	0.66
10	complement C3b		80.0	2.25	1.6	1.4
11	pigment epithelium-derived factor	multifunctional factor with neurotrophic, anti-oxidative, anti-inflammatory, and anti-angiogenic properties (skin homeostasis and wound healing)[30]	51.0	2.23	1.67	1.33
12	insulin-like growth factor binding protein	regulation of cell growth and survival[31]	89.3	0.64	0.78	0.82
13	IgM	primary immune response to antigens[32]	83.7	0.25	0.12	2.13
14	IgM		83.0	0.20	0.13	1.61
15	IgM		82.2	0.33	0.29	1.12

C – patients with bladder tumors; H – healthy controls; IgM – immunoglobulin M; Mr – molecular mass; S – patients suspected of having cancer; * because the spot was not detected in the normal plasma sample, the given value corresponds to the value of the mean relative abundance (see Table 1).

Discussion

The proteomic analyses of plasma samples of the patients with urothelial bladder cancer and cystitis allowed us to visualize 15 differentially expressed protein spots, which were identified as 9 proteins (Fig. 1, 2 and Tables 1, 2). The identified proteins showed some discrepancies between the calculated molecular masses (Mr) (Table 1) and the theoretical SwissProt/NCBI database matches. Such differences, also reported for some urine and plasma bladder cancer proteins, might be related to post-translational modifications (glycosylation and phosphorylation) and may have resulted from pathological tissue conditions (glycoxidation, proteolytic cleavage or association with other molecules), possibly influencing the size, properties and electrophoretic mobility of the affected molecule(s).[18,22,33–35]

Our results showed some changes between the 2 cancer groups in relation to the normal group, but none between group C patients with active urothelial carcinoma and group S patients with acute cystitis. However, the patients from group S had undergone a transurethral resection of a urothelial bladder tumor 2 years earlier, so they might have recurrent malignancy, still unidentified. On the other hand, the synthesis of the identified proteins is associated with the inflammatory state that is always present in cancer and of course in cystitis.

The protein identification of the 2DE pattern of spots (Fig. 1 and Table 1) revealed the presence of 2 molecular forms of haptoglobin (β chain: 43.1 kDa and 42.9 kDa), 3 of IgM (heavy chain: 83.7 kDa, 83.0 kDa, 82.2 kDa), and moreover, 1 of a low molecular mass form of transferrin (54.9 kDa), which were absent in the normal plasma. They probably represent several glycovariants of these glycoproteins. The relative abundance of haptoglobin, transferrin and IgM molecular forms in bladder cancer patients' plasma samples showed significant differences compared to those of the normal group (Table 2). Such differences might be associated with some defects in glycan structures of glycoproteins, which in malignancy may take a variety of forms, such as hypo- and hyper-glycosylated forms, leading to the occurrence of glycoforms with incomplete truncated oligosaccharide parts, heavily sialylated, fucosylated or branched structures, and even to the loss of expression or the appearance of novel structures.[36] On the other hand, the presence of several spots of fibrinogen gamma (54.8 kDa, 54.1 kDa and 51.7 kDa) with evidently lower molecular masses than the untouched native form might reflect the presence of proteolytic degradation products rather than the occurrence of carbohydrate-deficient variants.

The proteomic analyses of bladder cancer plasma samples (Table 1) allowed us to distinguish 3 groups of plasma proteins, whose presence might be of value as

potential bladder cancer markers. The 1st one comprised the isoforms of proteins absent in the normal group, but occurring abundantly in the plasma of the cancer group (Table 1). They were 54.9 kDa plasma transferrin, 51.7 kDa fibrinogen gamma and 125.4 kDa complement C3b. The 2nd group consisted of glycoproteins released into circulation in high concentrations during immunoinflammatory processes. They are well-known members of the acute phase glycoprotein group, such as haptoglobin and alpha-2-macroglobulin, as well as 2 multifunctional glycoproteins, vitamin D-binding protein and pigment epithelium-derived factor (51.0 kDa). The 3rd group of potential bladder cancer markers comprised 3 molecular forms of IgM (heavy chain of IgM: 83.7 kDa, 83.0 kDa, 82.2 kDa), which may be involved in tumor immunity and biological changes in the tumors.[37]

Interestingly, our results show that vitamin D-binding protein is evidently associated with bladder cancer. Its relative abundance in patients' plasma samples was 8 times higher than in the normal plasma samples (Table 2). That multifunctional glycoprotein, besides its role in transporting vitamin D and circulating metabolites, is involved in the elimination of dead or injured cells, macrophage activation and neutrophil chemotaxis under inflammatory conditions.[25,38] However, its involvement in the carcinogenesis of bladder cancer is controversial and requires further analysis.[39]

Among the selected differentially expressed glycoproteins, there are prognostic and predictive markers associated with several other cancers. An elevated concentration of transferrin has been described in the urine of bladder cancer patients.[28] It not only takes part in normal iron metabolism, but is also involved in iron transport to highly proliferative cancer tissues, requiring high amounts of iron ions as a co-factor for ribonucleotide reductase enzyme for DNA synthesis.[28] An elevated fibrinogen concentration in the plasma of bladder cancer patients has been described as an independent indicator of the late stage of the disease.[27] The low abundance of IgM observed by us might reflect the disturbances in maintaining immunocompetence in specific recognition and elimination of precancerous, cancerous and age-related lesions in tissues.[32] Simultaneously, in contrast to the decrease of the IgM level, the high abundance of complement C3b reflects the activation of innate immunity. To recapitulate, the parameters described by us are mostly related to bladder cancer-linked inflammatory responses, which are known to play decisive roles at different stages of tumor development.[40]

Conclusions

The results of proteomic analyses of the plasma samples of patients with urothelial bladder cancer allowed us to select 9 differentially expressed glycoproteins as potential bladder cancer markers. We recommend vitamin D-binding protein, haptoglobin, transferrin, fibrinogen, IgM, complement C3b, alpha-2-macroglobulin, and pigment epithelium-derived factor as a group of markers associated with bladder cancer. They might be considered crucial parameters for the development of tests useful in clinical practice. However, in our opinion, the evaluation of haptoglobin, transferrin and IgM glycovariants, or various cleavage products of fibrinogen and complement C3b might be more promising and more sensitive and specific than the evaluation of their total respective protein concentrations for bladder cancer diagnosis and disease progression monitoring.

Considering the complexity of the proteomic technique we used and the future usefulness of the test in clinical practice, the results should be confirmed by less expensive and time-consuming methods in a larger group of patients.

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Adropin and irisin: New biomarkers of cardiac status in patients with end-stage renal disease? A preliminary study

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2019;28(3):347–353

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Funding sources
None declared

Conflict of interest
None declared

Received on August 31, 2017
Reviewed on December 9, 2017
Accepted on December 22, 2017

Published online on December 7, 2018

Abstract

Background. The new polypeptide hormones adropin and irisin have a broad impact on human metabolism and energy homeostasis. They could be potential biomarkers of cardiac injury. In end-stage renal disease (ESRD), the clinical importance of adropin and irisin is yet to be investigated.

Objectives. The aim of this study was to determine the relationship between these peptides and cardiac status in ESRD patients.

Material and methods. Seventy-nine ESRD patients on hemodialysis (HD), peritoneal dialysis (PD) or after renal transplantation (Tx), and 40 healthy, age- and sex-matched controls (CON) were included in this study. Serum concentrations of adropin and irisin were measured with enzyme-linked immunosorbent assay (ELISA). Cardiac status was estimated by transthoracic echocardiography and the plasma concentration of N-terminal pro-brain natriuretic peptide (NT-proBNP) and cardiac troponin T (cTnT).

Results. The levels of irisin were significantly lower in HD patients as compared to CON. During HD sessions, the concentrations of adropin did not change significantly, whereas the concentrations of irisin increased with borderline significance. Positive correlations were evident between adropin concentration and cTnT as well as NT-proBNP. Adropin was also correlated with left ventricular systolic internal diameter (LVIDs) ($r = 0.375$, $p = 0.045$) and relative wall thickness (RWT) ($r = -0.382$, $p = 0.034$). Irisin was correlated with right ventricular diameter (RVd) ($r = -0.363$, $p = 0.045$). No correlations were found between irisin and adropin, and blood pressure (BP) measurements.

Conclusions. Adropin could be a new candidate marker of cardiac dysfunction in HD patients. The cause of low levels of irisin found in HD patients is still unclear. These 2 myokines should be further investigated as potential prognostic markers of cardiac status in HD patients.

Key words: hemodialysis, end-stage renal disease, irisin, adropin, cardiac status

Cite as
Kałużna M, Pawlaczyk K, Schwermer K, et al. Adropin and irisin: New biomarkers of cardiac status in patients with end-stage renal disease? A preliminary study. *Adv Clin Exp Med*. 2019;28(3):347–353. doi:10.17219/acem/81538

DOI
10.17219/acem/81538

Introduction

End-stage renal disease (ESRD) leads to the development of a variety of hormonal and metabolic disorders.[1] Myokines and adipokines, as regulators of metabolism, have recently gained increasing interest. Cardiovascular events remain the most common cause of death in patients treated with chronic hemodialysis (HD) or peritoneal dialysis (PD).[2] The pathophysiology behind the increased relevance of cardiovascular disease (CVD) in dialyzed patients is still unclear. To prevent cardiac events and mortality, identification of high-risk cardiovascular patients is necessary. Therefore, determination of new and efficient markers of cardiovascular function in HD patients will be valuable.

Irisin and adropin, newly discovered peptide hormones, have broad implications in energy homeostasis and metabolism in humans.[3–8] Adipokines and myokines can potentially provide a link between CVD and other features of metabolic syndrome (MS). Adropin is a polypeptide hormone that participates in the regulation of metabolism and energy homeostasis in humans.[9] Recent data indicates that adropin contributes to the development of coronary atherosclerosis and heart failure (HF).[5,10,11] This is supported by the fact that adropin appears to be a potential biomarker for endothelial dysfunction and CVD.[11–19] Low plasma adropin levels have been reported in obesity, diabetes, insulin resistance, atherogenesis, cardiac syndrome X (CSX), and aging.[6,10,13,18,19] The relationship between adropin level and kidney function has not been satisfactory established.

Irisin is a 112 amino acid protein of 12 kDa molecular weight, and a product of fibronectin type III domain 5 (FNDC5) cleavage.[20] Irisin converts white adipose tissue into brown adipose tissue, and is believed to improve glucose metabolism and repeal visceral obesity.[20–22] Irisin has been proven to play a role in cardiomyoblast metabolism and blood pressure (BP) control.[23,24] It has been proposed to be a possible marker of HF and myocardial infarction (MI).[25–27] In contrast to other adipokines, irisin is not eliminated by renal excretion.[28] Its concentration positively correlates with estimated glomerular filtration rate (eGFR), making it a potential predictor of chronic kidney disease development and progression.[28,29] Interestingly, both irisin and adropin are produced locally in the heart.[30,31]

The aim of this study was to compare serum adropin and irisin levels in ESRD patients treated with HD, PD and kidney transplantation (Tx), and to determine the relationship between these peptides and cardiac status in HD patients. We hypothesized that adropin and irisin levels could be disturbed in ESRD patients and associated with cardiovascular complications and comorbidities, especially with altered cardiac function. We also expected that the level of these peptides could be altered by HD.

Material and methods

Subjects

Seventy-nine ESRD patients: 41 treated with HD (12 women, 29 men, median age 55 years, range 26–84 years), 18 patients treated with continuous ambulatory PD (14 women, 4 men, median age 57 years, range 25–78 years) and 20 ESRD patients after Tx (6 women, 14 men, median age 40.5 years, range 21–75 years, 6–256 months after Tx) at Heliodor Święcicki Clinical Hospital (Poznań, Poland) were recruited in January and February 2015 into this study. The exclusion criteria consisted of the following: diabetes mellitus or glucose intolerance, acute or chronic hepatic failure, hematologic disorders, active autoimmune disease or neoplasm, history of acute infection within the previous 7 days, and MI during the last 6 months. Forty healthy, age- and sex-matched people were also enrolled into the study as a control group (CON). The Bioethics Committee at Poznan University of Medical Sciences approved the study protocol. Patients gave their written informed consent to participate in the study.

Blood chemistry measurements

For HD patients, blood samples were collected before (HD1 group) and after the midweek HD (HD2 group) session, which means that ultimately, there were 5 groups of patients: HD1 group, HD2 group, PD group, Tx group, and CON. In all patients and controls, the sera were obtained following fasting (excluding HD patients after dialysis session) and stored at –80°C until analysis. Serum levels of adropin were measured using enzyme-linked immunosorbent assay (ELISA) in duplicate and the results were averaged. The Human Adropin (ENHO) Elisa Kit (Cusabio Technology LLC, College Park, USA) was used. The sensitivity of this assay was below 0.39 pg/mL. The same analytic method was used for serum irisin measurements with the AdipoGen Elisa Kit (AdipoGen Life Sciences, Liestal, Switzerland). The lowest detected concentration for this assay was 1 ng/mL. In addition, serum creatinine, ferritin, N-terminal pro-brain natriuretic peptide (NT-proBNP), and cardiac troponin T (cTnT) were measured in HD patients prior to HD. These measurements were performed in the accredited and quality-controlled Central Laboratory of Heliodor Święcicki University Hospital, using NT-proBNP (pg/mL, Elecsys® assay, Roche Diagnostics, Basel, Switzerland) and cardiac troponin T levels (cTnT, Elecsys® Troponin T fourth generation assay, Roche Diagnostics, Basel, Switzerland).

Examination of cardiovascular status

Blood pressure measurements were performed in all HD patients prior to, during and after HD sessions. Cardiovascular function was assessed by 2-dimensional (2-D), M-mode and Doppler echocardiography approx. 30 min

after a routine HD session in 31 patients.[32] The echocardiographic analyses were performed with a commercially available ultrasound system (M5S probe, Vivid E9; GE Healthcare, Milwaukee, USA). The M-mode and 2-D tracings were obtained with the subjects in the left lateral recumbent position, in accordance with the standards of the American Society of Echocardiography (ASE) and European Association of Cardiovascular Imaging (EACVI).[33] Parasternal long- and short-axis views, as well as apical 4- and 2-chamber views, were used for evaluation of the functions of the left ventricle (LV) and heart valves. One investigator performed all the echocardiographic examinations. Few patients did not agree to the echocardiographic examination. Endocardial border detection was enhanced by the use of second harmonic imaging. The left ventricular end-diastolic (LVEDd) and end-systolic (LVEDs) cavity dimensions, interventricular septal wall thickness (IVSd), left ventricular posterior wall thickness (LVPWTd), and left atrial dimension (LAD) were measured. Chamber systolic function was assessed as left ventricular fractional shortening (LVFS) at the endocardium. Left ventricular fractional shortening was calculated as:

$$LVFS = [(LVEDd - LVEDs)/LVEDd] \times 100$$

Left ventricular ejection fraction (LVEF) was calculated according to the Teichholz method.[33] Left ventricular mass (LVM) was calculated using the American Society of Echocardiography formula modified by Devereux et al.[34]:

$$LVM = 0.8 \times [1.04 \times (LVEDd + IVSd + LVPWTd)^3 - LVEDd^3] + 0.6\ g$$

The LV-mass index (LVMI) was determined by dividing the LVM by the body surface area of the patient. The upper normal limit is 115 g/m^2 for men and 95 g/m^2 for women.[33]

Doppler measurements of LV diastolic filling were performed by placing the pulsed Doppler sample volume at the mitral leaflet tips to obtain maximal velocities of early diastolic transmitral flow (E-wave peak velocity) and late transmitral flow (A-wave peak velocity). The E-wave deceleration time was measured, and the ratio between E- and A-wave peak velocities was subsequently derived. Mitral annular velocities at the septal corner and lateral corner of the mitral annulus were also recorded to determine peak early diastolic annular velocity. Left ventricle diastolic function was estimated according to EACVI recommendations on the basis of LV mitral inflow, peak early diastolic mitral annular velocity and the presence of left atrium (LA) enlargement.[35]

Statistical analysis

Statistical calculations were performed using STATISTICA v. 12 (StatSoft, Inc., Tulsa, USA). The Shapiro–Wilk test was used to determine whether the data followed a normal distribution. Results were expressed as median (interquartile range) and minimum–maximum range. The level of significance (p-value) was set below 0.05 for all analyses. In order to determine the differences between groups, the Kruskall-Wallis test was used. A possible association among measured parameters was quantified using Spearman's rank correlation coefficient r.

Table 1. Baseline characteristics of HD population – HD and laboratory parameters

Parameter	Median (interquartile range)
V urea [L]	33.8 (18.5–51.8)
Kt/V	1.44 (0.65–1.84)
Time of HD session [min]	240 (225–240)
Ferritin [µg/L]*	1545 (155–3305)
cTnT [ng/L]*	50 (13–325)
NT-proBNP [pg/mL]*	4077 (2330–35,000)

HD – hemodialysis; V urea – distribution volume of urea; Kt/V – number used to quantify hemodialysis treatment adequacy; cTnT – cardiac troponin T; NT-proBNP – N-terminal pro-brain natriuretic peptide; * parameters measured prior to HD session.

Results

Clinical characteristic of hemodialysis patients

The baseline characteristics of the HD population are summarized in Table 1. The HD vintage of patients varies from 92 to 7183 days. Hemodialysis patients had high levels of NTproBNP and cTnT. Of the 31 patients from the HD group in whom the echocardiography study was performed, the majority (9 women and 12 men) presented LV concentric hypertrophy. Two male patients had eccentric LV hypertrophy. In the remaining 8 patients, LV was normal. Normal LV systolic function, assessed by calculation of LVFS and LVEF, was found in 27 patients (7 women and 20 men). Abnormal LV relaxation was diagnosed in 3 patients (2 women and 1 man). The LA was enlarged in 27 patients, whereas 3 women and 1 man had normal LA diameter. The right ventricular dimension was normal in all examined patients (Table 2).

Adropin and irisin before and after hemodialysis session, and in peritoneal dialysis, control and kidney transplantation group

The levels of adropin did not change significantly before (HD1 group) and after (HD2 group) HD sessions (3.95 vs 4.26 ng/mL, not significant (NS)) (Table 3). A negative correlation was observed between adropin concentrations and HD session duration (r = –0.421, p = 0.0085) (Table 4). There were no significant differences in the levels of adropin in all the groups – CON, PD, Tx, HD1, and HD2 (4.02 vs 4.74 vs 4.73 vs 3.95 vs 4.26 ng/mL, NS) (Table 3).

Table 2. Echocardiographic parameters in HD patients*

Parameter	Median (interquartile range)
RVd [mm]	30 (22–36)
IVSd [mm]	14 (10–27)
PWd [mm]	13 (10–25)
LAD [mm]	47 (34–62)
LVIDs [mm]	30 (20–44)
LVIDd [mm]	47 (34–62)
LVFS [%]	36 (17.02–50.00)
LVEF [%]	65.4 (35.6–81.8)
RWT [mm]	03 (0.39–1.04)
MV E [cm/s]	73 (33–142)
MV A [cm/s]	80.50 (42–186)
MVE/MVA [ms]	0.88 (0.52–1.82)
MV E DT [ms]	200 (130–485)
Mit E/E' [ms]	8.8 (5–24)
LV mass [g]	272 (133–744)
LV mass I [g/m^2]	147 (83–327)

HD – hemodialysis; RVd – right ventricular diameter; IVSd – interventricular septal end diastole dimension; PWd – posterior wall thickness at end-diastole; LAD – left atrium dimension; LVIDd – left ventricular internal diameter in diastole; LVIDs – left ventricular internal diameter in systole; RWT – relative wall thickness; LVFS – left ventricular fractional shortening; LVEF – left ventricular ejection fraction; MV E – mitral valve early (E) filling velocity; MV A – mitral valve atrial (A) filling velocity; MV E DT – deceleration time of mitral valve early (E) filling velocity; Mit E/E' – the ratio between early mitral inflow velocity and mitral annular early diastolic velocity; LV mass – left ventricular mass; LV mass I – left ventricular mass index; *echocardiographic parameters were measured after HD session to avoid the influence of overhydration.

The median levels of irisin were significantly lower in HD1 patients compared to CON (4.32 vs 7.85 μg/mL; $p < 0.000001$), PD patients (4.32 vs 9.73 μg/mL, $p = 0.0015$) and Tx patients (4.32 vs 6.96 μg/mL, $p < 0.000001$). The levels of irisin during HD sessions (HD1 vs HD2) increased with a borderline statistical significance (4.32 vs 5.01 μg/mL; $p = 0.081$). There were no differences between levels of irisin in the Tx, PD and CON groups (Table 3). The detailed statistical analysis of adropin and irisin levels in all studied groups is presented in Table 3.

There was no correlation between irisin or adropin concentrations and HD vintage ($p < 0.05$), nor between adropin or irisin levels and time after Tx ($p < 0.05$).

Cardiac status indicators

Positive correlations were found between adropin pre-HD concentration and cTnT ($r = 0.325$, $p = 0.046$) as well as NT-proBNP ($r = 0.355$, $p = 0.034$) (Table 4). There was no correlation between adropin and BP measured before, during or after HD sessions. Irisin levels were not correlated with cTnT, NT-proBNP or BP.

Post-HD adropin levels were correlated to left ventricular internal systolic diameter (LVIDs) ($r = 0.375$, $p = 0.045$) and relative wall thickness (RWT) ($r = -0.382$, $p = 0.034$). Post-HD irisin levels were correlated with RVd ($r = -0.363$, $p = 0.045$). Absolute differences in adropin concentration before and after the midweek HD session were closely related to RVd ($r = -0.336$, $p = 0.064$) (Table 4).

Ferritin

There was a negative correlation between pre-HD levels of irisin and ferritin ($r = -0.335$, $p = 0.046$) (Table 5). There was no correlation between adropin and ferritin concentration in HD patients.

Discussion

Renal failure

Adropin and irisin are hormones produced locally in many tissues. The renal metabolism of both myokines is not well understood. So far adropin has been isolated

Table 3. Adropin and irisin levels in studied groups. Results are expressed as median (min–max range)

Groups chosen for comparison	Adropin [ng/mL] median concentration (interquartile range) for both groups	p-value	Irisin [ug/mL]	p-value
HD1 vs CON	3.95 (1.49–6.41) vs 4.02 (1.23–7.84)	NS	4.32 (2.51–11.95) vs 7.85 (5.34–16.80)	p < 0.0001
HD1 vs HD2	3.95 (1.49–6.41) vs 4.26 (1.67–7.23)	NS	4.32 (2.51–11.95) vs 5.01 (3.20–14.11)	NS
HD1 vs PD	3.95 (1.49–6.41) vs 4.73 (2.64–8.50)	NS	4.32 (2.51–11.95) vs 6.96 (4.50–13.55)	p = 0.0015
HD1 vs Tx	3.95 (1.49–6.41) vs 4.74 (2.76–9.01)	NS	4.32 (2.51–11.95) vs 9.73 (6.27–20.00)	p < 0.0001
HD2 vs CON	4.26 (1.67–7.23) vs 4.02 (1.23–7.84)	NS	5.01 (3.20–14.11) vs 7.85 (5.34–16.80)	p < 0.0001
HD2 vs PD	4.26 (1.67–7.23) vs 4.73 (2.64–8.50)	NS	5.01 (3.20–14.11) vs 6.96 (4.50–13.55)	NS
HD2 vs Tx	4.26 (1.67–7.23) vs 4.74 (2.76–9.01)	NS	5.01 (3.20–14.11) vs 9.73 (6.27–20.00)	p < 0.0001
PD vs CON	4.73 (2.64–8.50) vs 4.02 (1.23–7.84)	NS	6.96 (4.50–13.55) vs 7.85 (5.34–16.80)	NS
Tx vs CON	4.74 (2.76–9.01) vs 4.02 (1.23–7.84)	NS	9.73 (6.27–20.00) vs 7.85 (5.34–16.80)	NS
PD vs Tx	4.73 (2.64–8.50) vs 4.74 (2.76–9.01)	NS	6.96 (4.50–13.55) vs 9.73 (6.27–20.00)	NS

HD – hemodialysis; CON – median value in healthy controls; HD1 – median value prior to HD session; HD2 – median value after HD session; PD – median value in peritoneal dialysis patients; Tx – median value in patients after renal transplantation; NS – not significant.

Table 4. Correlation coefficient r between serum adropin concentration and HD, laboratory and ECHO parameters

Variables	Adropin prior to HD	Adropin after HD
V urea	r = −0.707, p < 0.0001	r = −0.587, p = 0.0002
Kt/v	r = 0.308, NS	r = 0.039, NS
HD session duration	r = −0.421, p = 0.0085	r = −0.403, p = 0.012
Ferritin	–	r = 0.117, NS
cTnT	–	r = 0.325, p = 0.0460
NT-proBNP	–	r = 0.496, p = 0.0020
RVd	–	r = −0.201, NS
LVIDs	–	r = 0.375, p < 0.0001
RWT	–	r = −0.382, p = 0.0340

HD – hemodialysis; V urea – distribution volume of urea; Kt/V – number used to quantify hemodialysis treatment adequacy; cTn-T – cardiac troponin-T; NT-proBNP – N-terminal pro-brain natriuretic peptide; RVd – right ventricular diameter; LVIDs – left ventricular internal diameter in systole; RWT – relative wall thickness; r – correlation coefficient; NS – not significant.

Table 5. Correlation coefficient r between serum irisin concentration and HD parameters, laboratory and ECHO parameters

Variables	Irisin prior to HD	Irisin after HD
V urea	r = 0.026, NS	r = −0.168, NS
Kt/v	r = −0.215, NS	r = −0.061, NS
HD session duration	r = 0.007, NS	r = 0.116, NS
Ferritin	r = −0.335, p = 0.0460	r = −0.252, NS
cTnT	–	r = 0.027, NS
NT-proBNP	–	r = 0.102, NS
RVd	–	r = −0.363, p = 0.0450
LVIDs	–	r = −0.025, NS
RWT	–	r = −0.116, NS

HD – hemodialysis; V urea – distribution volume of urea; Kt/V – number used to quantify hemodialysis treatment adequacy; cTn-T – cardiac troponin-T; NT-proBNP – N-terminal pro-brain natriuretic peptide; RVd – right ventricular diameter; LVIDs – left ventricular internal diameter in systole; RWT – relative wall thickness; r – correlation coefficient; NS – not significant.

in the liver, muscle, intestines, kidney, heart, pancreas, brain, umbilical vein, and salivary glands.[6,9,19,31,36,37] In rats with isoproterenol (ISO)-induced MI, the expression of adropin dramatically increased in the kidney tissues.[12] Therefore, we hypothesized that the adropin levels might be altered in HD patients, as renal insufficiency is usually accompanied by CVD. However, we found no statistically significant difference in adropin levels when comparing HD patients and CON. Also, no statistically significant difference was observed between the adropin level prior to and after the HD session. The results of our study revealed that adropin is dialyzable during HD. Changes of adropin level should be interpreted in accordance to eGFR. However, the low sample size of HD and PD patients could have interfered with the results of the study.

We found a borderline statistically significant rise in irisin level during HD sessions, which was not related to UF and/or HD adequacy measured by Kt/V. This is not consistent with the recent results of Ebert et al., who found a significant 23% decrease in irisin level after HD.[28] The increase in concentration in our study could be attributed to decreased intravascular volume; however, this finding questions the dialyzable nature of irisin. Hemodialysis itself does not seem to be the reason for low irisin concentrations in HD patients. A study by Wen et al. showed that uremic toxins have a negative influence on FNDC5 expression, which is the precursor of irisin.[29] Studies using larger samples are required to validate the dialyzable nature of irisin.

Other factors such as decreased physical activity, malnutrition and cachexia in HD patients could be the possible reasons of the low irisin level in the group of HD patients. However, just as our results, finding on these topics are inconclusive. Perhaps the negative correlation between irisin and ferritin, a marker of iron stores in dialysis patients and a positive acute-phase reactant in our study may show a connection between chronic inflammation and low irisin levels in HD patients. The pathophysiological significance of low irisin levels in HD patients needs to be further investigated.

Cardiac function

Cardiac dysfunction is accompanied by a number of changes in the neuroendocrine system.[38] Adropin immunoreactivity was found in the endocardium, myocardium and epicardium.[31] In our study, there was a significant positive correlation between adropin level, cTnT and NT-proBNP. These findings demonstrate a probable and complex relation between adropin concentration and the cardiac status of HD patients. Many studies have displayed the role of cTnT and NT-proBNP as significant predictors of cardiovascular and all-cause mortality in ESRD patients.[39–41] However, in ESRD patients, interpretation of cTnT and NT-proBNP can be confusing as an elevation of these biochemical parameters can be the effect of renal insufficiency and overhydration.

The available data on the relationship between adropin and altered cardiac function is inconsistent. Lian et al. found that patients with HF are characterized by higher adropin levels than healthy controls and implied a potential role of high adropin levels in the development of the HF.[11] The elevation in adropin was proportional to the severity of the heart disease according to New York Heart Association (NYHA) classification.[11] A negative relationship between adropin and LVEF (r = −0.710, p < 0.001) was also observed in their study.[11] In our study, we did not observe any correlations between adropin and LVEF after HD. This could be attributed to our sample, as the majority of the HD patients had normal LV systolic function and only 4 patients had diminished LVEF. We found a positive correlation between adropin concentration and LVIDs and a negative correlation between irisin and RVd. The correlation

between adropin level and RWT observed in our study may suggest that adropin concentration is related to concentric LV hypertrophy; however, we did not observe any correlations between adropin and the LVMI. To establish this fact, further studies with a larger and more diversified group of patients regarding LV function is needed.

Other researchers have noted that increased adropin concentration could be a potential biomarker of cardiac status. Aydin et al. in their animal study, proposed using elevated adropin levels in conjunction with elevated troponin as biomarkers of MI.[12] The synthesis of adropin in rats was increased within 24 h after MI (induced by isoproterenol) in most tissues, including cardiac muscle cells.[12] However, opposing results were observed in humans. Serum adropin level in patients with MI was significantly lower in comparison to subjects with stable angina pectoris or controls in a study by Yu et al.[15] Furthermore, lower adropin level was found to be an independent predictor for MI in coronary artery disease (CAD) patients.[15] Interestingly, no correlation between serum adropin levels and cTnT or NT-proBNP was observed,[15] contrasting with the findings of our study.

A decrease in adropin concentration is considered as a new marker of coronary atherosclerosis.[10,18,42,43] The data suggests low serum adropin level as a predictor of stable CAD, and an association with the severity of coronary atherosclerosis.[17,44] Low concentrations of adropin were described to be related to coronary atherosclerosis in both diabetic and non-diabetic groups of patients.[42] Lower serum adropin level was also found to be an independent risk factor for CSX.[13]

Conflicting data on levels of adropin in cardiac dysfunction most likely suggests a complex role of adropin in different types of CVD and varying groups of patients. Factors such as concomitant diseases or additional hormonal and metabolic factors that could have overlapped with the study cannot be excluded. The diagnostic and prognostic value of adropin in the field of CVD and events in ESRD patients is still unclear. We cannot extrapolate the results from animal studies on adropin to humans. The relationship between adropin level and cardiac dysfunction in ESRD patients requires further investigation in order to consider it as a cardiac biomarker and improve cardiovascular risk assessment of ESRD patients in the future.

Low irisin levels were described in patients with HF.[25,26] We reported decreased irisin levels in HD patients. Lack of irisin upregulation in insulin resistance and other components of metabolic syndrome might hypothetically suggest the development of underlying cardiac insufficiency in ESRD patients treated with HD. However, we did not find a relation between irisin and cardiac markers in the subjects examined. This could be attributed to the small sample or the fact that the majority of our subjects had normal LV systolic function.

The main limitation of this study was the small number of patients included. The small sample size in our study may have prevented our results from reaching a more significant level. Further prospective longitudinal studies with a larger number of patients are needed to highlight the role of adropin in the metabolic homeostasis of ESRD patients.

Conclusions

Hormonal imbalances can be both the reason and the result of advancing ESRD and its complications. This study is the first to indicate an association between the levels of 2 myokines, adropin and irisin simultaneously, and the cardiac status of HD patients.[31] The significant positive correlation between adropin, cTnT and NT-proBNP demonstrates a probable and complex relation between adropin level and the cardiac status of HD patients. Our findings suggest that adropin may be involved in the pathogenesis of cardiac dysfunction in HD patients, but it certainly needs further detailed analysis and studies on the pathogenic mechanism. Of the 3 groups studied (HD, PD and Tx), HD patients had the lowest concentration of irisin. Nevertheless, adropin and irisin have to be interpreted carefully with regard to the patient's individual residual renal function and concomitant diseases. The new myokines, especially adropin, should be under further investigation as potential prognostic markers of cardiac status and clinical status in HD patients.

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DNA methylation analysis of selected genes for the detection of early-stage lung cancer using circulating cell-free DNA

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2019;28(3):355–360

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
This study was supported by the Project of Research and Demonstration Applications in Social Development, Jiaxing, China (2015AY23018) and a research fund from the Jiaxing Innovation Team of Early Diagnosis and Comprehensive Therapy for Lung Cancer.

Received on July 12, 2017
Reviewed on November 6, 2017
Accepted on January 31, 2018

Published online on December 5, 2018

Cite as
Yang Z, Qi W, Sun L, Zhou H, Zhou B, Hu Y. DNA methylation analysis of selected genes for the detection of early-stage lung cancer using circulating cell-free DNA. *Adv Clin Exp Med*. 2019;28(3):355–360. doi:10.17219/acem/84935

DOI
10.17219/acem/84935

Abstract

Background. Lung cancer is still the deadliest cancer in the world, but early diagnosis cannot be achieved because of the limitations of diagnostic methods. DNA methylation has been proven to be a potentially powerful tool for cancer detection and diagnosis over the past decade.

Objectives. We explored whether free DNA methylation in plasma can be a reliable biomarker for noninvasive lung cancer detection.

Material and methods. We detected the methylation of 8 genes in plasma-free DNA of patients with pulmonary space-occupying lesions using real-time quantitative methylation-specific polymerase chain reaction (QMSP). Among the 50 selected patients, 39 were confirmed using pathological analysis as having early lung cancer and 11 had an inflammatory pseudotumor.

Results. The QMSP detection showed that the methylation levels of 8 genes in the patients were significantly higher than in the non-lung cancer group. The methylation level of *CALCA* was the highest and the methylation level of *HOXA9* was the lowest. Methylation of *RASSF1A*, *CDKN2A* and *DLEC1* occured only in lung cancer patients, while methylation of *CALCA*, *CDH13*, *PITX2*, *HOXA9*, and *WT1* occured not only in lung cancer patients, but also in non-lung cancers. The specificity reached 95~100%, whether for a single gene or overall, but the sensitivity was relatively low for each gene. The sensitivity can reach 72% if the methylation of any of the 8 genes is positive and the overall specificity was 91%. The positive and negative predictive values were 96% and 60%, respectively.

Conclusions. Quantitative detection of DNA methylation in plasma is a potential method for early diagnosis of lung cancer.

Key words: lung cancer, early diagnosis, QSMP, plasma-free DNA

Introduction

The lung cancer mortality is 19.4%; it accounts for 22.83% of all male tumors, while its incidence rate in female tumors is 14.32%.[1] The 5-year survival rate of patients with stage I lung cancer is 60–90%, while it is only 5–20% in patients with stage IIIb and stage IV.[2] Most lung cancers are detected in middle and late stages due to the lack of effective diagnostic methods. Therefore, early detection and treatment of lung cancer play an important role in controlling its mortality. Chest radiography and sputum cytology are economical and practical methods in current early screening methods; however, their sensitivity and specificity are not very high. Low-dose computed tomography (CT) can detect small lesions of only a few millimeters in the lung and its sensitivity is high, but the specificity is poor; it can also cause a mental and financial burden to patients with benign tumor nodules, and may even inflict unnecessary trauma to their bodies.[3]

DNA methylation is an important epigenetic phenomenon that can affect gene expression without changing the DNA sequence. Aberrant methylation of the promoter 5'—C—phosphate—G—3' (CpG) island leads to silencing of tumor suppressor genes, which is one of the important mechanisms of tumorigenesis.[4] Studies have shown that DNA abnormal methylation exists before the patient shows clinical symptoms or before X-ray evidence is obtainable. Moreover, it exists in many bodily fluids such as plasma, urine, semen, and feces.[5–7] These findings suggest that DNA methylation may serve as a biomarker for noninvasive diagnosis, and is useful in the early diagnosis of cancer. Methylation of the *CALCA*, *HOXA9* and *PITX2* genes is an effective biomarker for lung cancer.[8–11] However, the sensitivity of single gene methylation detection is only about 60%, which fails to meet the standard for early diagnosis of lung cancer. One study found that the combined detection of multigene methylation can increase the sensitivity to 93%, but the specificity is decreased.[12] Even so, the concentration and integrity of free DNA in patients with cancer are higher than in healthy subjects, and detecting the methylation of lung cancer-associated genes by extracting free DNA in plasma is the trend in the early diagnosis of lung cancer in recent years.

In this study, the methylation of 8 genes (*CDH13*, *WT1*, *CDKN2A*, *HOXA9*, *PITX2*, *CALCA*, *RASSF1A*, and *DLEC1*) in plasma was analyzed by fluorescent real-time quantitative methylation-specific polymerase chain reaction (QMSP), and the results of a histopathological diagnosis and gene methylation were compared and analyzed. We determined whether free DNA methylation in plasma can be a reliable biomarker for noninvasive lung cancer detection, which could provide clinical data support for free DNA methylation as a biomarker for large-scale early noninvasive screening of lung cancer.

Material and methods

Subjects

A total of 50 patients with pulmonary space-occupying lesions found using chest CT who underwent surgery in the First Affiliated Hospital of Jiaxing University, Zhejiang, China, between June 2015 and June 2017 were enrolled into the study. The selected patients met the following criteria:

- chest CT suggested radiographic abnormalities with pulmonary space-occupying lesions, including nodules and masses 5–20 mm in size;
- absence of related antineoplastic treatments such as surgery, chemotherapy, radiotherapy, targeted therapy, or biological immunotherapy;
- absence of diseases not suitable for venous blood collection, such as blood diseases and acute infectious diseases that can be transmitted by blood.

Patients with lung cancer recurrence, accompanied by other malignancies, pregnant or breast-feeding women, and patients with autoimmune diseases were excluded. The demographic characteristic of the patients is shown in Table 1.

The study protocol was reviewed and approved by the Research Ethics Committee of the hospital. Written informed consent was obtained from each of the participants or their legal guardians.

Pathological features

The lung tissue was resected surgically and fixed with 4% paraformaldehyde to prepare paraffin sections and hematoxylin and eosin (H&E) staining. Lung cancer was diagnosed by 2 experienced pathology experts. A total of 39 cases of stage I lung cancer were identified by pathological analysis among the 50 selected cases, which included 25 cases of adenocarcinoma, 12 cases of squamous cell carcinoma and 2 cases of mucinous carcinoma. The remaining 11 cases were inflammatory pseudotumors.

Table 1. Comparison of demographic characteristics of the 2 groups

Variables	Lung cancer patients (n = 39)	Non-lung cancer patients (n = 11)	p-value
Gender (%)			
male	24 (61.5)	7 (63.6)	0.09
female	15 (38.5)	4 (36.4)	
Age (mean + SD)	51 ±11.62	54 ±7.47	0.11
Smoking status (%)			
smokers	29 (74.4)	8 (72.7)	0.29
non-smokers	10 (25.6)	3 (27.3)	
Stage I of pTNM (%)	39 (100)	–	–

SD – standard deviation; pTNM – TNM Classification of Malignant Tumours (tumour–node–metastasis) based on histopathologic examination of a surgical specimen.

Plasma DNA extraction

Samples of anticoagulant peripheral blood were collected before the operation and plasma was separated by centrifugation at 1000 rpm for 10 min. The isolated plasma was cryopreserved at –80°C for the extraction of plasma-free DNA and used for methylation detection.

The DNA was extracted from 500 µL of plasma of the subjects using a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was amplified using a *GAPDH* gene primer (F: AGGTCGGAGTCAACGGATTTG, R: GTGATGGCATGGACTGTGGT). The conditions for the PCR were as follows: 5 min at 95°C, then 15 s at 95°C, 30 s at 60°C, 30 s at 72°C for 38 cycles, and finally 10 min at 72°C. Polymerase chain reaction amplification products were observed after 2% agarose gel electrophoresis using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Marlborough, USA) gel imaging system, and the specimens not amplified for the desired PCR product were eliminated.

DNA modified by sodium bisulfite

Plasma-free DNA was modified by sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen), according to the manufacturer's instructions. DNA (1 µg) was added to Bisulfite Mix Solutions (85 µL; Qiagen) and DNA Protect Buffer (35 µL; Qiagen) – the final volume was 140 µL – by adding RNase-free water. They were incubated at 60°C for 5 h, then heated to 95°C and rapidly quenched in an ice bath. The single-stranded DNA solution was purified by an EpiTect Spin Column (Qiagen) and DNA modified by sodium bisulfite was obtained.

Real-time quantitative methylation-specific polymerase chain reaction

The modified DNA was amplified using real-time quantitative methylation-specific PCR (QMSP) as described by Nawaz et al.[12] The target genes included *CDH13*, *WT1*, *CDKN2A*, *HOXA9*, *PITX2*, *CALCA*, *RASSF1A*, and *DLEC1*. The relative methylation level of the target genes was determined with β-actin as a reference gene. The sequences of primers and probes used in this study are shown in Table 2. The QMSP reaction system contained 600 nM of primers, 200 nM of probe, 0.75 U of platinum Taq polymerase, 200 mM of deoxyribonucleotide triphosphate (dNTP), 16.6 mM of ammonium sulphate, 67 mM of Tris, 6.7 mM of magnesium chloride ($MgCl_2$), 10 mM of mercaptoethanol, 0.5 µL of 0.1% dimethyl sulfoxide (DMSO), and 50 ng of modified DNA.

The conditions for the QMSP reaction were as follows: 1 min at 95°C; 15 s at 95°C and 1 min at 60°C for 50 cycles. DNA of healthy human leukocytes treated with Sss I methylase (New England Biolabs, Inc., Ipswich, USA) was used as a positive control.

Statistical analysis

The SPSS v. 19.0 software (IBM Corp., Armonk, USA) was used for statistical analysis. The association between the pathological features and the demographic characteristics was evaluated with a χ^2 test. The level of statistical difference was defined at 0.05. The sensitivity and specificity of plasma DNA methylation level detection to diagnose lung cancer were calculated. Sensitivity was positive for positive methylation/total cancer cases and specificity was negative for negative methylation/total benign cases.

Results

The QMSP results showed that the methylation levels of 8 genes in 39 lung cancer patients were significantly higher than those in the non-lung cancer group. The methylation level of *CALCA* gene was the highest, followed by *CDH13*, *RASSF1A*, *DLEC1*, *WT1*, *CDKN2A*, and *PITX2*, and the methylation level of *HOXA9* gene was the lowest. Methylation of *RASSF1A*, *CDKN2A* and *DLEC1* occurred only in lung cancer patients, while the methylation of *CALCA*, *CDH13*, *PITX2*, *HOXA9*, and *WT1* occurred not only in lung cancer patients, but also in benign lung patients. Methylation levels of each gene in lung cancer and non-lung cancer patients are shown in Fig. 1 and Table 3.

Table 2. QMSP primer and probe sequences

Genes	Primer sequence (5'-3')	Probe sequence (5'-3')6FAM-DNA sequences-6TAMRA
β-Actin	F: TGGTGATGGAGGAGGTTTAGTAAG T R:AACCAATAAAACCTACTCCTCCCTTAA	ACCACCACCCAACACACAATAACAAAC ACA
CDH13	F: TCGCGGGGTTCGTTTTTCGC R: GACGTTTTCATTCATACACGCG	CGCCCACCCGACCTCGCA T
WT1	F:GCGTCGGAGGTTAAGGTTGTT R: CTCTCCAAAATTACCGTACGCG	AACTCGCTCGCCCGCCGAA
HOXA9	F: TTTAGGGTTTTAGTGGTGGTTAT R: TTCCCCCCCCATACCACCAAATTATTACA	AATTATTACATAAAATCTACAATT
PITX2	F: GGATAGTCGGATCGAGTTAACGTC R: CCCTCCCAAACGCCGA	TTCGGTAATTCGTAGCGGTAGGGTTTGG
CALCA	F: GTTTTGGAAGTATGAGGGTGACG R: TTCCCGCCGCTATAAATCG	ATTCCGCCAATACACAACAACCAATAAACG
RASSF1A	F: GGGATTAGAATTTTTTATGCGAGTTGT R: TACCCCGACGATACCCAAAC	TGTCGAGAACGCGAGCGATTCG
DLEC1	F: GGTTTGTTAAAAGGGGATGT R: ACACCCTCCCCCCCTTAAT	GTTTTATAATATTTGGGATTGA
CDKN2A	F: TTATTAGAGGGTGGGGCGGATCGC R: GACCCCGAACCGCGACCGTAA	TAACTAAAAATTCACCTACCGAC

Quantitative methylation tests results were compared with histopathological findings, and the specificity and sensitivity of the detection of plasma-free DNA methylation levels in the diagnosis of lung cancer were determined. As shown in Table 4, the specificity reached 95~100%, whether for a single gene or overall, but the sensitivity was relatively low for each gene. The sensitivity can reach 72% if the methylation of any of the 8 genes is positive and the overall specificity was 91%. The positive and negative predictive values were 96% and 60%, respectively.

Discussion

Lung cancer mortality has decreased by 20% due to the widespread use of low-dose CT screening recently.[1–3] This technique may be the main method for the diagnosis of lung cancer in the future, and more patients with early-stage lung cancer will be diagnosed.[3] However, low-dose CT screening cannot distinguish lung cancer from benign lung lesions. Therefore, in this study, we used the QMSP technique to detect the methylation levels of 8 genes (*WT1*, *CDKN2A*, *HOXA9*, *PITX2*, *CALCA*, *RASSF1A*, *CDH13*, and *DLEC1*) in the plasma-free DNA of lung cancer-affected patients diagnosed by low-dose CT screening. The results were compared with the pathological results of lung tissue to observe whether the level of plasma-free DNA methylation could be used for the diagnosis of early lung cancer.

DNA methylation is common in human cancers; it has been observed that methylated DNA can enter the plasma from cancer cells, and methylation of DNA in plasma suggests the presence of cancer.[14–17] DNA has a stable molecular structure and provides better detection stability. DNA methylation can be accurately quantified using qPCR analysis. In this study, we detected the methylation of 8 genes in the plasma-free DNA of patients with pulmonary space-occupying lesions using QMSP. It was found that there were significant differences in the methylation levels of the 8 genes in lung cancer and non-lung cancer patients. Different levels of plasma DNA methylation can be detected in 70% of lung cancer patients. Compared to the regression analysis results of pathology, the specificity of the diagnosis of lung cancer reached 95~100% with the analysis of the plasma DNA methylation level of 8 genes. DNA methylation may be a molecular marker for the early diagnosis of lung cancer.

The *CDH13* gene is one of the members of the cadherin gene family. It is a tumor suppressor gene. Its expression is reduced or absent in a variety of tumors. The methylation of the *CDH13* promoter is the major cause of its downregulation.[18]

The *WT1* gene encodes human Wilms tumor protein; it is a transcriptional regulatory factor that has a bidirectional regulatory effect on cancer. Hypermethylation and overexpression of *WT1* gene are present in many tumors.[19]

CDKN2A encodes p16 protein and is also an important tumor suppressor gene; it belongs to the cyclin-dependent kinase inhibitory factor family. The *CDKN2A* gene plays a negative regulatory role in the cell cycle. It causes the cell cycle to stagnate at the G1/S phase by inhibiting the activity of cyclin-dependent kinases 4 and 6. *CDKN2A* methylation levels increase in a variety of malignancies.[20]

The *HOXA9* gene is a member of the homeobox (HOX) gene family. Its protein is an important transcription regulator, which plays an important role in controlling embryonic development and regulating cell differentiation. The methylation of the *HOXA9* promoter and its abnormal expression are closely related to acute leukemia, malignant glioma, ovarian cancer, lung cancer, and breast cancer.[9]

PITX2 belongs to the transcription factor of the paired-bicoid protein family. It plays an important role in embryonic development and is abnormally expressed in breast cancer, nonfunctional pituitary adenoma, nephroblastoma, and colorectal cancer.[21]

CALCA is a polypeptide secreted by the parathyroid gland and forms a complex with calcitonin. It has been found that elevated levels of *CALCA* promoter methylation

Table 3. Positive rate of plasma DNA methylation in patients

Genes	Lung cancer*	Non-lung cancer*
CDH13	66.7 (26/39)	27.3 (3/11)
WT1	30.8 (12/39)	0 (0/11)
CDKN2A	28.2 (11/39)	0 (0/11)
HOXA9	20.5 (8/39)	9.1 (1/11)
PITX2	28.2 (11/39)	9.1 (1/11)
CALCA	84.6 (33/39)	45.5 (5/11)
RASSF1A	41.0 (16/39)	0 (0/11)
DLEC1	41.0 (16/39)	0 (0/11)
All 8 genes	94.9 (37/39)	63.6 (7/11)

* % (number of methylation-positive cases/total number of cases).

Table 4. Specificity and sensitivity of DNA methylation in diagnosis of lung cancer

Genes	Sensitivity	Specificity	Cut-off values	Ranges of methylation	
				lung cancer	non-lung cancer
CDH13	31 (12/39)	91 (10/11)	18.0	0.000–75.513	0.000–26.912
WT1	31 (12/39)	100 (11/11)	0.0	0.000–181.576	0.000–0.000
CDKN2A	28 (11/39)	100 (11/11)	0.0	0.000–21.321	0.000–0.000
HOXA9	21 (8/39)	100 (11/11)	3.1	0.000–39.405	0.000–3.041
PITX2	28 (11/39)	100 (11/11)	2.0	0.000–19.059	0.000–1.811
CALCA	51 (20/39)	100 (11/11)	38.0	0.000–425.447	0.000–37.004
RASSF1A	41 (16/39)	100 (11/11)	0.0	0.000–19.059	0.000–0.000
DLEC1	41 (16/39)	100 (11/11)	0.0	0.000–24.146	0.000–0.000
All 8 genes	72 (28/39)	91 (10/11)	N/A	N/A	N/A

N/A – not applicable; % (number of methylation-positive cases/total number of cancer cases); specificity: % (number of methylation-negative cases/total number of benign cases).

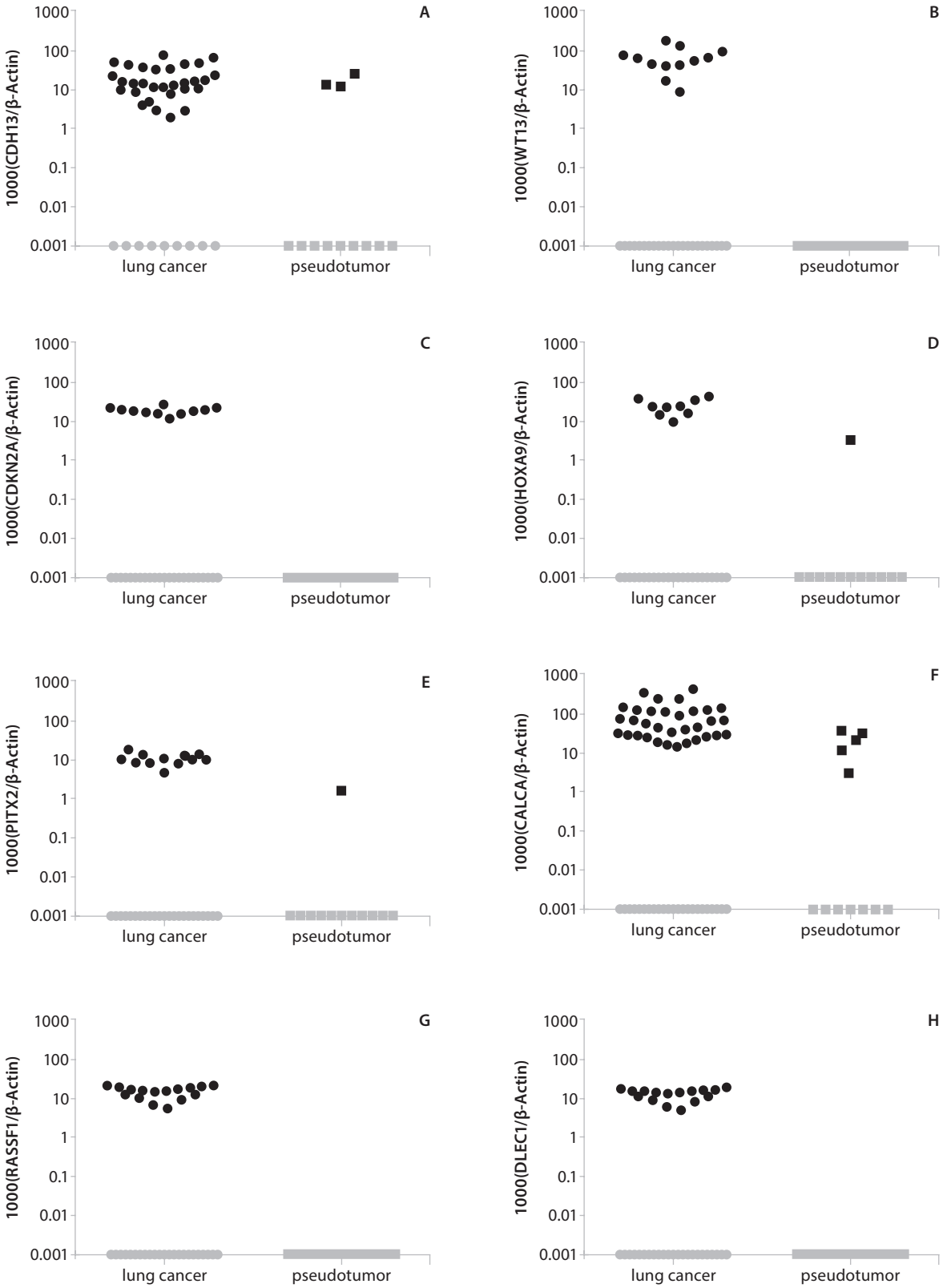

Fig. 1. Comparison of plasma DNA methylation levels of 8 genes in patients with lung cancer and without lung cancer. The methylation level of *CALCA* was the highest, followed by *CDH13*, *RASSF1A*, *DLEC1*, *WT1*, *CDKN2A*, and *PITX2*, while the methylation level of *HOXA9* was the lowest in patients. Methylation of *RASSF1A*, *CDKN2A* and *DLEC1* occurred only in lung cancer patients

A – *CDH13*; B – *WT1*; C – *CDKN2A*; D – *HOXA9*; E – *PITX2*; F – *CALCA*; G – *RASSF1A*; H – *DLEC1*.

may be an important marker of malignant tumors such as esophageal cancer, colon cancer and thyroid cancer.[22]

RASSF1A is a novel tumor suppressor gene cloned from the short arm of human chromosome 3. It is low-expressed or not expressed in various tumor tissues. The reason for its nonexpression may be the specific methylation of CpG island in the promoter region.[23] *DLEC1* is a tumor suppressor gene located at the 3p21.3 region. It can inhibit the growth of lung cancer, esophageal cancer and the renal carcinoma cell line. The specific methylation of CpG island in the promoter region can cause downregulation or nonexpression of *DLEC1*, which is associated with the development of multiple tumors.[24]

It has been shown that the methylation of *CDKN2A*, *RASSF1A*, *APC*, *RARβ*, *DLEC1*, *DAPK*, and *CDH13* genes in peripheral plasma-free DNA may be a potential marker for the diagnosis of lung cancer.[25,26] The methylation of *CDKN2A* and *RASSF1A* are also associated with the prognosis of lung cancer.[8,27] The methylation of these genes has good sensitivity and specificity in lung cancer.[23,28] Our results showed that there were significant differences in the methylation levels of the 8 genes between lung cancer patients and non-lung cancer patients. Methylation of *RASSF1A*, *CDKN2A* and *DLEC1* occurred only in lung cancer patients, while the methylation of *CALCA*, *CDH13*, *PITX2*, *HOXA9*, and *WT1* occurred not only in lung cancer patients, but also in non-lung cancer patients. The sensitivity and specificity of methylation detection of these 8 genes in the diagnosis of lung cancer are lower than those reported in other studies. This may be due to the fact that all the subjects examined in this study are stage I lung cancer patients, or due to the small sample size.

We explored the utility of quantitative detection of plasma DNA methylation in the diagnosis of early lung cancer. The distinction between early lung cancer and benign lung lesions is highly specific and sensitive by detecting the methylation levels of 8 genes. This study also showed that the accuracy of the detection is increased with the increase of the number of detection genes; therefore, finding more and more meaningful markers will also be an important task in further studies. It was found that quantitative detection of DNA methylation in plasma is a potential method for early diagnosis of lung cancer.

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Could infrared thermal imaging be a new diagnostic tool for acute appendicitis?

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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*. 2019;28(3):361–367

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Funding sources
This study received financial support as an individual research project from Adıyaman University, Turkey (Project No: TIPBAP/2012-0004).

Conflict of interest
None declared

Acknowledgements
The authors want to thank Assoc. Prof. Dr. Bulent Celik for statistical analysis and interpretation of data.

Received on September 23, 2016
Reviewed on January 12, 2017
Accepted on March 21, 2018

Published online on January 17, 2019

Abstract

Background. Appendicitis is the most frequent acute abdominal disease and there are actual effective diagnostic tools for its detection.

Objectives. The objective of this study was to determine whether a thermal imaging camera is a useful tool for diagnosing acute appendicitis on the basis of abdominal skin surface temperature changes which reflect intra-abdominal inflammation.

Material and methods. The prospective data consisting of surgical and pathological findings of 51 patients who had undergone appendectomy between January 2013 and December 2014 with the diagnosis of acute appendicitis was collected, as well as thermal imaging camera recordings. A handheld infrared (IR) thermal imaging camera (ITIC) was used to take measurements.

Results. Of the 51 patients studied, 30 were male and 21 were female. Of these, 12 had their highest temperature measurement in the epigastric and 17 in the umbilical areas. These 2 groups constituted 56.9% of the patients. Regarding the lowest temperature measurement, 10 patients had the lowest temperature in the right inguinal and 15 in the hypogastric area. These 2 numbers constituted 49% of the patients.

Conclusions. This is the first report concerning the use of a thermal camera as a diagnostic tool for the evaluation of acute abdominal illness. Considering the results of our study, ITIC is not feasible as a new diagnostic tool for acute appendicitis. It may be suitable for determining superficial inflammation; however, it is not suitable for determining deep inflammation.

Key words: acute appendicitis, diagnostic tool, thermal camera, infrared imaging, acute inflammation

Cite as
Hatipoglu S, Abdullayev R. Could infrared thermal imaging be a new diagnostic tool for acute appendicitis? *Adv Clin Exp Med*. 2019;28(3):361–367. doi:10.17219/acem/87114

DOI
10.17219/acem/87114

Background

All the objects over –273°C (0 K) emit thermal energy with an infrared (IR) wavelength. This radiation is proportional to the vibration and rotation of the molecules of the object. The amount of radiation is proportional to the fourth power of the temperature; hence, minor changes in the temperature result in substantial changes in the amount of radiation.[1,2] This observation has given rise to the concept of observing IR radiation as an indicator of the temperature changes. Infrared radiation is invisible to the naked eye, which is why thermal cameras are used for visualization.

A thermal camera is a device that senses the thermal model of an object in the IR wavelength spectrum, without physical contact. It consists of an optical mechanical unit, detector and processing unit. A lens collects the IR light and focuses it on an IR detector, after which the processing unit creates an electronic image. This image contains a color spectrum from blue to yellow according to the temperature, where blue represents the coldest area and yellow the hottest (Fig. 1). Infrared thermography (IRT) has been used for many years in the military, aerospace research, construction industry, textile field, other industrial sectors, and veterinary medicine.[3,4] Although its use in medicine dates back to 1960s, it has only recently gained popularity.[5] With the technological developments in IRT, it is increasingly being used in many fields of medicine for diagnostic purposes.[3,6]

Abdominal pain constitutes 4–8% of adult admissions to the emergency surgery service, and appendicitis is the most frequently considered diagnosis among these cases.[7] Suspicion of appendicitis is one of the most common results of surgical consultations in the outpatient or emergency room setting. Late diagnosis is associated with increased risk of morbidity and mortality, which is why early diagnosis is crucial.[7,8] The diagnosis of appendicitis is based on clinical examination, laboratory findings and abdominal ultrasonography (USG). Scoring systems, abdominal X-ray, computed tomography (CT), magnetic resonance imaging (MRI), and diagnostic laparoscopy are also used in the diagnosis.[7,8] These diagnostic instruments have different advantages and disadvantages as well as specificity and sensitivity.

The aim of this study was to analyze abdominal wall IR thermal images of patients with intra-abdominal inflammation and evaluate if this method could be a good diagnostic tool for acute appendicitis.

Material and methods

A total of 51 patients undergoing emergency appendectomy under general anesthesia at the Department of General Surgery of Adıyaman University Research and Educational Hospital, Turkey, between January 2013 and December 2014 were recruited into the study. The Clinical Trials Ethics Committee of Adıyaman University approval was obtained. Prospective data of the patients consisting of surgical and pathological findings was collected, as well as thermal camera images. The research adhered to the tenets of the Declaration of Helsinki and informed consent was obtained from all patients.

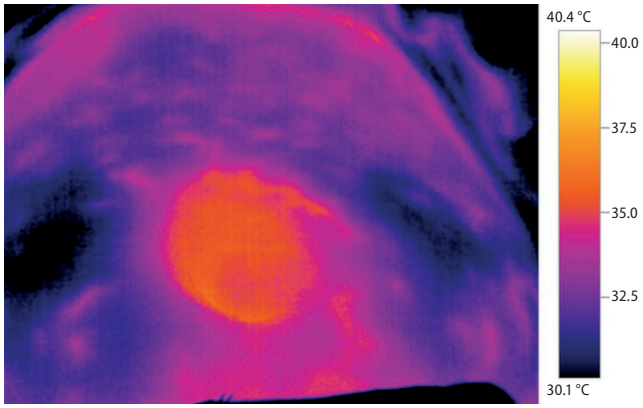

Fig. 1. Thermal camera images contain a color spectrum from blue to yellow according to the temperature, where blue represents the coldest area and yellow the hottest

The data consisted of the first findings obtained at admission and included body temperature, preoperative thermal imaging camera observations of abdominal skin surface, perioperative, and pathological findings. Acute appendicitis was diagnosed by history and physical examination, as well as laboratory and radiological examinations, and then thermal camera images of anterior abdominal skin surface were taken preoperatively. Thermal camera images were co-evaluated with operational data and histopathological findings.

Preoperative surgical evaluation of the patients included serum electrolyte levels, standard liver function tests, enzymes, complete blood count, coagulation tests, serology tests for HIV and hepatitis B and C viruses, electrocardiography, and chest radiography.[7,8] Emergency USG and CT imaging were not routinely performed in these patients due to an insufficient access to radiological consultation out-of-shift. The first examination and surgery in these patients were performed by the same general surgeon. The patients received preoperatively a prophylactic dose of 2nd generation cephalosporin (1 g iv.) and underwent an open approach appendectomy via a McBurney incision under general anesthesia.[7] A laparoscopic approach was not performed due to technical inadequacy. Diagnosis of appendicitis was made by perioperative macroscopic evaluation. All microscopic evaluations of appendectomy specimens from patients were evaluated in the hospital pathology unit. Histopathologically negative appendectomy specimens for appendicitis were excluded from the study.

Application and properties of infrared thermal imaging

A number of factors were standardized to minimize variability, including pre-measurement time lag, patient positioning, camera distance, room temperature,

and humidity. The patients were transported to the operation room 10 min before the measurement to give time for temperature equilibration between core and skin. The patients were placed in supine position and anteroposterior IR images were obtained with thermal cameras placed 30 cm from the patient's abdominal wall. Images were obtained in the room shielded from direct sunlight. Temperature was kept between 20°C and 22°C, and humidity between 40% and 60%. We used a handheld IR thermal camera (Testo 882; Testo Electronics and Thermal Imaging Systems, Istanbul, Turkey) with a resolution of 320 × 240 pixels and thermal sensitivity <0 mK (i.e., temperature changes of 0.05°C were detected). Thermal maps of the abdominal wall were recorded and the data was stored, after which it was analyzed by dividing the abdominal wall into 9 quadrants (right hypochondriac, epigastric, left hypochondriac, right lumbar, umbilical, left lumbar, right iliac, hypogastric, and left iliac), and extracting minimum and maximum temperature values from the thermal maps as indicated in Fig 2. Figure 3 presents an example of the thermal map of one of the patients.

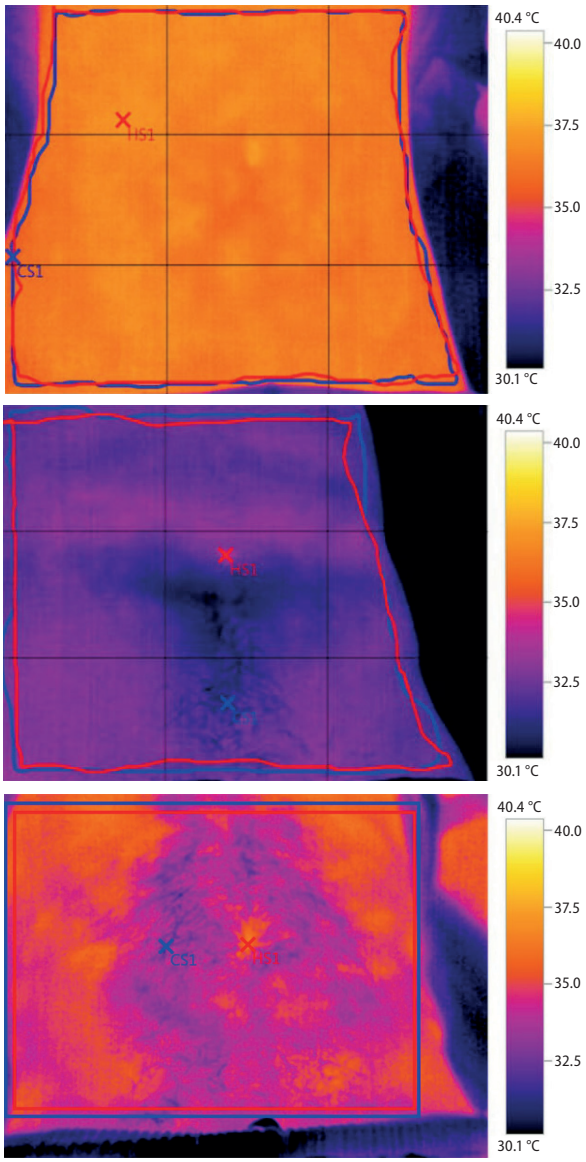

Fig. 2. Abdominal wall quadrants (right hypochondriac, epigastric, left hypochondriac, right lumbar, umbilical, left lumbar, right iliac, hypogastric, and left iliac) and minimum and maximum temperatures from the thermal maps (CS: Coldest Spot, HS: Hottest Spot)

Statistical analysis

Data analysis was performed using Statistical Package for the Social Sciences v. 15.0 (SPSS Inc., Chicago, USA). Highest and lowest temperature values in respective areas were presented as mean, standard deviation (SD), and minimum and maximum values. Also, the frequency and percentages of temperature measurements obtained from the areas are presented in the Tables 1 and 2. The data was interpreted using these percentages, means and SDs.

Results

Fifty-one consecutive open appendectomies were performed during the study period. All of the patients were classified using American Society of Anesthesiologists (ASA) physical fitness classification system as I–II E. The mean age was 27.4 ±7.5 years (range: 18–55 years); 21 of the patients were women, whereas 30 were men.

Tables 1 and 2 present the highest and lowest temperature measurements of the abdominal wall regions. Of these 51 patients, 12 had their highest temperature measurement in the epigastric and 17 in the umbilical

Table 1. Regions of the abdomen with highest temperature values

Areas	n	%	Temperature			
			mean	SD	min	max
Right hypochondrium	8	15.7	36.9	1.1	35.1	38.4
Epigastric region	12	23.5	36.8	1.1	34.9	39.2
Left hypochondrium	2	3.9	37.3	1.3	36.3	38.2
Umbilical region	17	33.3	36.6	1.0	34.1	38.0
Hypogastric region	6	11.8	36.1	1.0	34.7	37.6
Left inguinal region	6	11.8	36.2	1.4	34.3	38.0

Table 2. Regions of the abdomen with lowest temperature values

Areas	n	%	Temperature			
			mean	SD	min	max
Right hypochondrium	5	9.8	33.1	1.6	31.1	35.4
Left hypochondrium	2	3.9	32.1	0.3	31.9	32.3
Right colic region	9	17.6	33.5	2.0	30.1	35.3
Umbilical region	1	2.0	30.9	0.0	30.9	30.9
Left colic region	1	2.0	34.7	0.0	34.7	34.7
Right inguinal region	10	19.6	33.4	1.9	29.8	35.5
Hypogastric region	15	29.4	32.6	1.6	30.0	35.4
Left inguinal region	8	15.7	32.3	1.4	30.8	34.3

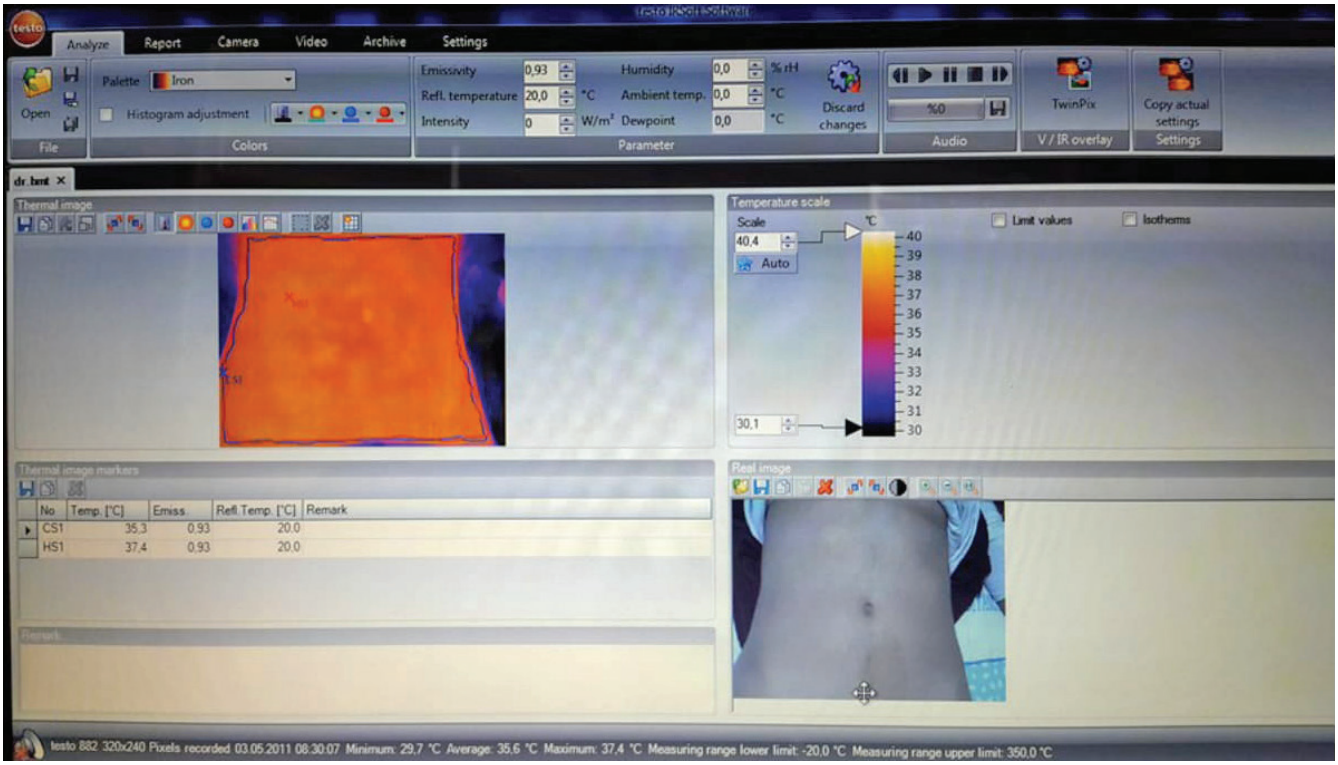

Fig. 3. An example of the thermal screen of infrared thermal imaging obtained from one of the patients

areas. These 2 groups constituted 56.9% of the patients. Regarding the lowest temperature measurement, 10 patients had their lowest temperature measurements in the right inguinal and 15 in the hypogastric area. These 2 groups constituted 49.0% of the patients.

Discussion

The main finding of our study was that IR thermal imaging of abdominal wall in the patients with acute appendicitis does not help in precise diagnosis of this disease. The temperature of the abdominal wall skin does not reveal deeper pathologies.

Infrared thermal imaging becomes more and more common diagnostic technique in medicine. It is used for diagnostic purposes in veterinary medicine.[9,10] Its use in medicine comprises the evaluation of side effects and the reliability of surgical devices and procedures,[11–15] animal experiments[16] and, of course, diagnostic purposes. Carpal tunnel syndrome, varicocele, thyroid diseases, breast cancer, diabetic wounds, surgical site infections, compartment syndrome, and rheumatic diseases are diagnosed using this technique, among others.[1,17–23]

Infrared thermal imaging is a non-invasive, painless assessment tool. Its non-invasive nature makes it convenient for serial measurements. Minor changes in the temperature, even as small as 0.01°C, can be detected. It can give objective information about pain. The physiological structure of the investigated area is observed rather than the anatomical, and thermal map of the region can be generated. Real time assessment is another advantage of this method.[6] Thermal cameras can only sense superficial temperature, so measurements can only be made beyond 1–2 mm of the epidermis.[24] The advantages of thermal camera as a diagnostic tool besides its non-invasive nature are its ease of application, non-subjective results, cost-effectiveness, and the fact that it can be applied by every physician and/or specialist staff. Also, it is a temperature measuring technique, capable of displaying real-time surface temperature distribution and measuring a wide range of temperature on the macro as well as on the micro levels.

Microcirculation and thermal radiation in human tissue are influenced by inflammatory, metabolic and toxic factors, and are mediated through sympathetic tonus and vasoactive agents.[25] Many pathological processes manifest themselves as local changes in heat production and also as changes in blood flow pattern in affected organs or tissues. Owing to the blood flow and conductive transfer of heat from the interior of the body to the surface areas, IR images are believed to be capable of reflecting thermal processes deep in the body.[26] Despite standard measurements like flow and resistance, IRI can also provide data about homogeneity of blood flow in the organs.[27]

Acute appendicitis is an acute inflammatory condition resulting from early response of appendicular tissue to injury. Acute inflammation is non-specific and may be evoked by any injury.[28] It is a short-term process, occurring before the immune response becomes established, usually appearing within few minutes or hours and ceasing upon

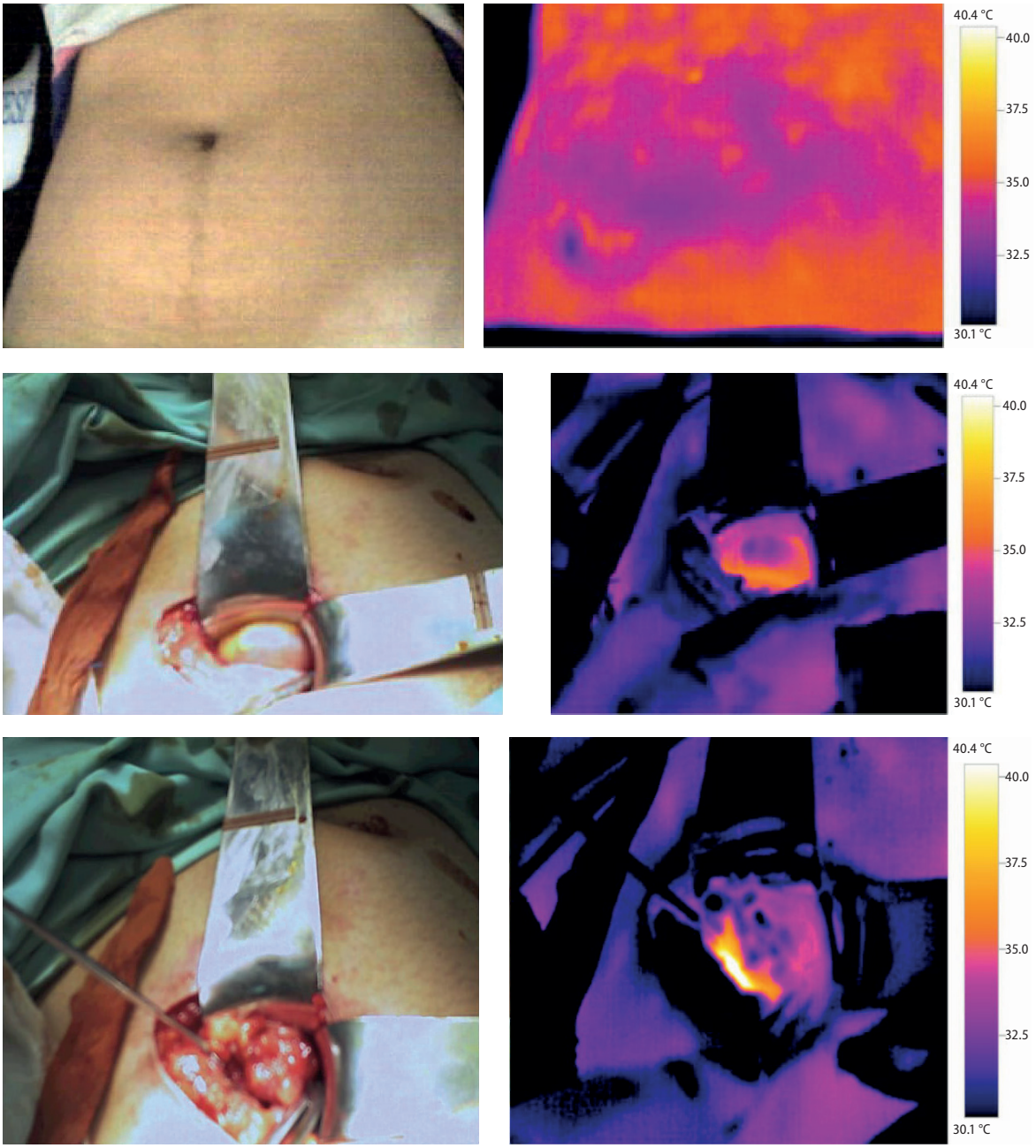

Fig. 4. This preoperative and peroperative examination was carried out according to EN 13187 using a thermal image from one of the patients' respectively

Picture parameters: Emissivity: 0.93; Refl. temp. [°C]: 20.0; Lens: Standard 32°

the removal of the injurious stimulus. Acute inflammation may be regarded as the first line of defense against tissue injury and is characterized by changes in the microcirculation, exudation of fluid and emigration of leukocytes from blood vessels to the area of injured tissues.[28] Acute inflammation normally resolves by mechanisms still remaining somewhat elusive.[29] Physical examination, laboratory tests and radiological evaluations are being carried out during the diagnosis of acute appendicitis.[7,8] Sometimes USG and CT examinations are mandatory for proper diagnosis, yet subjective interpretations may yield some problems. The purpose of this study was to evaluate IRI as an alternative to other radiodiagnostic tools in the diagnosis of acute appendicitis. The hypothesis was based on the possible abdominal skin wall temperature changes after intra-abdominal inflammatory process (Fig. 4).

Clinically, acute inflammation is characterized by 5 cardinal signs: rubor (redness), color (increased heat), tumor (swelling), dolor (pain), and functio laesa (loss of function).[28] The first 4 were described by Celsus; the 5th was a later addition by Virchow and these 5 signs are manifested when acute inflammation occurs on the surface of the body, but not all of them may be apparent in acute inflammation of internal organs.[28]

The increased heat of inflamed skin is due to the entry of a large amount of blood at body core temperature into the naturally cooler skin. When inflammation occurs internally – where tissue is normally at body core temperature – no increase in heat is apparent.[28] On the other hand, it is not clear whether there is a meaningful temperature increase on the abdominal skin surface because of the acute appendicitis. A thorough understanding of the physiology of the acute inflammation of appendix is essential. Some questions must be addressed. Is there a meaningful increase in the temperature of local inflammation area? How can this temperature change be reflected in the abdominal skin? How can we precisely measure it? Is it possible that detection of this local temperature increase can be helpful for the diagnosis of acute abdominal disease, especially for acute appendicitis? There are no studies in the literature regarding the use of a thermal imaging camera. Therefore, we aimed to find answers to these questions.

About 57% of the patients had their highest temperature values recorded in the epigastric and umbilical areas. None of the patients had their highest temperature recordings in their right inguinal area. Even though our initial expectations were that the highest temperature recordings would be found in the right inguinal area, about half of the patients had the lowest temperature measurements in their right inguinal and hypogastric areas. Anatomical proximity of the large vessels and abdominal skin wall thicknesses can be an explanation.

Temperature changes in the inflammation area can be recorded using deep tissue thermal cannula. Sasaki used subdermal thermal cannula for comparison of subdermal tissue temperature with skin temperature.[12] Further studies should be devoted to this method. A possible positive correlation between deep and superficial temperature can lead to a hypothesis about the thermal conduction. Temperature changes in the skin due to blood flow are complex and will follow vascular anatomy. Moreover, a minimal temperature increase on the abdominal skin surface may be precisely determined with the development of medical technology and technological advances in devices such as non-invasive thermal imaging camera.

There were several limitations in this study. Optimal time for obtaining IR image is not clearly determined, so we took IR images of the patients after a 10-minute waiting period in the operation room. Ring has proposed a 15-minute waiting time for temperature equilibration between skin and body core.[6] Nonetheless, the time was standardized for all the patients. Another limitation was the lack of data record and standardization of the patients' body mass index (BMI). Taking into consideration possible temperature conduction from deeper tissues besides blood flow, patients with a thick abdominal wall will have different conductive properties compared to patients with a lower BMI. Intra-abdominal or subdermal temperature measurements via cannulas could have been made for the sake of comparison, as done by Sasaki.[12] Because of the limited technical capacity of the camera used in our study, temperature differences below 0.05°C could not have been detected and the low resolution of the camera was another shortcoming.

In conclusion, IR thermal imaging does not meet the requirements for a good diagnostic tool for acute appendicitis. Despite its significance in many areas of medicine, its usefulness in acute intra-abdominal inflammation is limited because of its inability to evaluate the body's interior. However, this method might be promising if temperature changes in deeper tissues are visualized with improved technology.

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Hair cortisol concentration in a population without hypothalamic–pituitary–adrenal axis disorders

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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*. 2019;28(3):369–373

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Funding sources
None declared

Conflict of interest
None declared

Received on November 22, 2017
Reviewed on December 9, 2017
Accepted on April 18, 2018

Published online on December 5, 2018

Abstract

Background. Measuring hair cortisol seems to be a good alternative to laboratory tests used thus far in routine endocrine diagnostics, primarily because it is independent of the circadian rhythm of cortisol. Due to the average hair growth of 1 cm per month, the results are related to the average blood cortisol levels over the previous weeks, months or years (depending on the length of the hair sample).

Objectives. The aim of this study is an attempt to apply hair cortisol concentration (HCC) measurements to clinical endocrine diagnostics, based on reference cortisol concentrations in the blood in a population without disorders of the hypothalamic–pituitary–adrenal axis (HPA).

Material and methods. In the final selection process, 44 patients were enrolled in the study, all with negative interviews regarding disorders of the HPA and with reference levels of cortisol concentration obtained in routine laboratory tests. In the pre-analytic phase, we used 1 cm proximal hair strands cut from the posterior vertex area of the head, followed by the incubation of a 20 mg hair sample in methanol. The final cortisol measurement was done using an enzyme-linked immunosorbent assay (ELISA).

Results. The results of HCC ranged from 2 pg/mg up to 51.63 pg/mg. The diurnal decrease in cortisol levels was significantly lower in females than in males ($p = 0.031$), but we do not consider that difference to be clinically significant. The difference in the HCC between males and females was not statistically significant ($p = 0.767$). The linear regression coefficient for age was not statistically significant ($p = 0.847$). Neither the regression coefficients for gender nor the gender and age interactions were statistically significant ($p = 0.815$).

Conclusions. Hair cortisol concentration measurement, unlike other endocrinological tests, gives information about the cortisol concentration in the long-term perspective. The results obtained in this study may be used as a reference for further research aimed at determining normal values of HCC.

Key words: measurements, validity, hair cortisol, reference

Cite as
Cieszyński Ł, Jendrzejewski J, Wiśniewski P, Owczarzak A, Sworczak K. Hair cortisol concentration in a population without hypothalamic–pituitary–adrenal axis disorders. *Adv Clin Exp Med*. 2019;28(3):369–373. doi:10.17219/acem/90038

DOI
10.17219/acem/90038

Introduction

The aim of this study was an attempt to apply measurements of cortisol concentrations in hair to clinical endocrine diagnosis, based on reference cortisol concentrations in the blood in a population with no disorders of the hypothalamic–pituitary–adrenal axis (HPA).

Routine endocrine diagnostics for patients with HPA axis disorders entail a series of blood, urine and saliva tests. These include daily serum cortisol, urine free cortisol (UFC), saliva cortisol, and several hormonal dynamic tests using stimulation, such as the corticotropin-releasing hormone (CRH) stimulation test, or inhibition test, e.g., the low/high dose dexamethasone suppression test. The numerous methods of cortisol assessment arise from the necessity of collecting reliable results that correlate with a clinical picture. Their interpretation is often impeded due to variations in the method of determination, environmental factors, physiological variations in cortisol levels, drugs/xenobiotics taken, and pathological conditions secondarily affecting the HPA axis. For this reason, there is no single test that will produce a credible result, and therefore, at least a few tests must be performed to provide a basis for a proper diagnosis.[1–3]

Publications related to hair cortisol concentrations (HCC) mainly refer to non-primary endocrine disorders, including depressive-anxiety disorders, stressful events, shift work, physical exertion, alcohol abuse, smoking, chronic illnesses, or doping control.[4–12] Among the most relevant endocrine disorders in the context of HCC assays are Cushing's syndrome, Cushing's subclinical syndrome, Addison's disease, secondary adrenal insufficiency, and cortisol/hydrocortisone substitution.[13–20]

Measurement of HCC seems to be a good alternative to the laboratory tests used thus far in diagnosing endocrine disorders, primarily due to its independence from the circadian rhythm of cortisol. Given the average hair growth of 1 cm per month, the results are related to average blood cortisol levels over the past weeks, months or years, depending on the length of the hair sample.[21–23]

The presence of cortisol in hair, as well as other steroid hormones, is most probably a result of the passive diffusion of free cortisol from blood to the hair matrix during its formation in hair follicles. Another possible explanation for the presence of cortisol in hair is its presence in sweat and sebum. Hence, its concentration may depend on additional factors such as gender (stimulation of sweat and sebum production by androgens), age (slower hair growth with age), hair dyeing (possible flushing out of cortisol by chemicals, interference with HCC assays), physical activity, hygienic habits (influence of hair washing frequency), or exposure to sunlight.[11,24–31] Additional benefits of HCC assessment are its non-invasive sample collection procedure and the possibility of sample storage at room temperature for a long period of time without prior processing.

Hair cortisol concentration measurement is most often based on immunological methods, such as the enzyme-linked immunosorbent assay (ELISA) or liquid chromatography tandem mass spectrometry (LC-MS/MS).[27,32–37] Measurement of HCC in humans was initiated by J.S. Raul in 2004.[36] The population analyzed in his study consisted of 44 volunteers recruited on the basis of a questionnaire. The HCC measurement was done using chromatography, and the amount of cortisol in the samples ranged from 5 pg/mg to 91 pg/mg. Since the first report, a number of publications analyzing the HCC measurement have involved different populations of subjects recruited on the basis of demographic and anthropometric factors.[24] The reliability of HCC measurement is limited due to the heterogeneity of the populations analyzed, the various methodologies of the assays, comparative assessment without initial laboratory diagnostics regarding blood cortisol concentrations, and the lack of commonly accepted HCC reference values. An attempt to establish reference values of HCC in healthy subjects who have also been evaluated for cortisol levels using standard laboratory tests remains ongoing.

Material and methods

We included 88 patients who were hospitalized in the Department of Endocrinology and Internal Medicine at the Medical University of Gdańsk, Poland, for the evaluation of hormone levels during the course of an incidental adrenal tumor. All participants signed a written informed consent to participate in the study. The study was approved by the Bioethical Committee of the Medical University of Gdańsk. Enrollment in the project was carried out in 2016.

The routine diagnostic protocol consisted of measurement of morning plasma adrenocorticotropic hormone (ACTH) (ACTH8am), morning plasma cortisol (MPC8am), evening plasma cortisol (EPC8pm), 24-hour urinary free cortisol excretion (UFC24h), and the 1 mg overnight dexamethasone suppression test (1mgONDST). These measurements were performed at the Central Laboratory of the University Hospital in Gdańsk. Patients taking glucocorticosteroids during the preceding year and those with conditions likely to affect the results of the tests – in particular depression, alcoholism, acute/chronic inflammatory disease, and primary or secondary HPA disorders – were excluded from the study. In the final selection process, 44 patients were enrolled in the study, all with reference levels of ACTH8am (reference: <46 pg/mL), MPC8am (reference: 101–535 nmol/L), EPC8pm (reference: 79–478 nmol/L), UFC24h (reference: 12–486 nmol/24 h), a regular diurnal decrease of cortisol (DDC; reference: EPC < MPC more than 50%), and proper suppression of cortisol after 1mgONDST (reference: <50 nmol/L).

A hair sample was collected in the examination room from the posterior vertex area of the head, using a sterile

Table 1. Characteristics of the population. Data is presented as mean ± standard deviation (SD) and median ± interquartile range (IQR)

Variable	Total	Female	Male	p-value
Number	44	31	13	n/a
Age [years]	63.3 ±10.3	63.6 ±8.8	62.5 ±13.8	0.757
Morning plasma cortisol [nmol/L]	338.0 ±113.8	323.7 ±109.4	375.0 ±120.3	0.175
Evening plasma cortisol [nmol/L]	111.1 ±46.8	115.2 ±46.8	101.4 ±47.1	0.379
Diurnal decrease of cortisol [%]	–65.5 ±11.9	–63.0 ±10.6	–71.4 ±13.1	0.031
Urine free cortisol [nmol/24 h]	204.3 ±130.2	201.7 ±127.9	210.7 ±140.7	0.837
ACTH [pg/mL]	29.95 ±20.01 (22.8 ±15.7)	27.67 ±19.11 (22.0 ±14.4)	35.39 ±21.81 (26.8 ±19.1)	0.095
1mgONDST [nmol/L]	32.27 ±6.07 (28.0 ±8.0)	33.23 ±6.61 (30.0 ±9.0)	30.00 ±3.83 (28.0 ±3.0)	0.1374

1mgODST – 1 mg overnight dexamethasone suppression test; ACTH – adrenocorticotropic hormone; n/a – not applicable.

scalpel to cut an approximate quantity of 100–200 hair strands as close as possible to the scalp. The samples obtained were stored in dry envelopes until further analysis. Next, 1 cm segments of the hair strands were cut away near the follicles, and following weighing, exactly 20 mg of the sample was used in the study. The preparation of the hair samples was performed at the Department of Clinical Nutrition and Dietetics at the Medical University of Gdańsk. First, the hair samples were placed in 10 mL plastic tubes and flushed with 2 mL of methanol. Next, they were transferred to 5 mL tubes and incubated at 50°C in 1 mL of methanol for 24 h. The extract was then transferred to 3 mL tubes, after which it was evaporated. Finally, the residue was dissolved in 250 µL of phosphate-buffered saline (PBS) by incubation for 1 h.

The final HCC measurement was done at the Central Laboratory of the University Hospital in Gdańsk using an ELISA Cortisol Assay (IBL International GmbH, Hamburg, Germany; catalog No. RE 52611). The assay is reported to demonstrate cross-reactivity with prednisolone (30%), 11-desoxy-cortisol (7%), cortisone (4.2%), prednisone (2.5%), corticosterone (1.4%), and less than 1% with other tested substances. The limit of detection and functional sensitivity are calculated to be 0.005 ug/dL and 0.030 ug/dL, respectively. A detailed description of the assay is available on the manufacturer's website (http://www.ibl-international.com/en/cortisol-saliva-elisa). For each sample, the HCC was measured twice, and the average value was used in the statistical analysis.

Standard descriptive statistics were prepared. Conformity to a normal distribution was assessed using a histogram analysis. Some of the dependent variables underwent a logarithmic transformation before they were included in the parametric analysis. The arithmetic mean of the 2 HCC assessments was analyzed. The results of HCC are presented as pg/mg according to the cortisol concentration in the analyzed volume of the solution (250 µL) based on the amount of incubated hair (20 mg). Comparisons in subgroups were evaluated using the t-test or the Mann–Whitney U test. A multivariate regression (general linear) analysis was used to evaluate the relationship between age and HCC. The regression model also included gender and analyzed the interaction between age and gender. P-values <0.05 were considered statistically significant. All the calculations were performed using the STATA Statistical Package v. 13.1 (StataCorp LLC, College Station, USA).

Results

The size of the population, demographic data and the parameters of the pituitary-adrenal axis that were evaluated are presented in Table 1. The diurnal decrease in cortisol levels was significantly lower in females than in males (p = 0.031). However, we consider that difference not to be clinically significant. There were no statistically significant differences between other variables.

The results of HCC ranged from 2 pg/mg up to 51.63 pg/mg. The difference in HCC between males and females was not statistically significant (p = 0.767). The detailed characteristics of the HCC results are presented in Table 2. We also analyzed the relationship between age and HCC. The results are shown in Fig. 1. The linear regression coefficient for age was not statistically significant (p = 0.847). The regression coefficients for gender and for gender and age interactions were also not statistically significant (p = 0.815).

Table 2. Hair cortisol concentration (HCC) results [pg/mg]

Variable	Total (n = 44)	Female (n = 31)	Male (n = 13)
Mean	7.17	7.74	5.82
SD	10.40	12.05	4.63
Median	3.38	3.25	3.63
IQR	2.97	2.56	6.19
Minimum	2.00	2.00	2.13
Maximum	51.63	51.63	17.06

SD – standard deviation; IQR – interquartile range.

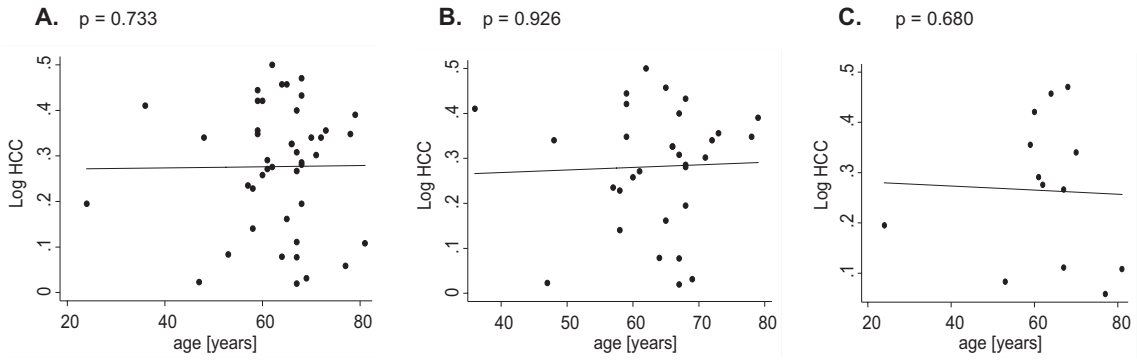

Fig. 1. Association between hair cortisol concentration (HCC) and age

A – all; B – females; C – males.

Discussion

Hair cortisol concentration assessment is a multistep process, starting with proper hair sampling, followed by extraction of cortisol at the pre-analytical stage and concluding with final laboratory evaluation using one of the reference methods. Each of these steps can have a significant impact on the final result. Hence, the various phases of HCC assessment have been subjected to evaluation in numerous studies. In our study, the number of collected hair strands from a given individual was largely a result of a compromise between the need to obtain as much material as possible and esthetic issues, especially among the female population. In most studies, the amount of hair analyzed ranges from 2.5 mg to 150 mg, with the most widely used amount being 10–20 mg.[6,7,33,34,38] The optimal site for hair sampling is the vertex posterior area of the head; possible individual variation in HCC can be as high as 24%, depending on the site of collection, as demonstrated by drug and xenobiotic studies.[39] Incubation of a total hair sample (1-centimeter fragments) paradoxically results in higher HCC than milled hair, as it has been shown in 1 work describing HCC methodology.[40] Extraction of cortisol using a methanol solution is one of the most commonly used methods.[4,24,41] Analyses of various methods of HCC determination indicate the superiority of ELISA in terms of the sensitivity of cortisol measurement.[40,42] This assay is very useful due to its widespread availability as well as its ease of implementation. The correlations between HCC measurements with ELISA and LC-MS/MS in interlaboratory comparisons have been found to be strong.[42]

The essential problem in HCC analysis is to determine the degree of cortisol extraction from hair samples. Generally, we assumed close to 100% cortisol recovery by methanol extraction, which might not be fully true based on the latest reports. One publication has demonstrated higher extraction efficiency by methanol and acetone over methanol alone.[40] However, most published works used cortisol extraction by methanol as a reference method.

Unlike previous studies, our work focused on the deep characteristics of the study population. The recruited volunteers may be regarded as a reference group because of their extensive screening for regular levels of cortisol. Thanks to the recruitment protocol, the impact of anthropometric and demographic parameters possibly affecting the HPA axis during hair collection (e.g., gender, body weight, hair color, race) has largely been excluded. We believe that higher maximal HCC in females (n = 31, HCC = 51.63 pg/mg) vs males (n = 13, HCC = 17.06 pg/mg) may possibly be due to the small male population tested and may not be related to other factors. Most studies exclude gender as a potential confounding factor in adult participants.[13,27,33,36] The issues outlined in Material and methods section meant that only 44 patients out of 88 met the final inclusion criteria based on the study protocol (incorrect DDC and 1mgONDST were the most common reasons for exclusion). An interview that excluded the enrollment of patients with primary or secondary HPA disorders in the period >1 year prior to sampling further strengthens our results since analyzed hair samples reflected an average cortisol concentration of past month only.

Conclusions

The results of the study indicate the possibility of using HCC measurement in all subjects under the condition of receiving a sufficient hair sample. Cortisol extraction using a methanol medium is easy to perform with basic laboratory equipment. Commercial ELISA assays have high sensitivity and specificity with a very low cortisol detection threshold.

The study was conducted on a homogeneous group of participants who did not display corticotropic axis disorders. The HCC ranged from 2 pg/mg to 51.63 pg/mg. The results obtained in the present work may be used as a reference for further research.

Hair cortisol measurement may be a new diagnostic tool in clinical practice. Further research on HCC may contribute to improvement of the diagnosis and treatment of patients with HPA axis diseases. Hair cortisol concentration measurement, unlike other endocrinological tests, provides information about cortisol concentration in the patient's long-term history.

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Safety assessment during initiation and maintenance of propranolol therapy for infantile hemangiomas

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2019;28(3):375–384

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Funding sources
None declared

Conflict of interest
None declared

Received on July 4, 2018
Reviewed on July 26, 2018
Accepted on August 9, 2018

Published online on January 15, 2019

Abstract

Background. Propranolol is an effective method of treatment for infantile hemangiomas (IH). A recent concern is a shift of the therapy into outpatient settings.

Objectives. The aim of the study was to evaluate the safety of initiating and maintaining propranolol therapy for IH.

Material and methods. The study involved 55 consecutive children with IH being treated with propranolol. The patients were assessed in the hospital at the initiation of the therapy and later in outpatient settings during and after the therapy. Each time, the following monitoring methods were used: physical examination, cardiac ultrasound (ECHO), electrocardiography (ECG), blood pressure (BP), heart rate (HR), and biochemical parameters: blood count, blood glucose, aspartate transaminase (AST), alanine transaminase (ALT), and ionogram. The therapeutic dose of propranolol was 2.0 mg/kg/day divided into 2 doses.

Results. Four children were excluded during the qualification or the initiation of propranolol; a total of 51 patients were subject to the final analysis. All the children presented clinical improvement. There was a significant reduction in the mean HR values only at the initiation of propranolol. There were no changes in HR during the course of the therapy. Blood pressure values were within normal limits. Both systolic and diastolic values decreased in the first 3 months. Bradycardia and hypotension were observed sporadically, and they were asymptomatic. Electrocardiography did not show significant deviations. The pathological findings of the ECHO scans were not a contraindication to continuing the therapy. There were no changes in biochemical parameters. Apart from 1 symptomatic case of hypoglycemia, other low glucose episodes were asymptomatic and sporadic. The observed adverse effects were mild and the propranolol dose had to be adjusted in only 6 cases.

Conclusions. Propranolol is effective, safe and well-tolerated by children with IH. The positive results of the safety assessment support the strategy of initiating propranolol in outpatient settings. Future studies are needed to assess the benefits of the therapy in ambulatory conditions.

Key words: infantile hemangioma, propranolol, ECG monitoring, blood pressure, adverse effects

Cite as
Babiak-Choroszczak L, Giżewska-Kacprzak K, Dawid G, Gawrych E, Bagłaj M. Safety assessment during initiation and maintenance of propranolol therapy for infantile hemangiomas. *Adv Clin Exp Med*. 2019;28(3):375–384. doi:10.17219/acem/94136

DOI
10.17219/acem/94136

Infantile hemangiomas (IH) are the most common benign tumors of infancy, affecting about 10–12% of infants, including 20–30% of babies born prematurely.[1] Their natural development includes a phase of intensive growth (proliferation) lasting up to 18 months of age and a phase of slow disappearance (involution). In 40% of children, the IH does not leave traces or minimal residues.[2] However, in most cases, lesions involute leaving telangiectasia, scars, excess skin or so-called fibrofatty residua. Extensive or ulcerated IHs with localizations impairing vital functions (such as periocular IH), causing a significant cosmetic defect or directly threatening life with no access for surgical excision are an important clinical challenge, classified as inoperable. Attempted chemotherapeutic approaches have included, among other things, steroids or interferon α, with unsatisfactory results. In 2008, a revolutionary successful pharmacological therapy was discovered: propranolol.[3] Thanks to its rapid and spectacular results with minor side effects, propranolol is currently considered the first-line drug in the treatment of IH.

Research is still being carried out to understand the exact mechanism of action of propranolol on IH, to determine effective doses and the optimal algorithm of the therapy. Recently, the safe introduction of the drug in outpatient settings is being discussed.[4,5]

The aim of the study was to evaluate the safety of initiating and maintaining propranolol for IH in children based on cardiology and biochemical studies and the occurrence of adverse effects.

Material and methods

The analysis included 55 consecutive cases of infants with IH treated between 2011 and 2014. The criteria for inclusion were lesions that were inoperable, extensive, ulcerative, that impaired important vital functions, caused a significant cosmetic defect, or affected internal organs.

The Bioethics Committee at the Pomeranian Medical University (Szczecin, Poland) approved the study protocol and consent forms. Written informed consent was obtained from the legal guardians of all of the study participants and recorded in the patient files.

Methods

After taking patient histories regarding the coexistence of risk factors for IH, the following clinical interventions were applied before treatment, during treatment and at the follow-up 1.5 months after treatment:

1. Physical examinations including assessments of the location, size, color, and consistency of the lesion.

2. Cardiac examinations including electrocardiography (ECG) and echocardiography with Doppler (ECHO), carried out by the same pediatric cardiologist, along with blood pressure (BP) and heart rate (HR) measurements.

3. Basic laboratory tests: blood count, ionogram, blood glucose concentration, aspartate transaminase (AST), and alanine transaminase (ALT).

4. The protocol of the initiation of propranolol, divided into 2 doses (at 8:00 am and 8:00 pm), was 0.5 mg/kg/day on day 1, 1 mg/kg/day on day 2, 1.5 mg/kg/day on day 3, and 2.0 mg/kg/day on day 4.

The treatment was carried out in the hospital for 4–5 days with HR, BP, saturation, and 4-fold blood glucose monitored over 24 h; the treatment was then continued on an outpatient basis. The parents were taught to measure the pulse and glucose at home.

The therapy was continued until the child was at least 1-year old, when complete involution was observed or there was no further improvement. The drug was discontinued gradually, reducing the dosage over the next 1–3 months.

Statistical analysis

All continuous variables were checked for the normality of distribution using the Kolmogorov-Smirnov test. These variables were described as averages, medians, standard deviations, quartiles, and minimum and maximum values. The statistical differences between the 2 groups were tested with Student's t-test and the Mann-Whitney test. For multiple groups, multiple analysis of variation (MANOVA), analysis of covariance (ANCOVA) or the Kruskal-Wallis test were used. Discontinuous variables were described by the number and frequency of occurrence. Pearson's χ^2 test was used for discontinuous variables. The statistical analysis was carried out using Stata v. 11 statistical software (StataCorp LLC, College Station, USA).

Results

Four children were excluded during the qualification or during the initiation of the therapy, for the following reasons: increased liver parameters (AST and ALT), worsening dyspnea symptoms due to laxity of the larynx, and arrhythmia in the form of bradycardia and paroxysmal tachycardia in 2 children. Extended cardiac diagnostics in these children did not show any abnormalities, but the parents did not agree to resume the treatment. A total of 51 patients were subject to the final analysis (Table 1). The corrected age at the time of initiating treatment in prematurely born children was after newborn age. Bronchopulmonary dysplasia observed in prematurely born children was not a contraindication for the inclusion in the study, as the patients with this conditions did not require pharmacological treatment.

The total number of IHs present in 51 patients was 79. Infantile hemangiomas complicated with ulceration were found in 12 children (15%).

All the children showed clinical improvement after the treatment. The detailed results were the subject of a previous publication.[6] The best therapeutic effect was obtained

in the group of the youngest children, who started the treatment up to the 6th month of life. The final therapeutic effect in the form of complete atrophy of the IH was noted in 69% of the children in this group. In the 6–12-month-old children, complete involution was observed in 56%, while among patients who started treatment after 1 year of age, complete involution was seen in 25%.

Cardiac examination

The ECG examinations performed before, during and after the treatment were within the normal range.

The ECHO scans performed before the treatment was induced showed patent foramen ovales (PFOs) in 24 children and an atrial septal defect (ASD II) in 3 cases. In 1 child, the defect in the ASD coexisted with a perimembranous ventricular septal defect (VSD). The defects found in the ECHO scans were not contraindications for treatment with propranolol. In the control examinations after the full therapeutic dose was reached, a PFO was observed in 1 child, tricuspid regurgitation of the 1st degree without hemodynamic significance in 1 case and accelerated flow in the pulmonary artery in 1 case. In the next examination performed during treatment (after 1 month), ASDs II were present in 2 children, VSDs in 1 and PFOs in 19 children. At the follow-up 1.5 months after treatment was ceased, there were ASDs II in 2 children, a VSD in 1 and PFOs in 5 children.

The HR was between 117 bpm and 160 bpm in all patients, while after switching to the full therapeutic dose, it dropped by 18 bpm (98–136 bpm on average). The decrease in the HR after the full dose was reached was statistically significant in relation to the parameters before the treatment (p = 0.0001) (Fig. 1). Further HR values in the ambulatory controls remained lower than the initial pretreatment parameters. However, in the maintenance period after 2 weeks of treatment, mean HR values increased in relation to the HR just after the full therapeutic dose was reached (p = 0.0039). Changes in mean HR values after discontinuation of the drug were not statistically significant, but showed a slight increase. During all the outpatient measurements,

Table 1. Characteristics of the study group

Characteristics	Value
Male, n [%]	15 (29.4)
Female, n [%]	36 (70.6)
Birth weight, range <2,500 g, n [%] 2,500–3,000 g, n [%] 3,001–4,000 g, n [%] >4,000 g, n [%]	1,700–4,460 8 (15.5) 7 (13.5) 32 (63) 4 (8)
Gestational age <37 w, n [%], range [w] > = 37 w, n [%], range [w]	 7 (14), 32–36 44 (86), 37–41
Age at initiation of propranolol, average (range) 7 w–6 m, n [%] 6–12 m, n [%] 18.5–21.8 m, n [%]	5.8 m (7 w–21.8 m) 39 (75) 9 (17) 3 (8)
Weight at initiation of propranolol, average (range) <5,000 g, n [%] 5,000–6,000 g 6,001–7,000 g 7,001–13,500 g	7,000 (3,740–13,500) 6 (12) 16 (31) 15 (29.5) 14 (27.5)

w – weeks; m – months.

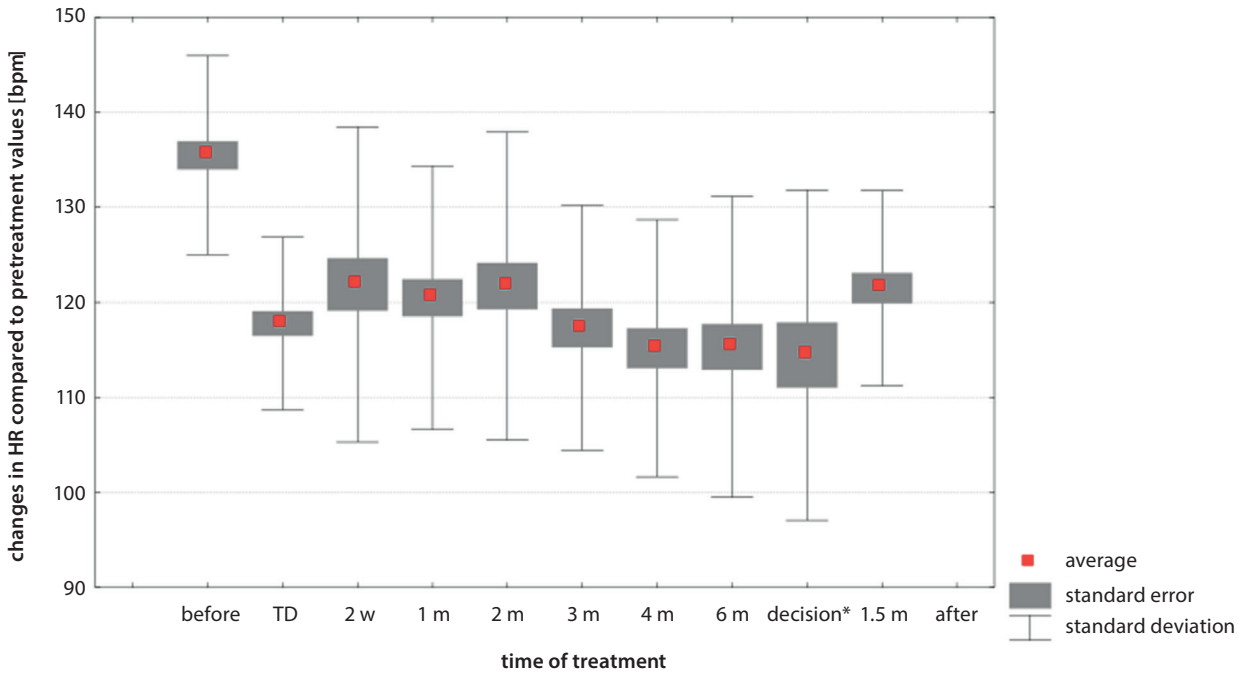

Fig. 1. Changes in the heart rate after induction of the full therapeutic dose and during subsequent outpatient monitoring

TD – therapeutic dose; HR – heart rate; *decision to gradually withdraw propranolol; w – weeks; m – months.

the mean HR parameters were normal in relation to age (114–122 bpm), with a minimum value of 80 bpm. One patient presented an episode of bradycardia during an ambulatory examination by a family doctor; this was not confirmed during a later cardiac check-up. Heart rate reference values were based on the recommendations of a group of European experts (Table 2).[5]

No hypotension was observed in any of the children before treatment (BP > 5th percentile). Reference values were interpolated for age from 1 month to 1 year as follows: systolic blood pressure (SBP): P5 = 70 mm Hg, P90 = 105 mm Hg; diastolic blood pressure (DBP): P5 = 36 mm Hg, P90 = 66 mm Hg.[7]

Mean SBP was 103 mm Hg (80–124 mm Hg), whereas after treatment with the full therapeutic dose it dropped by an average of 10 mm Hg to a mean SBP of 93 mm Hg (74–119 mm Hg). The mean SBP was significantly reduced relative to pretreatment parameters only after the full therapeutic dose was reached (p = 0.00001) and in the first 3 months of treatment (p < 0.039) (Fig. 2). The average SBP during all outpatient monitoring was normal (92–102 mm Hg), reaching lower values in the first 3 months of treatment. After 4 months of treatment, a significant increase in the average SBP (by 7 mm Hg) was observed (p = 0.0106). After the drug was discontinued, an increase in the mean SBP was noted, but it was not statistically significant in comparison to the results of the preceding check-up.

The parameters of the mean SBP were within normal limits, both before and after treatment. Values of SBP below the standard in individual measurements were sporadic (7 SBP measurements (2%) were below the 5th percentile) and were asymptomatic.

Table 2. Reference values for heart rate (HR) and blood pressure (BP)[5,7]

Age [months]	HR [bpm]	BP (systolic/diastolic) [mm Hg]
0–3	100	65/45
3–6	90	70/50
6–12	80	80/55
12–24	70	80/55

Mean DBP was 61 mm Hg (39–84 mm Hg), while after reaching the full therapeutic dose of the treatment, it decreased by an average of 8.7 mm Hg to a mean DBP of 52.4 mm Hg (40–72 mm Hg). The average DBP values were significantly reduced compared to pretreatment parameters only after the full therapeutic dose was reached (p = 0.00001) and during the first 3 months of treatment (p = 0.0468) (Fig. 3). In the following months of treatment, a statistically significant increase in the average DBP (7.0–9.8 mm Hg) was observed in relation to the DBP after the therapeutic dose was reached (p < 0.048). After the drug was discontinued, the changes observed in DBP were not significant compared to the results of the pre-withdrawal check-up.

The parameters of mean DBP were normal both before treatment, during outpatient monitoring and after completion of the therapy. Lower DBP values were observed sporadically (3 DBP measurements (0.9%) were below the 5th percentile) and were asymptomatic.

A detailed analysis of the results of HR and BP measurements during the gradual increase to the full therapeutic dose over 3–4 days during the patient's hospital stay is presented in Table 3. On average, each patient underwent

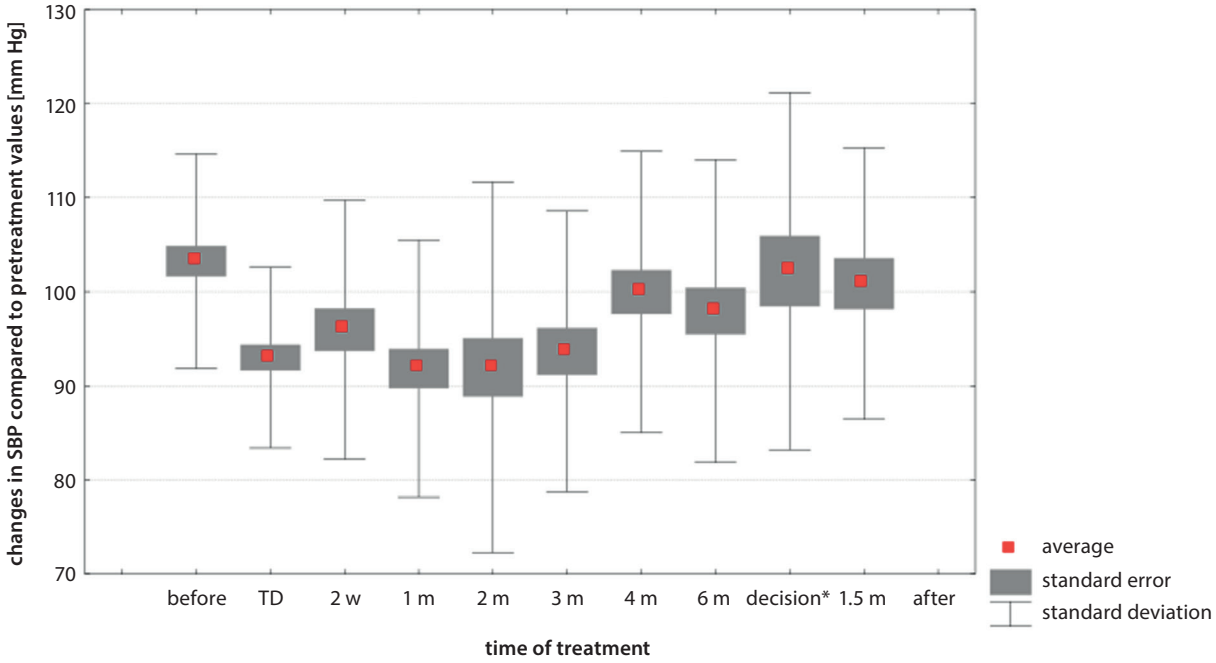

Fig. 2. Changes in systolic blood pressure after induction of the full therapeutic dose and during subsequent outpatient monitoring

TD – therapeutic dose; SBP – systolic blood pressure; *decision to gradually withdraw propranolol; w – weeks; m – months.

5 measurements each day (1 h, 2 h, 4 h, 6 h, 8 h, and 10 h after the dose). In 17 children, the full therapeutic dose was reached on the 3rd day of hospitalization.

The average HR values in all the children were within reference values. It was only on the 1st day that the mean HR values were significantly different from those on other days ($p < 0.02$). The mean HR values were significantly different in the youngest children (1–3 months of age ($p < 0.03$) and 3–6 months of age ($p < 0.009$)) (Fig. 4). The average minimum values of HR in the following days of treatment were also normal (112–105 bpm), with the lowest HR being a single measurement of 78 bpm. There were 6 episodes below the norm; bradycardia was therefore sporadic, as well as asymptomatic.

Minimal HR depended on the day of treatment; it was significantly lower on day 1 in relation to other days ($p < 0.0063$). It did not depend on the age of the child.

The values of SBP and DBP before treatment (including mean SBP and DBP as well as minimal and maximal values) did not change during treatment days. There were no significant changes in any of the age groups analyzed. During the introduction of propranolol, no average SBP was below the 5th percentile, whereas there was 1 average DBP below the 5th percentile.

Laboratory tests

Slightly elevated AST values (max 77U/L with the laboratory reference <32 U/L) were present in 39 children; this was not a contraindication to proceeding with the therapy. During treatment, these values were reduced in 30 children, while they remained elevated in 9. After the end of the treatment, slightly elevated AST values (max 59 U/L) were present in 32 patients. There was a mild elevation in ALT (by 3–14 U/L) in 10 children, but the results were still within the reference values (<39 U/L).

The mean serum glucose concentration was within the normal range before the treatment. Mean glycemia during the 4 days of the drug initiation phase was normal (97.3–98.8 mg/dL). In the study group, only 2 single

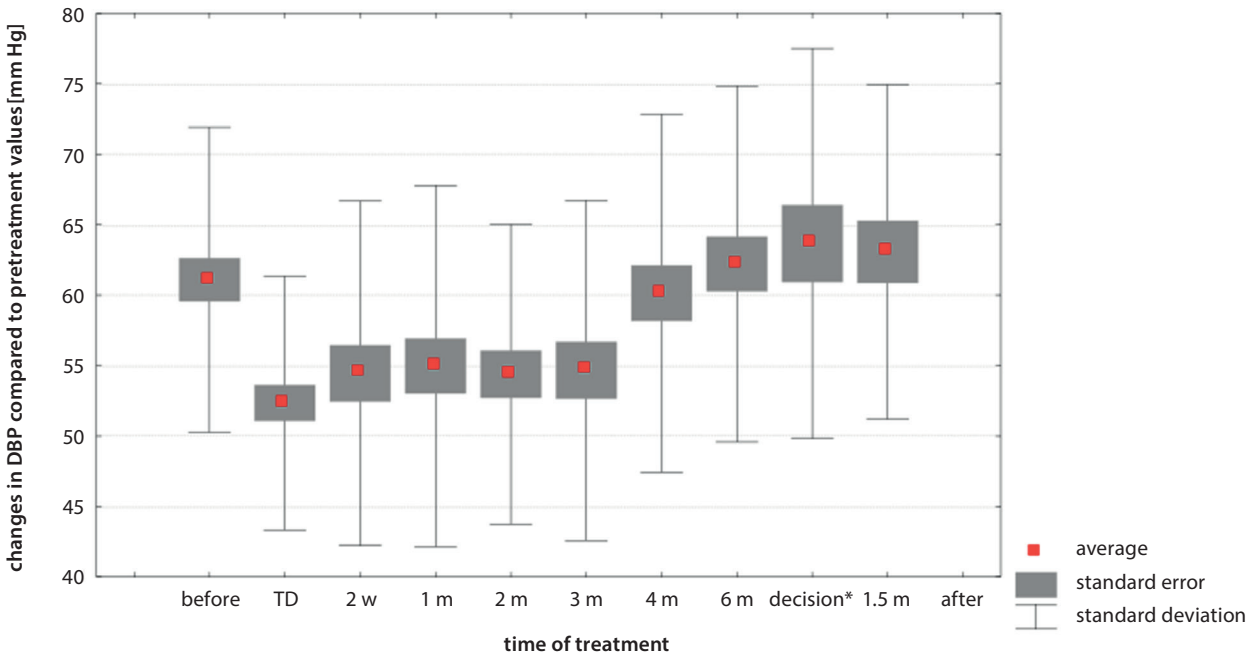

Fig. 3. Changes in diastolic blood pressure after induction of the full therapeutic dose and during subsequent outpatient monitoring

TD – therapeutic dose; DBP – diastolic blood pressure; *decision to gradually withdraw propranolol; w – weeks; m – months.

Table 3. Analysis of the induction of propranolol therapy

Day of treatment	Average dose [mg/kg/day]	Decrease in HR [bpm]	Average HR (range) [bpm]	Episodes of bradycardia (n)	Decrease in SBP	Average SBP (range) [mm Hg]	Decrease in DBP	Average DBP (range) [mm Hg]
1	0.56 ±0.24	7.2	128.4 (93.5–150.3)	1	4.4	97.9 (75.3–131.7)	8.2	53.4 (36.0–76.0)
2	1.13 ±0.24	11	124.6 (104.8–142.8)	0	4.9	97.4 (74.3–124.8)	8.7	52.9 (33.0–77.3)
3	1.64 ±0.22	14	121.6 (100.5–136.4)	4	5	97.3 (77.0–116.3)	7.9	53.7 (37.0–72.0)
4	1.93 ±0.14	15.6	120.1 (100.2–135.4)	1	5.4	96.1 (76.8–115.0)	13.4	50.3 (36.5–68.8)

HR – heart rate; SBP – systolic blood pressure; DBP – diastolic blood pressure.

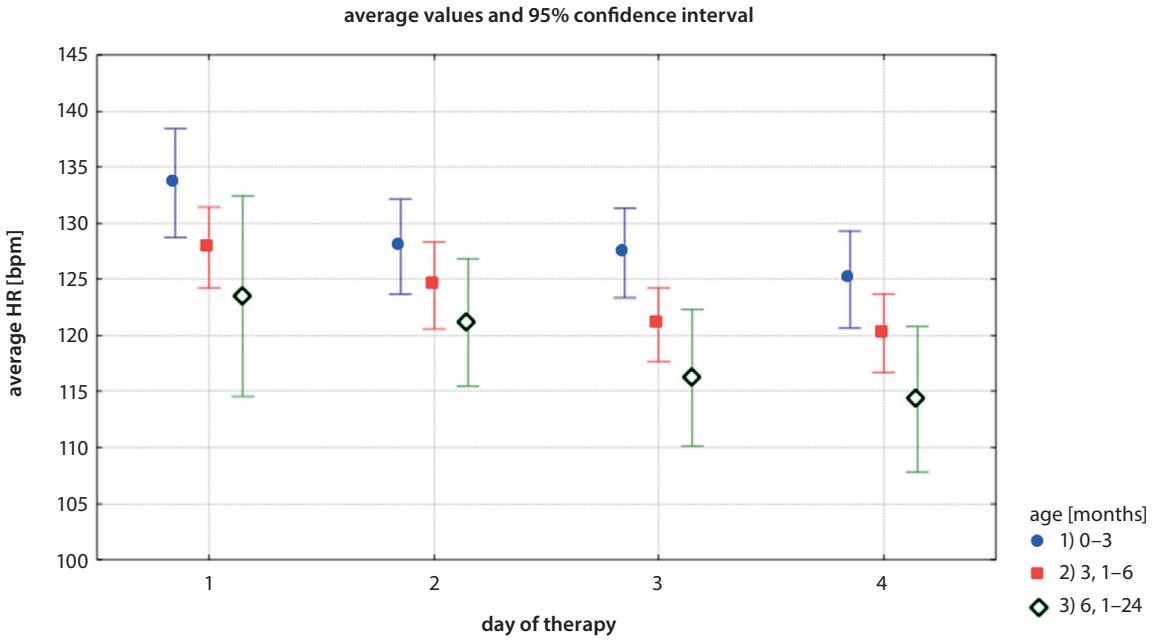

Fig. 4. Average values of heart rate (HR) during the induction of propranolol therapy, with divisions into age groups

measurements had below-standard glycemia <60 mg/dL: 56 mg/dL in a child younger than 3 months on the 2nd day and 58 mg/dL in a child 3–6-month old on the 4th day. No outbreaks of hypoglycemia were observed during outpatient monitoring. In 3 patients, episodes of asymptomatic hypoglycemia (41–50 mg/dL) were observed during parental home measurements. One child had symptomatic hypoglycemia of 44 mg/dL, manifested by sweating, drowsiness, apathy, and vomiting and requiring hospitalization in the 2nd week of treatment. These symptoms resolved after a dose reduction.

No ion abnormalities were detected in any of the children.

Evaluation of the treatment proceedings

Drug dosage

The target therapeutic dose was 2 mg/kg/day (1.3–2.2 mg/kg/day) divided into 2 daily doses. In 6 children, the therapy started with a lower dose (1.3–1.6 mg/kg/day) due to younger age, low body weight and low serum glucose levels, as well as lower BP and HR values. Moreover, extensive skin changes associated with atopic dermatitis and the risk of developing asthmatic symptoms were also reasons for lowering the initial dose. In 5 of these children, the dose was increased to the target dose after 1 month without side effects. In addition, 6 other children required a therapeutic dose reduction to 0.9–1.6 mg/kg/day due to adverse reactions (frequent spitting and vomiting, sleep disturbances, frequent respiratory infections, symptomatic hypoglycemia, reflux, and suspected bronchial asthma). In 4 of these children, the dose reduction resulted in a slightly worse final effect.

Treatment period

The full therapeutic dose period was on average 8.7 months; in the cases of periocular lesions, it was 11.4 months. The total length of treatment, including the gradual withdrawal of the drug (1.5–3 months), was 12 months on average.

Adverse effects

Mild adverse reactions occurred in 17 (33%) children. In this group, single episodes of asymptomatic hypoglycemia (41–50 mg%) were observed in 3 patients, symptomatic hypoglycemia (44 mg%) in 1 patient and sleep disorders in 2 children. Frequent respiratory infections were noted in 3 cases, bradycardia in 1 child, worse body weight gain in 4 children and excessive drowsiness in 1 child. Constipation was observed in 1 patient. In 2 children, it was necessary to perform additional diagnostics for asthma; in 1 of these children, the diagnosis was extended to gastroesophageal reflux.

Discussion

The development of IH is a long process. It is considered that about 30% of untreated lesions involute within a child's first 3 years of life and 50% within 5 years.[8] Accordingly, in about 2 years of observation, IH should disappear in 20% of patients.[9] In the study group, a 3-fold higher rate of hemangioma atrophy was achieved within 8.7 months of treatment with propranolol. This indicates that propranolol contributes significantly to the involution of IH.

It is worth emphasizing that the early introduction of propranolol treatment slows down the phase of intensive IH growth, which reduces the risk of cosmetic defects and the need for complementary treatment such as excision or laser therapy.[4] This is supported by the fact that the best results in our study were observed among the patients that were the youngest at the induction of propranolol. This justifies the importance of research on the safety of propranolol, especially for the youngest patients, who are potentially the main beneficiaries of the therapy.

Hengst et al. were the first to present the results of BP, HR and glucose monitoring in 109 children at the average age of 2.8 months during the induction of propranolol in hospital setting.[7] In 3–4 days, the dose was gradually increased from 0.5 mg/kg/day to 2 mg/kg/day. They continued the assessment up to 6 months. However, in contrast to our study, they excluded prematurely born children and those with confirmed heart defects from the study. They observed no hypotension after 3 months of treatment. No clinical signs of hypotension, bradycardia or other adverse events were present. As in our study, HR and laboratory parameters were normal. The authors concluded that in healthy infants, 2 mg/kg/day of propranolol divided into 3 doses is well tolerated and that monitoring of BP during long-term treatment is not necessary.[7]

Puttgen et al. also assessed HR, BP and glycemia during the initiation of propranolol treatment.[10] In a study of over 50 patients at a median age of 3.4 months, the treatment was started at a dose of 1 mg/kg/day; on day 2 it was increased to 2 mg/kg/day. All the patients had at least 1 low SBP or DBP, 2/3 (76%) had at least 1 measurement of both low SBP and low DBP, and 14% of the children had an episode of bradycardia. Despite the high incidence of these deviations, they were asymptomatic and did not affect the therapeutic dose. They also showed that children over the age of 6 months presented bradycardia more often than younger infants ($p < 0.001$). Mean SBP, DBP and HR were significantly reduced between day 1 and day 2 ($p = 0.004$) but did not change from day 2 to day 3.[10] In our study, only a decrease in HR was observed between the 1st day of therapy and the remaining days.

In 31 children at the average age of 4.6 months, Liu et al. observed a decrease in SBP during the induction phase by 4 mm Hg, without statistically significant changes in DBP or glycemia.[11] The drug was introduced immediately in the full therapeutic dose of 2 mg/kg/day divided into 3 doses, without a gradual increase. The study suggests that a 24-hour hospitalization with monitoring may not be necessary for otherwise healthy infants.[11]

Hypotension is most frequently observed in the first 2 h after administration of the drug.[12] A low incidence of asymptomatic systolic hypotension during the induction phase was observed by Hermans et al.[13] However, the study group was heterogeneous, with a high proportion of premature babies (22.4%); the dose ranged from 1 mg/kg/day to 3 mg/kg/day; and some children were administered steroids.[13] However, in a study by Filippi et al., 19.2% of premature babies treated with propranolol at doses of 1–2 mg/kg/day developed symptomatic hypotonia, bradycardia or both.[14] Marqueling et al. published a large meta-analysis of 41 reports on 1,264 children (mean age 6.6 months, dose 2.1 mg/kg/day, therapy time 6.4 months), recording BP decreases in 39 children (3%), in 5 of whom (0.4%) it was symptomatic.[15] In the study group, hypotension was asymptomatic and occurred sporadically during both the initiation and maintenance periods. However, on the 2nd day of the therapy, bradycardia and hypotension were the reasons for discontinuing the drug in 1 child. Another patient was disqualified due to tachycardia.

In our study, a significant reduction in HR was observed during the induction of therapy in comparison to the values before the treatment. The average HR values during the gradual increase in the dosage were all within the normal range. The average HR value on the 1st day were significantly different from the values on the following days; this was the only statistically significant difference in HR values. The reported cases of bradycardia were sporadic and asymptomatic, as in reports by other authors.[10,11] The average SBP and DBP values did not change over time and did not significantly differ during the subsequent days of therapy. There was no decrease in SBP below the 5th percentile, and in the case of mean DBP, such a decrease was recorded only once. These results demonstrate a high level of safety in the introduction of propranolol.

Bradycardia is usually observed in the first 1–3 h of drug administration.[4] Therefore, observation for such a period of time may be sufficient in outpatient conditions. In the study group, asymptomatic bradycardia was observed in only 1 child in the 1st month of treatment, immediately after administration of the drug. Bradycardia was not registered either in the next Holter examination or during outpatient supervision.

Blood pressure was not monitored outside the induction phase in any of the abovementioned studies except Hengst et al., whose results suggest that BP screening should be performed only in the initial phase of treatment.[7] European and American expert groups recommend monitoring BP in healthy infants only when starting treatment with propranolol and increasing its dose by >0.5 mg/kg/day.[4,5,7]

Our observations show that continuing treatment at a dose of 2 mg/kg/day is safe. The reduction in HR observed after induction was compensated after 2 weeks, as a result of possible drug tolerance rather than as a result of a relative reduction in the dose as children gained weight. Therefore, it would be reasonable to introduce the drug in ambulatory conditions. Moreover, a few days long break between dosage increases can ensure the safety of the process. The statistically significant decrease in mean SBP and DBP values observed in the first 3 months of our study still remained within the normal range. The hypotensive episodes observed were rare and asymptomatic. This indicates a high degree of safety of propranolol therapy.

In the past, ECGs, ECHO scans and consultations with pediatric cardiologists before, during and after propranolol treatment were obligatory.[16,17] Some authors, however, question the need for ECHO before starting treatment.[4,18] Liu et al. recommend performing this test in cases of a heart murmur or a positive interview.[11] Blei et al. analyzed the results of 239 children under 1 year evaluated by a cardiologist before treatment with propranolol.[19] In 21% of the children, ECHO heart abnormalities were found, but as in our study, the ECHO findings were not a contraindication for propranolol therapy. Cardiac contraindications for propranolol are rare (congestive heart failure with myocarditis, second- and third-degree heart block with bradycardia, and hypotension), but cardiac anomalies are more common in the IH population than in the healthy population.[19] At present, the recommended approach is to perform ECG and cardiac consultations in cases of a positive medical history or arrhythmia.[5] Routine ECHO scans are not necessary, except for specific clinical indications, since all other circulatory disorders that are contraindications to propranolol treatment can be assessed during the physical examination and history taking.[5] Recent pharmacokinetic studies showed that propranolol reaches its balance in newborns after 48 h.[14] A retrospective analysis of 24-hour Holter exams conducted 48 h after the 1st dose of propranolol did not reveal any persistent arrhythmias.[20]

Pretreatment laboratory tests allow us to detect possible value disturbances before therapy. Numerous studies have confirmed the low usability of these tests in monitoring treatment due to the low frequency of disturbances, for example hyperkalemia.[21] There are also no indications for routine serum glucose screening during the course of propranolol therapy due to the varied and unpredictable time of occurrence of hypoglycemic episodes. The indications for blood glucose is prematurity or hypertrophy, with growth disorders and hypoglycemia.[5]

The first recommendations of propranolol treatment for IH were published in 2013.[4] Hospital treatment was indicated for infants under 8 weeks of corrected age and in the absence of adequate social support, the occurrence of a concomitant pathology of the heart and/or lungs, and glycemic disorders. According to the 2015 recommendations of European experts, the introduction of the drug takes place in a hospital setting.[5] This strictly applies to children under 8 weeks of age, children with a body weight <3.5 kg, premature babies (because the risk of bradycardia and low BP in premature babies is high), children with life-threatening hemangiomas, PHACE syndrome and coexisting cardiovascular and respiratory diseases, impaired glycemic levels, and those with insufficient care in the home environment.[4,14] Older infants can be hospitalized for part of a 1-day stay. Treatment of older children or a resumption of propranolol treatment can be started on an outpatient basis, with a gradual dosage increase once a week, starting with a dose of 1 mg/kg/day, and ending with a dose of 2 (or 3) mg/kg/day, with BP and HR monitoring before each dose increase and 1 h or 2 h after administration.

The aforementioned group of European experts stated that the therapeutic dose should be in the range of 2 mg/kg/day to 3 mg/kg/day, administered in 2 or 3 doses.[5] If propranolol is administered in 2 doses, the average serum concentrations of the drug are about 12% higher than with 3 doses per day.[4] In our study group, the treatment was started from 0.5 mg/kg/day and gradually increased to 2 mg/kg/day divided into 2 doses, with high clinical effectiveness.

Adverse effects during propranolol therapy are extremely rare. Transient hypoglycemia is observed in 0.9–11.4% of patients.[4] Inhibition of glycogenolysis, gluconeogenesis and lipolysis by propranolol is considered the most likely cause. Preterm children are particularly predisposed. In our study, asymptomatic hypoglycemia (41–50 mg/dL) in the monitoring performed by parents at home occurred in 1 preterm child and 2 other children. Early initiation of treatment in 1 patient (at 7 weeks of age) and co-infection in the others were regarded as the probable causes. Hypoglycemia has been reported in various periods of propranolol treatment, regardless of age. The condition is probably caused by overly long interruptions in feeding or by co-infection.[4] Therefore, parent education in the diagnosis of hypoglycemic symptoms, the importance of frequent feeding, avoiding long breaks between meals, administration of the drug with a meal, and special care in cases of overlapping infections, local infections, vomiting, and/or diarrhea seems to be sufficient, and glucose testing in the treated children seems unnecessary. According to Drolet et al., special care should be taken when introducing treatment before the 8th week of age.[4] A typical symptom of hypoglycemia is tachycardia, which can be masked by propranolol. Therefore, attention should be paid to other symptoms, such as sweating, drowsiness, loss of appetite, seizures, apneas, fainting, and hypothermia, which may also indicate low blood sugar levels. Fusilli et al. recorded the lowest value of glycemia (15 mg/dL) in a 6-month-old boy during therapy, and convulsions were the first symptom observed.[22] Only a few cases of symptomatic hypoglycemia have been described.[22–25] In our study group, symptomatic transient hypoglycemia was observed in 1 patient in the 2nd week of treatment, accompanied by excessive sleepiness, pallor and loss of appetite, which resolved after reducing the daily dose of the drug during hospitalization. It was a unique case report. Perhaps the reason for the hypoglycemia in that child was a coexisting local infection of an ulcerated hemangioma. Accidentally detected episodes of hypoglycemia observed at home were sporadic and were seen in the youngest participants, those prematurely born or with comorbid infections. Our results suggest that performing frequent glycemic measurements during the introduction of propranolol may be unnecessary, which concurs with other authors.[4,5]

Drolet et al. emphasized the possibility of sporadic occurrences of other transient adverse effects, such as bradycardia (0.9–8.7%), respiratory tract infections (1.4–8%), sleep disturbances (3.7–13.5%), low blood pressure (3.1–21%), and gastroesophageal reflux (0.7–6%). Asthma and allergic conditions are contraindications to the use of propranolol, which, as a non-selective inhibitor of β-adrenergic receptors, can cause bronchospasm. The appearance of wheezing or shortness of breath without the symptoms of infection is an indication for discontinuation of propranolol therapy.[4]

Symptoms of the central nervous system in the form of sleep disorders or excessive irritability probably result from the easy penetration of the drug into the nervous system.[4] Propranolol is very lipophilic, which in theory may be associated with adverse effects on neurological or cognitive development.[5,26] The sleep disorders and anxiety observed in 2 of our patients resolved after treatment.

The necessity of discontinuing propranolol treatment due to complications is extremely rare. Only single cases of discontinuing therapy due to life-threatening symptoms are described in the literature. The most frequently mentioned are persistent bradycardia, bronchospasm in the course of respiratory tract infections, asthma, and sudden drops in blood pressure.[27–30]

The fairly high percentage of mild and transient adverse reactions observed in our study (18%) is consistent with the observations of other authors reporting on large groups of patients.[31,32] Adverse events in long-term ambulatory monitoring should be reported.

Propranolol is an effective and safe drug, which leads to involution of IH in a short time. The numerous benefits and safety of propranolol have been demonstrated in studies involving a large number of patients[13,15,33] and 2 randomized trials.[34,35] Our results indicate a strong basis for shifting propranolol therapy to outpatient settings.

Conclusions

Propranolol is effective, safe and well-tolerated by children with infantile hemangiomas. The positive results of the safety assessment in hospital conditions support the strategy of initiating and maintaining propranolol therapy in the outpatient setting in children with IH. Future studies are needed to assess the benefits of introducing the therapy in the outpatient setting.

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Expression of the *PIM2* gene is associated with more aggressive clinical course in patients with chronic lymphocytic leukemia

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2019;28(3):385–390

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Funding sources
The study was financially supported by the statutory funds of Wroclaw Medical University (ST.A080.17.035).

Conflict of interest
None declared

Received on April 11, 2017
Reviewed on April 29, 2017
Accepted on May 5, 2018

Published online on December 7, 2018

Abstract

Background. The *PIM2* gene belongs to the PIM family, which encodes serine/threonine kinases involved in cell survival and apoptosis. The relation between the expression of the *PIM2* gene and the course of chronic lymphocytic leukemia (CLL) has not been fully determined.

Objectives. The aim of the study was to evaluate the role of the *PIM2* gene as a marker of CLL malignancy and its importance as a predictive and prognostic factor.

Material and methods. Sixty-seven patients, 35 females and 32 males, aged 49–90 years, with de novo CLL, and 14 healthy individuals were enrolled in the study. Expression of the *PIM2* gene was analyzed using TaqMan RQ-PCR assay and western blot test.

Results. Median *PIM2* gene expression in CLL patients was higher than in controls. Patients with high expression of the *PIM2* gene had shorter progression-free survival and time to first treatment than patients with low *PIM2* expression. It was found that patients with CR had lower expression of the *PIM2* gene than patients without complete remission (CR). Notably, associations between high PIM2 expression and rapid lymphocyte doubling time, the percentage of malignant lymphocytes with ZAP70 expression and the Rai stage were revealed.

Conclusions. We found that the *PIM2* gene is associated with a more aggressive clinical course of CLL.

Key words: *PIM2* gene, chronic lymphocytic leukemia, prognostic factor

Cite as
Kapelko-Słowik K, Dybko J, Grzymajło K, et al. Expression of the *PIM2* gene is associated with more aggressive clinical course in patients with chronic lymphocytic leukemia. *Adv Clin Exp Med*. 2019;28(3):385–390. doi:10.17219/acem/90771

DOI
10.17219/acem/90771

Introduction

Chronic lymphocytic leukemia (CLL) is a disease strongly connected with an accumulation of neoplastic cells. Suppression of apoptosis in vivo of leukemic lymphocytes is the most characteristic feature inducing their accumulation in CLL patients. The extensive research regarding intercellular paths leading to cell life prolongation and resistance to pro-apoptotic stimuli is still in progress. In recent years, this research has led to the discovery of an anti-apoptotic kinase PIM2. PIM2 kinase was identified as taking part in murine lymphoid cell transformation.[1] PIM2 along with PIM1 and PIM3 belong to the serine/threonine kinase family encoded by corresponding oncogenes.[2,3] *PIM2* gene expression is regulated at both the mRNA and protein level by cytokines (e.g., interleukin 3 – IL-3) involved in the differentiation of hematopoietic cells. This expression has been detected in spleen thymus and in proliferating hematopoietic cells. Constitutive expression of PIM2 extends cellular lifespan and makes the cells insensitive to proapoptotic stimuli or deficiency of exogenous growth factors.[4] Elevated expression of the *PIM2* gene was revealed in cell lines derived from human solid (SW480, A549, G361) and hematological tumors (HL60, K562, RAIJ), acute myeloid leukemia, as well as lymphoid malignant cells.[5,6] This indicates a possibility of this factor playing a significant role in neoplastic growth pathogenesis.[7–9] In previous studies, increased expression of the *PIM2* gene in CLL and follicular and large-cell lymphoma was found. *PIM2* mRNA expression was significantly higher in CLL lymphocytes than in normal B-lymphocytes and was correlated with advanced stage as well as shorter lymphocyte doubling time (LDT).[10] Therefore, the aim of this study was to further evaluate *PIM2* expression in ex vivo CLL cells and to evaluate its possible predictive and prognostic value in this disease as assessed by the response to chemotherapy and survival analysis.

Material and methods

Patients

Sixty-seven patients with newly diagnosed CLL from the Department of Hematology, Wroclaw Medical University, Poland, were enrolled in the study. Among them were 35 females and 32 males, aged 49–90 years (median age was 66 years). Peripheral blood samples were collected from all patients at the time of diagnosis, whereas only samples with monoclonal lymphoid cells representing more than 80% of total cellularity were assessed. The follow-up period of the patients was 11–71 months. During that period the indications for cytostatic treatment, according to the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) guidelines, appeared in all patients.[11] Twenty-two patients under 60 years of age with an estimated glomerular filtration rate >70 mL/min and without clinically significant comorbidities received 4–6 cycles of FC-R (fludarabine, cyclophosphamide, rituximab), 33 patients received 6–9 cycles of LP (chlorambucil, prednisone) and 12 patients received 4–6 cycles of RB (rituximab, bendamustine). Complete remission (CR) was assessed according to the standard criteria.[12] Thirty-two patients reached CR and 11 patients partial remission (PR). Thirteen patients reached CR after FC-R therapy, and 10 patients after LP or RB. In 16 patients peripheral blood lymphocytes were additionally analyzed in CR following the chemotherapy. The control group consisted of 14 healthy individuals, patients of the Department of Ophthalmology, Wroclaw Medical University, matched by age and sex to the examined group. The clinical data of CLL patients and controls is presented in Table 1.

Methods

Peripheral blood was obtained from the patients at the time of diagnosis and control subjects by venipuncture. Mononuclear cells (MNC) were separated by centrifugation over Gradisol L lymphocyte separation medium (Aqua Medica, Warszawa, Poland). The isolated cells were stored at –80°C as dry pellets.

Real-time PCR

Total RNA was isolated from 6 × 10^6 MNC using TriRaegent® Solution (Life Technologies, Carlsbad, USA) according to the producer's standard. DNA was removed from the isolated RNA samples by DNase using DNA-free TM reagent (Life Technologies). Two micrograms of RNA were reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, USA). Expression of the *PIM2* gene was assessed by TaqMan RQ-PCR assay using predesigned TaqMan Gene Expression Assays Hs00179139_m1 for *PIM2*), on a 7500

Table 1. Clinical data of patients with CLL and the control group

Parameters	Patients with CLL	Control group
n	67	14
Age (median)	66 (49–90)	59 (47–88)
Sex F/M	35/32	7/7
CR/NCR	32/35	NA
Rai stage 0 I II III IV	 0 18 24 9 16	NA
LDT ≥12 months <12 months	 20 47	NA

CLL – chronic lymphocytic leukemia; F – females; M – males; CR – complete remission; NCR – no complete remission; LDT – lymphocyte doubling time; NA – not applicable.

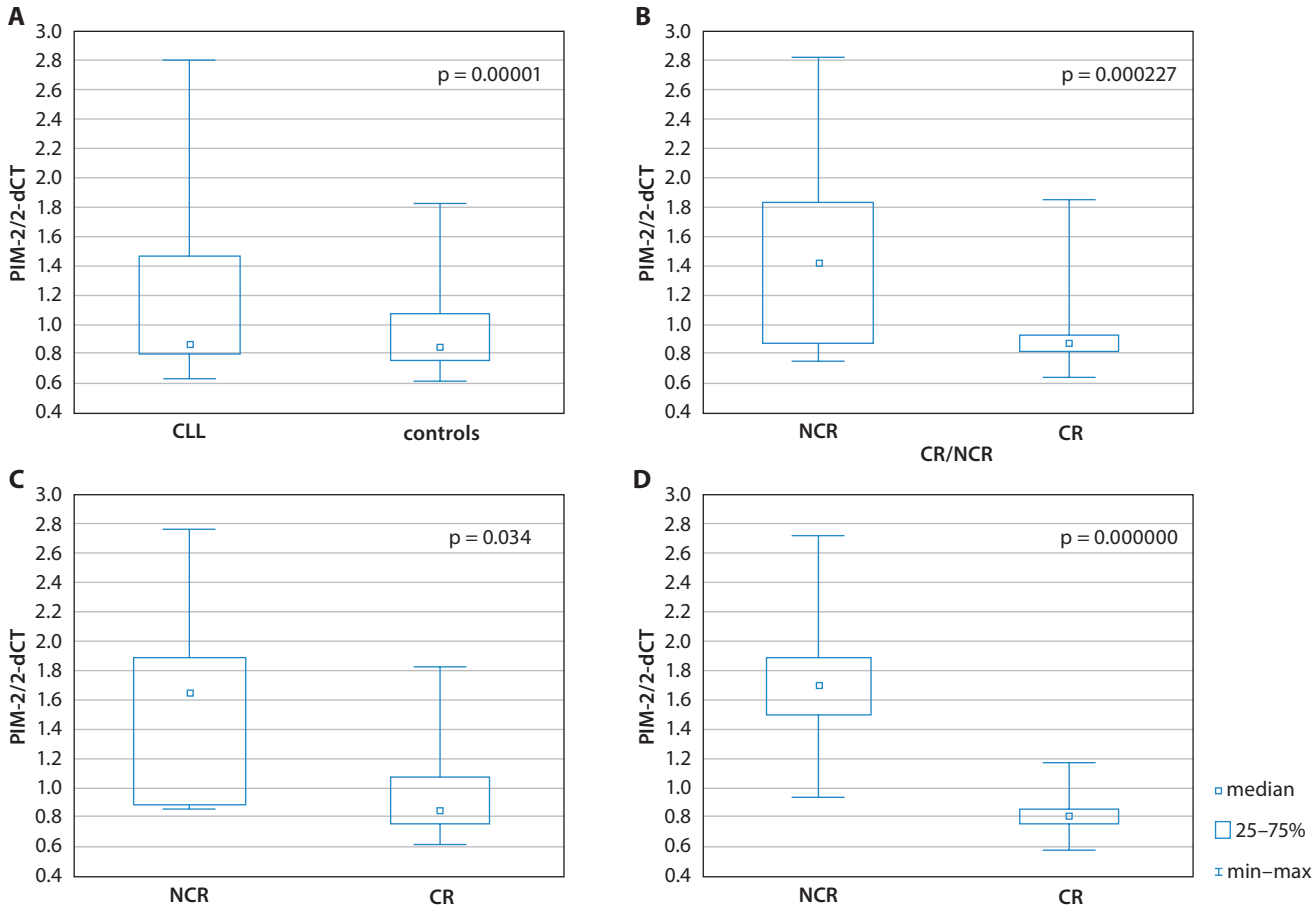

Fig. 1. Comparison of median *PIM2* expression between patients with CLL and healthy controls (A); between patients who achieved CR vs NCR (B); between patients who reached CR vs NCR after FC-R treatment (C) and patients with LDT <12 months (a) and ≥12 months (b) (D)

RQ-PCR System (Applied Biosystems, Foster City, USA). The β-glucuronidase gene (*GUS*) was used as a control gene (TaqMan Gene Expression Assay: Hs99999908_m1). The relative gene expression level was measured as the difference between the C_t values of the *PIM2* gene and the *GUS* gene (ΔC_t) and expressed as $2^{-\Delta Ct}$.

Western blot

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 10,000 × g, 10 min, 4°C. Clear supernatant was collected and the protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma–Aldrich, St. Louis, USA). Cell lysates (40 µg per line) after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel were transferred to nitrocellulose (Bio-Rad, Hercules, USA), blocked for 1 h with 5% fat-free dry milk in tris-buffered saline (TBS) and incubated overnight at 4°C with the appropriate primary antibody. The following primary antibodies were used: rabbit monoclonal antibody against PIM2 (Cell Signaling Technology, Leiden, the Netherlands) and rabbit polyclonal antibody against β-actin (Abcam, Cambridge, UK). Immunodetection was performed using secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). After washing, the membrane was briefly incubated using Lumi-Light^{PLUS} western blot substrate (Roche, Basel, Switzerland) and exposed to Bio-Light film (Eastman Kodak, Rochester, USA). The films were photographed using a Canon Power Shot A640 camera and analyzed with ImageJ 1.45S software (Wayne Rasband, National Institutes of Health, USA) using β-actin as a control. The integrated density was calculated for each line.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann–Whitney U test for independent samples. The correlation between quantitative variables was tested with Spearman's rank correlation test. Progression-free survival (PFS) and time to first treatment (TTFT) analysis was performed using the Wilcoxon test. The result was considered statistically significant when $p \leq 0.05$.

Results

Median *PIM2* gene expression in leukemic cells of CLL patients was higher than in peripheral blood lymphocytes of the control group: 0.88 (0.63–2.81) vs 0.73 (0.63–0.86),

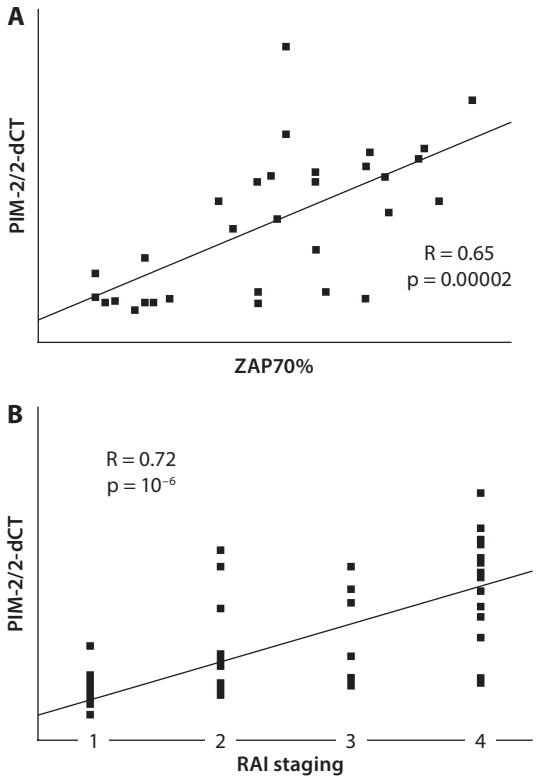

Fig. 2. Positive correlation between *PIM2* gene expression and the percentage of malignant lymphocytes with ZAP70 expression, R = 0.69, p = 0.00002 (A) and between *PIM2* gene expression and Rai stage, R = 0.72, p = 0.00000 (B)

p = 0.000001. Chronic lymphocytic leukemia patients with LDT shorter than <12 months showed higher *PIM2* expression than patients with LDT ≥12 months: 1.79 (0.95–2.81) vs 0.85 (0.63–1.25), p = 0.00000 (Fig. 1A,D). Notably, a positive correlation between *PIM2* gene expression and the percentage of malignant lymphocytes with ZAP70 expression and the Rai stage was revealed: R = 0.69, p = 0.00002 and R = 0.72, p = 0.00000, respectively (Fig. 2A,B). Chronic lymphocytic leukemia patients with high expression levels of the *PIM2* gene (above the median) had shorter PFS than patients with low *PIM2* expression, 11 months (4–38) and 22 (5–60), respectively, p = 0.01 (Fig. 3A). Moreover, TTFT was longer in patients with *PIM2* expression below median than in patients with higher *PIM2* expression, 90 days (30–180) and 30 (30–180) respectively, p = 0.0003 (Fig. 3B). In addition, it was found that patients who achieved CR had significantly lower expression of the *PIM2* gene than patients with no CR (NCR): 0.84 (0.63–1.84) vs 1.41 (0.74–2.81), p = 0.000227 (Fig. 1B). *PIM2* expression in peripheral blood lymphocytes of CLL patients in CR was lower than in leukemic lymphocytes of those patients analyzed before the treatment, and was comparable to healthy controls (data not shown). Notably, we found that in patients with CR after R-FC treatment, median *PIM2* expression was lower than in patients with NCR, 0.086 (0.83–1.84) and 1.67 (0.88–2.81), respectively, p = 0.034 (Fig. 1C). To confirm the results at the *PIM2*, mRNA level the cell lysates from 9 CLL patients were analyzed by western blot. Protein bands were found in all analyzed cell lysates. The number of bands differed between the samples, which reflected the presence of different isoforms of the protein (Fig. 4A,B). As expected, the densitometric analysis revealed high PIM2 expression in CLL patients when compared with the controls (Fig. 4C). Moreover, a positive correlation between mRNA and protein level was found, R = 0.98, p = 0.000002 (data not shown).

Discussion

PIM (1, 2 and 3) (provirus integration site for Moloney murine leukemia virus) family proteins are highly conserved serine/threonine kinases, which are considered to be involved in cancer progression and the development of resistance to chemotherapeutic agents. Their elevated expression has been observed in solid tumors and hematological malignancies.[6,13] The activation of PIM kinases modulates a broad range of cellular phenomena, including apoptosis, progression of the cell cycle, differentiation, protein translation, and interactions with

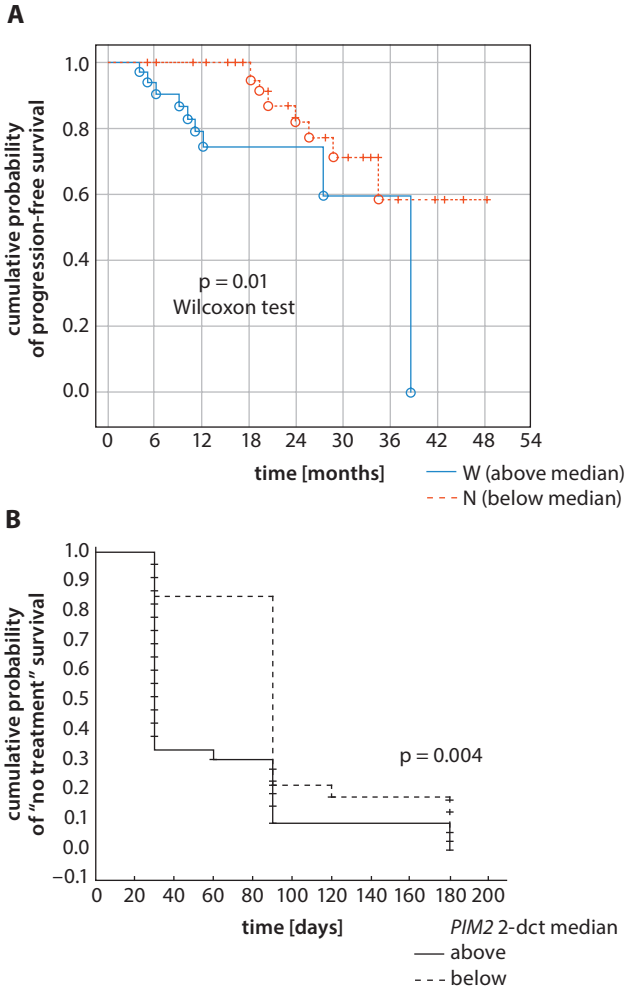

Fig. 3. Kaplan–Meier PFS (A) and TTFT (B) curves with regard to *PIM2* gene expression in CLL patients stratified as above or below the median value of *PIM2* gene expression (0.88)

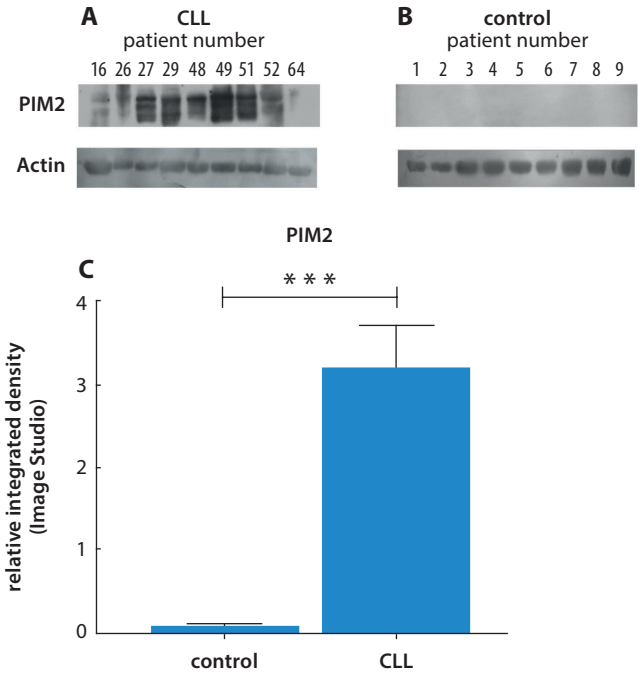

Fig. 4. Western blot analysis of PIM2 in CLL patients and normal controls (A, B), densitometric analysis of PIM2 expression in mononuclear cells of CLL patients and normal controls, ***p < 0.0001 (C)

the microenvironment.[5] Their expression is mediated by JAK-STAT and NFκB signaling pathways after the stimulation of growth factors, cytokines and TNF family receptors.[14] These kinases have also been demonstrated to play a role in the development of hematological malignancies, since their expression was found in both cell lines and malignant cells obtained from patients with different myeloid and lymphoid malignancies.[5,15]

The inhibition of mechanisms leading to in vivo apoptosis is considered as one of the most important factors leading to the accumulation of leukemic lymphocytes of patients with chronic lymphocytic leukemia. Extensive research conducted on this topic has led to the identification of intracellular phenomena involved in the prolonged lifespan of CLL lymphocytes, such as the BCR-induced pathway and antiapoptotic protein BCL-2 hyperexpression.[16] However, our knowledge of the mechanisms responsible for the inhibition of apoptosis in CLL cells needs to be extended, as well as the research of factors which would predict the risk of rapid progression of this disease in order to single out the patients who may benefit from early institution of the treatment.

The results of some studies published so far strongly suggest that PIM kinases act as survival-promoting factors for CLL cells, since their inhibition by an imidazo [1,2-b] pyridazine small molecule, SGI-1776, led to the in vitro apoptosis of those cells accompanied by reduction of the *MCL1* gene transcript.[17] Recently, several pan-PIM kinase inhibitors were developed.[18,19] The antiapoptotic effect of the inhibition of PIM kinases by 3 different inhibitors was also demonstrated by Decker et al.[7]

PIM2 kinase attracted particular attention for the research on the biology of CLL cells. The serine/threonine kinase PIM2 is an oncogenic factor that has been shown to promote cell survival by activating the expression of *NF*κ *B*-dependent gene expression following a number of proliferation-inducing signals.[4,20,21] As demonstrated by knockdown experiments, this kinase acts in CLL cells as a survival-promoting factor, but in contrast to PIM1, it does not affect the surface expression of the CXCR4 receptor.[7] Increased expression of the *PIM2* gene as compared with normal lymphocytes was reported by several authors.[7,10,22] Cohen et al. found that higher expression of this gene was correlated with such negative prognostic factors as shorter lymphocyte doubling time and higher Binet stage.[10] Moreover, Hüttmann et al. demonstrated that *PIM2* mRNA expression ratio in highly purified CLL lymphocytes, when calculated using unpurified mononuclear cells of healthy donors as a template, was higher not only in advanced Rai stages, but also in $CD38^+/ZAP70^+$ cells as compared to double-negative ones.[22] In contrast, Decker et al. did not find a relationship between *PIM* transcript levels or PIM protein expression levels and such risk factors in CLL as clinical stage or IgVH mutational status.[7]

Given the biological function of PIM2 kinase as a survival-promoting factor and the above-quoted findings which suggest a possible relationship between its elevated expression and some prognostically unfavorable clinical and biological factors of CLL, we decided to investigate whether expression of the *PIM2* gene is also related to a more aggressive course of the disease and whether it has an impact on the response to cytostatic treatment.

In line with previous studies, we found that *PIM2* mRNA content in circulating CLL lymphocytes was higher than in normal peripheral blood lymphocytes obtained from healthy donors. We also demonstrated that increased expression of the *PIM2* gene was related to shorter LDT, more advanced clinical stage and higher percentage of ZAP70-positive cells. Western blot analysis of PIM2 protein content in 9 representative samples demonstrated a correlation between mRNA and protein content, which suggests transcriptional regulation of expression of this factor. An original aspect of our study is the finding that high *PIM2* expression was associated with a short TTFT and short PFS after the successful frontline treatment. This correlation between high *PIM2* expression and aggressive clinical course of CLL is consistent with the relationship between expression of this kinase and the abovementioned negative prognostic factors. It therefore remains to be determined whether this negative prognostic value of *PIM2* expression is independent or related to other factors known to negatively influence the clinical course of CLL.

We also found that the CR rate was lower in patients who strongly express the *PIM2* gene, as compared to low expressors of the gene in question. This negative predictive value of high expression of the *PIM2* gene was found

both for the whole group of patients who underwent immunochemotherapy and for the subgroup in whom a CR was obtained using the FC-R regimen. To our knowledge, this is the first observation published so far concerning the impact of expression of the *PIM2* gene in CLL cells on the results of immunochemotherapy. Our findings may suggest that *PIM2* decreases the sensitivity of CLL cells to purine analogue-based immunochemotherapy. It is thus possible that PIM2 kinase survival-promoting activity compensates the apoptosis pathway triggered by purine analogues, alkylating drugs and rituximab. It is noteworthy that the overexpression of PIM kinases has been reported to confer resistance to rapamycin, an mTOR (mammalian target of rapamycin) inhibitor, and another drug active in hematological malignancies.[23,24]

Our findings warrant further studies on the value of expression of the *PIM2* gene as a prognostic factor associated with a more aggressive clinical course of CLL, and a predictive factor of the response to immunochemotherapy administered as frontline treatment. These findings also support the idea of studying the PIM kinases as therapeutic targets in lymphoid malignancies.

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A hypothesis for the mechanism of urine incontinence in patients after radical prostatectomy due to urinary bladder hypertrophy

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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*. 2019;28(3):391–395

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
The authors would like to thank Miss Izabella Ostrowski, currently studying neuroscience at the University of Vermont, Burlington, USA, for English linguistic support.

Received on March 12, 2017
Reviewed on August 16, 2017
Accepted on November 8, 2017

Published online on August 29, 2018

Abstract

Prostate cancer is one of the most common neoplasms in elderly males in Europe and is rapidly becoming a major health burden throughout the world. Radical prostatectomy is a first-line treatment in the case of organ-confined prostate cancer and in selected cases of locally advanced disease. Recent studies have suggested that the urinary bladder wall thickness (BWT) is an important predictor of an overactive bladder (OAB). This article focuses on a hypothesis for the mechanism of urine incontinence in patients after radical prostatectomy due to urinary bladder hypertrophy. To verify the possible influence of changes in the urinary bladder structure on urine incontinence development, we designed an experiment in which patients with prostate cancer were qualified for radical prostatectomy. Our hypothesis that urinary bladder hypertrophy influences urine incontinence development in patients after radical prostatectomy may provide an insight toward the prevention and control of urine incontinence after surgery by oral pharmacotherapy or intravesical onabotulinumtoxinA injections in pre-radical prostatectomy mode.

Key words: urinary incontinence, hypertrophy, urinary bladder, prostatectomy, structural changes

Cite as
Juszczak K, Ostrowski A, Bryczkowski M, Adamczyk P, Drewa T. A hypothesis for the mechanism of urine incontinence in patients after radical prostatectomy due to urinary bladder hypertrophy. *Adv Clin Exp Med*. 2019;28(3):391–395. doi:10.17219/acem/79935

DOI
10.17219/acem/79935

Introduction

Prostate cancer is the most common neoplasm in elderly males in Europe and is a major health burden, especially in countries with a higher proportion of elderly men in the general population. Epidemiological data suggests that the highest incidence of this cancer can be found in Northern and Western Europe (approx. 200 cases per 100,000 men). Moreover, a continuous increase of prostate cancer morbidity can be observed in Eastern and Southern Europe.[1] Radical prostatectomy is a first-line treatment in the case of organ-confined prostate cancer. Additionally, surgical treatment should be considered an option in all patients with locally advanced disease. The goal of radical prostatectomy in any approach is the eradication of the disease while preserving continence and, whenever possible, potency.[2] However, some groups of patients require an adjuvant therapy (e.g., radiotherapy) after radical prostatectomy due to the onset of poor prognostic factors, as well as in cases of biochemical recurrence. Despite this, the 10-year survival of patients after radical prostatectomy is very high, reaching survival rates of 95–100%. Radical prostatectomy is associated with postoperative complications (e.g., erectile dysfunction, urinary incontinence, etc.). Most of the patients who experience urinary incontinence after radical prostatectomy often have stress urinary incontinence. However, in some cases, the post-surgical urinary incontinence can also manifest due to urgency caused by the development of an overactive bladder (OAB), which is defined as urge urinary incontinence. Recent clinical observations suggest that men can also develop OAB after radical prostatectomy. Moreover, due to storage lower urinary tract symptoms (LUTS) (frequency, urgency, etc.), many patients with OAB usually reduce fluid intake and limit daily activity to avoid the discomfort of the symptoms.[3] These facts significantly affect the quality of life of patients who receive radical prostatectomy treatment.

The hypothesis

We hypothesized that urinary bladder hypertrophy influences urine incontinence development by causing OAB in patients with prostate cancer after radical prostatectomy. The primary outcome of the study is defined as an increased incidence of OAB with or without urge urinary incontinence in patients after radical prostatectomy, which is characterized by increased urinary bladder weight and wall thickness. The secondary outcome is that urinary bladder outlet obstruction (BOO) increases the risk of OAB with or without urge urinary incontinence in patients after radical prostatectomy.

Evaluation of the hypothesis

Radical prostatectomy and urinary incontinence

Radical prostatectomy remains the standard treatment of choice in the cases of organ-confined prostate cancer and in selected cases of locally advanced prostate cancer.[4] The radical prostatectomy procedure consists of removing the prostate gland and seminal vesicles, ideally with negative surgical margins. Thus, radical prostatectomy is associated with an increased risk of developing urinary incontinence. Urinary incontinence after radical prostatectomy represents a troublesome issue in some patients. Many factors determine urinary incontinence after radical prostatectomy, including anatomical and biological factors. The pathophysiology of post-surgical urinary incontinence is multifactorial. Excluding anatomical determinants (e.g., changes in the urethral sphincter complex and/or structures related to the membranous urethra, damage to neural components, etc.), some biological factors have been defined. Several clinical features seem to be crucial predictors of post-prostatectomy urinary incontinence, including age, body mass index (BMI), comorbidity index, lower urinary tract symptoms, and prostate volume. Urinary incontinence in patients after radical prostatectomy is primarily due to urethral sphincter incompetence and weakness, known as stress urinary incontinence.[5–7] Urge urinary incontinence, in some cases, can also be due to urinary bladder dysfunction, which leads to pure urge urinary incontinence or mixed urinary incontinence, exacerbating co-existing stress urinary incontinence. Previous studies suggest that an onset of detrusor overactivity after radical prostatectomy seems to be a significant biological risk factor, which is related to urinary incontinence after surgery.[7] An overactive bladder negatively impacts quality of life and often requires pharmacotherapy. A study by Blaivas et al. revealed that about 20% of men who developed OAB symptoms were previously treated for prostate cancer.[8] Another study by Porena et al. observed detrusor overactivity in patients after radical prostatectomy in about 2–77% of cases during urodynamic testing.[9] Boettcher et al. found that patients after radical prostatectomy and radiotherapy had OAB 3 years after the therapy in approx. 13% and 30% of cases, respectively.[10] The data suggests that patients with prostate cancer after radical prostatectomy are at a higher risk of developing OAB. Hosier et al. revealed that OAB and storage LUTS are quite common in patients after radical prostatectomy.[11] Additionally, adjuvant or salvage radiotherapy increases the risk of OAB in patients primarily treated with radical prostatectomy. In about 4% of cases, detrusor overactivity leads to urge urinary incontinence in patients after radical prostatectomy. In about 42% of cases, detrusor overactivity is also associated with mixed urinary incontinence after radical prostatectomy.[12] The results from Song et al. showed that

more than 51% of patients presented with detrusor overactivity at a 3-year follow-up after radical prostatectomy.[13] It is well-known that radical prostatectomy may affect proper innervation and vascularization of the urinary bladder, thus causing a functional alteration of the urinary bladder, such as detrusor overactivity and/or decreased urinary bladder compliancy.

Considering the number of factors that potentially affect urinary continence after radical prostatectomy, it can be inferred that post-prostatectomy urinary continence is an unpredictable and clinically demanding condition. Thus, the proper evaluation of these potential factors in patients after radical prostatectomy is crucial in predicting the risk and severity of urinary incontinence development. A better understanding of the etiopathogenesis of urinary incontinence in relation to radical prostatectomy will enable physicians to more thoroughly inform patients about the risks associated with urinary incontinence, along with potential treatment options for the symptoms they are experiencing.

Bladder outlet obstruction and overactive bladder

Benign prostate enlargement is a common urological problem mainly seen in older men, causing LUTS, such as nocturia, frequency, urgency, etc. It is known that BOO, depending on the severity and duration of the obstruction, leads to structural changes in the urinary bladder. A significant proportion of patients with benign prostate enlargement require treatment due to the discomfort caused by LUTS. In patients with benign prostate enlargement, about 60% of symptomatic patients and 52% of asymptomatic patients experience urinary BOO. Although urodynamics is an invasive, expensive and time-consuming procedure, it still remains the most valuable test for making a final diagnosis of urinary BOO. Therefore, new methods of evaluating urinary BOO are required. So far, the following clinical factors were defined as predictors for urinary BOO: 1. International Prostate Symptom Score (IPSS) >19 points; 2. maximal urine flow (Q_{max}) <15 mL/min; and 3. post-void residual volume >100 mL. In addition, a study by Güzel et al. suggested that the urinary bladder wall thickness (BWT) increases simultaneously with increasing urinary BOO.[14] The authors of this study showed that the measurement of BWT is a simple, fast and reproducible procedure predicting the severity of urinary BOO. Hakenberg et al. revealed that men with LUTS and benign prostate enlargement presented with a moderate increase in BWT.[15]

Based on clinical experience, many patients with prostate cancer have co-existing benign prostate enlargement with or without BOO. Thus, this relationship may predispose a patient to develop OAB and urinary incontinence, especially following radical prostatectomy. Therefore, it is crucial to identify obstructed patients who seem to be at a higher risk of developing post-prostatectomy storage LUTS, especially urge urinary incontinence, which is due to OAB development.

Urinary bladder ultrasonographic features may predict overactive bladder

Ultrasonography is crucial in diagnosing functional urinary bladder disorders. The BWT parameter is useful in OAB diagnosis. Previous studies have revealed that an increased BWT is a predictor of detrusor overactivity in patients diagnosed with OAB.[16] An increased BWT seems to be a consequence of increased intensity of detrusor muscle contractions against a competent urethral sphincter, ultimately leading to urinary bladder hypertrophy. Yang and Huang reported that a thickened urinary bladder wall is common in women with lower urinary tract symptoms.[17] A study by Khullar et al. found the average BWT to be 5 mm.[18] This mean value was important in the creation of a sensitive screening method for diagnosing detrusor overactivity in symptomatic women without urinary bladder outflow obstruction. A study by Ali et al. suggested that an evaluation of BWT in ultrasonography is a very sensitive and specific technique for a diagnosis of detrusor overactivity.[19] The sensitivity and specificity of this test amount to about 91.9% and 86.1%, respectively.

Study design

To test the hypothesis, we have decided to conduct an experiment to investigate the effect of urinary bladder structural changes and urinary BOO on OAB and urinary incontinence development in patients after radical prostatectomy. The study is designed for patients with prostate cancer who qualified for radical prostatectomy. Currently, the study is still ongoing. Before surgery, each patient is strictly evaluated for OAB and BOO. After that, all patients are assigned to one of the following study groups: group 1 – patients with no OAB symptoms and without BOO; group 2 – patients with no OAB symptoms and with BOO; group 3 – patients with OAB symptoms and with BOO; and group 4 – patients with OAB symptoms and without BOO. Additionally, as a reference experimental group (control group), we enrolled 30 healthy, young volunteers with no OAB symptoms and without benign prostate enlargement or BOO. All patients with prostate cancer who qualified for radical prostatectomy are evaluated. Before surgery, the patients fill in the IPSS questionnaire, which assesses LUTS. Overactive bladder symptoms are analyzed using King's questionnaire, as well as 2 different OAB symptom scores (OABSS) designed by Blaivas et al. and Homma et al.[20–22] Each patient undergoes a transabdominal ultrasonographic evaluation of the prostate and urinary bladder structure. The width (W), height (H), length (L), and volume of the prostates are recorded. In each patients the ultrasonography is done when normal desire to void is achieved. The BWT and detrusor

muscle thickness (DWT) are measured in the sagittal plane in the anterior bladder wall. The means of 3 measurements of BWT and DWT are calculated. The urinary bladder weight – Ultrasound Estimated Bladder Weight (UEBW) – is also estimated. Ultrasound Estimated Bladder Weight is calculated from the urinary bladder surface area (SA), BWT and specific gravity of the urinary bladder wall (δ), using the following formula: UEBW = SA × BWT × δ.[22–24] The urinary bladder wall has a specific gravity of 1 g/cm^3. After ultrasonography, each patient undergoes uroflowmetry with post-void residual estimation. The same procedures are carried out in healthy subjects, as control, for the exclusion of benign prostate enlargement, BOO and OAB symptoms. Based on the protocol described above, all patients are divided into aforementioned 4 study groups. The study is going to be continued until each experimental group includes at least 30 patients. The study has been approved by the Local Ethical Committee of Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland..

The inclusion criteria for the patients in the study are as follows: 1. adult men over 40 years of age with prostate cancer who qualified for radical prostatectomy; 2. the control group comprised of 30 healthy men aged 20–40 years without any urological conditions and with no LUTS. The exclusion criteria are as follows: 1. neoadjuvant (any time before) or adjuvant radiotherapy or brachytherapy (within the follow-up period of 12 months); 2. macroscopic infiltration of the urinary bladder (pT4); 3. neurological deficiencies (e.g., stroke, spinal cord injury, multiple sclerosis, Parkinson's disease, etc.) which would cause an inability to control the urinary bladder; 4. urethral strictures; 5. previous surgery, such a transurethral resection of the prostate (TURP) or adenomectomy, performed within 12 months prior to radical prostatectomy; 6. any form of constant catheterization (an indwelling catheter, clean intermittent catheterization); 7. any bowel dysfunction (especially constipation, irritable bowel syndrome (IBS), etc.); and 8. taking a medication affecting urine production (e.g., diuretics).

After the initial evaluation prior to surgery, the control evaluation is set for 1, 3, 6, 9, and 12 months postoperatively in groups 1–4 in order to define LUTS after radical prostatectomy in relation to pre-surgical patient clinical features.

Our study was entitled "Effects of urinary bladder hypertrophy on urge urine incontinence after radical prostatectomy" and was registered at the website https://clinicaltrials.gov (ID number: NCT03061760).

Implications

The possible impact of urinary bladder hypertrophy on OAB with or without urge urinary incontinence in patients after radical prostatectomy may point at potential new methods for the prevention and control of OAB and urinary incontinence after surgery. Some possible methods are oral pharmacotherapy or intravesical onabotulinumtoxinA injections in pre-prostatectomy mode in the cases of patients with urinary bladder hypertrophy.

Consequences of the hypothesis

The data and evidence presented in this paper support the idea that fluctuations in BWT can predict the development of OAB. Thus, we hypothesized that urinary bladder hypertrophy influences the development of urine incontinence in patients with prostate cancer after radical prostatectomy through the occurrence of OAB. This provides a better method of classification of patients with a higher risk of developing urge urinary incontinence after radical prostatectomy.

In conclusion, it would be beneficial to conduct further studies to confirm whether urinary bladder hypertrophy is a sensitive predictor of OAB and/or urge urinary incontinence in the general population, as well as in patients after radical prostatectomy.

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Diseases of the oral cavity in light of the newest epigenetic research: Possible implications for stomatology

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(3):397–406

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Funding sources
None declared

Conflict of interest
None declared

Received on April 7, 2017
Reviewed on May 9, 2017
Accepted on July 24, 2017

Published online on October 2, 2018

Abstract

Epigenetics is the study of inheritable changes in gene expression without changes in the underlying deoxyribonucleic acid (DNA) sequence. The main mechanisms of epigenetic regulation include DNA methylation, modifications in histones, and micro-ribonucleic acids (miRNA). Recent research evidence has shown that environmental and lifestyle factors dynamically interact with the genome, influencing epigenetic changes, from development to the later stages of life. This happens across a spectrum, from physiological to pathological conditions, such as genetic defects, developmental disorders, infectious or inflammatory processes, cancers, mental disorders, and substance abuse. Epigenetic studies have been conducted in various medical disciplines (e.g., oncology, internal medicine or psychiatry), adding valuable insight to standard medical approaches. However, in stomatology, epigenetic research is still in its infancy; thus, this review is aimed at presenting the role of epigenetic mechanisms in diseases of the oral cavity, including periodontal diseases, caries, developmental anomalies, and oral carcinoma. In addition, this paper reveals new insights into epigenetic biomarkers that can be helpful in the detection, early diagnosis, prognosis, and treatment of different oral diseases. Moreover, this review is focused on the possible clinical implications (diagnostic and therapeutic) of epigenetics, in the form of some noninvasive methods that can possibly be used in the future for the screening, work-up, outcome prediction and novel treatments of some dental diseases. Finally, this paper highlights that an epigenetic approach can be useful for designing novel interventions that will improve the management of oral malignancies or developmental abnormalities.

Key words: periodontal diseases, dental caries, epigenetics, developmental anomalies, oral squamous cell carcinoma

Cite as
Jośko-Ochojska J, Rygiel K, Postek-Stefańska L. Diseases of the oral cavity in light of the newest epigenetic research: Possible implications for stomatology. *Adv Clin Exp Med*. 2019;28(3):397–406. doi:10.17219/acem/76060

DOI
10.17219/acem/76060

Introduction

One of the biggest challenges in contemporary medicine is a deeper understanding of disease pathophysiology, since this knowledge can create novel diagnostic and therapeutic methods to potentially reduce the prevalence of various chronic diseases or developmental defects. Deoxyribonucleic acid (DNA) has so far been regarded as the only source of genetic information. If, however, genes which encode proteins are so important, why do they constitute only 2% of the human genome? In an attempt to explain this topic, recent research has indicated that in our cells, in addition to the genome (or the "first code"), there is also an epigenome (or the "second code"), which is a controlling superstructure of the "first code".[1]

Moreover, a modern approach to disease pathophysiology combines the current knowledge of genomic and environmental influences, and this connection is the main subject of a new medical science – epigenetics – that explores non-genomic inheritance.[1]

Since environmental and lifestyle factors can interact with the genome and can influence epigenetic changes, epigenetics can shed new light on the etiopathology of some genetic defects, developmental anomalies, mental or behavioral disorders, infectious or inflammatory changes, precancerous or cancerous conditions, and many other abnormalities.[2] Due to the fact that epigenetic mechanisms are reversible, they can be affected by different factors, such as diet, medications, physical activity, mental or physical stress, traumatic events, environmental toxins, and addictive substances (e.g., nicotine and alcohol).[2,3]

Epigenetics is mostly a domain of genetics, biology, internal medicine, oncology, or psychiatry. In contrast, in stomatology, epigenetic research is still in its infancy. However, it should be noted that epigenetic mechanisms play an important role in gene expression during dental development (from the 6th week of gestation to approx. the 20th year of life), and that the potential impact of many environmental factors, over such a long period of time, can significantly disturb normal developmental processes and can influence the development of various oral diseases. Therefore, an understanding of epigenetic mechanisms is essential to the future implications for research and practice in the area of dentistry.[3,4]

This review aims to describe the role of epigenetic mechanisms in diseases of the oral cavity, including periodontal diseases, dental caries, developmental anomalies, and oral carcinomas. Moreover, it presents some insight into epigenetic biomarkers, which can be beneficial in the early detection, diagnosis, prognosis, and treatment of different oral diseases. Finally, it highlights that epigenetics (in addition to current standard treatments) can be useful for designing novel interventions that will improve the management of some inflammatory processes, developmental abnormalities or malignancies in stomatology.

Epigenetic mechanisms and their relevance to oral diseases

Epigenetic mechanisms are "in charge" of transcriptional control (which regulates gene expression), and their interactions modify the structure and function of chromatin. Epigenetic modifications occur across the lifespan of the individual (from intrauterine environment to advanced age), and by altering gene expression patterns, they contribute to various cellular phenotypes.[4–6] The key epigenetic mechanisms – including DNA methylation, histone modification and non-coding RNAs – and their relevance to oral diseases are briefly presented below.

DNA methylation

The process of DNA methylation, which represents the most characterized type of chromatin modification, involves the transfer of a methyl group (CH3) from S-adenosyl methionine (SAM) to cytosines present in cytosine-phosphate-guanine (CpG) dinucleotides of the DNA chain. The CpG sequences that are located throughout the genome are usually highly methylated and germline-specific. The CpG methylation results in transcriptional repression.[4,5] Abnormal methylation states can therefore lead to disease development. For instance, hypomethylation changes in DNA are related to chromosome instability that may lead to cancer. Based on recent studies in stomatology, DNA methylation patterns can be changed by inflammatory processes. Furthermore, alterations in DNA methylation patterns and cytokine gene expression can be seen in chronic periodontitis. Also, methylation patterns can differ between healthy and inflamed dental pulp.[4,5]

Histone modifications

A basic unit of chromatin, the nucleosome, consists of a DNA segment and 8 core histones. Such an octamer has 2 copies of H2A, H2B, H3, and H4, and creates a rigid structure to chromatin. During post-translational modification of the core histones, chromatin can be condensed or relaxed. This is a key epigenetic mechanism that regulates gene transcription. The modification of histones predominantly occurs at the N-terminal tails of the protein. Acetylation of the core histones leads to an "open" chromatin structure that enables transcription (e.g., the acetylated N-termini promote a more relaxed conformation of the chromatin, which permits the recruitment of the basic transcription factors). In contrast, histone deacetylases remove the acetyl groups, so that the chromatin become more condensed (which represses gene transcription). Histone methylation can cause either an activated or a repressed chromatin state.[4,5]

Recent studies have reported that histone modifications may induce differentiation and mineralization in dental pulp stem cells. Histone acetylation and deacetylation play a crucial role in the regulation of gene expression. This, in turn, can promote pulp repair and regeneration, and thus, may be useful in restorative stomatology.[4]

Noncoding RNAs

Noncoding RNAs are RNA molecules that do not encode for protein. They include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), and short-interfering RNAs (siRNAs). Both miRNAs and siRNAs can regulate gene expression without altering the DNA sequence.[4] It has been reported that noncoding RNAs are involved in oral diseases such as oral cancer. Also, it has been demonstrated that miRNAs play essential roles in odontoblast differentiation.[4,5]

Periodontal diseases

Periodontal diseases are pathological processes involving the periodontium (including the gums [gingiva], the alveolar bone [alveolar process], the dental cementum, and the periodontal ligament). They represent a significant public health problem worldwide and affect up to 70% of the population, regardless of gender or age. The main causes of periodontal diseases include poor oral hygiene, bruxism, malocclusion, and some general medical diseases, as well as improper fillings and prosthetic appliances. Recently, it has been considered that the epigenetic mechanisms contribute to the development of periodontal diseases, since they often occur during inflammation, are localized (e.g., on the border of the biofilm of the gums around the teeth) and differ depending on the location.[7]

Chronic periodontitis

Chronic periodontitis is an inflammatory disease affecting the tooth support structures. It is usually caused by interactions between periodontal pathogens and an immunological response of the host. Epigenetic mechanisms might contribute to the development of chronic periodontitis (Table 1), by influencing the expression of the genes participating in the immunological and inflammatory responses.[8]

In particular, according to the study by de Faria Amormino et al., it was shown that the toll-like receptors (TLR) play an important role in the response to bacterial infections. These receptors are mostly located in the areas that are potential "gates" for infection.[9]

In addition, the authors of the study compared the methylation status and the expression of the *TLR2* gene in samples of gum tissue derived from patients with chronic periodontitis (study group) and from healthy individuals (control group). The results showed that in patients with periodontitis, there is hypermethylation and low expression of the *TLR2* gene, comparing to the control group. In addition, a correlation was found between the methylation level and the degree of inflammation (e.g., less vs more advanced).[9]

According to some earlier studies, a decreased expression of E-cadherin is characteristic of certain carcinomas, including breast, lung, prostate, stomach, and colon cancers.[10] Likewise, an altered E-cadherin expression plays a role in the progression of chronic periodontitis.[11] In addition, translational research studies conducted by Loo et al. explored some epigenetic changes in E-cadherin and cyclooxygenase 2 (COX-2) that occur simultaneously in cancer and in chronic periodontitis. In particular, it was found that in the patients with breast cancer and in the ones with chronic periodontitis, both E-cadherin and COX-2 were hypermethylated, in contrast to the control group. The authors emphasize that chronic periodontitis can, to some degree, be associated with hypermethylation of DNA, which is related to cancer risk factors. In addition, they believe that further studies on similar epigenetic changes might be very useful in diagnosing and treating chronic periodontitis.[12]

Similarly, the results of another study have revealed that an increase of acetylation in the histone H3K9 and in the promotor CBP/p300, and a decrease in the activity of histone deacetylase caused an increased expression of proinflammatory cytokines (such as IL-1, IL-2, IL-8, and IL-12), and the development of chronic periodontitis.[13] In some other studies, it was also found that in chronic periodontitis, hypermethylation of the promoter of the prostaglandin-endoperoxide synthase 2 (PTGS2) resulted in reduced COX-2 expression.[14] In this way, some epigenetic biomarkers relevant to hypermethylation or hypomethylation have been suggested as being helpful for a better understanding of the pathophysiology of chronic periodontitis. They include: 1. hypermethylation and decreased expression of the tumor necrosis factor (TNF-α) and cyclooxygenase 2 (COX-2) and 2. hypomethylation and increased expression of interferon γ (IFN-γ), transcriptional factors (e.g., nuclear factor κB [NF-κB]), and signal transducer and activator of transcription (STAT) proteins.[15,16]

Aggressive periodontitis

Aggressive periodontitis, including localized aggressive periodontitis (LAP) and generalized aggressive periodontitis (GAP), is a type of periodontal disease with some specific features (Table 1). Although it is much more rare than chronic periodontitis, it usually affects younger patients, who are otherwise in good health. In contrast to chronic periodontitis, the main characteristics of aggressive periodontitis (both LAP and GAP) include rapid bone destruction, a discrepancy between the amounts of microbial deposits and the severity of the periodontal tissue destruction,

Table 1. Epigenetic mechanisms in common diseases of the oral cavity and their potential clinical implications: emerging epigenetic biomarkers for prediction or diagnosis

Diseases of oral cavity	Epigenetic mechanisms regulating gene expression/transcriptional control; type of disease and comments regarding epigenetic biomarkers	Author, year of publication and reference number
Periodontal diseases: – chronic periodontitis – aggressive periodontitis	hypermethylation and low transcription of *TLR2* gene; chronic periodontitis	de Faria Amormino et al., 2013[9]
	hypermethylation and decreased expression of E-cadherin; hypermethylation and decreased expression of cyclooxygenase 2 (COX-2); chronic periodontitis	Loo et al., 2010[12]
	hypermethylation and decreased expression of tumor necrosis factor α (TNF-α); hypomethylation and increased expression of interferon γ (IFN-γ), nuclear factor κB (NF-κB), signal transducers and activators of transcription (STAT) proteins; chronic periodontitis	Lindroth et al., 2013[15]
	DNA methylation in chemotactic cytokine 25 (*CCL25*) and interleukin 17C (*IL17C*) genes; aggressive periodontitis	Schulz et al., 2016[17]
Dental caries Pulpitis	DNA methylation in alkaline phosphatase (ALP) gene; regulation of ALP in human osteoblastic cells	Delgado-Calle et al., 2011[24]
	hypomethylation and increased expression of metylotransferase DNMT1 and TET proteins; amelogenesis process	Yoshioka et al., 2015[27]
	DNA methylation, histone modifications and altered activity of miRNA – interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukins (IL-1, IL-6, IL-8, IL-10, IL-17); dental pulp inflammation/pulpitis	Hui et al., 2017[29]
	methylation of NF-κB, FOXP3, STAT and IRF; epigenetic regulation in dental pulp inflammation	Hui et al., 2017[29]
Pulpitis	histone deacetylase inhibitors (HDACis) – increased expression of bone morphogenetic protein 2 (BMP-2) HDACis epigenetically promote reparation in primary dental pulp cells (DPC)	Duncan et al., 2013[33] and Qiu et al., 2017[34]
Cleft lip/palate	DNA methylation in *Clf2* gene; an epigenetic component in the multifactorial etiology of cleft lip and palate	Plamondon et al., 2011[37]
	all-trans retinoic acid (ATRA) – DNA methylation within CpG islands; maternal exposure to ATRA and cleft lip/palate	Kuriyama et al., 2008[40]
	valproic acid – inhibition of histone; deacetylase	Ornoy et al., 2009[44]
Skeletal malocclusion	increased expression of molecular motor *MYO1C*, acetyltransferase *KAT6B* and osteogenetic transcription factor *RUNX2* genes in human masseter muscle; contributing factors to the development of malocclusion	Desh et al., 2014[46]
	increased expression of *KAT6B* and *HDAC4* genes; epigenetic influence of *KAT6B* and *HDAC4* in the development of malocclusion	Huh et al., 2013[50]
	altered DNA methylation patterns in RUNX2 and alkaline phosphatase (ALP) genes; gingival fibroblast/osteoblast transdifferentiation through epigenetic mechanisms	Cho et al., 2017[51]
Hypodontia Anodontia	increased DNA methylation in 6636 genes; DNA methylation as critical factor for tooth agenesis (e.g., anodontia, hypodontia)	Wang et al., 2016[53]
Oral squamous cell carcinoma (OSCC) Emerging epigenetic biomarkers	DNA methylation in gamma-aminobutyric acid B receptor 1 – *GABBR1* (cg21022792) gene; innovative DNA methylation target in oral rinse samples as prediction of survival in OSCC	Langevin et al., 2014[57]
	DNA methylation in *WT1*, *MSH6*, *GATA5* and *PAX5* genes; possible epigenetic biomarkers in OSCC	Ribeiro et al., 2016[58]
	DNA methylation in *PTEN* and *p16* genes; possible epigenetic biomarkers in OSCC	Sushma et al., 2016[59]
	DNA methylation in γ-synuclein (*SNCG*) gene; γ-synuclein expression as a Malignant Index in OSCC	Cheng et al., 2016[60]

impaired neutrophil functions, abnormal chemotaxis and phagocytosis processes, hyperresponsive macrophage phenotypes (e.g., elevated levels of prostaglandin E2 [PGE2] and interleukin 1β [IL-1β]), as well as the typical involvement of a certain tooth (e.g., numbers 1–2 and 6–7).[17] Epigenetic modifications influence the immunological response that plays a key role in aggressive periodontitis. In a study of patients with acute periodontitis, Schulz et al., for the first time, examined the methylation of CpG in 22 inflammatory gene candidates (*ATF2, CCL25, CXCL14, CXCL3, CXCL5, CXCL6, FADD, GATA3, IL10RA, IL12A, IL12B, IL13, IL13RA1, IL15, IL17C, IL17RA, IL4R, IL6R, IL-6, IL7, INHA*, and *TYK2*). They found that the methylation of CpG chemotactic cytokine 25 (CCL25) and interleukin

17C (IL17C) was substantially lower than healthy periodontal tissues. Therefore, the decrease of CpG methylation probably accompanies an increase of expression of these genes. That, in turn, can lead to the increased availability of CCL25 (which is relevant to T-cell development) and IL17C (which regulates innate epithelial immune responses), causing pro-inflammatory reactions and contributing to a loss of attachment of the tooth in the alveolar fossa. Such epigenetic modifications can be triggered by many different external and internal factors (e.g., bacteria and demographic or socio-economic factors).[17]

Dental caries

Dental caries is a localized destruction of the tooth surface, initiated by decalcification of the enamel and followed by enzymatic lysis of organic structures, leading to cavity formation. If this condition is left unchecked and untreated, the cavity can penetrate the enamel and dentin, and then extend to the pulp.[18] Tooth formation begins around the 34th day of fetal life, and at this moment the process of amelogenesis (enamel formation) also begins. Amelogenesis is influenced by several internal (e.g., fever, hypoxia, lack of nutrients, and toxins) and external factors (e.g., antibiotics, environmental pollution and socio-economic status).[18] Interestingly, some additional, novel factors, such as the impact of traumatic experiences of a pregnant mother on the development of the fetus, and later on, the child's dental structures, including early childhood caries (ECC), have been proposed.[19]

Furthermore, based on a study of 314 pairs of twins, aged from 1.5 to 8 years, it was found that if a pregnant mother suffers from depression, then the risk of ECC in her child increases.[20] In addition, in a study by Hughes et al., in which 1,200 pairs of twins were examined using modern molecular methods, it was determined that epigenetic and environmental factors influenced tooth development and oral health. In particular, it was confirmed that genetic factors strongly influenced variation in the timing of primary tooth emergence. Also, ongoing follow-up clinical examinations of the twins are being conducted to examine whether those who become colonized earlier with decay-forming bacteria will develop dental decay at an earlier age. In this way, a comparison within and between monozygotic and dizygotic twin pairs will help to explain the interactions between genetic, epigenetic and environmental factors, and their impact on oral health and disease.[21]

It should be highlighted that the concept of induced pluripotency (an "instrument" to obtain patient-specific stem cells) may provide some insight into the inter-relationships between transcription factors and chromatin structure and dynamics, which could be useful in stomatology. In particular, during cell differentiation, methylation of DNA might be the last step in the direction toward stabilization of cellular "destiny" (cellular reprogramming).[22]

This process is catalyzed by DNA methyltransferases (DNMTs) DNMT1 and DNMT3. DNMT1 is responsible for maintaining methylation in de novo formatted DNA strands, post replication, while DNMT3a and DNMT3b participate in de novo methylation during the embryonal processes of development and cell differentiation.[23]

DNA methylation contributes to the regulation of the functions of the osteoblasts, osteocytes, osteocalcin, and sclerostin. Moreover, DNA methylation plays a crucial role in controlling the expression of the alkaline phosphatase (ALP) gene, which regulates the transition of osteoblasts into osteocytes.[24,25] Recent studies have revealed that the ten-eleven translocation (TET) protein family (TET1, TET2, and TET3) plays a main role in the demethylation of DNA. In particular, they interact with proteins of complexes participating in histone modifications, and by influencing their activity and ability to bind to chromatin, they can change the methylation profile or histone acetylation. It should be noted that a decreased expression of the TET genes is related with the development and progression of different types of malignancies, and that an increased expression of the TET genes is related with the amelogenesis process.[26,27] In addition, it has been revealed that the methylation of cytosine correlates with enamel development, especially during early developmental stages (e.g., the expression levels of methyltransferase DNMT1 during the early developmental stages were almost 3 times higher than those during the late stages). A similar correlation was found in regard to the expression levels of the TET protein family (e.g., the levels of expression of TeT1 were 3 times higher in the early developmental stages than in the late stages). Since the DNMT methyltransferases and the TET protein family appear to be the main factors for epigenetic reprogramming (Table 1), it is highly probable that the dynamic changes of cytosine methylation can be essential in the regulation of amelogenesis.[27]

In their studies on the correlations between epigenetic changes and the development of dental caries in children, Fernando et al. examined 3,000 families (from 2006 to 2011). In each of these families, the periods of pregnancy, childhood and adulthood were assessed and for the epigenetic tests, saliva samples were collected. However, until now, only pilot studies have been conducted, and due to the small sample sizes, it was impossible to draw meaningful conclusions. However, there is hope that in the future these pioneering studies on epigenetic variability in dental caries in children may provide some evidence of associations between epigenetic variability and social or environmental factors causing caries in the pediatric population.[28]

Pulpitis

Pulpitis is a complication of untreated dental caries that develops due to the harmful action of some strains of bacteria causing decay, as well as to trauma or other adverse

factors. The inflammatory reaction of pulp is regulated by epigenetic modifications, in which environmental factors play an enormous role, as proinflammatory triggers. According to some recent research in stomatology, epigenetic changes (e.g., DNA methylation) can occur due to environmental changes, both external and internal, including inflammatory lesions, dysbiosis, immune system compromise, or abnormalities in cytokine regulation.[4,15]

According to the studies by Hui et al., it was determined that the epigenetic regulation of dental pulp inflammation occurs via the processes of DNA methylation, histone modification and changed activity of miRNA. At these 3 levels, the dynamic modulations of interferon γ (IFN-γ), TNF-α and a number of interleukins (e.g., IL-1, IL-6, IL-8, IL-10, and IL-17) play a main role in pulp inflammation.[29,30]

It should be noted that over the last 10 years, tissue engineering has gained a lot of attention, since it can offer an innovative approach to dental pulp regeneration, after damage due to an inflammation or other types of injury. These innovative strategies, aimed at regaining the tissue and restoring its function, are related to de novo dental pulp regeneration (e.g., filling the canal with vital tissues rather than filling it with artificial materials). In particular, in the case of pulp regeneration, the goal is to reestablish pulp and dentin tissues in the canal space. This includes revitalization or revascularization. Although this is difficult to achieve, some novel approaches have been proposed, such as cell-based (e.g., transplanting exogenous cells into the host) and non-cell-based revitalization procedures. However, there are still some unresolved issues that have to be investigated in research studies prior to the possible introduction of these techniques into dental practice.[31,32]

Current research has revealed that histone modification might induce the differentiation and regeneration of stem cells of the dental pulp via hypoacetylation, exerted enzymatically by the histone deacetylase (HDAC). The HDAC inhibitors (HDACIs) inhibit the activity of HDAC and alter the level of histone acetylation (Table 1). This may represent a potential new approach to the treatment of many dental diseases, (e.g., via promoting reparative processes in primary dental pulp cells).[33] Furthermore, in recent neuropsychopharmacological studies, 2 inhibitors of the histone deacetylase – valproic acid (VPA) and trichostatin A (TSA) – were examined, focusing on their influence on inducing the reparative responses in stem cell cultures of primary dental pulp cells (DPC).[34] For instance, it was found that the administration of HDACIs stimulates, to a large degree, osteopontine and the expression of a growth factor – bone morphogenetic protein 2 (BMP-2). In addition, the HDACIs also promote differentiation of the pulp's stem cells without cytotoxic effects. These new data underscore the potential related to the possible use of low HDACI concentrations in the treatment of pulpitis. Moreover, it was found that HDACIs might be used as potentially new medications in monotherapy, or in combination with other pharmacological agents.[34]

It should be underscored that epigenetic mechanisms such as the acetylation and deacetylation of histones play a key role in the regulation of gene expression and might promote reparative changes of the pulp, offering the possibility of an innovative dental therapy in the future.[35]

Birth defects

Cleft lip and/or palate

Cleft lip and cleft palate (or facial cleft) is a group of conditions including cleft lip, cleft palate and both together. A cleft lip is an opening in the upper lip, extending into the nose, and a cleft palate is a condition where the roof of the mouth has an opening into the nose. Cleft lip and palate are birth defects that are caused by the improper joining of the tissues of the face during development. These defects can cause feeding difficulties, speech abnormalities, hearing impairment, and frequent ear infections. Cleft lip and/or palate, is one of the most prevalent birth defects in the human population (e.g., it occurs in approx. 1 per 1,000 births in the developed world and in 2–3 per 1,000 births in Poland).[36]

An animal model of human cleft lip and palate are the A/WySn mouse strain, in which the cleft occurs in 20% of the population. Plamondon et al. have found that in mice, epigenetic modifications in the form of methylation in the *Clf2* gene contribute to the development of the cleft.[37] In humans, genetic (e.g., maternal diseases) and environmental factors (e.g., maternal nutritional habits and substance use or dependence disorders, such as tobacco smoking, alcohol, addictive medications, or illegal drug use) are responsible for the development of this defect. These environmental factors, predominantly including tobacco smoking, influence the gene expression.

Based on a meta-analysis by Little et al. that included data from 32 case-control and cohort studies, it was reported that there is a statistically significant correlation between maternal tobacco smoking and cleft lip, with or without cleft palate, and also between maternal tobacco smoking and cleft palate. The authors suggest that this evidence is so strong that it can support anti-nicotine campaigns.[38] On the other hand, according to the results of a study by Grosen et al. that examined a population of twins with cleft lip/palate from the Danish Twin Registry (including 9,146 people who were born in the years 1936–2004), such a correlation was not determined.[39] Furthermore, there is growing evidence indicating that epigenetic processes play a role in abnormal craniofacial formation. For instance, in 2008, Kuriyama et al. showed that in newborn mice, methylation of DNA – in specific dinucleotide sequences of CpG – induced the formation of cleft palate (Table 1), when their pregnant mothers were given a preparation of all-trans retinoic acid (ATRA).[40] Also, it should be noted that epigenetic mechanisms are

related not only to the changes of DNA and histones, but also occur at the level of microRNA (miRNA), which participates in the regulation of 30% of human genes, contributing to normal cell development. Recent studies have shown that miRNA regulates the processes of proliferation, differentiation and apoptosis of cells which are necessary to normal embryonic development.[41] Moreover, it has been shown that in animal models, during the development of maxillary and facial tissues, different types of miRNA play a key role in ontogenesis (e.g., regulating a protein cytoskeleton, influencing growth factors, and modulating signal transduction and transcriptional factors).[42,43] In addition, the influence of valproic acid used by pregnant mothers on the development of cleft palate in their children was examined by Ornoy (Table 1). The results of this study suggest that valproic acid (which inhibits the histone deacetylase) causes changes in gene expression and contributes to the development of this defect.[44]

Skeletal malocclusion

A skeletal malocclusion is a misalignment or incorrect relationship between the teeth of the 2 dental arches when they approach each other as the jaws close. Malocclusion is classified as follows:

– class I, neutrocclusion, in which the molar relationship of the occlusion is normal (e.g., for the maxillary 1st molar), though the other teeth have some abnormalities (e.g., spacing, crowding, and over- or under-eruption);

– class II, distocclusion (retrognathism or overbite), where the mesiobuccal cusp of the upper 1st molar is not aligned with the mesiobuccal groove of the lower 1st molar (it is anterior to it); and

– class III, mesiocclusion (prognathism, anterior crossbite or underbite), in which the upper molars are not placed in the mesiobuccal groove, but posteriorly to it (the mesiobuccal cusp of the maxillary 1st molar lies posteriorly to the mesiobuccal groove of the mandibular 1st molar) and the lower front teeth are usually more prominent than the upper front teeth (e.g., a large mandible or a short maxillary bone).

The skeletal muscles that are involved in movements of the jaws are very flexible, and able to react or adapt to different physiological stimuli, via contraction or release. According to the pioneering studies by Pandorf et al. on the epigenetic mechanisms affecting these skeletal muscle fibers, it was revealed that histone modifications at the myosin heavy chain (MHC) genes occur in the locus of these genes (e.g., in response to muscle unloading).[45] It should be highlighted that the main transcription factor regulating osteogenetic processes is the Runt-related transcription factor 2 (*RUNX2*), and its expression in osteoblasts and osteoprogenitor cells is crucial for these processes. In particular, in patients who underwent a surgical treatment of class II or class III malocclusion in their masseter muscle, the expression of the *MYO1C* gene (which encodes myosin 1C) and of acetyltransferase KAT6B (which activates RUNX2) were examined (Table 1). The findings of this study indicated that the abovementioned epigenetic modifications contributed to the development of malocclusion.[46] Furthermore, it was determined that there were highly significant associations between MYO1C and the KAT6B expressions. Therefore, the change in expression of the myosin genes confirms that the differences in fiber type in the masseter muscle are important contributors to osteogenesis and to the development of malocclusion.[47] Over the last 10 years, research related to epigenetic control of skeletal muscle fiber types and osteogenesis has rapidly emerged.[48] These epigenetic mechanisms often include acetylation of lysine residues in the chromatin through acetyltransferases (KAT) and deacetylases (HDAC) of the histones.[49] In a study by Huh et al., conducted among patients undergoing surgical procedures for malocclusion, biopsies of the masseter muscle were obtained, and myosin genetic variability (contributing to the development of class III malocclusion) was examined. In particular, 2 functionally connected enzymes – KAT and HDAC – were compared, to determine their correlations with the muscular–skeletal system during the development of malocclusion (Table 1). The data from this study support the results of some other published reports related to the epigenetic regulation of muscle and bone growth. In particular, it was shown that the expression of the *KAT6B* and *HDAC4* genes was a few times higher in the masseter muscles of patients with a "deep bite" than in the ones with an "open bite." In addition, in patients with class III malocclusion, the expression of these genes was significantly higher than in patients with class II malocclusion. Therefore, the authors suggest that epigenetic regulation through coordination of the actions of both KAT6B and HDAC4 can be essential for the entire motor complex of mastication, during the development of skeletal malocclusion.[50]

One of the unresolved issues in stomatology is alveolar bone resorption, due to periodontal diseases, inflammatory processes, or traumatic injuries. To address this problem, Cho et al. analyzed the osteogenic potential of human gingival fibroblasts (HGFs) via a direct transdifferentiation (stem cell reprogramming and differentiation, not only to tissues of origin, but also to other tissues) from HGFs to functional osteoblasts, through epigenetic modification and osteogenic signaling, with bone morphogenetic protein 2 (BMP2). It turned out that HGF treatment with 5-aza-2'-deoxycytidine (5-aza-dC) induced demethylation in the hypermethylated CpG islands of the osteogenic lineage marker genes *RUNX2* and ALP. Furthermore, subsequent BMP2 treatment drove the fibroblasts to osteoblast lineage.

The osteoblastic alterations, mediated by epigenetic modifications, demonstrated the changed methylation patterns in the RUNX2 and ALP promoter regions, as well as their effect on gene expression (Table 1). Based on these

findings, the authors concluded that the epigenetic modification permits the direct programming of HGFs into functional osteoblasts, indicating that this approach could initiate a potential new therapeutic direction in alveolar bone regeneration.[51]

Anomalies of dental development

Developmental dental anomalies represent marked deviations from the normal number, shape, size, and degree of tooth development. Both local and systemic factors can contribute to these abnormalities, and can be inflicted before or after birth. Aberrations in the normal number of teeth include hyperdontia (supernumerary or excess teeth), hypodontia (a congenital lack of 1 or more teeth or missing teeth) and oligodontia (a developmental absence of 6 or more teeth, excluding the 3rd molars). Anomalies in the shape of teeth include microdontia (teeth that are smaller in size) and macrodontia (teeth that are larger in size than normal). In addition, various anomalies in shape can be present (e.g., dens invaginatus, talon cusp, dens evaginatus, gemination, fusion, root dilacerations, taurodontism, and concrescence). Such anomalies, in addition to their appearance, often create different dental problems, as well as difficulties during dental treatment.[52] Hypodontia is caused by complex interrelations between genetic, epigenetic and environmental factors during dental development. In a pilot study that examined 6,636 human genes, conducted by Wang et al., it was found that there are significant differences in the level of genome methylation between people with hypodontia, in whom methylation increased, and the control group (people without hypodontia) (Table 1). Since the DNA samples in this study were only collected from the oral epithelial cells, the authors suggest that in the future additional studies focused on some other types of cells participating in odontogenesis would be merited. Furthermore, a larger sample size is needed to explore in depth the epigenetic mechanisms related to the development of hypodontia. Although the study by Wang et al. was only a pilot study, it highlighted the key role of DNA methylation in hypodontia.[53] Similarly, studies on monozygotic pairs of twin, in which there were differences in the number of absent or additional teeth, also confirmed the impact of epigenetic modifications.[54–56]

Oral cavity carcinomas

In general, the prevalence of oral cavity carcinomas in children is much lower than in adults, and the majority of them develop in the oral mucosa (about 70%) (e.g., on the tongue), in the maxillary and mandibular bones (very rarely), in odontogenic tissues (about 25%), and in the salivary glands (about 5%). In general, the prevalence of oral cavity carcinomas increases with age and men are more predisposed to them.

Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is the most common malignant epithelial neoplasm of the oral cavity. It is often characterized by a heterogeneous clinical picture and an aggressive course. For these reasons, an early diagnosis and effective therapy are essential, including the emerging epigenetic approaches on top of the standard medical care (Table 1).

In their study, Langevin et al. identified in oral rinse samples of patients with OSCC new prognostic epigenetic biomarkers which can predict overall survival in OSCC. In particular, they identified 7 novel DNA methylation loci, 1 of which was validated using a custom pyrosequencing assay. The authors found that DNA methylation occurs in the gene encoding for gamma-aminobutyric acid B receptor 1 (GABBR1) (cg21022792). Hopefully, in the future, the application of this noninvasive test can help predict the survival of patients with OSCC.[57] Furthermore, in a recent study by Ribeiro et al., some additional epigenetic biomarkers for the potential prediction of survival in patients with OSCC were indicated. For instance, it was found that the methylation of the WT1 gene promoter is related to a better survival outcome in patients with OSCC. In contrast, the methylation of MSH6 and the GATA5 gene promoter is related with a worse survival outcome in OSCC patients (Table 1). In addition, the authors have shown that the methylation of the PAX5 gene promoter is linked with carcinoma of the tongue.[58]

According to a study on the South Indian population, it has been reported that some other epigenetic changes might contribute to the development of OSCC. In particular, it has been determined that the phosphatase and tensin homolog (*PTEN*) and *p16INK4a* (*p16*) genes are tumor suppressor genes associated with epigenetic alterations. In this study, the authors found that the low level of expression of *PTEN* and *p16* genes due to DNA methylation might contribute to the development of carcinoma, and thus, it can be useful in the prognosis of OSCC (Table 1). Moreover, the authors suggested that epigenetic changes in these genes might represent a valuable biomarker for the early detection of OSCC.[59]

Similarly, the expression of γ-synuclein (SNCG) is related to the development of various carcinomas, including OSCC (Table 1). In their study, Cheng et al. examined a correlation between DNA methylation in the *SNCG* gene and the development of OSCC, including the clinical symptoms of this malignancy. It was found that positive *SNCG* expression in patients with OSCC significantly correlated with cancer staging and lymph node metastasis. However, *SNCG* methylation did not correlate with its genetic expression or clinical-pathological variables in OSCC tissues. In addition, the authors suggested that DNA methylation in *SNCG* might cause progression in OSCC.[60] The results of a meta-analysis by Singh et al., including studies published from 2000 to 2015, have revealed that the research

on epigenetic mechanisms can provide useful knowledge on novel biomarkers for early diagnosis, prognosis and treatment of oral cavity carcinomas or some other oral lesions.[2]

The role of epigenetics in future research and implications for dental practice

Future research exploring the role of epigenetics in oral diseases will broaden our knowledge of how epigenetic patterns affect the phenotypical expression of diseases such as periodontitis, dental caries, congenital orofacial malformations, and oral cancer.[3]

In particular, this research will help us to understand how the "interplay" between genes and the oral microbiome or local biofilm can affect epigenetic mechanisms (in the periodontal tissues and oral mucosa), in order to modify the inflammatory or immune responses. Moreover, the relevant epigenetic biomarkers need to be evaluated in preclinical and clinical studies.[4]

Furthermore, epigenetic remodeling of cells (e.g., dental pulp) to a pluripotent state creates the potential for epigenetic reprogramming that can possibly be useful in healing local tissue injuries. Unquestionably, future studies are necessary to address these issues and to explore the impact of epigenetics on dental health and diseases. This, in turn, will allow the development of innovative, safe and effective therapies, so that dental practice can benefit from new opportunities for the diagnosis, treatment and prevention of oral diseases that are difficult to manage.[5] Furthermore, there is a chance to develop an epigenetic profile of an individual patient in order to deliver personalized dental care. Finally, the possibility of screening for potential oral health problems (starting from early childhood) might prevent diseases, attenuate their course, or improve the patient's functional level and quality of life.[6]

Conclusions

The field of epigenetics is rapidly developing and there is emerging evidence that environment and lifestyle can interact with the genome to influence epigenetic changes. Epigenetics plays an important role in gene regulation via the main mechanisms, such as DNA methylation, histone modifications and non-coding RNAs. These mechanisms affect gene expression. Exogenous factors (e.g., diet, physical activity, medications, toxins, psychophysical stress, traumatic experiences, and substance abuse), as well as inflammatory processes, can cause alterations in epigenetically regulated gene expression. In addition, epigenetic changes can contribute to the development and progression of certain diseases, such as periodontal disease, dental caries, congenital orofacial defects, and oral cancer. In summary, epigenetic modifications are potentially reversible and can be influenced by several lifestyle factors. Therefore, a deep understanding of these modifications will help to determine novel therapeutic targets in dental diseases, guided by appropriately indicated epigenetic biomarkers. Hopefully, these emerging biomarkers will allow the early detection, diagnosis, prognosis, and therapy of some common oral diseases.

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Treatment of ovarian endometrial cysts in the context of recurrence and fertility

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online) *Adv Clin Exp Med*. 2019;28(3):407–413

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Funding sources
None declared

Conflict of interest
None declared

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Received on April 4, 2017
Reviewed on May 6, 2017
Accepted on May 5, 2018

Published online on January 15, 2019

Abstract

An approach to ovarian endometrial cysts has changed considerably during recent years, especially in regard to treatment of recurrent endometriosis, fertility sparing and infertility management. Surgical treatment is the primary therapeutic option. The most efficient types of treatment are radical procedures involving adhesiolysis, removal of the cyst along with its capsule and any remaining endometriotic foci. However, small asymptomatic cysts should not be treated surgically, especially in patients older than 35 years. Surgical treatment can be considered in infertile women and those who failed to get pregnant despite 1–1.5 years of trials, as well as in cases in which in vitro fertilization is not an option. Also large cysts, with more than 4 cm in diameter, should be treated surgically due to the risk of their rupture or torsion. The most efficient preventive measure for recurrent ovarian endometriosis is unilateral oophorectomy with sparing the contralateral ovary. Such a procedure should be considered in women who are no longer interested in childbearing or present with another endometriotic cyst in the same ovary. The role of pharmacotherapy is fairly limited; it should be considered in patients in whom diffuse endometriosis is associated with pain. Therapeutic agents from the following groups can be used: estrogen-progestin preparation, gestagens, including progesterone-releasing intrauterine systems and gonadotropin-releasing hormone agonists. Women with infertility should get pregnant as soon as possible, and in patients who failed to get pregnant and/or are older than 35 years, in vitro fertilization should be the treatment of choice.

Key words: endometriosis, pharmacotherapy, surgical treatment, endometrioma

Cite as
Nowak-Psiorz I, Ciećwież S, Brodowska A, Starczewski A. Treatment of ovarian endometrial cysts in the context of recurrence and fertility. *Adv Clin Exp Med.* 2019;28(3):407–413. doi:10.17219/acem/90767

DOI
10.17219/acem/90767

Introduction

Endometrial ovarian cysts are one of the more common gynecological disorders found in women of reproductive age. They are frequently the cause of surgical interventions, undertaken not only by gynecologists but also by pelvic surgeons. Therefore, at least in this context, endometrial ovarian cysts should be considered an interdisciplinary problem.

Endometriosis is a chronic benign estrogen-dependent disease. It is observed primarily in patients of reproductive age, and its prevalence in this population is estimated at 5–10%. Endometriosis is defined as the presence of active endometrial tissue outside the uterine cavity, usually on the peritoneum of the minor pelvis, in the myometrium, ovaries and fallopian tubes, as well as extraperitoneally. Endometriotic lesions can be also found in the intestines, urinary bladder, lungs, and even in the brain. Based on the localization of the lesions, the disease is classified as peritoneal, ovarian or deep infiltrating endometriosis.[1]

Etiopathogenesis of endometriosis is still not fully understood. There are several theories on the etiology of this condition. The most widely accepted one is Sampson's theory according to which formation of ectopic endometrial tissue is a consequence of retrograde menstruation. During this process, some of the endometrial debris leaves the uterus with small volumes of menstrual blood, reaches abdominal cavity via the fallopian tubes and is implanted into peritoneum, usually within the pelvis.[2] Furthermore, immune and genetic factors are postulated to play a crucial role in the etiopathogenesis of endometriosis.[3]

The most common manifestations of endometriosis include painful menstrual periods with heavy menstrual bleeding, pelvic pain, dyspareunia, infertility, and sometimes pain during voiding or defecation. Ovarian endometriosis is the most common form of this condition. Ovarian endometrial cysts (endometriomas) are found in 20–55% of women with endometriosis.[4]

An ovarian mass can be qualified as an endometrial cyst based on its ultrasonographic presentation, using the criteria that have been published by the International Ovarian Tumor Analysis (IOTA) collaboration in 2013.[5] These criteria include size, shape, echogenicity of the lesion, structure of its capsule, presence of any projections to the cyst's lumen, vasculature, and relationship with surrounding anatomical structures. Currently, differential diagnosis of endometrial cysts and ovarian malignancies can be conducted promptly with the aid of an online application available on the IOTA website.[6] Another parameter that can be used in the differential diagnosis is the concentration of CA-125, which is usually slightly elevated in patients with this condition.[7]

The approach to ovarian endometrial cysts has changed considerably during recent years, especially regarding the treatment of recurrent endometriosis, fertility sparing and infertility management. Current recommendations of international scientific bodies are based on the results of published meta-analyses and randomized trials.

Watchful waiting

There is a general consensus that small ovarian endometrial cysts, up to 3 cm in diameter, should be left untreated.[1,8] However, 2 questions arise: what is the maximal duration of the expectant management and should it be considered in all patients with endometriosis? With no doubt, the expectant management is excluded in women with pelvic pain; in such cases, surgery is the treatment of choice.[9] However, is it the same with asymptomatic cases? It was shown that the formation of ovarian endometrial cyst results in a decrease in ovarian reserve, which progresses further if the lesion persists longer or enlarges. This may impair fertility, i.e., due to significantly lower ovulation rate and premature menopause.[10] Furthermore, the presence of endometrial cysts results in the formation of, frequently massive, solid adhesions between the ovary, fallopian tube and broad ligament of the uterus, which also decrease the likelihood of pregnancy. All this data supports surgical treatment, especially in young women with infertility.[11] However, surgical treatment is also known to decrease the ovarian reserve, thus promoting reproductive aging and accelerating the onset of menopause,[12] especially in the case of repeated surgeries. Moreover, one should consider the risk of postoperative adnexal adhesions that may considerably reduce the mobility of the fallopian tubes or result in their complete obliteration.

Treatment of infertile women above 35 years of age should be discussed separately. Surgical management of such patients will result in a decrease in their already low ovarian reserve. In such cases, especially in women with advanced endometriosis, with mechanical or male factor infertility, in vitro fertilization is an efficient option. According to Tsoumpou et al.,[13] surgical treatment of patients prior to implementation of assisted reproductive technologies (ART) does not improve their outcomes. The only exception pertains to large endometrial cysts that hinder access to ovarian follicles.[13]

To summarize, one should be cautious when considering surgical treatment of small ovarian endometrial cysts. Small asymptomatic cysts should not be treated surgically, especially in patients older than 35 years. Surgical treatment should be considered in infertile women and patients who failed to get pregnant despite 1–1.5 years of trials, as well as in cases in which in vitro fertilization is not an option.[8] Also large cysts, with more than 4 cm in diameter, should be treated surgically due to the risk of their rupture or torsion.

Surgical treatment

Surgical treatment is the principal method of endometrial cyst management. The least efficient technique, nowadays considered mostly obsolete, involves puncturing the cyst under ultrasonographic guidance or during

laparoscopy, aspiration of its contents, irrigation and, whenever necessary, administration of a sealant. However, this procedure frequently led to complications, such as the formation of abscesses and peritoneal adhesions. Moreover, up to 80–90% of patients present with recurrence already 6 months after the procedure.[14] Another surgical procedure, still used in many centers, is a cystectomy with irrigation of the lumen of the cyst and coagulation of its capsule.[15] Bipolar coagulation, optimally argon plasma coagulation, or laser coagulation should be used to spare normal ovarian tissue.[16] Unfortunately, this technique also does not guarantee the complete destruction of the capsule of the cyst. Saleh and Tulandi[14] showed that more than a half of patients presented with recurrent cysts as early as 2 years after the procedure, and 42 months post-surgery the recurrence rate increased to 60%.

Currently, laparoscopy is considered the "gold standard" in the management of ovarian endometrial cysts. The laparoscopic procedure includes freeing the ovary from adhesions, cystectomy, irrigation of the cyst, and complete excision of its wall with least possible injury to normal ovarian tissue. Bleeding vessels should be coagulated with a bipolar electrode, optimally using argon plasma coagulation. Also, all other endometriotic foci present in the pelvis should be coagulated carefully whenever feasible.[17,18] The abovementioned technique is associated with the highest pregnancy rates in patients with infertility.[18,19] Moreover, the removed ovarian cyst can be subjected to a histopathologic examination, which is of vital importance, considering that ca. 0.8–0.9% of endometriomas turn out to be malignant. In one study, up to 13% of endometriomas were eventually identified as borderline ovarian tumors.[20] The ovarian-sparing treatment with complete removal of the capsule of the cyst results in a considerable decrease in the endometrioma recurrence rate. However, between 10% and 40% of the patients may present with recurrent cysts, and the recurrence rate was shown to increase with the time elapsed since surgery.[21,22]

The severity of endometriosis turned out to be a key determinant of sustained remission after surgical treatment. The severity of the disease is determined with revised American Society for Reproductive Medicine (rASRM) scoring system, and many previous studies showed that the higher the rASRM score and severity of endometriosis, the greater the risk of its recurrence.[21,23,24] Liu et al. showed that the rASRM severity grade correlates with the recurrence rate of endometriosis, but its prognostic value is relatively poor.[25] However, this hypothesis is not supported by all the authors. For example, Koga et al. did not find a significant association between the severity of endometriosis and its recurrence rate.[26] Yun et al. analyzed the role of various components of the rASRM scoring system as potential risk markers for recurrent endometriosis.[27] They showed that the risk of recurrence increases considerably in patients with adhesions involving the ovaries and/or fallopian tubes and/or with complete obliteration of the cul-de-sac.[27] Consequently, the presence of adhesions, especially involving adnexa and cul-de-sac, seems to be an important predictor of recurrence. This hypothesis was also confirmed by other authors.[24,25,27]

Bilateral endometrial cysts are generally considered to be a prognostic factor of recurrence, although not all previous studies confirmed this relationship.[26] An association between the localization of the cyst and the risk of recurrence is unclear. However, the results of some studies suggest that endometriomas located in the left ovary are more likely to recur.[28] Also, the available data on the influence of the diameter of the cyst on the recurrence rate is inconclusive. Nevertheless, a larger diameter of the cyst is generally considered an unfavorable prognostic factor.[21,26,29] However, a minimum diameter of the lesion associated with unfavorable prognosis has not been established thus far. Moreover, Ghezzi et al. showed that the diameter of the cyst has no influence on the recurrence rate.[28] Similar findings were also reported by Porpora et al.[24]

Similarly, no consensus was reached regarding the influence of the patient's age on the risk of recurrence. According to many researchers, young age has an unfavorable effect on the duration of remission after surgical treatment. This is probably related to the fact that younger women present with more aggressive forms of endometriosis and higher postoperative blood concentrations of estrogens.[21,25] However, Koga et al.[26] did not find an association between the patient's age and the duration of remission, and according to Parazzini et al.,[30] the risk of recurrence increases with age.

Pregnancy was shown to exert a beneficial effect and to reduce the risk of recurrent endometriosis, and as such is even considered a protective factor. Elevated concentrations of progesterone in pregnancy may inhibit the growth of endometriotic foci and attenuate related inflammation.[23,31] A relatively less often considered prognostic factor of recurrent endometriosis is the preoperative level of CA-125. A high preoperative level of CA-125 was postulated to predispose to recurrence. Due to its low specificity, this antigen is not useful as a diagnostic marker of the disease, but still may play an important role in the monitoring of its treatment.[7] In turn, no associations were found between the presence of uterine leiomyoma or adenomyosis and recurrence rate.[26]

Nowadays, molecular background of endometriosis is a subject of ongoing research. Considering the estrogen-dependence of the disease, estrogen receptor (ER), present in 2 forms, ER-α and ER-β, was an obvious target of research. Some studies showed an association between ER-α polymorphism and the risk of recurrence.[32] Another risk marker for recurrent endometriosis may be cyclooxygense-2. Increased activity of this enzyme, catalyzing synthesis of prostaglandins, was observed in endometriotic lesions in women who later presented with recurrence.[33]

The most efficient preventive measure for recurrent ovarian endometriosis is unilateral oophorectomy with sparing the contralateral ovary. Such a procedure should

be considered in women who are no longer interested in childbearing or present with another endometriotic cyst in the same ovary. Importantly, the procedure should be extended to adhesiolysis and the removal of all remaining endometriotic foci. Namnoum et al.[34] demonstrated that residual adhesions and endometriotic foci are associated with an 8-fold increase in reoperation risk. Hysterectomy with the removal of adnexa can be an option in perimenopausal women with endometrial cysts, diffuse peritoneal endometriosis or deep infiltrating endometriosis, especially with concomitant pain.[35]

Pharmacotherapy

Currently, pharmacotherapy is considered to play a secondary role during the postoperative period, and as such is implemented only in selected cases. Furthermore, preoperative pharmacotherapy was shown to exert an unfavorable effect on ovarian endometrial cysts. Koga et al.[26] demonstrated that pharmacotherapy constitutes a risk factor for postoperative recurrence, since causing atrophy and fibrosis may hinder intraoperative identification of all endometriotic foci. In such cases, resection is more likely to be incomplete and is more technically demanding.[26] Similar conclusions were also reported by Muzii et al.[36] Moreover, preoperative pharmacotherapy was shown to be associated with an increase in the proportion of dyskariotic cells and to slow down development of eukaryotic cells within the cyst's wall.[37]

Postoperative pharmacotherapy is recommended after incomplete resection of endometriotic foci or in patients with concomitant pain. Endometriosis is an estrogen-dependent condition and, therefore, the principal aim of pharmacotherapy is to suppress ovarian function and to promote the atrophy of endometriotic lesions. Therapeutic agents from the following groups can be used: estrogen-progestin preparation, gestagens, including progesterone-releasing intrauterine systems and gonadotropin-releasing hormone (GnRH) agonists. Due to its multiple side effects, danazol is no longer recommended in pharmacotherapy of endometriosis.[38] Frequently, the agents mentioned above are simultaneously used as contraceptives, and, therefore, specific agents should be selected on an individual basis with their potential side effects taken into consideration.

Two-component oral contraceptives play an important role in the prevention of recurrent endometriotic lesions and the attenuation of pain. Beneficial effects of these preparations are associated with the inhibition of ovulation. Less intensive menstrual bleeding and lack of retrograde menstruation prevent endometrial debris spreading outside the uterus. Furthermore, estroprogestin contraceptives suppress the proliferation of endometriotic tissue. Their superiority to other therapeutic options is associated with their small number of side effects, good tolerance and reasonable price.

Due to the frequent use of oral contraceptives, their efficacy was a subject of many previous studies. During a 24-month postoperative follow-up, Seracchioli et al.[39] found recurrent endometriomas in 29% of women who did not use any contraceptives, as well as in 14.7% and 8.2% of patients who used cyclic and continuous contraceptives, respectively. The same group showed that the diameter of recurrent endometrial cysts in women who used hormonal preparations was markedly smaller than in patients who did not receive this form of therapy. Also, the growth rate of recurrent lesions in the former group was reduced. The most beneficial effects were observed in patients taking continuous contraceptives.[40,41] Zorbas et al.[42] compared the effects of continuous and cyclic contraceptives. The risk of recurrence turned out to be markedly higher in patients using cyclic contraceptives (16.6%) than in those undergoing continuous therapy, probably due to the fact that the latter resulted in constant suppression of inflammatory processes.[42] Cucinella et al.[43] analyzed the efficacy of oral contraceptives depending on the type of progestin they contained. Contrary to other authors, they showed that the type of progestin (desogestrel, gestodene, dienogest) exerted no effect on the efficacy of oral contraceptives in the prevention of recurrent endometriosis. In contrast, the results of other studies point to more beneficial effects of dienogest, but this may be a consequence of its synergy with endogenous estradiol.[44] Surprisingly, however, Muzii et al.[45] showed that postoperative administration of low-dose contraceptives does not exert a significant effect on the recurrence rate of endometriosis,[45] but may only prolong the time to recurrence. The absence of significant effects of treatment with contraceptives on the recurrence rate of endometriosis was also reported by Koga et al.[26] However, the latter authors analyzed the effects of short-term administration of oral contraceptives (<12 months) and it is generally believed that it is the long-term therapy that shows the most evident benefits. Furthermore, the protective effect of oral contraceptives dramatically decreases after their withdrawal. Consequently, continuation of the therapy is recommended until the patient decides to get pregnant.[24,31]

Of all gestagens, dienogest is used is monotherapy most often. It inhibits the growth of endometriotic foci, markedly attenuates pain and rarely produces side effects when administered at 2 mg per day.[44,46] The results of previous studies suggest that dienogest therapy may also result in a lower recurrence rate of endometriosis. According to Yanase et al.,[47] a 6-month cyclic therapy with this gestagen after previous surgical treatment of endometriosis protects against the recurrence of the disease, and this protective effect may last for approx. 4 years. Similar findings were also reported by Ouchi et al.[22]

Also levonorgestrel-releasing intrauterine systems found an application in the adjuvant treatment of endometriosis. Their principal advantages include the possibility to achieve and maintain high local concentrations of levonorgestrel

without concomitant suppression of the ovary. The system turned out to be particularly effective in the control of pain, but its role in the prevention of recurrent endometriosis is put into question. The only documented beneficial effect was a longer time to recurrence.[48] Another study compared the efficacy of a levonorgestrel-releasing intrauterine system and dienogest-containing oral contraceptives. While the latter turned out to be markedly more effective in attenuating pain, they were only slightly more effective in preventing recurrence.[49]

GnRH analogues are another group of therapeutics that have been tested for their potential application in the prevention of recurrent endometriosis. Their beneficial effects were observed if they had been administered for at least 6 months post-surgery. However, a 3-month therapy exerted a markedly less pronounced effect on the recurrence rates. Aside from reducing the recurrence rate, GnRH analogues were also shown to prolong the time to recurrence. However, the potential side effects of the therapy need to be emphasized, as it was shown that even a 6-month therapy with GnRH analogues may promote bone demineralization.[50]

Aromatase inhibitors are a novel group of therapeutics. Alborzi et al.[51] compared the efficacy of aromatase inhibitors and GnRH analogues in the prevention of recurrent endometriosis after laparoscopic treatment. Both agents were administered for 2 months, and then the patients were followed up for another 12 months. The recurrence rate in patients treated with aromatase inhibitors was similar as in women who received GnRH analogues. Surprisingly, a similar recurrence rate was also observed in the controls (patients without any pharmacotherapy). However, it is the duration of the therapy which probably has a considerable effect on its outcome.

Management of endometrial cysts in women with infertility

Management of ovarian endometrial cysts in women with infertility should be discussed separately. As mentioned above, endometrial cysts undoubtedly compromise fertility.[52–54] On the other hand, surgical treatment is associated with a decrease in ovarian reserve and the induction of peritoneal adhesions. Concentration of anti-Müllerian hormone was demonstrated to decrease markedly immediately after surgery and remain at a lower level for 6–9 months.[55,56] Surgical treatment results in a permanent, ca. 10%, decrease in antral follicle count.[57] The influence of surgical treatment on ovarian reserve is particularly evident in the case of cysts larger than 4 cm in diameter.[57,58] Consequently, surgical treatment is not recommended in patients with small cysts and in women older than 35 years. Such patients should get pregnant as soon as possible, and whenever needed they can be subjected to the induction of ovulation and intrauterine insemination,[8] although the effectiveness of the latter procedure in this setting is often put into question.[59,60] In patients who failed to get pregnant and in women older than 35 years, in vitro fertilization is the treatment of choice.[8,59,60] Surgical treatment should be limited solely to the large cysts that may hinder ovarian puncture,[13] and to the lesions being suspicious for malignancy based on their ultrasonographic appearance.

Take-home message

In this paper, we have reviewed the results of studies conducted at various centers over a 30-year period, to demonstrate the efficacy of the treatment for ovarian endometrial cysts in the context of their recurrence. To the best of our knowledge, the number of similar reviews is sparse, although such publications would with no doubt raise the awareness of the appropriate treatment for this most common type of endometriosis among gynecologists, and would support them in the selection of the most effective therapeutic option. Still, coagulation of the capsule of the endometrial cyst with the state-of-the-art devices, such as lasers or plasma knifes, is preferred at some centers, without consideration of recurrence rates after such procedures. The published data and our own clinical experiences suggest that surgery should constitute the primary treatment for ovarian endometriosis. The most efficient types of surgery are radical procedures involving adhesiolysis, removal of the cyst along with its capsule and any remaining endometriotic foci. The role of pharmacotherapy is fairly limited; it should be considered in patients in whom diffuse endometriosis is associated with pain. Women with infertility should get pregnant as soon as possible, and in patients who failed and/or are older than 35 years, in vitro fertilization should be the treatment of choice.

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Response to the article "Perioperative standards for the treatment of coagulation disorders and usage of blood products in patients undergoing liver transplantation used in the Clinic for Transplant Surgery in Wrocław"

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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*. 2019;28(3):415–416

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Funding sources
None declared

Conflict of interest
None declared

Received on September 24, 2018
Accepted on February 18, 2019

Published online on March 12, 2019

Abstract

We read with interest the article by Łukaszewski et al. published in Advances in Clinical and Experimental Medicine (*Adv Clin Exp Med*. 2018;27(9):1211–1215, published online on July 18th, 2018, as ahead of print). As enthusiasts of promoting global assays of hemostasis, we would like to commend the authors for their commitment and effort in their implementation and clinical application. As the authors rightly pointed out in the article, perioperative care of liver transplantation (OLTx) patients is challenging for transplant team members due to the risk of severe changes in global hemostasis. Łukaszewski et al. presented a single center experience in using rotational thromboelastometry (ROTEM) to monitor hemostasis during liver transplantation. In our center, this method has been used routinely since 2008. So far it has been used in over 400 patients undergoing OLTx. Considering the potential contribution to thrombotic complications (including portal vein thrombosis after liver transplantation), we believe that antifibrinolytic treatment should be reserved for patients with active bleeding and hyperfibrinolysis confirmed by ROTEM. The available literature indicates an increased risk of thrombotic complications in patients receiving antifibrinolytic therapy. This raises an important question for the authors about the reason for using Exacyl® in all 12 of the cases presented, even in patients who did not require any blood product transfusion. We hope that our letter will open up further discussion on this subject, which is undoubtedly crucial for OLTx patients' safety.

Key words: hemostasis, liver transplantation, thromboelastometry, hyperfibrinolysis, tranexamic acid

Cite as
Pluta J, Cieniewicz A, Trzebicki J. Response to the article "Perioperative standards for the treatment of coagulation disorders and usage of blood products in patients undergoing liver transplantation used in the Clinic for Transplant Surgery in Wrocław". *Adv Clin Exp Med*. 2019;28(3):415–416. doi:10.17219/acem/104539

DOI
10.17219/acem/104539

Response to the Article "Perioperative standards for the treatment of coagulation disorders and usage of blood products in patients undergoing liver transplantation used in the Clinic for Transplant Surgery in Wrocław"

We read with interest the article "Treatment of Coagulation Disorders" by Łukaszewski et al. published in Advances in Clinical and Experimental Medicine (*Adv Clin Exp Med*. 2018;27(9):1211–1215, as ahead of print on July 18th, 2018). As enthusiasts of promoting global assays of hemostasis, we would like to commend the authors for their commitment and effort in their implementation and clinical application.

As the authors rightly pointed out in the article, perioperative care of liver transplantation (OLTx) patients is challenging for transplant team members due to the risk of severe changes in global hemostasis. Liver failure leads to specific disorders in the coagulation and fibrinolysis system, referred to by Lisman et al. as "rebalanced hemostasis".[1] Hemostasis assessment in OLTx patients is extremely difficult – often impossible when using only routine coagulation screening tests. Global assays of hemostasis such as rotary thromboelastometry (ROTEM) or thromboelastography (TEG) allow better understanding of existing coagulation abnormalities, which facilitates appropriate hemostatic management.

In their article Łukaszewski et al. presented a single center experience in using ROTEM to monitor hemostasis during liver transplantation. In our center, this method has been used routinely since 2008.[2] So far it has been used in over 400 patients undergoing OLTx. We fully agree with Łukaszewski et al. regarding the benefits of implementing this method. It allows early recognition of hyperfibrinolysis, which is undetectable using classical laboratory coagulation tests. Treatment, if necessary, includes administration of antifibrinolytics, currently tranexamic acid (Exacyl®).

Łukaszewski et al. stated that they introduced antifibrinolytic treatment when the maximum clot lysis (ML) in the EXTEM test was above 15%. An important issue during ML evaluation is the time when lysis occurred (the time elapsed from the beginning to the end of measurement): it has to be specified. The Essen Algorithm, mentioned by Łukaszewski et al., includes the clot lysis index after 60 min (CLI60); administration of antifibrinolytics should be considered at values below 85%.[2,3] The clot lysis index describes the percentage of maximum clot firmness (MCF) in relation to the ML value in a particular minute of the test. Maximum clot lysis values over 15% may be regarded as physiological if the test duration is significantly longer than 60 min.

Additionally, we want to highlight that the occurrence of hyperfibrinolysis in liver cirrhosis patients during OLTx, especially late hyperfibrinolysis (over 30 min of ROTEM measurement) after reperfusion, is a self-limiting process that does not require antifibrinolytic therapy.[3] Inhibition of fibrinolysis is recommended only in cases of clinical manifestations of coagulopathy.

In their results section, the authors stated that all the patients with confirmed hypofibrinogenemia received fibrinogen concentrate and Exacyl®. We share their opinion that fibrinogen concentrate is the safest fibrinogen substitute in cases of confirmed hypofibrinogenemia. However, our concerns were raised about the prophylactic administration of Exacyl® described in the article. According to the previously mentioned Essen Algorithm, routine administration of tranexamic acid is suggested only for patients with hepatitis fulminans with no history of thrombosis, or patients with MCF (EX) ≤35 mm or CT (EX) >80 s.[4,5]

Considering the potential contribution to thrombotic complications (including portal vein thrombosis after liver transplantation), we believe that antifibrinolytic treatment should be reserved for patients with active bleeding and hyperfibrinolysis confirmed with ROTEM. The available literature indicates an increased risk of thrombotic complications in patients receiving antifibrinolytic therapy.[4,6] This raises an important question for the authors about the reason for using Exacyl® in all 12 of the cases presented, even in patients who did not require any blood product transfusion (Table 1, page 4).

We hope that our letter will open up further discussion on this subject, which is undoubtedly crucial for OLTx patients' safety.

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