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Original papers

Reviews

Decoy receptor 3 alleviates hepatic fibrosis through suppressing inflammation activated by NF-κB signaling pathway

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Abstract

Background. Hepatic fibrosis is a reversible pathological process. Inflammatory responses are the prevailing reactions during hepatic fibrosis. Decoy receptor 3 (DcR3) has been reported to have an anti-inflammatory effect.

Objectives. The aim of the study was to investigate the preventive effects of DcR3 on hepatic fibrosis.

Material and methods. Hepatic fibrosis was induced in rats by administering intraperitoneally (ip.) 1% dimethylnitrosamine (DMN). DcR3 plasmid was delivered into rats by intravenous injection. After 4 weeks, the expression of DcR3, TNF-like molecule 1A (TL1A) and α-SMA of the liver tissue were checked. The levels of inflammatory cytokines such as TNF-α, IL-6 and IL-1β were detected using western blotting and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Masson's trichrome staining for histopathological changes of the liver tissue was observed. Finally, the activity of NF-κB in the liver was examined by enzyme-linked immunosorbent assay (ELISA).

Results. A higher expression of DcR3 was observed in rats treated with DcR3 ($p < 0.05$). Histological results showed that DcR3 significantly attenuated pathology in hepatic fibrosis rats. Consistently, mRNA and protein levels of α-SMA, TL1A, TNF-α, IL-6, and IL-1β were repressed in the liver tissue after treatment with DcR3 ($p < 0.05$). Moreover, DcR3 also inhibited the activation of NF-κB in the liver tissue ($p < 0.05$).

Conclusions. This study demonstrated that DcR3 attenuated liver injury and inflammatory responses in rats with hepatic fibrosis. We suggest DcR3 may be a prophylactic and promising therapeutic agent in the treatment of hepatic fibrosis.

Key words: decoy receptor 3, hepatic fibrosis, inflammatory response, NF-κB, TNF-like molecule 1A

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Introduction

Hepatic fibrosis is a wound-healing response to chronic liver injury. It is a reversible pathological process, but results in cirrhosis or liver failure if inappropriately treated.[1] The pathological characteristic of hepatic fibrosis is sustained liver damage leading to chronic inflammation and liver death, followed by the release of cytokines by activated Kuppfer cells and endothelial cells, which leads to the formation of liver fibrosis by excessive accumulation of scar tissue and deposition of extracellular matrix (ECM) proteins as a result of further activation of hepatic stellate cells (HSCs).[2–6] Therefore, inhibiting inflammation and liver cell apoptosis after liver damage is the key point to reverse liver fibrosis.

Decoy receptor 3 (DcR3), a soluble protein, belongs to the tumor necrosis factor receptor (TNFR) superfamily.[7] DcR3 is found in humans but not in mice, and its encoding gene is located on human chromosome 20q13.3.[8] DcR3 competitively interacts with its primary ligands, including Fas ligand (FASLG), T lymphocytes (LIGHT) and TNF-like molecule 1A (TL1A), to negatively regulate their ligand-receptor downstream signaling.[9–11] It has been reported that DcR3 was overexpressed in various human cancers by negatively regulating Fas-mediated apoptosis.[8,12] Moreover, growing evidence has demonstrated that DcR3 is involved in the regulation of immune responses by activating "reverse signaling" after binding with their receptors.[13] Treatment with DcR3 protein reduces the levels of cytokines, including TNF-α, IL-6 and IFN-γ in the blood and peritoneal lavage fluid of mice with sepsis.[14] Another study discovered that DcR3 reduced the TLR2-induced cytokine production by B cells.[15] All this evidence suggests that DcR3 plays an important role in the inflammatory response.

TL1A has been identified as a ligand of death receptor 3 (DR3) and DcR3. The major downstream signaling pathway of DR3/TL1A is the NF-κB pathway, which is involved in the treatment of inflammation by regulating the secretion of proinflammatory cytokines like TNF-α, IL-6 and IL-1β. More importantly, TL1A can promote the survival and secretion of proinflammatory cytokines by activating T cells in vitro.[10] Shu et el. found that the inhibition of MAPK and NF-κB signaling pathways alleviated carbon tetrachloride (CCl_4)-induced liver fibrosis in mice with Toll-like receptor 5 (TLR5) deficiency.[16] A proteome-wide quantitative phosphoproteomic analysis showed that DcR3 modulates the activity of key kinases critical for the activation of MAP kinases, and NF-κB activation.[17] DcR3 can bind with TL1A by competing with DR3, but the role of DcR3 in the development of liver fibrosis is still unknown. Since DcR3 plays a pivotal role in the inflammatory response by binding to TL1A, we hypothesize that DcR3 may moderate liver fibrosis progress via attenuating inflammatory responses and liver injury.

DcR3 has been supposed to negatively regulate the DR3/TL1A pathway by a competitive combination with TL1A. In the present study, we evaluated the role of the DcR3 gene in the process of 1% dimethylnitrosamine (DMN)-stimulated fibrosis of liver in rats. Our results show that the DcR3 gene may be involved in the progress of fibrosis via the inhibition of NF-κB signaling pathways activated by the DR3/TL1A pathway.

Material and methods

Establishment of hepatic fibrosis rat model

Male Wistar rats (wild-type, WT), weighing 180–220 g, were purchased from the experimental animal center of Jilin University (China). All rats were housed in a temperature- (25°C) and humidity-controlled environment with food and water provided in the cages. All rats were randomly divided into 3 groups as follows: 1. the control group (WT group); 2. the 1% DMN + empty vector group (EV group); 3. the 1% DMN + DcR3 vector group (DcR3 group). DMN (Zhenzhun Ltd., Shanghai, China) was dissolved in saline and finally diluted to 1%. After 1-week adaptation the EV and DcR3 group rats were administered by intraperitoneal (ip.) injection with 1 μL/g body weight 1% DMN 3 times every week for 4 weeks. Meanwhile, WT group rats were similarly injected with an equal volume of saline. The study was approved by the Ethics Committee on Animal Research at the Jilin University Animal Care and Use Committee (No. SYXK (Ji) 2008-0010/0011).

Decoy receptor 3 gene therapy

The pEF1α-IRES-DsRed-Express2-DcR3 was constructed as described previously.[18] Briefly, the DcR3 gene was isolated by the real-time reverese transcription-polymerase chain reaction (RT-PCR) using the forward primer 5'GTCGACATGAGGGCGCTGGAGG3' and reverse primer 5'GGATCC TCAGTGCACAGGGAGGAA3'. The amplified product was cloned into pEF1a-IRES-DsRed-Express2 vector (Takara, Dalian, China) to produce the vectors pEF1α-IRES-DsRed-Express2-DcR3.

The DcR3 group rats were injected with DcR3 plasmid by tail intravenous (iv.) injection, while the EV group rats were similarly administered with an equal EV. All the rats were sacrificed 4 weeks after vector injection. The liver tissues were carefully harvested and stored appropriately until analysis.

Histopathologic examination of liver tissues

The liver tissues were fixed with 4 paraformaldehyde, cut into 4–5 μm thick sections and mounted on slides. Masson's trichrome staining was conducted to evaluate the pathological changes such as collagen deposition in the

liver tissue. The sections were stained in iron hematoxylin solution for 10 min after dewaxing. After wash, the sections were stained in Biebrich scarlet-acid fuchsin solution for 10–15 min. Then, the slices were washed and differentiated in phosphomolybdic-phosphotungstic acid solution for 10–15 min. Next, the sections were transferred into aniline blue solution and stained for 5–10 min, rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2–5 min. Finally, dehydration and mount were performed after wash. The method was described in the handbook of Trichrome Stain (Masson's) Kit (Sigma-Aldrich, St. Louis, USA). All the tissue sections were observed under a microscope.

Determination of DcR3, TL1A, α-SMA, TNF-α, IL-6, IL-1β, and β-actin in the liver by western blot analysis

To detect the expression of DcR3, TL1A, α-SMA, TNF-α, IL-6, IL-1β, and β-actin, total protein of the liver was extracted using RIPA buffer, then the nuclear and cytoplasmic proteins were extracted using a nuclear/cytoplasmic isolation kit (Takara, Dalian, China). The protein concentration was determined by a BCA Protein Assay Kit (Takara, Dalian, China). The protein was separated by 15% SDS-PAGE and transferred onto a PVDE membrane (Bio-Rad Laboratories Inc., Hercules, USA). The membrane was blocked with 5% skim milk powder for 2 h and incubated overnight with the primary antibody. Each membrane was washed and incubated with the secondary antibodies for 1.5 h. The immunoblots were developed using an ECL Advanced Western Blotting Detection Kit (Invitrogen, Carlsbad, USA).

Determination of DcR3, TL1A, α-SMA, TNF-α, IL-6, IL-1β, and β-actin in the liver by real-time reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate the mRNA transcriptional levels of DcR3, TL1A, α-SMA, TNF-α, IL-6, and IL-1β in the livers of each group, the total RNA was isolated using trizol reagent (Takara, Dalian, China) according to the manufacturer's instructions. Then, total RNA was reverse-transcribed to cDNA using the reverse transcription kit (Takara Biological Company, Dalian, Japan). RT-PCR was performed using Eppendorf AG-5341 fluorescence quantitative instrument. The procedure of qPCR was administered as following: 95°C for 3 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 3 min; and a final extension period at 72°C for 10 min. All primers were synthesized by Shanghai Sangon (Shanghai, China) (Table 1). The results were calculated using the $2^{-\Delta\Delta Ct}$ method, and the gene GAPDH was used as an internal control.

Table 1. Primer nucleotide sequence of the liver tissue *DcR3*, *TL1A*, *α-SMA*, *TNF-α*, *IL-6*, *IL-1β*, and *β-actin*

Genes	Nucleotide sequence of primer (5'–3')	Product size (bp)
DcR3	F:#CGCTGGTTTCTGCTTGGAG	122
	R:#AGCTGCTGGCTGAGAAGGTG	
TL1A	F:#TCTACTCCCAGATCACATTCCG	180
	R:#ACCAGTTGCTGCTTATTTCACAC	
α-SMA	F:#AGGAGGATTCCGTGCTGTTC	312
	R:#TGGGCTTGATGTTATCTGATTT	
TNF-α	F:#CCCCTTTATCGTCTACTCCTC	134
	R:#TTCAGCGTCTCGTGTGTTTC	
IL-6	F:#CTTCGGTCCAGTTGCCTTCT	228
	R:#GCCTCTTTGCTGCTTTCACA	
IL-1β	F:#TTACAGTGGCAATGAGGATG	131
	R:#TGTAGTGGTGGTCGGAGATT	
β-actin	F:#CGGCTACAGCTTCACCACCA	143
	R:#CGGGCAGCTCGTAGCTCTTC	

DcR3 – decoy receptor 3; *TL1A* – TNF-like molecule 1A; *α-SMA* – smooth muscle actin; *TNF-α* – tumor necrosis factor; *IL-6* – interleukin 6; *IL-1β* – interkeukin 1 beta; F – forward primer; R – reverse primer.

Expression of NF-κB by enzyme-linked immunosorbent assay (ELISA)

The production levels of NF-κB in the liver were assayed by enzyme-linked immunosorbent assay (ELISA) kit (Takara, Dalian, China) according to the manufacturer's protocols.

Statistical analysis and software

The data was analyzed by SPSS 19.0 software, and comparisons between multiple groups were performed by a one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison tests to determine statistical significance. The data was expressed as mean ±SD (standard deviation) and a p-value of <0.05 was considered statistically significant. The homology analysis was performed by BLAST and the structure of protein was predicted by SMART online tool.

Results

DcR3 decreased the inflammatory cytokines levels by suppressing the NF-κB signaling pathway

Inflammation of the liver was reported to contribute to the development of liver fibrosis. This study aimed to investigate the effect of DcR3 on inflammatory cytokines in hepatic fibrosis, and, therefore, the TNF-α, IL-6 and IL-1β levels were detected in the liver. The results showed that the TNF-α, IL-6 and IL-1β mRNA levels in the liver

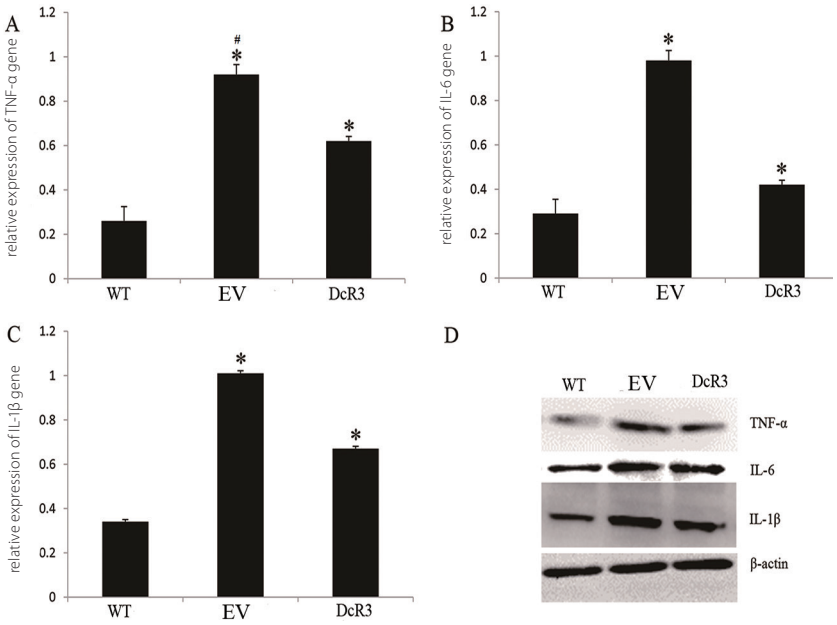

Fig. 1. DcR3 decreased the inflammatory cytokines levels in the liver tissue of 1% DMN-induced hepatic fibrosis rats. A, B and C – analysis of the TNF-α, IL-6 and IL-1β mRNA expression by qRT-PCR, respectively. D – analysis of the TNF-α, IL-6 and IL-1β protein expression by western blot, respectively. Data is shown as mean ±SD of 10 rats in each group

WT – control group (wild-type); EV – 1% DMN + empty vector group; DcR3 – 1% DMN + DcR3 vector group; * $p < 0.05$ compared with WT group; # $p < 0.05$ compared with EV group; DcR3 – decoy receptor 3; DMN – dimethylnitrosamine.

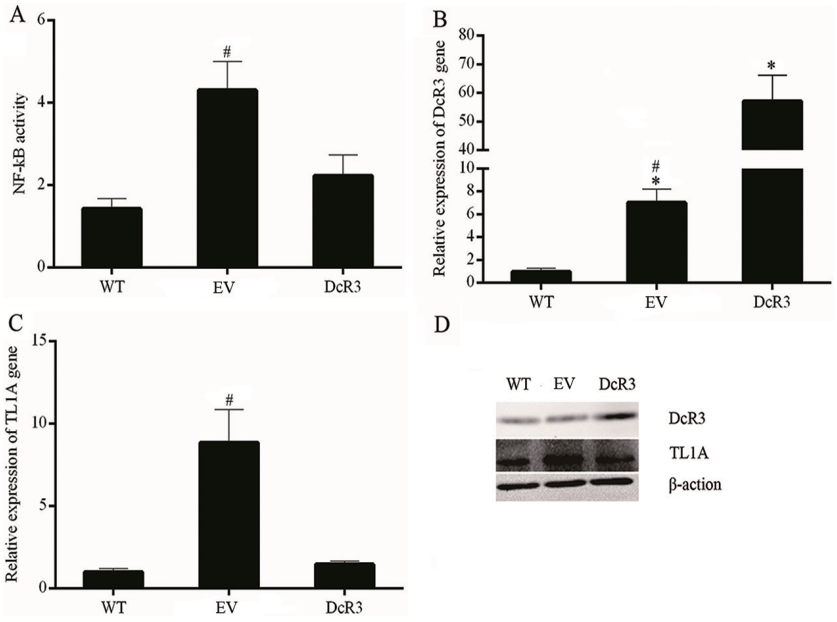

Fig. 2. DcR3 decreased the TL1A levels and inhibited NF-κB activation in the liver tissue of 1% DMN-induced hepatic fibrosis rats. A – analysis of NF-κB activity by ELISA. B and C – analysis of the DcR3 and TL1A mRNA expression by qRT-PCR, respectively. D – analysis of the DcR3 and TL1A protein expression by western blot, respectively. Data is shown as mean ±SD of 10 rats in each group

WT – control group (wild-type); EV – 1% DMN + empty vector group; DcR3 – 1% DMN + DcR3 vector group; * $p < 0.05$ compared with WT group; # $p < 0.05$ compared with EV group; DcR3 – decoy receptor 3; TL1A – TNF-like molecule 1A; ELISA – enzyme-linked immunosorbent assay; DMN – dimethylnitrosamine.

dramatically decreased in the DcR3 group compared with the EV group ($p < 0.05$) (Fig. 1A–C). In addition, western blot analysis also showed that rats treated with DcR3 exhibited lower TNF-α, IL-6 and IL-1β protein levels than the EV group (Fig. 1D). These results indicated that DcR3 alleviated the 1% DMN-induced inflammatory responses in rats.

We found that DcR3 could inhibit the expression of pro-inflammatory cytokines, and then we further studied whether DcR3-mediated protection against liver fibrosis was associated with alterations in NF-κB activation. ELISA tests showed that rats treated with DcR3 secreted lower NF-κB levels compared with EV rats ($p < 0.05$) (Fig. 2D). On the basis of these results, we suggest that DcR3 inhibited the TNF-α, IL-6 and IL-1β production through suppressing the NF-κB signaling pathway.

DcR3 decreased the inflammatory cytokines levels by a combination with TL1A

TL1A was recognized as a ligand of DR3 and DcR3 which negatively regulated the downstream signaling cascades of DR3/TL1A such as NF-κB activation. Therefore, we next wondered whether DcR3 inhibited the production of inflammatory cytokines by binding with TL1A. First, we validated the DcR3 overexpression in hepatic fibrosis rats by western blot analysis and RT-PCR. The results showed that DcR3 increased after injection with DcR3 vector ($p < 0.05$) (Fig. 2A, C). We also examined the expression of TL1A in hepatic fibrosis rats. TL1A protein and mRNA expression was obviously decreased in the DcR3 group compared with the EV group ($p < 0.05$) (Fig. 2B, C). Our results suggest that DcR3 might interact with TL1A to

negatively regulate the downstream signaling cascades of DR3/TL1A.

DcR3 inhibited the inflammatory cell infiltration and collagen accumulation in hepatic fibrosis rats

Next, we evaluated the morphological characteristics of the rat livers by Masson's trichromic staining. No pathological changes or immune reactions were observed in the liver tissue in the WT group. However, the cell swelling, fatty infiltration, extensive accumulation of collagen, cell death, and leukocyte infiltration became overt in the liver tissue in the EV group (Fig. 3A), whereas DcR3 markedly attenuated these pathological changes (Fig. 3A). These results indicate that hepatic fibrosis rats had marked fibrotic changes in the livers after the treatment with DcR3.

DcR3 inhibited the expression of α-SMA in the rat livers

The expression of α-SMA is associated with the activity of HSCs and the degree of hepatic fibrosis. Western blot analysis and RT-PCR showed a great decrease in the α-SMA expression in the DcR3 group compared with the EV group (Fig. 3B, C). Combined with histological observation, our results suggest that DcR3 can alleviate hepatic fibrosis induced by 1% DMN.

Homology analysis and the prediction of DcR3 domain

We predicted the DcR3 domain structure and analyzed the homology in humans and rats by online software. The DcR3 protein has 4 TNF receptor repeats in extracellular domain (Fig. 4A). The structure of the DcR3 protein is quite similar to rat TNFR1-α, and a homology analysis indicates high conservation between DcR3 and TNFR1-α in rats and humans (Fig. 4B).

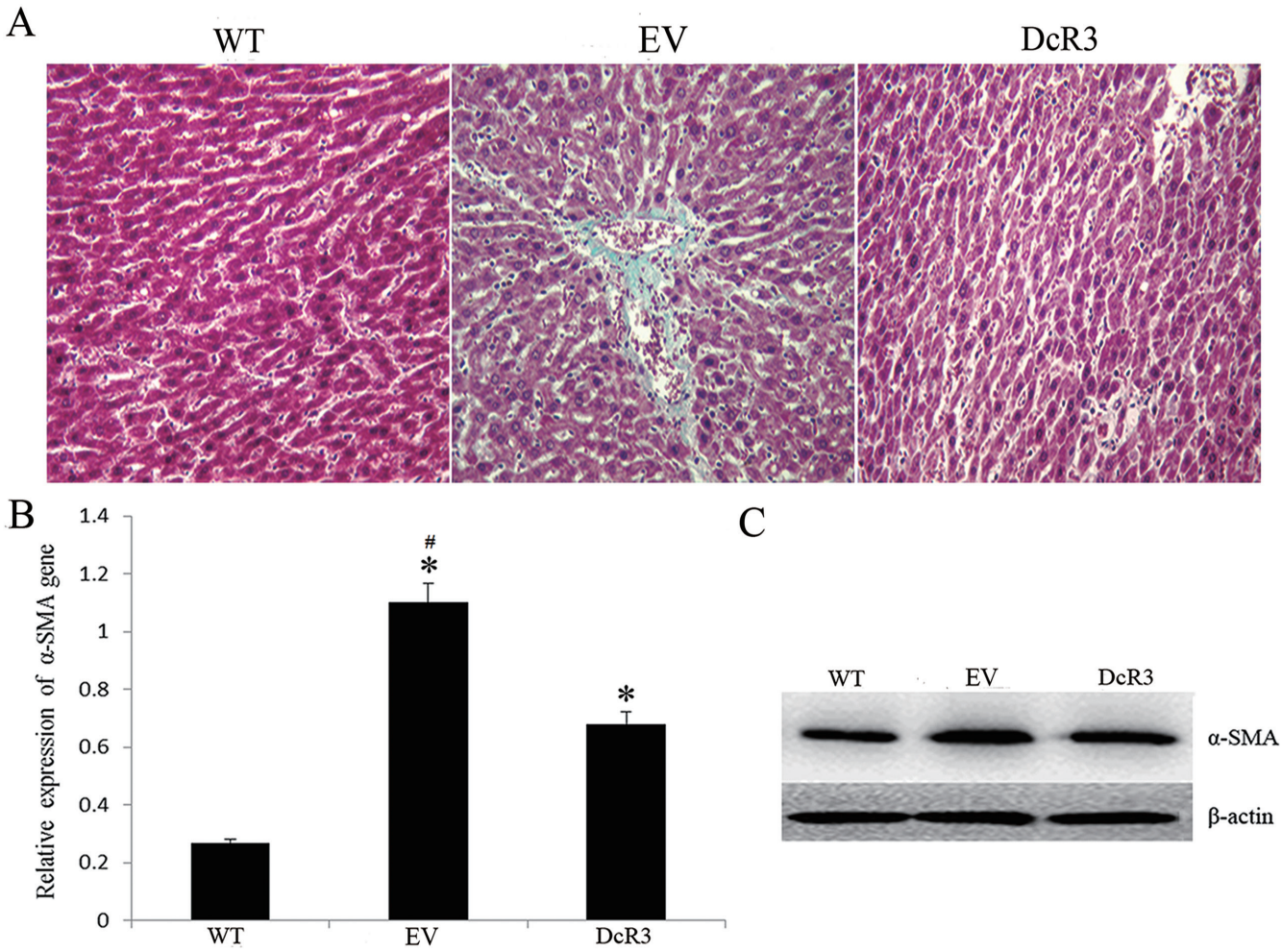

Fig. 3. Histopathological changes of fibrosis and the expression of α-SMA in 1% DMN-induced rats. A – Masson's trichromic staining of the liver tissue. B – analysis of the α-SMA mRNA expression by qRT-PCR. C – analysis of the α-SMA protein expression by western blot. Data is shown as mean ±SD of 10 rats in each group

WT – control group (wild-type); EV – 1% DMN + empty vector group; DcR3 – 1% DMN + DcR3 vector group; * $p < 0.05$ compared with WT group; # $p < 0.05$ compared with EV group; DcR3 – decoy receptor 3; DMN – dimethylnitrosamine.

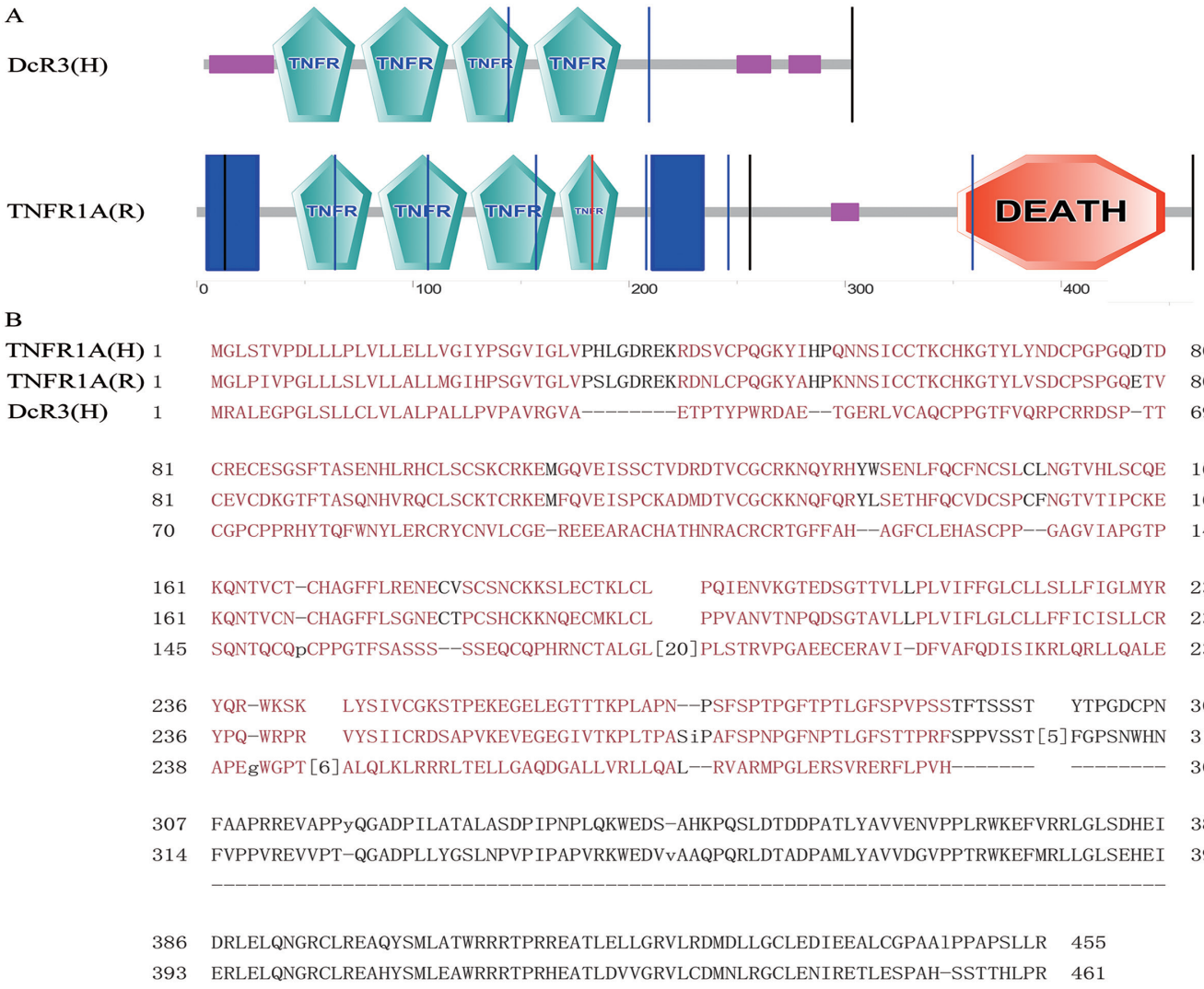

Fig. 4. Domain prediction and homology analysis of DcR3. A – the structure of DcR3 and rat TNFR1-α; B – the homology analysis among DcR3, human TNFR1-α and rat TNFR1-α

TNFR1-α – tumor necrosis factor receptor 1 alpha; H – human; R – rat; DcR3 – decoy receptor 3.

Discussion

Hepatic fibrosis has been evidenced to develop into liver cirrhosis with high morbidity and mortality. Some evidence has suggested that hepatic fibrosis caused by sustaining liver injuries is a reversible process.[19] A growing number of treatment and therapeutic approaches have been identified and used to prevent hepatic fibrosis.[20,21] However, there are no useful therapies targeting the primary stimuli of fibrogenesis such as a blockade of proinflammatory pathways. In this study, we demonstrated that the DcR3 gene attenuated hepatic fibrosis by anti-inflammatory effects in rats. The DcR3 gene is a member of TNF superfamily; it is also known as TNFSF6B in humans. The DcR3 protein is similar to the extracellular part of rat TNFR1-α, with 4 TNF receptor repeats. However, it lost the trigger functional domain. DcR3 competitively binds with Fas and TL1A, but does not recruit a downstream ligand. This may explain the suppression function of DcR3.

Meanwhile, the DcR3 gene may suppress liver inflammation by suppressing the NF-κB pathway. The hepatic inflammation is a hallmark of fibrosis.[22] Some studies also reported that TNF-α, IL-1β and IL-6 were associated with many liver diseases such as hepatotoxic-induced liver injures.[23] A study showed that the anti-fibrosis efficacy could be improved by decreasing the TNF-α level.[24] In addition, overexpressed DcR3 decreased the TNF-α, IL-1β and IL-6 levels in mice.[17] Our data showed that cytokines in the liver, including TNF-α, IL-1β and IL-6, were significantly upregulated in the EV group compared with the WT group, indicating increased inflammatory damage to the liver. However, treatment with DcR3 significantly reduced the TNF-α, IL-1β and IL-6 expression of the liver tissue in 1% DMN-induced liver fibrosis rats. Combined with histology results, DcR3, as a heterogenous protein, did not cause an immunoreaction in rats. In contrast, it suppressed the inflammatory cytokines expression. These results suggested that DcR3 could alleviate liver fibrosis

caused by 1% DMN partly through the suppression of the inflammatory response.

Accumulating evidence has demonstrated that binding of DR3/TL1A is associated with 2 downstream signaling cascades. One results in apoptosis and cell death, the other leads to cell proliferation and activation as well as secretion of cytokines. Ma et al. found that TL1A could promote the expression of inflammatory cytokines, such as IL-6, on fibroblast-like synoviocytes of rheumatoid arthritis (RA) patients by the NF-κB and JNK signaling pathway.[25] Huang et al. showed that the DcR3 overexpression mitigated the IAV-induced release of pro-inflammatory cytokines by suppressing the IAV-activated NF-κB pathway.[17] In our study, we found that DcR3 decreased the levels of inflammatory cytokines in rats with liver fibrosis. Then, we detected NF-κB activity. As expected, an increase in NF-κB activity was observed in rats with liver fibrosis when compared with WT rats, while NF-κB activation was inhibited by DcR3, implying that the inhibition of NF-κB activation was tightly involved in the anti-inflammatory action of DcR3. Our studies indicated that DcR3 attenuated hepatic fibrosis severity via the suppression of the NF-κB pathway, which was activated by DR3/TL1A. Nevertheless, further studies are required to define the exact mechanism underlying the anti-inflammatory effects of DcR3.

In addition, we also examined the effects of DcR3 on 1% DMN-induced liver fibrosis in rats by means of histological examination. Masson's trichromic staining showed that DcR3 significantly inhibited 1% DMN-induced liver fibrosis in rats (Fig. 3). Furthermore, we also examined the α-SMA expression. The α-SMA expression markedly activated HSCs, which play a critical role in liver fibrogenesis.[26] RT-PCR and western blot analyses showed that DcR3 could reduce the α-SMA expression in hepatic fibrosis rats. These results confirmed the conclusions presented below. Therefore, our findings provided novel evidence of the protective effect of DcR3 in 1% DMN-induced hepatic fibrosis rats.

Conclusion

This study showed that treatment with DcR3 was beneficial in terms of antifibrotic effects on inflammatory cytokines induced by 1% DMN. The protective effects of DcR3 against hepatic fibrosis may be due to the suppression of the NF-κB pathway, which was activated by the DR3/TL1A signaling pathway. This study suggests that DcR3 could be a possible prophylactic for the prevention or therapy of liver fibrosis in humans.

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Interleukin 6, osteoprotegerin, sRANKL and bone metabolism in inflammatory bowel diseases

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Abstract

Background. Cytokines are mediators of inflammatory processes in the course of inflammatory bowel disease (IBD) and participate in the bone metabolism. Interleukin 6 (IL-6) initiates osteoclastogenesis by modulating the activity of soluble receptor activator of nuclear factor kappa B ligand (sRANKL) and osteoprotegerin.

Objectives. The aim of the study was to evaluate bone mineral density (BMD) by densitometry and the concentration of interleukin 6, osteoprotegerin (OPG) and sRANKL protein (sRANKL) by ELISA in patients with IBD in relation to the control group; to assess the relationship between IL-6, OPG, sRANKL and BMD; and to assess the impact of disease duration and number hospitalization on BMD.

Material and methods. The studied group included 37 patients with Crohn's disease (I – CD), 37 patients with ulcerative colitis (II – UC) and 37 healthy subjects – control group (III – CG).

Results. The prevalence of osteoporosis and osteopenia was as follows: in I – CD, 18.92% and 32.43% in L2–L4; 13.51% and 35.13% in the neck, and in II – UC, 2.7% and 37.84% in L2–L4; 2.7%, and 29.73% in the femoral neck. The concentration of IL-6 correlated negatively with T-scores in the neck for the whole group, and in group I – CD, there was a significant positive correlation between serum OPG and IL-6.

Conclusions. The incidence of osteopenia and osteoporosis in patients with IBD is high and increases with the duration of the disease and the number of hospitalizations. Patients with CD are at a higher risk of skeletal pathology than patients with UC. IL-6 can modulate bone mineral density in the femoral neck especially in the course of CD.

Key words: cytokines, osteoporosis, inflammatory bowel disease

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Introduction

Inflammatory bowel diseases (Crohn's disease and ulcerative colitis) cause the development of bone pathologies, like osteopenia and osteoporosis, whose pathogenesis is not fully understood. There are several risk factors for osteoporosis in patients with inflammatory bowel diseases (IBD), including glucocorticoid therapy, reduced physical activity, bone resorption intensified by inflammation (elevated levels of proinflammatory cytokines such as interkeukin-6, interleukin 1 and tumor necrosis factor alpha), poor dietary calcium intake (related to lactose intolerance), magnesium deficiency in the diet, vitamin D deficiency, decreased albumin concentration, and impaired intestinal absorption.[1,2] Patients with inflammatory bowel diseases, in whom inflammation is quenched, have higher bone mineral density (BMD) in remission. The overall risk of bone fractures in IBD patients, measured as 1 per 100 patient-years, is 40% higher than in the general population and this risk increases with age.[1,3] Cytokines are a system that governs the functioning of many systems. They are mediators of inflammatory processes in the course of inflammatory bowel diseases and are involved in resorption and bone formation. The interrelation and regulation of the cytokine pathway may play a role in bone pathology in patients with IBD. Interleukin 6 (IL-6) is one of the pro-inflammatory cytokines that initiates and enhances inflammation, and also stimulates bone resorption by developing osteoclast progenitors. IL-6 is a glycosylated polypeptide of molecular weight 21–28 kDa, composed of 4 long α helices connected by loops. It is a typical secretory protein, which is produced together with the N-terminal signal peptide. The gene encoding IL-6 is located on chromosome 7 and contains 5 coding segments (exons). IL-6 is the main stimulus for the production of most acute phase proteins.[4] Schulte et al. has demonstrated that the polymorphism of IL-6 does not affect bone loss in patients with IBD.[5] IL-6 induces a variety of factors, mainly interleukin-1 (IL-1), but also tumor necrosis factor alpha (TNF-α).[6] The gene promoter has sequences that bind transcription factors such as nuclear factor kappa B (NF-κβ), which regulate gene transcription of IL-6 in a manner dependent on the cell type and the activating agent. It is worth noting that attempts have already been made to use antibodies against IL-6 in Crohn's disease.[7] IL-6 is one of the proinflammatory cytokines that intensifies osteoclastogenesis, modulating activity of soluble receptor activator of nuclear factor kappa β ligand (sRANKL), which, contrary to osteoprotegerin, does not have osteogenic effects.[8] IL-6 is produced by macrophages, monocytes, endothelial cells, and T and B lymphocytes. It shows biological activity only when combined with a specific receptor located on the membrane surface of the target cell and it is a potent stimulator of inflammatory processes. The receptor for IL-6 is composed of 2 subunits: glycoproteins of 80 kDa and 130 kDa. The importance of the osteoprotegerin-sRANKL system increases, as it can be modulated by cytokines, particularly interleukin-6, the concentration of which in serum correlates negatively with BMD.[7]

Material and methods

The study group consisted of 37 patients with CD (I – CD) aged 31.7 ±8.0 years on average, including 15 women and 22 men; 37 patients with ulcerative colitis (II – UC) aged 40.6 ±15.1 years on average, including 21 women and 16 men; and 37 healthy volunteers aged 29.6 ±8.0 years on average, including 18 women and 19 men, who constituted the control group (III – CG). The inclusion criteria were as follows: age between 18 and 60 years, diagnosis of IBD based on cross-sectional imaging and/or endoscopy with histopathological confirmation, disease duration >1 year, lack of any other conditions (e.g., rheumatoid arthritis, chronic renal failure), lack of actually biological and steroids therapy which could affect the cytokines profile.

Densitometry of the lumbar spine with L2–L4 assessment and densitometry of the proximal epiphysis of the femur with the assessment of the femoral neck was carried out on all patients, using the Dual Energy X-ray Absorptiometry (DEXA-Lunar DPX-IQ; GE Healthcare Lunar, Boston, USA) technique. The analysis took into account the values of BMD as well as the T-score and Z-score indices. Each patient filled a specially designed questionnaire concerning the current progress and duration of the disease, number of exacerbations and hospitalizations, time and type of pharmacological treatment.

Serum samples for cytokin determinations were stored at –25°C for an average period of 2 months. The samples were not thawed until assay. Quantitative sandwich enzyme immunoassay method (enzyme-linked immunosorbent assay, ELISA) with monoclonal antibody specific for each cytokine/interleukin (IL) specified below was employed. The serum concentration of interleukin 6 was measured by ELISA kits (R&D Systems Inc., Minneapolis, USA) on microplater reader Sunrise™ (Tecan Group Ltd., Männedorf, Switzerland), with a sensitivity of 0.70 pg/mL. The following intra-assay and inter-assay coefficient of variations (CV) were calculated for IL-6: 1.8% and 3.4%. The serum concentrations of sRANKL and OPG were measured by ELISA kits by a sandwich immunoassay method including monoclonal antibodies (BioVendor – Laboratorni medicina a.s., Brno, Czech Republic) on microplater reader Sunrise™ (Tecan Group Ltd., Männedorf, Switzerland), with a sensitivity of 0.1 pmol/L, while intra-assay and inter-assay coefficient of variations were: 6.5% and 6.9% for sRANKL and 6.0% and 7.2% for OPG, respectively. Statistical analysis was carried out using the Kruskal-Wallis test with Dunn's post hoc test to distinguish homogeneous groups. The relationship between the analyzed parameters was assessed using Spearman's rank method. The osteopenia and osteoporosis prevalence in analyzed groups was compared

with test for proportions. The analysis was carried out using STATISTICA PL v. 10 software (StatSoft, Tulsa, USA). All test were considered significant at $p < 0.05$.

Approval for the conduct of the study was obtained from the Bioethics Committee at the Poznan University of Medical Sciences (consent No. 92/09). Informed consent was obtained from every participant.

Results

The aim of the study was to evaluate bone mineral density (BMD) and the prevalence of osteopenia and osteoporosis and to determine the concentration of interleukin-6, osteoprotegerin (OPG) and sRANKL protein (sRANKL) in patients with inflammatory bowel diseases in relation to the control group, and to assess the relationship between IL-6 and OPG, RANKL and s-BMD. The research objective was also to assess the impact of disease duration and number of hospitalizations on BMD. Characteristics of Crohn's disease patients (A) ulcerative colitis (UC) and the control group (B) is included in the table (Table 1).

The analyzed groups were not homogeneous with respect of age. The oldest was group of patients with UC (41 years). This group significantly differs from CD (32 years; $p < 0.0001$) and control group (30 years; $p = 0.0002$). No significant difference was observed between CD and control group with respect of age.

The characteristics of patients and control groups are presented in Table 1.

The prevalence of osteoporosis and osteopenia in group I – CD and II – UC in L2–L4 and the femoral neck are presented in Table 2.

I – CD patients group is characterized by a significantly higher rate of osteoporosis compared to II – UC patients group 18.92% vs 2.7%, $p = 0.0247$. Neck BMD and T-score in I – CD group differs significantly from III – CG group ($p < 0.05 = 0.0007$), but is not significantly different from II – UC group. The mean concentrations of IL-6 (pg/mL), OPG (pmol/L) and sRANKL (pmol/L) of patients and control groups are presented in Table 3.

The level of OPG (pmol/L) differed significantly between all analyzed groups. The highest level was observed in the I – CD patients group. For both I – CD and II – UC group the IL-6 (pg/mL) level was significantly higher compared to III – CG (I – CD vs III – CG group $p < 0.0001$; II – UC vs III – CG $p < 0.0001$). No significant differences in IL-6 (pg/mL) level were observed between the I – CD and the II – UC group (Table 3).

The concentration of IL-6 correlated negatively with neck T-scores for the whole group ($r = -0.33$; $p = 0.0004$), and there was a significant positive correlation ($r = 0.51$; $p = 0.0017$) between OPG and IL-6 in group I – CD. In patients with CD and UC, the mean concentrations of IL-6 were higher than in CG. This difference was statistically significant ($p < 0.0001$). There was no significant difference in disease duration between analyzed groups 8.05 ±5.29 in group I – CD, and 8.03 ±7.92 in group II – UC. Disease duration correlated with neck T- and Z-scores ($r = -0.40$; $p < 0.0001$ and $r = -0.24$; $p = 0.0120$). A similar correlation

Table 1. Characteristics of Crohn's disease patients, ulcerative colitis patients and control group parameters values presented as means and standard deviations

Parameters	Crohn's disease n = 37	Ulcerative colitis n = 37	Control group n = 37	p-value
	mean ±SD	mean ±SD	mean ±SD	
Age [years]	31.76 ±8.06^{a,b}	40.65 ±15.11^{b}	29.57 ±8.01^{a}	0.0014
Weight [kg]	62.81 ±13.8^{a}	66.89 ±15.3^{a,b}	74.57 ±14.61^{b}	0.0054
BMI	20.98 ±3.26^{a}	23.05 ±4.26^{a,b}	24.74 ±3.54^{b}	0.0001
Disease duration [years]	8.03 ±7.92	8.05 ±5.29	–	0.4174*
L2–L4 BMD	1.11 ±0.19^{a}	1.17 ±0.16^{a,b}	1.22 ±0.08^{b}	0.0074
T-score L2–L4	−0.95 ±1.56^{a}	−0.42 ±1.2^{a,b}	0.11 ±0.72^{b}	0.0030
Z-score L2–L4	−0.42 ±1.41	−0.03 ±1.28	0.04 ±0.66	0.1152
Neck BMD	0.92 ±0.2^{a}	0.97 ±0.16^{a}	1.09 ±0.16^{b}	0.0007
T-score neck	−0.86 ±1.49^{a}	−0.44 ±1.19^{a}	0.49 ±1.04^{b}	0.0001
Z-score neck	−0.41 ±1.28^{a}	0.03 ±1.1^{a,b}	0.42 ±0.99^{b}	0.0068

^{a,b} – groups followed by the same letter do not differ statistically significantly; SD – standard deviation; BMI – body mass index; * Mann-Whitney U test.

Table 2. Prevalence of osteoporosis and osteopenia in IBD (CD, UC)

Prevalence	CD	UC	p-value	CD	UC	p-value
	L2–L4			neck		
Osteoporosis [%]	18.92	2.7	0.0247	13.51	2.7	0.0884
Osteopenia [%]	32.43	37.84	0.6260	35.13	29.73	0.6198

IBD – bone mineral density; CD – Crohn's disease; UC – ulcerative colitis.

Table 3. Mean serum concentrations of tested cytokines, Crohn's disease, ulcerative colitis and control group (comparison of the studied groups)

Cytokine	Crohn's disease group n = 37	Ulcerative colitis n = 37	Control group n = 37	p-value
	(mean ±SD)	(mean ±SD)	(mean ±SD)	
IL-6 [pg/mL]	6.73 ±5.23^{b}	4.93 ±4.61^{b}	1.41 ±1.32^{a}	<0.0001
OPG [pmol/L]	8.76 ±3.22^{a,b}	6.02 ±2.51^{a}	9.42 ±2.01^{b}	<0.0001
sRANKL [pmol/L]	284.87 ±213.05	223.82 ±118.14	236.84 ±111.63	0.5856

^{a,b} groups followed by the same letter do not differ statistically significantly; SD – standard deviation; OPG – osteoprotegerin; sRANKL – soluble receptor activator of nuclear factor kappa B ligand.

has been demonstrated for the number of hospitalizations – T-score ($r = -0.41$, $p < 0.0001$) and Z-score ($r = -0.29$, $p = 0.0027$), respectively.

Discussion

The study shows that the incidence of osteoporosis and osteopenia in Polish patients with IBD is high, just as in other study populations; however, further population research is needed. The incidence of osteoporosis is greater in patients with CD than in UC patients, as evidenced by other researchers.[9] However, no statistically significant differences in BMD between the CD and UC groups have been demonstrated. A similar incidence of osteoporosis and osteopenia has been described in a Tunisian population of patients with IBD. Neck osteoporosis was observed in 31.8% of patients with CD and 13% of UC subjects, while L2–L4 osteoporosis was found in 40.9% of CD and 8.7% of UC patients, respectively. The incidence of neck osteoporosis was higher in CD patients. A study of 200 IBD patients in the Iranian population demonstrated an equally high incidence of osteoporosis (24.1%) and osteopenia (50.3%).[10] The loss of bone mass in the course of IBD is regulated by immune system mediators, such as proinflammatory cytokines (TNF-α, IL-1, IL-6, IFN-ɣ). Other TNF-related cytokines, such as RANK/RANKL/OPG are important mediators of inflammation in the intestine, and they participate in the pathophysiology of bone loss leading to the development of osteoporosis. There are certainly many mechanisms that lead to the development of osteoporosis in the course of IBD, but inflammation is a very important factor. The key mediator of inflammation in IBD is TNF-α, also involved in the inflammatory process in the intestine and bone metabolism, thus it can be hypothesized that TNF-α neutralizing therapy can improve the therapeutic strategies in the treatment of secondary osteoporosis associated with inflammation also in the course of IBD.[11–13] IL-6, whose levels increase with the severity of inflammation in the course of IBD, as a factor in osteoclastogenesis may affect the OPG/RANKL pathway. Further research is needed to confirm the effect of proinflammatory cytokines on the OPG/RANKL pathway in the course of IBD and thus to investigate the effect of these cytokines on bone loss and osteoporosis in the course of these diseases.[12,13] Interleukin 6 promotes destructive processes, affecting osteoclastogenesis. Interleukin 6, as well as IL-1β, TNF-α and IL-17, stimulates the production of cytokine RANKL, increases its cellular expression and induces bone resorption.[14]

Our study has also several limitations. The most important one is that we did not perform an association analysis between bone mineral density and the severity of the disease flare. Nevertheless, since all patients enrolled into the study had active disease, we decided not to divide the study group into another clinical subcategories in order to obtain more conclusive results. Our results are different as they indicate that OPG may be stimulated by IL-6. This relationship, therefore, requires broader research, perhaps in larger groups of patients. Experimental studies raise the importance of proinflammatory cytokines including IL-6, IL-1 and TNF-α as important factors that regulate bone resorption and may play a role in bone loss associated with age, estrogen deficiency (postmenopausal osteoporosis), and in the course of IBD. However, further research in this area is necessary.[15] According to other authors, the concentrations of IL-6 in patients with IBD and osteoporosis are increased, and it is thus believed that IL-6 plays a role in the pathogenesis of bone loss, but the mechanism is not fully understood and requires further studies.[16] The findings suggest that the concentration of IL-6 in the serum of patients with IBD in the Croatian population is a clinically important parameter and correlates with the activity of inflammatory disease.[17] IL-6, as a pro-inflammatory cytokine, adversely affects bone turnover by influencing the activity of osteoclasts. The negative effect of IL-6 on bone tissue has been shown in patients with rheumatoid arthritis.[18] An increased activity of IL-6 accelerates the process of bone resorption through the activity of osteoclasts. Other authors indicate that IL-6 concentrations were higher in postmenopausal women with osteoporosis than in the control group. This allows for the conclusion that this cytokine has destructive effects on bone tissue.[19,20] Our results are similar to the study conducted by Polinska et al.[21] The study demonstrated that the serum concentrations of IL-6 in UC was 3 times higher than in healthy group. Clinical studies of tocilizumab, a humanized monoclonal antibody against IL-6 receptors, have shown its high efficiency in the treatment of rheumatoid arthritis.[22,23] Therefore, these studies may contribute to the development and implementation of new secondary osteoporosis therapy in the course of IBD.

Conclusions

The incidence of osteopenia and osteoporosis in patients with inflammatory bowel diseases is high and increases with the duration of the disease and the number of hospitalizations. Patients with Crohn's disease are at a higher risk of skeletal pathology than patients with ulcerative colitis. Interleukin 6, as a proinflammatory cytokine, can modulate bone mineral density in the femoral neck, which can cause a loss of bone mass, especially in the course of Crohn's disease. The effect of interleukin 6, which modulates the OPG/sRANKL system, on bone mass density in the course of inflammatory bowel diseases requires further study.

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Levels of EGF and VEGF in patients with primary and secondary Sjögren's syndrome

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Abstract

Background. Aberrant angiogenesis plays a role in the pathogenesis of Sjögren's syndrome (SS).

Objectives. The aim of this study was to compare the levels of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) in stimulatory parotid saliva and in serum in healthy subjects (HS), patients with primary SS (pSS) and secondary SS (sSS) and to evaluate the expression of *EGF*, proangiogenic $VEGF_{165}$ and antiangiogenic $VEGF_{165}b$ mRNA isoforms. Additionally, we determined the salivary levels of serine/arginine splicing factor (SRSF1), which regulates $VEGF_{165}$ and $VEGF_{165}b$ expression.

Material and methods. The study comprised 34 women (16 with pSS and 18 with sSS) and healthy subjects for blood and saliva sampling. EGF and VEGF levels in saliva and serum and salivary SRSF1 levels were determined by enzyme-linked immunosorbent assay (ELISA). The expression of $VEGF_{165}$, $VEGF_{165}b$ and *EGF* in peripheral blood mononuclear cells (PBMC) was evaluated by quantitative polymerase chain reaction (qPCR).

Results. There were no differences in the levels of EGF, VEGF, SRSF1 and in the expression of the *EGF*, $VEGF_{165}$ and $VEGF_{165}b$ between HS and SS patients, or between pSS and sSS patients. The salivary levels of $VEGF_{165}$ and EGF were significantly higher in pSS, sSS and HS than serum levels. Levels of SRSF1 correlated positively with VEGF and EGF levels. Levels of EGF, VEGF and SRSF1 correlated with each other.

Conclusions. The balance of VEGF isoforms is not disturbed in SS. Saliva is more sensitive for the detection of EGF and VEGF than serum, but salivary levels of those proteins are not representative for SS.

Key words: Sjögren's syndrome, angiogenesis, vascular endothelial growth factor, epidermal growth factor

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Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by periductal mononuclear cell infiltrate in the salivary and lachrymal glands, autoimmunization, injuries to endothelial cells and their subsequent apoptosis.[1,2] Inflammatory cells secrete growth factors, proteases and cytokines that induce extracellular matrix degradation, endothelial cell growth and migration promoting angiogenesis. Neovessels may contribute to the perpetuation of inflammation by recruiting more inflammatory cells to the inflammation site. Angiogenic factors induce endothelial cells to express adhesive molecules, cytokines and chemokines, which have additional stimulatory effects on chronic inflammation.[3]

One of the major proangiogenic regulators is vascular endothelial growth factor (VEGF). It promotes the migration of inflammatory cells into the extracellular matrix by inducing vascular permeability and endothelial cell expression of adhesion molecules, of which elevated levels are observed in SS.[3] VEGF exerts its biological function by binding to its receptors: VEGFR-1, VEGFR-2 and VEGFR-3.[4,5] The main isoform $VEGF_{165}$ has 165 amino acids in the mature structure. Two families of VEGF proteins are formed by an alternative splice-acceptor-site to give to 2 distinctive C-terminal sequences differing in their angiogenic properties.[6–8] These 2 isoforms bind to the VEGFR-2 with the same affinity, but the binding of $VEGF_{165}$b results in an insufficient tyrosine phosphorylation/activation of VEGFR-2 and incomplete or transient downstream signaling, which leads to an impaired angiogenic response.[6,9] The isoforms $VEGF_{165}$ and $VEGF_{165}$b account for a substantial proportion of the total VEGF.[9] The balance of the $VEGF_{xxx}$ (proangiogenic) and $VEGF_{xxx}$b (antiangiogenic) families may have a crucial role in controlling angiogenesis in health; an imbalance can underpin pathological angiogenesis. Primarily, the influence of $VEGF_{165}$ and $VEGF_{165}$b on cancerogenesis was examined.[4,10–12] Similar patomechanisms between pro- and antiangiogenic also seem to occur in autoimmune diseases and coexistent chronic inflammation influencing their development by stimulating or inhibiting angiogenesis. The mechanism regulating the expression of pro- and antiangiogenic isoforms of *VEGF* is not known. Many growth factors and other proteins can change the proportion of $VEGF_{165}$ and $VEGF_{165}b$ and regulate alternative splicing, e.g., insulin-like growth factor 1 (IGF-1), transforming growth factor alpha 1 (TGF-α1), transforming growth factor beta 1 (TGF-β1) and its co-regulator-serine-rich protein splicing factor 1 (SRSF1).[9] The serine/arginine rich proteins regulate binding to exon-splicing enhancers and silencers and intronic enhancers and silencers in pre-mRNA. Exon splicing depends on the balance of splicing factors activities.

So far, most studies have focused on the serum levels of VEGF and epidermal growth factor (EGF) and their local expression in salivary gland biopsy specimens in SS patients. Therefore, this is also the first trial assessing the possible role of *VEGF* splice variants: $VEGF_{165}$ and $VEGF_{165}b$ and SRSF1 in SS. In SS, pathological changes are more advanced locally in comparison to systemic ones. Proinflammatory cytokine production is disordered in salivary glands as well as in peripheral blood. In SS patients there are particular differences between leukocytes in the peripheral blood and in the salivary glands.[13]

The aim of this study was to compare the levels of VEGF and EGF in stimulated parotid saliva and in serum in patients with primary SS (pSS) and secondary SS (sSS) and in healthy subjects (HS), as well as to evaluate the expression of *EGF* and isoforms $VEGF_{165}$ and $VEGF_{165}b$ in PBMC in pSS, sSS and HS. Furthermore, the levels of SRSF1 in parotid saliva were compared.

Material and methods

Study groups

The study comprised 34 women with SS (16 with pSS and 18 with sSS) aged 41.5 (interquartile ranges (IQR): 28.5) in pSS group and 56.0 (IQR: 21.0) in sSS group, respectively (Table 1), fulfilling the 2002 American-European Consensus Group (AECG) classification criteria. Our study diagnosis of pSS required 4 out of 6 criteria, including the presence of the antibody to SS-A/SS-B. The diagnosis of sSS has not yet been addressed by the AECG. In practice, we required the patients to fulfill the criteria for pSS and also to meet the American College of Rheumatology (ACR) criteria for an established connective-tissue disease (CTD), such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or mixed connective tissue disease (MCTD).[1,2,14] Patients were recruited consecutively in 2013 from the Department of Rheumatology and Clinical Immunology. Exclusions to the diagnosis of SS included previous radiotherapy to the head and neck, lymphoma, sarcoidosis, graft-versus-host disease, infections with hepatitis C virus (HCV), human T-cell lymphotropic virus type 1 (HTLV-I) and human immunodeficiency virus (HIV). The patient's history was taken and physical and dental examinations were performed in the Department of Oral Surgery at Poznan University of Medical Sciences by dentist for each subject. Laboratory assessments included routine measurements of erythrocyte sedimentation rate (ESR) (Westergren method)[15] and detection of antinuclear antibodies (ANA) by indirect immunofluorescence (IIF) on HEp-20-10 cells (Euroimmun, Lubeck, Germany) and their differentiation using ANA Profile3 (Euroimmun, Lubeck, Germany). Xerostomia (assessed by patients) was measured by a visual analogue scale (VAS) and Fox's test. To assess ocular sicca symptoms, Schirmer's test was carried out. Both eyes were tested at the same time. Schirmer's test was performed without anesthesia before the procedure. Special paper strips (Alcon Laboratory, Fort Worth, USA)

were placed under the lower eyelid of each eye lateral to the canthus away from the cornea. The patients kept their eyes closed for 5 min. Mechanical irritation resulted in the production of tears. After 5 min the paper was removed and measured to check how moist it was. The sign of normal tear production was indicated by the presence of more than 10 mm of moisture on the filter paper after 5 min. Both eyes normally release the same amount of tears. A score between 5–10 mm was a sign of mild to moderate dry eye. Less than 5 mm of moisture on the filter paper after 5 min was a sign of severe dry eye and was a diagnostic criterion for SS. To report the severity of dry eye we used the average of both eyes.[16,17]

In the study, 15 age-matched healthy donors from the Regional Centre of Blood Donation and Blood Treatment in Poznań formed the control group for serum sampling (healthy subjects, HS).

A total of 15 age-matched healthy donors of dental students and dentists formed the control group for saliva sampling (HS); it included 10 women and 5 men aged 23 (IQR: 5). Only patients without severe systemic diseases, who do not take any drugs, and without symptoms of dry mouth and xerostomia were classified to the control group.

The protocol for this study was approved by the Bioethics Committee of Poznan University of Medical Sciences, Poland (No. 211/2013). Written informed consent was obtained from every subject before any study procedure was carried out.

Blood and saliva sampling

Peripheral blood samples were collected from the antecubital vein into BD Vacutainer Rapid Serum Tubes (Becton; Dickinson and Company, New York, USA). Peripheral blood mononuclear cells (PBMC) were isolated from 3 mL of fresh EDTA whole blood with the use of Lymphocyte Separation Medium 1077 (PAA, Pasching, Austria), in accordance with the manufacturer's protocol. Next, the cells were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, USA).

Parotid saliva was collected directly from the parotid gland opening with Lashley cups into Eppendorf tubes after stimulation with 3% citric acid. Gauze swabs soaked with citric acid were put on the tongue every minute. Saliva sampling lasted as long as was needed to collect 2 mL saliva. Saliva was not centrifuged according to the instruction recommended in the applied tests. We did not add any proteinase inhibitors. About 2 h before sampling, the patients refrained from eating, drinking, rinsing their mouths and brushing their teeth. The saliva, sera and lysed PBMC were stored at –70°C.

RNA extraction, reverse transcription and quantitative polymerase chain reaction

Extraction of RNA, reverse transcription and quantitative polymerase chain reaction (qPCR) were carried out as described earlier.[18] The sequences used for *EGF* amplification were: F: 5'CCTGATGGGAAACGATGTC3', R: 5'GTGAGGAACAACCGCTAC3'. The sequences for $VEGF_{165}$ and $VEGF_{165}b$ were the same as described previously.[19]

Measurement of studied proteins

Circulating EGF and $VEGF_{165}$ levels in the saliva and serum were determined by ELISA (R&D Systems, Minneapolis, USA) with a sensitivity of less than 5.0 pg/mL for $VEGF_{165}$ and 0.7 pg/mL for EGF. Soluble SRSF1 levels in parotid saliva were determined by ELISA (EIAab SCIENCE, Wuhan, China) with a sensitivity of less than 0.078 ng/mL. Absorbance was measured with a ELx800 96-well Microplate Reader and KC junior 1.11 (Bio-Tek Instruments, Vermont, USA).

Statistical analysis

The calculations were carried out with Microsoft Excel 2010 and STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The distributions obtained at each step of data processing were evaluated for normality using the Shapiro-Wilk test. Depending on the number of groups analyzed, the differences between them were tested using the Mann-Whitney U test or the Kruskal-Wallis one-way analysis of variance (ANOVA) by ranks, followed by post hoc multiple comparisons of the mean ranks. Spearman's rank correlation analysis was used to find the associations between the levels of selected cytokines and other laboratory and clinical parameters of SS activity. All the data is expressed as medians with interquartile ranges (IQR). Differences were considered to be statistically significant at $p < 0.05$.

Results

Demography

All the subjects were of Caucasian origin. Table 1 and 2 present a demographic, laboratory and clinical profile of SS patients. The median age of the HS group for serum sampling (14 women and 1 man) was 49 (IQR: 14). The median age of the control group for saliva sampling (10 women and 5 men) was 23 (IQR: 5). The correlation between VEGF levels and age was studied in cancerogenesis. The VEGF expression was not correlated with age in patients with colon cancer.[20] There were no differences in VEGF levels between the young control group and the elder group with prostate patients.[21] There was no correlation between the age of healthy people without immunological, inflammatory and neoplastic disease and the VEGF concentration.[22] The main criterion to classify patients to the control group was the elimination of the possible role of inflammatory, immunological and other diseases and drugs on the VEGF levels.

Table 1. Characteristics of SS patients

Clinical and laboratory data	pSS (n = 16)	sSS (n = 18)
Age [years]	41.5 (28.5)	56.0 (21.0)
BMI [kg/cm^2]	24.65 (7.60)	23.55 (5.30)
Disease duration [years]	4.5 (4)	3 (9)
RA, n [%]	–	7 (38.89)
SLE, n [%]	–	4 (22.20)
Others, n [%]	–	7 (38.89)
Westergren ESR [mm/h]	23.5 (31)	19 (15)
ANA titer >1:160, n [%]	11 (68.75)	15 (83.33)
SS-A, n [%]	10 (62.50)	10 (55.55)
SS-B, n [%]	7 (43.75)	4 (22.22)
Ro-52	10 (62.50)	11 (61.11)
dsDNA	0	3 (16.67)
Sm	0	1 (5.55)
PCNA	0	1 (5.55)
Ribosomal-P-protein	0	3 (16.67)
Centromeres B	0	1 (5.55)
PM-Scl	0	1 (5.55)
Histones	0	1 (5.55)
Nucleosomes	0	1 (5.55)
RNP	0	1 (5.55)
Organ involvement, n [%]		
Articular	11 (68.75)	11 (61.11)
Peripheral nervous system	5 (31.25)	3 (16.67)
Cutaneous	4 (25.00)	3 (16.67)
Pulmonary	4 (25.00)	1 (5.55)
Lymphadenopathy	3 (18.75)	2 (11.11)
Glandular	1 (6.25)	1 (6.25)
Current treatment, n [%]		
MTX	0	5 (27.78)
NSAID	4 (25.00)	4 (22.22)
Methylprednisolone	4 (25.00)	6 (33.33)

pSS – primary Sjögren's syndrome; sSS – secondary Sjögren's syndrome; BMI – body mass index; MTX – methotrexate; NSAID – nonsteroidal anti-inflammatory drugs; RA – rheumatoid arthritis; SLE, – systemic lupus erythematosus; ESR – erythrocyte sedimentation rate. Unless otherwise stated, data is expressed as median (IQR).

Table 2. Oral and ocular characteristics of SS patients

SS patients (n = 34)	pSS (n = 16)	sSS (n = 18)
PtXer-VAS [mm]	46.5 (52.5)	28.0 (47.0)
Fox's test score [%]	45 (40)	55 (30)
Oral symptoms, n [%]		
Dysphagia	4 (25.0)	6 (33.33)
Xerostomia	9 (56.25)	12 (66.67)
Ocular symptoms		
Schirmer's test [mm] (pSS n = 11, sSS n = 12)	4 (8)	10.75 (12)
Dryness of the eyes (subjective assessment), n [%]	11 (68.75)	13 (72.22)

pSS – primary Sjögren's syndrome; sSS – secondary Sjögren's syndrome; PtXer-VAS – patient xerostomia assessment on visual analogue scale. Unless otherwise stated, data is expressed as median (IQR).

Expression levels of VEGF, EGF, SRSF1, and transcript levels of *VEGF$_{165}$*, *VEGF$_{165}$b* and *EGF* in pSS, sSS and HS levels of VEGF and EGF in saliva were significantly higher than in serum. There were no differences between the pSS and sSS group for EGF and VEGF levels. There was also no significant difference in salivary SRSF1 concentration between pSS, sSS and HS. *VEGF$_{165}$*, *VEGF$_{165}$b* and *EGF* mRNA expression in pSS patients, sSS patients and HS were at similar levels. Detailed results are presented in Table 3.

There was a statistically significant correlation between VEGF and EGF serum levels in SS patients (Fig. 1). The levels of SRSF1 in parotid saliva correlated positively with the salivary levels of VEGF and EGF (Fig. 2). No statistically significant correlations were found between the gene expression and the other SS activity parameters.

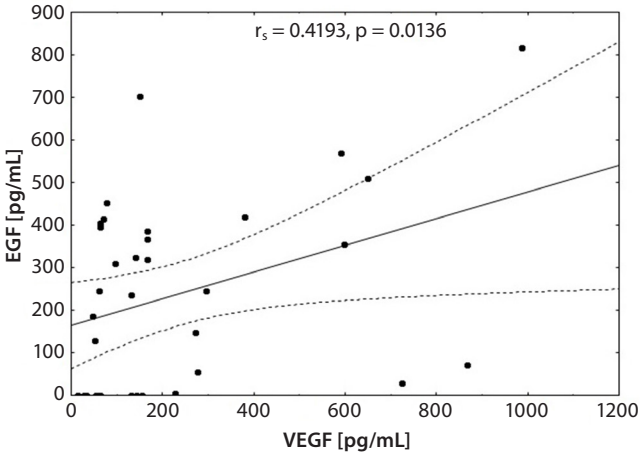

Fig. 1. A positive correlations between serum levels of EGF and VEGF in SS patients. The strength of the relationship was determined by the Spearman's rank correlation coefficient. A p-value of <0.05 was considered statistically significant

Discussion

VEGF contributes to the pathomechanism of SS in many aspects. Recent studies demonstrated a close interplay between VEGF and its receptors in various autoimmune diseases, including SLE, RA, and multiple sclerosis.[3,23,24] Increased levels of VEGF and VEGFR-1 in plasma were detected in patients with active SLE as compared to the levels in patients with inactive disease and in controls.[25] Moreover, VEGF was overexpressed in the skin in systemic sclerosis (SSc) and its increased levels correlated with the severity of nailfold capillary loss in SSc.[5] Considerable amounts of VEGF in both glandular epithelia and inflammatory cells of inflamed glands in SS patients as compared with those from HS were observed. Additionally, an immunohistochemistry examination revealed a strongly positive staining for VEGF and

VEGFR-2 proteins in the biopsy specimens from SS. Furthermore, anti-Ro/SS-A antibodies enhanced VEGF expression in SS salivary glands biopsy specimens.[26,27] We obtained contradictory results to the previous studies, because even sSS patients with SLE or RA had no increased levels of VEGF when compared to the controls. Levels of VEGF in parotid saliva were elevated compared to serum levels. This means that the increased levels of VEGF in salivary gland specimens are not reflected in their elevated serum levels. Levels of VEGF and EGF cannot be diagnostic parameters in SS.

Our results confirm that serum and salivary levels of VEGF, EGF as well as PBMC expression of $VEGF_{165}$ and $VEGF_{165}b$ are independent of the type of syndrome and other connective tissue diseases in sSS. In recent studies comparing pSS and sSS, it was found that the intensity and frequency of some symptoms can vary. Similar clinical, serological and histological features with the exception of perivascular infiltrates in the salivary gland biopsies suggests that pSS and sSS are of the same entity.[28,29] In previous studies, different levels of VEGF and its receptors in SSc and SLE were observed. Ambiguous results can arise from the different classification of sSS.[28] In our opinion, vasculitis and vasculitic changes are more important issues which should be taken into consideration when VEGF and its isoforms are compared in pSS and sSS. According to Manetti et al., increased plasma levels of $VEGF_{165}$b isoform are associated with the severity of capillary architectural derangement in SSc patients.[6] Elevated plasma levels of $VEGF_{165}b$ correlated significantly with the absence of microhemorrhages and the presence of ramified/bushy capillaries and avascular areas. The authors suggested that in SSc patients, the $VEGF_{165}$b might actively participate in the loss of microvessels. Additionally, the anti-angiogenic $VEGF_{165}b$ splice variant was selectively upregulated at both mRNA and protein levels in the skin biopsy samples from patients with SSc.[5] No significant difference in skin expression of $VEGF_{165}$b was found between patients with limited cutaneous SSc and those with diffuse cutaneous SSc.[6] In our study, the group of patients with vasculitis was too small to include this symptom in statistical analysis and comparison between patients.

In our study, serum levels of VEGF significantly corresponded with the serum levels of EGF. In previous studies, a similar role in the endothelial cell activation by VEGF and fibroblast growth factor 1 and 2 was observed.[3] Moreover, IGF-1 and TGF-β1 have previously been shown to enhance total VEGF expression.[9] According to Nowak et al.,

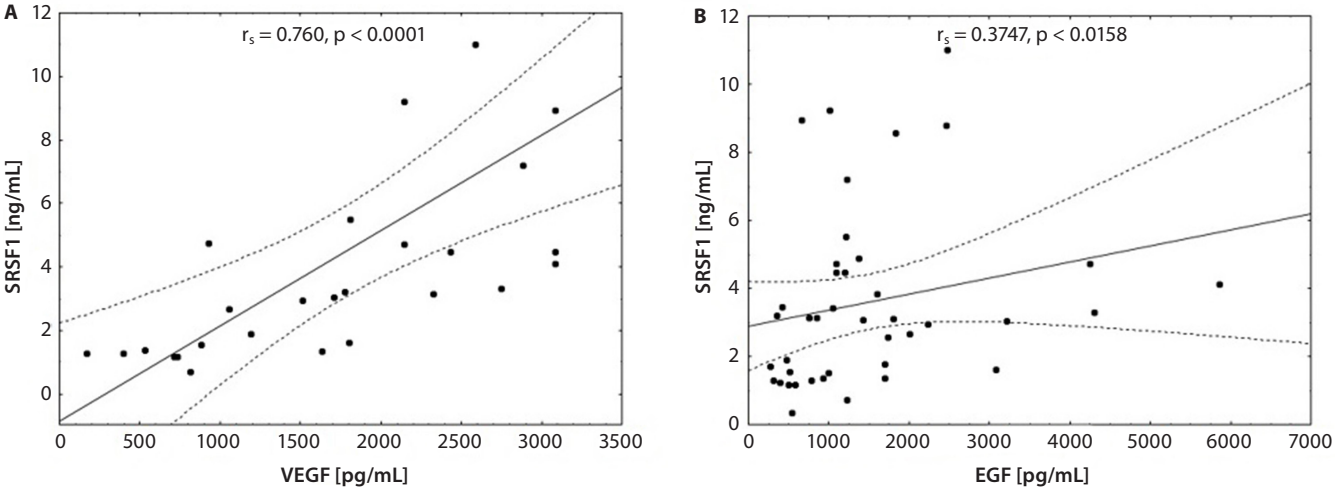

Fig. 2. A positive correlations between salivary levels of VEGF (A), EGF (B) and SRSF1 in SS patients. The strength of the relationship was determined by the Spearman's rank correlation coefficient. A p-value of < 0.05 was considered statistically significant

Table 3. Levels of VEGF, EGF in serum and saliva in pSS, sSS and HS. Salivary levels of SRSF-1 in pSS, sSS and HS. Expression of $VEGF_{165}$, $VEGF_{165}b$ and *EGF* in PBMC in pSS, sSS and HS

Studied groups and comparisons between pSS, sSS and HS	VEGF (pg/mL)			EGF (pg/mL)			SRSF-1 (ng/mL) saliva	$VEGF_{165}$ PBMC	$VEGF_{165b}$ PBMC	EGF PBMC
	serum	saliva	p[a]	serum	saliva	p[a]				
pSS	73.83 (108.47)*	1348.75 (1264.14)	<0.001	187.02 (390.09)	1003.25 (1571.62)	<0.001	2.94 (2.56)	2.17 (0.42)	2.25 (1.19)	2.29 (0.62)
sSS	212.58 (494.27)	2145.10 (1934.22)	<0.001	240.41 (376.28)	1215.40 (1890.94)	0.003	3.90 (7.57)	2.18 (0.42)	2.27 (0.84)	2.35 (0.41)
HS	23.20 (37.58)	1001.69(719.99)	<0.001	32.96 (79.82)	1371.64 (1205.48)	<0.001	3.12 (2.11)	2.03 (0.43)	1.70 (1.99)	2.26 (0.75)
p[b]	ns	ns		ns	ns		ns	ns	ns	ns

pSS – primary Sjögren's syndrome; sSS – secondary Sjögren's syndrome; HS – healthy subjects; ns – non-significant; * median (IQR); [a] comparisons between serum and saliva; [b] comparisons between pSS, sSS and HS.

IGF-1 and TGF-β1 and -β2 differentially affect the expression of $VEGF_{165}$ and $VEGF_{165}b$.[9] EGF is responsible for the growth of epithelial cells, which are the main targets in the initiation of inflammation and angiogenesis in SS.[30] Our results confirm that an evaluation of both EGF and VEGF is helpful for gaining a comprehensive view of angiogenesis in SS.

There were no differences in the levels of $VEGF_{165}$ and $VEGF_{165}b$ between pSS, sSS and HS. There were also no statistically significant correlations between the salivary levels of SRSF1 and PBMC expression of $VEGF_{165}$ and $VEGF_{165}b$. But SRSF1 levels correlated positively with total VEGF salivary levels. According to Manetti et al., TGF-β1 upregulates the expression of $VEGF_{165}b$ and serine/arginine protein 55 in both SSc and healthy microvascular endothelial cells.[6] The binding of them to *VEGF* pre-mRNA or their interaction with *VEGF* pre-mRNA sequences has been implicated in growth-factor-mediated alternate splice-site selection. Nowak et al. even suggested that known splicing factors differentially affect the expression of the $VEGF_{xxx}$ and $VEGF_{xxx}$b isoform families.[9] Additionally, VEGFR-1 and neuropilin modulate $VEGF_{165}$ signaling.[31] Furthermore, VEGFR-1, fibronectin and collagen also have differentially spliced inhibitory isoforms. A universal mechanism may, therefore, exist for the regulation of these antiangiogenic splicing events.[32]

There were no statistically significant differences in the salivary levels of the selected proteins determined in pSS and sSS patients. According to Hernández-Molina et al., the salivary levels of the pro-inflammatory cytokines were similar in pSS, sSS and pre-clinical SS and systemic autoimmune diseases, but there were differences between HS and patients with other autoimmune diseases.[33] Moriyama et al. observed higher levels of pro-inflammatory cytokines in the saliva of SS patients than in those from controls.[34] According to Bertorello et al., only increased salivary levels of interleukin 10 in SS patients correlate with the severity of the disease.[35] These differences in the salivary levels of the main cytokines can also stem from vasculitis in SS.[36] Thus, in saliva it is difficult to find a representative marker with a suitably high specificity to SS.

The limitations of this study can arise from the choice of the type of saliva and the methods of saliva sampling. The levels of salivary peptides vary depending on the type of salivary glands and methods of saliva sampling. Resting and mixed saliva seem to be the most representative oral fluids. On the other hand, mixed saliva consists of crevicular fluid, which can increase the total salivary concentration of proinflammatory cytokines and markers. Stimulation mainly produces parotid saliva, which is easily available for sampling, especially in dry mouth conditions in SS patients. Lack of the measurement of VEGFR2 levels does not provide a comprehensive view of the $VEGF_{165}$ and $VEGF_{165}$b relationship. In our study, a few patients were taking methylprednisolone, non-steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressive drugs. There were no naïve patients regarding both disease-modifying antirheumatic drugs (DMARDs) and NSAIDs. According to Nagashima et al., serum levels of VEGF are reduced by combined therapy with corticosteroids and MTX.[37] Current and previous therapy and co-existing diseases can interfere with our results, but it is not clear whether these drugs can affect serum and salivary VEGF and EGF levels in SS patients. The main limitations of this study arise from the small sample size. It should, therefore, be considered as a pilot study and our findings have to be verified in a larger SS cohort.

Conclusions

The balance of *VEGF* isoforms is not disturbed in SS. Saliva is more sensitive as a medium for detecting EGF and VEGF than serum, but salivary levels of these proteins are not representative for SS group.

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Caffeine alters the effects of bone marrow-derived mesenchymal stem cells on neutrophils

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Abstract

Background. It has been shown that mesenchymal stem cells (MSCs) express all four adenosine receptors' subtypes, and stimulation of these receptors plays an active role in bone marrow-derived mesenchymal stem cell proliferation and differentiation. The interaction between MSCs and immunocytes, such as neutrophils, has been investigated in some recent studies.

Objectives. This study was carried out to investigate the effects of caffeine as an adenosine antagonist on the effects of bone marrow-derived MSCs on neutrophils.

Material and methods. Mesenchymal stem cells were isolated from the bone marrow of rats and pulsed with different concentrations of caffeine (0.1, 0.5 and 1 mM) at different times (24, 48 and 72 h). Mesenchymal stem cells were co-cultured with neutrophils for 4 h and the functions of neutrophils were evaluated.

Results. The findings showed that MSCs pulsed with caffeine at low to moderate concentrations preserved the neutral red uptake by neutrophils and established the MSCs' ability to protect neutrophils from apoptosis. Mesenchymal stem cells treated with caffeine increased the phagocytosis of neutrophils and simultaneously diminished the production of potentially harmful reactive oxygen substances, more profound than MSCs without treatment. Nevertheless, a high concentration of caffeine could interfere with some aspects of the crosstalk between MSCs and neutrophils.

Conclusions. These findings may offer new insight into the potential mechanisms underlying the immunomodulatory effects of caffeine.

Key words: neutrophil, bone marrow-mesenchymal stem cell, caffeine

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Introduction

Caffeine (1, 3, 7-trimethylxanthine) is a natural product and a member of the methylxanthine family of drugs, which can be found in coffee, tea, soft drinks, chocolate, kola nuts, and certain medicines.[1,2] Caffeine possesses various effects on different body systems, including endocrine, cardiovascular, respiratory, urinary, gastrointestinal metabolism, immunity, and especially the central nervous system.[3,4] In fact, caffeine is the world's most widely and legally consumed psychoactive drug.[3] Caffeine is structurally similar to adenosine. Indeed, it is capable of binding to adenosine receptors without activating them, suggesting that caffeine is a competitive inhibitor of adenosine.[5,6] It also acts as a competitive, non-selective phosphodiesterase inhibitor and, therefore, raises intracellular cyclic adenosine monophosphate.[6]

Mesenchymal stem cells are plastic-adherent, fibroblast-like, and multipotent non-hematopoietic progenitor cells that differentiate into various mesenchymal lineages, including bone and cartilage.[7] Mesenchymal stem cells also showed a potent immunoregulatory activity, which can be a worthwhile strategy for the treatment of autoimmune diseases and graft-versus-host disease.[8–10] It has been observed that the MSCs' functions are under the control of a large number of signaling systems.[11–13] Interestingly, it has been shown that MSCs express all four adenosine receptors' subtypes, and stimulation of these receptors plays an active role in bone marrow-derived mesenchymal stem cell proliferation and differentiation.[14]

The interaction between MSCs and immunocytes, such as neutrophils, has been investigated in some recent studies.[15,16] Nonetheless, there is no information about the role of caffeine on the crosstalk between MSCs and neutrophils. The current survey was set out to investigate the effects of caffeine on the crosstalk between bone marrow-derived MSCs and neutrophils in rats.

Material and methods

Dextran was bought from Fresenius Kabi (Verona, Italy). Fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD). Moreover, caffeine, nitro blue tetrazolium (NBT), natural red (NR), dioxin, dimethyl sulfoxide (DMSO), tetradecanoyl phorbol acetate (TPA), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO). In addition, May-Grunwald-Giemsa stain was ordered from Merck (Darmstadt, Germany).

Isolation and proliferation of mesenchymal stem cells

Mesenchymal stem cells were isolated as described elsewhere.[17] In brief, bone marrow was aspirated from the tibias and femurs of deeply anesthetized Wistar rats. After 2 washings, the cells were plated in 75-cm^2 tissue-culture flasks with concentrations of 0.3 to 0.4 × 10^6 cells/cm^2 in the DMEM medium, supplemented with 15% fetal calf serum. The cells were incubated in a humidified 5% CO_2 at 37°C. Four days after primary culture initiation, non-adherent cells were removed and adherent cells were fed every other day. Mesenchymal stem cells were removed by trypsin/EDTA when the cultures reached 80% confluence. The cells were counted and passed in 1:3 ratios (about 1.5 × 10^6 cells/75-cm^2 flask). Cell passage was done up to subculture 3. Then, MSCs were incubated with different concentrations of caffeine (0.1, 0.5 and 1 mM) at different times (24, 48 and 72 h). Afterwards, the medium was aspirated and cells were washed three times with PBS.

Neutrophil isolation and incubation with mesenchymal stem cells

Blood samples were isolated under anesthesia by cardiac puncture in sodium citrate. The blood was centrifuged and the buffy coat was subjected to dextran sedimentation (1% w/v), followed by centrifugation on a Ficoll-Hypaque density gradient. The plasma and the mononuclear cell layer were removed, and erythrocytes were eliminated using hypotonic lysis. The neutrophils were washed and suspended in DMEM.[11] Following this procedure, the purity of neutrophils was 95%.

For co-culture experiments, 2 × 10^6 neutrophils were added to each well of 24-well flat-bottomed plates, containing 2 × 10^5 MSCs and incubated for 4 h at 37°C in a moist atmosphere of 5% CO_2. Afterwards, the neutrophils were isolated and used for the next experiments.

Evaluation of neutrophils viability

The viability of neutrophils was assessed by the MTT assay, similar to the procedures described earlier.[18,19] Briefly, 100 µL of the neutrophil suspension (2 × 10^6 cells/ml) was added to each well of 96-well microplates and pulsed with 20 µL of the MTT solution (5 mg/mL) for 4 h at 37°C. To dissolve the formazan crystals, 150 µL DMSO was added to each well of 96-well microplates and the plates were shaken vigorously. At the end, a microplate reader (Dynatech, Denkendorf, Germany) was used to determine the optical density (OD) at 550 nm. In addition, the experiments were performed in triplicate sets.

Neutral red uptake

Briefly, 100 µL of the neutrophil suspension (2 × 10^6 cell/mL) was added to each well of 96-well microplates and pulsed with 10 µL of the NR solution (0.33%) for 2 h at 37°C. At the end of the incubation period, the medium was discarded and the neutrophils were twice washed in PBS.

The internalized NR was solubilized for 30-min incubation by mixing 100 µL 10% acetic acid plus 40% ethanol solution. The optical density was measured at 550 nm.

Phagocytosis assay

This experiment was designed as previously described, with some modifications.[20] In brief, the cells were washed after stationary incubation of neutrophils with opsonized yeast at 37°C for 1 h, cytocentrifuged onto glass slides, and fixed in methanol. The slides were stained with May-Grunwald-Giemsa staining. Yeast ingestion was evaluated by light microscopy under oil immersion. Phagocytic activities of neutrophils were reported as percentage of neutrophils, internalized at least one yeast cell.

Respiratory burst

The NBT reduction assay was used to check the intracellular generation of reactive oxygen species (ROS) by neutrophils.[21] In brief, neutrophils were incubated for 20 min with 100 ng/mL TPA and 0.1% NBT. The unused NBT was discarded through washing and the reduced dye was extracted in dioxin and quantitated at 520 nm.

Statistical analysis

The normal distribution of data was confirmed with the Kolmogorov-Smirnov test. Next, the results were analyzed by one-way ANOVA plus Dunnett's post-hoc test and presented as means ±SD. The minimal level of significance was reported at p values of less than 0.05.

Results

The MTT test showed that MSCs could significantly increase the viability of neutrophils (Fig. 1). Moreover, MSCs pulsed with 0.5 mM of caffeine for 72 h and MSCs treated with 1 mM of caffeine for 24, 48 and 72 h significantly diminished the survivability of neutrophils, compared to the control group (the MSCs, which were not pulsed with caffeine) (Fig. 1). These findings suggested that caffeine at high doses can decrease the protective role of MSCs on the viability of neutrophils, so that MSCs treated with 1 mM of caffeine for 72 h significantly decreased the viability of co-cultured neutrophils compared to neutrophils alone.

As exhibited in Fig. 2, the NR uptake by neutrophils did not show any significant difference between neutrophils alone and neutrophils co-cultured with MSCs without treatment, or the MSCs pulsed with caffeine at concentrations of 0.1 and 0.5 mM. However, MSCs treated with caffeine at a concentration of 1 mM significantly lowered the NR uptake by co-cultured neutrophils (Fig. 2).

The phagocytic activity of neutrophils was significantly increased in the neutrophils co-cultured with the MSCs pulsed with caffeine or the MSCs alone, compared to neutrophils without treatment (Fig. 3). The gained results also demonstrated that the phagocytic activity of co-cultured neutrophils and MSCs treated with caffeine was significantly more pronounced than phagocytosis observed by the co-cultured neutrophils and MSCs alone (Fig. 3).

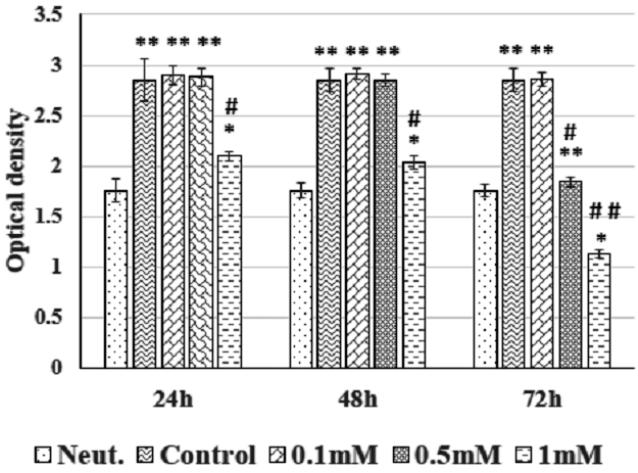

Fig. 1. Effect of caffeine on modulation of the viability of the neutrophils by mesenchymal stem cells (MSCs). MSCs were isolated from bone marrow of rats and pulsed with different concentrations of caffeine (0 (control), 0.1, 0.5 and 1 mM) at different times (24, 48 and 72 h). Then mesenchymal stem cells co-cultured with neutrophils for 4 h. Greater optical density indicates higher levels of viability. The results showed that caffeine at least at higher doses could significantly decrease the protective role of MSCs on neutrophils apoptosis. Results were shown as mean ± S.D. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (*p < 0.01, **p < 0.001 vs neutrophils alone; # p < 0.01, ## p < 0.001 vs control)

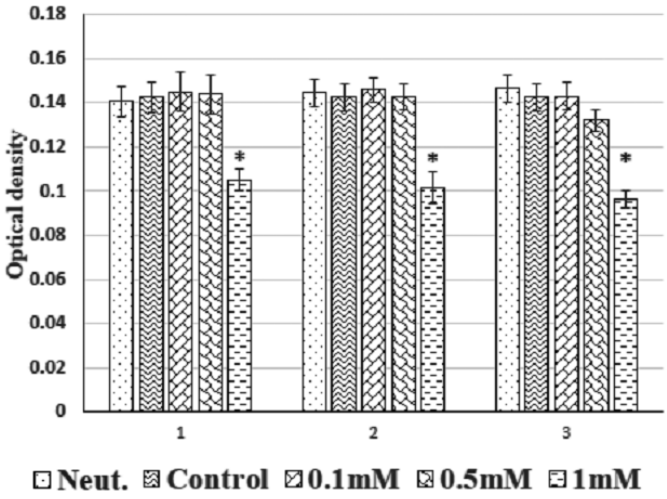

Fig. 2. Modulation of Neutral red uptake by neutrophils. Greater optical density shows higher levels of Neutral red uptake by neutrophils. Results indicated that NR uptake by neutrophils didn't show any significant difference between neutrophils alone and neutrophils co-cultured with MSCs without treatment or MSCs pulsed with caffeine at concentrations of 0.1 and 0.5 mM. However, MSCs treated with caffeine at concentrations of 1 mM significantly decreased the NR uptake by co-cultured neutrophils. The values were presented as mean ± S.D. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (*p < 0.001 vs neutrophils alone or control)

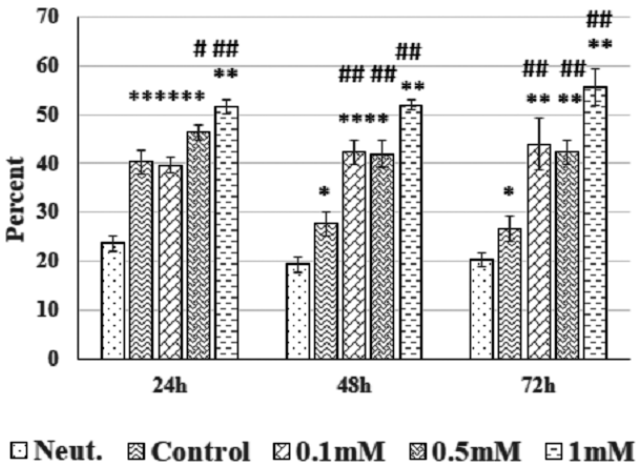

Fig. 3. Modulation of phagocytic ability of opsonized yeast by neutrophils co-cultured with MSCs. Neutrophils were incubated with opsonized yeast at a ratio of 1:10 for 0.5 h at 37°C. Greater present value indicates higher levels of phagocytic ability. The phagocytosis activity of neutrophils was significantly increased following co-culture with of MSCs treated with at least 0.5 mM of caffeine for 24 h compared with control group. Moreover, MSCs treated with caffeine at least at concentration of 0.1 mM significantly increased the phagocytosis activity of neutrophils after 48 h and/or 72 h. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (*p < 0.01, ** p < 0.001, *** p < 0.0001 vs neutrophils alone# p < 0 01, ## p < 0.001 vs control)

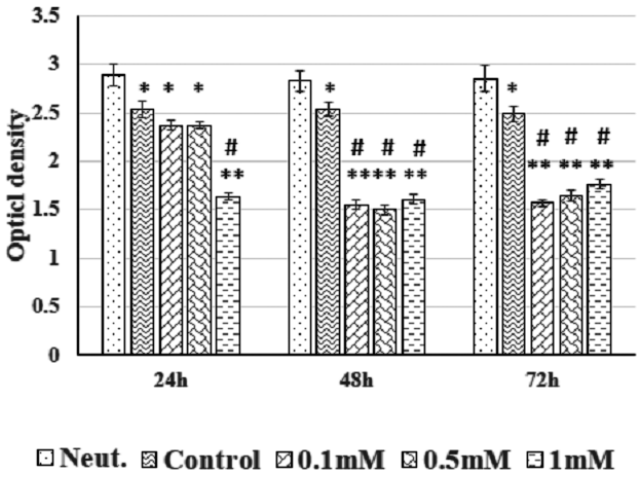

Fig. 4. Evaluation of neutrophils respiratory burst after opsonized yeast ingestion by caffeine treated MSCs. Lesser optical density indicates lower levels of respiratory burst. Compared to control group, MSCs pulsed with 1 mM of caffeine for 24 h and of MSCs treated caffeine even at 0.1 mM for 48 h and/or 72 h significantly diminished the rate of respiratory bust of co-cultured neutrophils. Neut. – neutrophils alone, Control – neutrophils co-cultured with of MSCs alone (*p < 0.01, ** p < 0.001 vs neutrophils alone; # p < 0.001 vs control)

The NBT reduction assay was used to measure the reactive oxygen species (ROS) activity in neutrophils.[22] The obtained findings expressed that the respiratory burst of neutrophils was significantly decreased in neutrophils co-cultured with the MSCs pulsed or without caffeine, compared to neutrophils without treatment (Fig. 4). The attained data also indicated that this reduction of the respiratory burst is more prominent in the neutrophils co-cultured with caffeine (except caffeine at concentrations of 0.1 or 0.5 mM for 24 h) than that observed after co-culture of neutrophils and MSCs alone (Fig. 4).

Discussion

It has been revealed that MSCs interestingly produce adenosine and express adenosine receptors (A1R, A2AR, A2BR, and A3R), which clearly indicates that adenosine and adenosine receptors play an autocrine or paracrine role in the proliferation and differentiation of MSCs.[14,23] Adenosine receptors are also differentially expressed in MSCs and involved in lineage-specific differentiation of MSCs. The A2B receptor is dominant in MSCs, and its expression and activity were transiently increased at the early stages of osteoblastic differentiation. During the later stages of osteoblastic differentiation, the expression of A2AR was increased.[23] On the other hand, differentiation of MSCs to adipocytes is associated with significant up-regulation in A1 and A2A receptors expression.[23] It has been known for a long time that the methylxanthine derivative, such as caffeine, can interfere with the adenosine/adenosine receptors biology.[5,6]

Neutrophils are the most prominent cell type of the innate immune system and are predominant in host tissues during acute inflammatory processes.[11,24] Neutrophils may also play an effective role in adaptive immunity.[24]

Mature neutrophils are normally found in the bloodstream and inflamed tissues, instead of bone marrow. Mesenchymal stem cells localized in the perivascular and periendothelial areas can directly crosstalk with neutrophils.[25,26] It is necessary to notice that MSCs localized in the perivascular area, derived from various tissues, have shown a phenotype similar to that of the bone marrow-derived MSCs.[26] Certainly, the isolation and expansion of MSCs from the bone marrow are easier than isolating MSCs from other tissues. Similar to the present study, some former studies also used the bone marrow derived MSCs to investigate the interaction between MSCs and neutrophils.[11,27]

Neutrophil homeostasis and turnover are highly regulated in the body. Circulating neutrophils have a short life span of 6-10 h, after which the cells undergo apoptosis.[28] It was shown that MSCs significantly protect neutrophils from apoptosis and increase the life span of these cells.[25,29] The MTT assay is a rapid test for assessing cell viability.[11] The findings showed that caffeine at higher doses could significantly decrease the protective role of MSCs on neutrophils apoptosis. Previous studies indicated that MSCs diminish the mitochondrial pro-apoptotic protein, Bax in neutrophils through IL-6 secretion.[27] It is also known that adenosine produced by MSCs can stimulate the A2B receptors through autocrine or paracrine routs, and potently stimulates IL-6 secretion.[23] According to the antagonistic effects of caffeine on adenosine receptor, it is possible that

caffeine at higher doses may interfere with IL-6 secretion by MSCs. However, the precise mechanisms involved in these effects are yet to be clarified.

Neutral red can be ingested and accumulated in the lysosomes of neutrophils depending on the level of cell activation. The neutral red uptake by neutrophils depends on different factors connected with cell viability, activity, and cell membrane integrity.[30]

Phagocytosis is an essential function of neutrophils, which participates in the uptake of pathogens, apoptotic bodies, and debris.[31] The phagocytic activity of neutrophils was markedly increased in neutrophils co-cultured with the bone marrow-derived MSCs compared to neutrophils without MSCs. Moreover, it has been demonstrated that the caffeine treated bone marrow-derived MSCs may cause a significant increase in the phagocytic ability of neutrophils more profound than MSCs alone.

Reactive oxygen species (ROS) are one of the important factors involved in the elimination of invading microbes by neutrophils.[22] In addition to encountering pathogens, different stimuli may induce the respiratory burst in neutrophils.[27] Nonetheless, when the production of ROS is excessive or inappropriate, ROS participate in severe host tissue damages and in different immunopathological conditions.[32] In this survey, it was observed that MSCs could significantly reduce the ROS production by neutrophils. In this regard, the former data indicated that the supernatant of MSCs could inhibit the basal and f-MLP-stimulated production of ROS by neutrophils.[27] The obtained data also indicated that MSCs pulsed with caffeine could profoundly inhibit the ROS production by neutrophils more pronounced than MSCs without treatment. Of note, higher phagocytic activity without the production of potentially harmful ROS can help phagocytes reduce inflammation.

Our in vitro findings suggest that at least some of the effects of caffeine on the interaction between MSCs and neutrophils may be different between the low to moderate and high concentrations of caffeine. Pervious works also confirmed that caffeine had dose-dependent effects on the osteogenic differentiation of MSCs: 0.1 mM caffeine significantly potentiated mineralization and alkaline phosphatase activity, and upregulated the osteogenic differentiation of MSCs. However, a concentration of caffeine greater than 0.3 mM diminished the osteogenic differentiation of MSCs.[3] A number of in vitro and in vivo studies have indicated that caffeine could modulate both innate and acquired immune responses.[6,33,34] Moreover, it has been demonstrated that the effects of caffeine may be partly related to the dose of caffeine.[6] Interestingly, some evidence has suggested that caffeine, even at the concentrations that are relevant to normal human consumption, may possess anti-inflammatory and immunomodulatory effects.[6] Based on the attained data, it has been proposed in this paper that some of the immunomodulatory and anti-inflammatory effects of caffeine may be due to the change in the interaction between MSCs and neutrophils.

Conclusions

The observations in this research suggest that bone marrow derived MSCs pulsed with caffeine at low (0.1 mM) to moderate (0.5 mM) concentrations preserve the basic activity of neutrophils and established the MSCs ability to protect neutrophils from apoptosis. Mesenchymal stem cells treated with caffeine increased the phagocytosis of neutrophils and simultaneously, diminished the production of reactive oxygen substances more profound than MSCs without treatment. Nevertheless, a high concentration of caffeine could interfere with some aspects of the crosstalk between MSCs and neutrophils. Overall, these findings may offer new insight into the potential mechanisms underlying the immunomodulatory and anti-inflammatory effects of caffeine.

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TEGDMA and UDMA monomers released from composite dental material polymerized with diode and halogen lamps

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Abstract

Background. More than 35 substances released from composite fillings have been identified. Among these, basic monomers and the so-called co-monomers are most often reported. The substances released from polymer-based materials demonstrate allergenic, cytotoxic, genotoxic, mutagenic, embryotoxic, teratogenic, and estrogenic properties.

Objectives. The aim of this study was to measure the amounts of triethylene glycol dimethacrylate (TEGDMA) and urethane dimethacrylate (UDMA) monomers released from composite dental fillings to citrate-phosphate buffer with the pH of 4, 6, 8 after 24 h and 6 months from the polymerization.

Material and methods. Ten samples for each polymerization method had been made from the composite material (Filtek Supreme XT, 3M ESPE, St. Paul, USA), which underwent polymerization using the following lamps: halogen lamp (Translux CL, Heraeus Kulzer, Hanau, Germany) (sample H) and diode lamp (Elipar Freelight 2, 3M ESPE), with soft start function (group DS) and without that function (group DWS).

Results. It has been demonstrated that the type of light-curing units has a significant impact on the amount of TEGDMA and UDMA released. The amount of UDMA and TEGDMA monomers released from composite fillings differed significantly depending on the source of polymerization applied, as well as the pH of the solution and sample storage time.

Conclusions. Elution of the monomers from composite material polymerized using halogen lamp was significantly greater as compared to curing with diode lamps.

Key words: light curing units, triethylene glycol dimethacrylate, urethane dimethacrylate, fluorescence spectrometry

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Introduction

In the oral cavity, chemical compounds from composite dental fillings can be released. Various factors contribute to this process, including a low degree of monomer conversion (DC%) of the polymer matrix (the reported DC% varies between 35 and 77%),[1,2] washing out with such fluids as saliva, gingival crevicular fluid, drinks consumed, an adequate light activation technique, the processes of enzymatic degradation of the material, the quality of light-curing unit used, the regime of restorative procedure, or wearing dental prostheses. The substances released from polymer-based materials demonstrate allergenic, cytotoxic, genotoxic, mutagenic, embryotoxic, teratogenic, and estrogenic properties.[3–11]

More than 35 substances released from composite fillings have been identified. Among these, basic monomers and the so-called co-monomers are most often reported. Substantial cytotoxic potential is observed in the basic monomers, such as bisphenol A-glycidyl methacrylate (Bis-GMA) and urethane dimethacrylate (UDMA), which inhibit cell growth in vitro, at the concentration of 0.1 mM.[3] Among the co-monomers, diethyleneglycol dimethacrylate (DEGDMA) and triethyleneglycol dimethacrylate (TEGDMA) exhibit the worst biocompatibility. It has been demonstrated that monomers also have sensitizing properties. Among the most allergenic ones, there are 2-hydroxyethyl methacrylate (HEMA), ethyleneglycol dimethacrylate (EGDMA) and TEGDMA. Authors describe allergic reactions on the oral mucosal membrane of patients; however, dentists are more prone to contact allergies.[3] The frequency of occurrence of allergy to metacrylates among dentists and their personnel varies between 1.3 and 14%.[12] Often, apart from skin lesions, asthma and sinusitis occur. Latex gloves do not protect against monomers, which penetrate through them within 1 min.[12] Some substances released from composite fillings may bind with estrogen receptors, mimicking natural hormones. They are called xeno-estrogens. Among them, there are: bisphenol A (BPA), bisphenol A-glycidyl methacrylate (Bis-GMA), bisphenol A-dimethacrylate (Bis-DMA), and TEGDMA.[13] Monomers have a damaging effect on cell genetic material; they disturb the regulation of cell growth cycle as well as the oxidation-reduction balance that may activate reactions leading to apoptosis.[3,7,14]

The type of light source used for photopolymerization has a considerable influence on the amount of monomers released from composite resin materials. Light-curing units used in dentistry include conventional quartz-tungsten-halogen (QTH, still in use), light-emitting diode (LED), plasma arc (PAC) lamps, and argon laser. The following parameters differentiate them: the light source and optic elements, the amount of thermal energy emitted, the efficiency of material polymerization and the intensity of curing, the performance efficiency, the existence of voltage stabilization system.[15]

The main feature which distinguishes LED lamps from halogen light-curing units is the source of light. A halogen lamp produces light by heating a metal filament wire to a high temperature until it glows. The spectrum of emitted waves is in the broad range of 360–560 nm, while the luminous intensity peak is in the range of 400–500 nm. Producers install special systems of filters to eliminate the useless radiation of ultraviolet or infrared ranges. Also, the elements of optical system and the bulb itself are subject of gradual degradation. As a result of those processes, the amount of emitted light is insufficient. It is assumed that halogen lamps function with 100% efficiency for some 50 h, which directly influences the increased presence of residual monomers, that is, their low level of conversion.[16]

In diode lamps, the sources of light emission are semiconductor p-n junctions or diodes (precisely light-emitting diodes, LEDs). The semiconductor used there is gallium nitride (GaN). The conduction of that compound is associated with the number of electrons and holes in the valence band of its atoms. The selection of specific semiconductor depends upon the selected range of emitted waves. The average spectra of diode lamps vary from 440 to 495 nm. The efficiency of LED lamps is approx. 10 times higher than that of halogen lamps, as the length of the emitted wave is better adjusted to the photoinitiators used in light-cured materials.[17] In the majority of composite materials, camphorquinone is the photoinitiator. It is activated by light of a wavelength of 468 nm. Better selectivity of diode lamps contributes to the reduction of the light intensity by 40–70% in the course of polymerization of dental materials in comparison with halogen lamps.[17] Due to the narrow radiation spectrum, producers eliminated the need of applying filter systems in those lamps. As a result, they can function with 100% efficiency for some 10,000 h.[17] However, not all dental materials may be cured with diode lamps. The above applies to materials in which the initiators or co-initiators of polymerization react to waves whose lengths are outside the emission range of LEDs.

The aim of the study was to assess the amount of TEGDMA and UDMA monomers released from composite dental filling materials to the solution, depending upon the light-curing units used for polymerization – halogen or diode ones, with and without the soft start function.

Material and methods

From Filtek Supreme XT composite material (3M ESPE, St. Paul, USA; dentinal shades A3D, A4D, B3D), 4 groups of 10 samples were prepared using standardized molds (the diameter of 15 ±1 mm and the thickness of 0.5 ±0.1 mm). The samples were polymerized using halogen (Translux LC, Heraeus Kulzer, Hanau, Germany – group H) and diode (Elipar FreeLight 2, 3M ESPE) light-curing units. A diode lamp was used with a soft start function (group DS) and the continuous light mode (group DWS). Soft start

polymerization means that irradiation is initiated with light of lower intensity and continued with higher irradiation compared to that in the continuous method. In the Elipar FreeLight 2 lamp, the intensity increases exponentially. The curing time was 40 s for all lamps. The control group consisted of 10 non-polymerized samples (group N). All samples were stored in Eppendorf tubes with 1 mL of citrate-phosphate buffer (No. P 4809, Perkin Elmer Inc., Waltham, USA) with different pH levels (4, 6 and 8) for 24 h and 6 months, at 36.6°C.[18,19] After 24 h and 6 months, the polymerized and non-polymerized materials were removed from the buffer. The samples examined after 24 h and 6 months (10 in each group) were separate collections of research material. The quantitative analysis of the residual monomer leached into solutions was carried out using a fluorescent spectrometer LS45 (Perkin Elmer Inc.), with a 0.6 mL quartz cuvette. The absorbance measurements of the samples were taken in reference to a buffer with corresponding pH. The optimum values of excitation (ex) and emission (em) for model substance (TEGDMA No. 90412 and UDMA No. 436909, Perkin Elmer Inc.) were determined using a spectrophotometer (Shimadzu UV-160A, Kioto, Japan). The values recorded were as follows: for TEGDMA λ_{ex} = 223 nm and λ_{em} = 290 nm, and for UDMA λ_{ex} = 219 nm and λ_{em} = 285 nm.

Statistical analysis of the data was performed using STATISTICA software v. 8.0 (StatSoft Inc., Tulsa, USA). Values in all groups were checked for normal distribution, applying the normality of the Shapiro-Wilk and Kolmogorov tests. If the distribution remained normal in both dependency tests, the data was analyzed using tests of 2 or multiple averages. In other cases, the Mann-Whitney U and the Kruskal-Wallis tests were used. If more than 1 sample has demonstrated differences in the tests (multiple averages or Kruskal-Wallis tests), post-hoc tests were performed. Statistical significance was set at $p \leq 0.05$.

Results

The mean concentrations of UDMA and TEGDMA monomers released into the citrate-phosphate buffer of different pH (4, 6, 8) after 24 h and 6 months, and a kind of distribution are presented in Tables 1 and 2. Between 24 h and 6 months, the mean concentrations of UDMA and TEGDMA significantly increased ($p < 0.001$) within all the groups, irrespective of the pH of the buffer (Table 3).

After 24 h, non-polymerized samples (N) released significantly more UDMA than samples polymerized using a diode lamp (DS and DWS) at pH 4 and samples cured using a diode lamp without the soft start function (DWS) in the solutions having pH 6 and 8. The release of UDMA occurred at a significant lower rate in samples cured with a diode lamp (DS and DWS) when compared with samples cured using a halogen lamp (H) in the solutions of pH 4 and 6. In the buffer pH 8 with the same residence time, there was significantly less ($p < 0.05$) UDMA when samples were cured with a diode lamp without the soft start function (DWS) compared with the samples polymerized using a halogen lamp (H).

After 6 months, no significant differences were found in the release of UDMA to the buffer pH 4 in all groups. In the buffer pH 6, the release of that monomer occurred to be significantly higher in uncured samples (N). However, when the pH of the buffer was 8, a reverse situation was noted – a significantly lower concentration of UDMA in group N as compared to other groups. In the buffer pH 8, a significantly higher release of that monomer was observed in the DWS group than in the N, DS and H groups ($p < 0.001$). In the H group, the amount of UDMA in the buffer was significantly lower when compared with the DS group ($p < 0.05$). The results are shown in Table 4.

After 24 h, in all groups, the mean concentration of UDMA in the buffer pH 4 was significantly higher than

Table 1. The amount of UDMA (μg/g) released from composite material into citrate-phosphate buffer at different pH level after 24 h and 6 months

Type of sample	Number of measurements	24 h (pH 4 / pH 6 / pH 8)				6 months (pH 4 / pH 6 / pH 8)			
		mean (μg/g)	standard deviation	standard normal distribution		mean (μg/g)	standard deviation	standard normal distribution	
				Shapiro-Wilk test	Kolmogorov test			Shapiro-Wilk test	Kolmogorov test
N	10/ 10/ 10	94.78/ 12.09/ 8.65	24.81/ 4.07/ 1.01	+/ +/ –	+/ +/ +	163.37/ 161.57/ 17.33	32.85/ 75.31/ 3.90	+/ +/ +	+/ +/ +
DS	10/ 10/ 10	60.03/ 8.94/ 7.64	9.25/ 1.74/ 1.15	–/ –/ +	+/ +/ +	364.96/ 118.52/ 100.33	247.65/ 38.16/ 15.30	–/ +/ +	+/ +/ +
DBS	10/ 10/ 10	62.73/ 7.83/ 7.28	8.91/ 1.14/ 1.26	+/ +/ +	+/ +/ +	349.66/ 95.12/ 142.92	260.23/ 38.07/ 38.49	–/ +/ +	–/ +/ +
H	10/ 10/ 10	86.78/ 12.11/ 8.99	25.51/ 2.32/ 1.26	–/ +/ +	+/ +/ +	392.37/ 115.00/ 81.97	333.16/ 19.52/ 8.28	–/ +/ +	+/ +/ +

N – group of samples non-polymerized; DS – group of samples polymerized using diode light-curing unit with soft start function; DBS – group of samples polymerized using diode light-curing unit without soft start function; H – group of samples polymerized using halogen light-curing unit.

Table 2. The amount of TEGDMA (μg/g) released from composite material into citrate-phosphate buffer at different pH level after 24 h and 6 months

Type of sample	Number of measure-ments	24 h (pH 4 / pH 6 / pH 8)				6 months (pH 4 / pH 6 / pH 8)			
		mean [μg/g]	standard deviation	standard normal distribution		mean [μg/g]	standard deviation	standard normal distribution	
				Shapiro-Wilk test	Kolmogorov test			Shapiro-Wilk test	Kolmogorov test
N	10/ 10/ 10	93.25/ 81.80/ 73.79	21.94/ 25.63/ 11.34	+/ +/ –	+/ +/ +	164.78/ 1002.26/ 151.77	25.37/ 450.30/ 39.27	+/ +/ +	+/ +/ +
DS	10/ 10/ 10	60.62/ 57.54/ 62.69	9.55/ 8.48/ 6.17	+/ +/ +	+/ +/ +	336.48/ 724.74/ 868.31	244.28/ 205.49/ 134.20	–/ –/ +	+/ +/ +
DBS	10/ 10/ 10	62.16/ 49.65/ 60.17	7.63/ 7.33/ 9.30	+/ +/ +	+/ +/ +	315.65/ 632.66/ 1250.57	265.82/ 217.71/ 337.96	–/ +/ +	–/ +/ +
H	10/ 10/ 10	79.84/ 73.32/ 74.82	19.03/ 12.89/ 7.86	+/ +/ +	+/ +/ +	404.61/ 704.31/ 712.55	393.20/ 135.78/ 74.55	–/ +/ +	+/ +/ +

N – group of samples non-polymerized; DS – group of samples polymerized using diode light-curing unit with soft start function; DBS – group of samples polymerized using diode light-curing unit without soft start function; H – group of samples polymerized using halogen light-curing unit.

Table 3. UDMA or TEGDMA monomers released from composite material, depending on the sample residence time in citrate-phosphate buffer – pH 4, pH 6, pH 8

Type of sample pH 4 / pH 6 / pH 8	UDMA 24 h–6 months t DA	UDMA 24 h–6 months t UM-W	TEGDMA 24 h–6 months t DA	TEGDMA 24 h–6 month t UM-W
N	a/a/–	–/–/a	a/a/–	–/–/a
DS	–/–/a	a/a/–	–/–/a	a/a/–
DBS	–/a/a	a/–/–	–/a/a	a/–/–
H	–/a/a	a/–/–	–/a/a	a/–/–

N – group of samples non-polymerized; DS – group of samples polymerized using diode light-curing unit with soft start function; DBS – group of samples polymerized using diode light-curing unit without soft start function; H – group of samples polymerized using halogen light-curing unit; a – $p < 0.001$; t DA – double average test; t UM-W – U Mann-Whitney test.

Table 4. UDMA monomer released from composite material into citrate-phosphate buffer (pH 4, pH 6, pH 8), depending upon polymerization light-curing unit used, after 24 h and 6 months

UDMA pH 4 / pH 6 / pH 8	24 h t K-W / t K-W / t K-W				6 months t K-W / t MA / t MA			
Type of sample ►	N	DS	DBS	H	N	DS	DBS	H
R or X ►	30.00/ 27.10/ 26.10	10.80/ 15.80/ 15.90	14.10/ 10.30/ 12.50	27.10/ 28.80/ 27.50	13.10/ 161.57/ 17.33	23.30/ 118.52/ 100.33	25.10/ 95.12/ 142.92	20.50/ 115.00/ 81.97
Type of sample ► ▼	N	DS	DBS	H	N	DS	DBS	H
N		$p < 0.001$/ $p = 0.0920$/ $p = 0.1532$	$p < 0.01$/ $p < 0.01$/ $p < 0.05$	$p = 1.0000$/ –/ –		–/ $p < 0.05$/ –	–/ $p < 0.01$/ –	–/–/–
DS	–/ –/ –		–/ –/ $p = 1.0000$	–/ –/ –	$p = 0.1532$/–/ $p < 0.001$		–/ $p = 0.1381$/ –	$p = 1.0000$/ $p < 0.05$/ $p < 0.05$
DBS	–/ –/ –	$p = 1.0000$/ –/ –		–/ –/ –	$p = 0.0652$/–/ $p < 0.001$	$p = 1.0000$/–/ $p < 0.001$		$p = 1.0000$/ $p = 0.4344$/ $p < 0.001$
H	–/ $p = 1.0000$/ $p = 1.0000$	$p < 0.01$/ $p < 0.05$/ $p = 0.0795$	$p < 0.05$/ $p < 0.01$/ $p < 0.05$		$p = 0.4708$/–/ $p < 0.001$	–/ –/ –	–/ $p = 0.1769$/ –	

N – group of samples non-polymerized; DS – group of samples polymerized using diode light-curing unit with soft start function; DBS – group of samples polymerized using diode light-curing unit without soft start function; H – group of samples polymerized using halogen light-curing unit; R – average t MA; X – average t K-W; t MA – multiple average test; t K-W – Kruskal-Wallis test.

Table 5. UDMA monomer released into citrate-phosphate buffer, depending on pH, after 24 h and 6 months

UDMA Sample N / DS / DBS / H	24 h t K-W / t K-W / t MA / t K-W			6 months t MA / t K-W / t K-W / t K-W		
pH value ►	4	6	8	4	6	8
R or X ►	25.50/ 25.50/ 62.74/ 25.50	13.00/ 13.20/ 7.83/ 14.50	8.00/ 7.80/ 7.28/ 6.50	163.37/ 25.30/ 24.30/ 25.10	161.57/ 12.30/ 7.90/ 15.10	17.33/ 8.90/ 14.30/ 6.30
pH value ► ▼	4	6	8	4	6	8
4		p < 0.01/ p < 0.01/ p < 0.001/ p < 0.01	p < 0.001/ p < 0.001/ p < 0.001/ p < 0.001		p = 0.4665/ p < 0.01/ p < 0.001/ p<0.05	p < 0.001/ p < 0.001/ p < 0.05/ p < 0.001
6			p = 0.3061/ p = 0.2553/ p = 0.4080/ p = 0.0632			p < 0.001/ p = 0.2553/ –/ p < 0.05
8					–/ –/ p = 0.1561/ –	

N – group of samples non-polymerized; DS – group of samples polymerized using diode light-curing unit with soft start function; DBS – group of samples polymerized using diode light-curing unit without soft start function; H – group of samples polymerized using halogen light-curing unit; R – average t MA; X – average t K-W; t MA – multiple average test; t K-W – Kruskal-Wallis test.

in buffers pH 6 and 8. On the other hand, for non-polymerized samples after 6 months, a significantly lower UDMA was noted when the pH of the buffer was 8 as compared to the solutions with lower pH values ($p < 0.01$) (Table 5).

The release of TEGDMA after 24 h was significantly higher in the non-polymerized group (N) in the buffer pH 4. At that time, no significant differences occurred in the release of this monomer from the samples cured using a diode lamp (DS and DWS) for all pH values. Regardless of the pH of the solution, the release of TEGDMA after 24 h was significantly lower when samples were cured with a diode lamp (DS and DWS) in relation to the samples cured with a halogen lamp (H). After 6 months, no significant differences were disclosed in the amount of TEGDMA released to the buffers pH 4 and 6 for all examined samples. However, when the pH of the solution was 8, a significantly lower concentration of this monomer was noted in the case of non-polymerized samples as compared with the polymerized ones, whatever the lamp type was ($p < 0.001$). Samples polymerized using a diode lamp without the soft start function (DWS) released significantly ($p < 0.001$) more TEGDMA to the buffer in comparison with the remaining samples. The results are shown in Table 6.

After 24 h, the release of TEGDMA from non-polymerized samples (N) to the buffer with pH 8 was significantly ($p < 0.05$) diminished in comparison with the solution of pH 4.

After 6 months, the mean concentration of TEGDMA released from samples N was significantly lower in solutions pH 4 and 8 in comparison with those of pH 6 ($p < 0.001$). The highest amount of TEGDMA was released in the DBS group in solutions of pH 8. The results are presented in Table 7.

Discussion

The quantitative and qualitative assessment of residual monomers released from composite fillings to external environment has been a subject of investigations by many authors.[1,18–25] The willingness to examine thoroughly what happens with the composite filling used, to what degree it is degraded and what influence the chemical compounds released from fillings have upon the human organism compelled the search for ever more accurate analytic methods. For the analysis of released monomers, the authors applied gas and liquid chromatography as well as fluorescence spectrophotometry. An intermediate method for examining the condition of fillings after curing, attesting to the degree of conversion of materials exposed to curing, is Raman or Fourier spectrometry.[26–30] These methods consist of a comparative analysis of double carbon bonds occurring in polymerized and non-polymerized material.

Polydorou et al. studied the release of Bis-GMA, UDMA and TEGDMA from Filtek Supreme XT restorative composite.[1] The cured and uncured samples were stored in 75% ethanol solution for 24 h, 7 days, 28 days, and 12 months. Elipar Highlight (3M ESPE) halogen lamp was used in the study, and the following polymerization time was applied: 0, 20, 40, and 80 s. Liquid chromatography-mass spectrometry was used for quantitative and qualitative analysis.

Table 6. TEGDMA monomer released from composite material into citrate-phosphate buffer (pH 4, pH 6, pH 8), depending upon polymerization light-curing unit used, after 24 h and after 6 months

TEGDMA pH 4 / pH / pH 8	24 h t MA / t MA / t K-W				6 months t K-W / t K-W / t MA			
Type of sample ►	N	DS	DBS	H	N	DS	DBS	H
R or X ►	93.25/ 81.81/ 26.30	60.62/ 57.54/ 15.40	62.16/ 49.65/ 11.60	79.84/ 73.32/ 28.70	19.20/ 27.20/ 151.77	20.20/ 20.10/ 868.31	20.80/ 16.50/ 1250.57	21.80/ 19.20/ 712.55
Type of sample ► ▼	N	DS	DBS	H	N	DS	DBS	H
N		p < 0.001/ p < 0.001/ p = 0.1112	p < 0.001/ p < 0.001/ p < 0.05	p < 0.05/ p = 0.1129/ –		–/ p = 0.5234/ –	–/ p = 0.1221/ –	–/ p = 0.2555/ –
DS	–/ –/ –		–/ p = 0.1297/ p= 1.000	–/ –/ –	p = 1.0000/ –/ p < 0.001		–/ p = 1.000/ –	–/ p = 1.000/ p < 0.05
DBS	–/ –/ –	p = 0.4146/ –/ –		–/ –/ –	p = 1.0000/ –/ p < 0.001	p = 1.0000/ –/ p < 0.001		–/ –/ p < 0.001
H	–/ –/ p = 1.000	p < 0.01/ p < 0.05/ p < 0.05	p < 0.01/ p < 0.001/ p < 0.01	–/ –/ –	p = 1.0000/ –/ p < 0.001	p = 1.0000/ –/	p = 1.0000/ –/ –	

N – group of non-polymerized sample; DS – group of sample polymerized using diode light-curing unit with soft start function; DBS – group of sample polymerized using diode light-curing unit without soft start function; H – group of sample polymerized using halogen light-curing unit; R – average t MA; X – average t K-W; t MA – multiple average test; t K-W – Kruskal-Wallis test.

Table 7. TEGDMA monomer released into citrate-phosphate buffer, depending on pH, after 24 h and 6 months

TEGDMA Sample N / DS / DBS / H	24 h t K-W / t MA / t MA / t MA			6 months t MA / t K-W / t K-W / t K-W		
pH value ►	4	6	8	4	6	8
R or X ►	19.50/ 60.62/ 62.16/ 79.34	16.00/ 57.54/ 49.65/ 73.32	11.00/ 62.70/ 60.17/ 74.82	164.78/ 6.90/ 7.50/ 9.20	1002.26/ 17.30/ 13.50/ 18.10	151.77/ 22.30/ 25.50/ 19.20
pH value ► ▼	4	6	8	4	6	8
4		p = 0.5610/ p = 0.2037/ p < 0.01/ p = 0.1538	p < 0.05/ –/ p = 0.2949/ p = 0.2155			p = 0.4561/ –/ –/ –
6			p = 0.3061/ –/ –/ –	p < 0.001/ p < 0.05/ p = 0.1913/ p < 0.05		p < 0.001/ –/ –/ –
8	–/ p = 0.2884/ –/ –	–/ p = 0.855/ p < 0.01/ p = 0.4060		–/ p < 0.001/ p < 0.001/ p < 0.05	–/ p = 0.3061/ p < 0.01/ p = 1.0000	

N – group of non-polymerized sample; DS – group of sample polymerized using diode light-curing unit with soft start function; DBS – group of sample polymerized using diode light-curing unit without soft start function; H – group of sample polymerized using halogen light-curing unit; R – average t MA; X – average t K-W; t MA – multiple average test; t K-W – Kruskal-Wallis test.

The release of monomers got significantly reduced with the extension of curing time.[1] The results of this study are in agreement with our report on monomers released from polymerized samples (40 s) and non-polymerized ones (0 s) made of the Filtek Supreme XT material, after 24 h of residence in citrate-phosphate buffer, whatever the pH level was, and after 6 months, when the pH of the buffer amounted to 6. The release of monomers, in accordance with above-mentioned Polydorou el al., also significantly differed in time. The amounts of free TEGDMA dropped, whereas those of the Bis-GMA monomer remained at a similar level.[1] This part of their results was different

from our study. This may be ascribed to a shorter observation time (6 months) as well as sample storage conditions (citrate-phosphate buffer with different pH levels: 4, 6, 8). The results of the release of the 3 monomers were presented by the authors quoted in the form of logarithmic function, which is why they prove difficult to refer to the results of this study.

Örtengren et al. analyzed the influence of the solution pH value and of time upon the release of monomers from the Z-100 composite material.[18] The samples were stored in citrate-phosphate buffer with different pH levels (4, 6, 8) for 24 h and for 6 months. For the sake of comparison, 2 methods were applied for the sample analysis: gas chromatography and fluorescence spectrophotometry. The highest levels of organic substances release were noted in the case of methacrylic acid, TEGDMA and camphoroquinone. The authors of this study applied identical conditions for sample storage (citrate-phosphate buffer, with the pH values of 4, 6, 8, sample storage time: 24 h and 6 months). Örtengren et al., in the case of 24 h storage, obtained the following results concerning TEGDMA eluted to the medium: at the pH of 4 – 100 μg/g, at the pH of 8 – 87 μg/g of sample (by fluorescence spectrophotometry).[18] After 6 months, the amount of TEGDMA increased significantly at the pH of 4 – 230 μg/g and at the pH of 6 – 320 μg/g of sample. In the analysis by means of gas chromatography, which, in accordance with the authors quoted, proved to be more accurate after 24 h and after 6 months of storage, a significantly lower level of TEGDMA has been noted at the pH of 8 in comparison with other pH values (4 and 6). Applying the other analytical method, the lowest values of monomer release were obtained at the pH of 6 after 24 h and at the pH of 4 after 6 months of storage.[18] The authors of this study applied the fluorescence spectrophotometry method and obtained similar results after 24 h of storage (the best convergence for samples cured using a halogen lamp), but in the case where the pH of the buffer amounted to 8, the average concentration of TEGDMA was higher than at the pH of 4 and 6. After 6 months of storage, the amount of released TEGDMA was higher than in the results obtained by Örtengren el al., using fluorescence spectrophotometry. One should also add that it increased with increasing pH of the buffer.

Örtengren el al. analyzed water adsorption and solubility of the samples, made of 6 composite materials, in aqueous solution. The analysis of monomers was carried out with liquid chromatography. The sample storage time amounted to 4, 24 h as well as 7, 60 and 180 days. They showed that composite materials containing hydrophilic monomers demonstrate higher sorption of water. The composite mass increased via chemical reaction between the filling and water. The monomer released in biggest quantities was TEGDMA, with the highest concentration after 7 days.[23]

Using the gas chromatography method, Moharamzadeh et al. analyzed the influence of various extraction solutions upon the release of TEGDMA, UDMA and Bis-GMA.[20] The extraction solutions used were: distilled water, isotonic salt solution, artificial saliva as well as Dulbecco medium, without serum and with 10% fetal calf serum. The results were indicative of a significant influence of the solution in which cured composite samples were stored on the release of monomers. The highest concentration of TEGDMA monomer was noted in the Dulbecco medium with serum. In other solutions, the concentration of this monomer was similar. The release of UDMA and Bis-GMA to the studied solutions was not observed. On the basis of results, the authors found that TEGDMA is leached in high quantities to aqueous solutions.

Sideridou and Achilias analyzed, by means of liquid chromatography, the influence of sample curing time and their storage time upon the release of Bis-GMA, ethoxylated bisphenol A glycol dimethacrylate (Bis-EMA), UDMA, and TEGDMA.[24] The samples were stored in 75% ethanol, at 37°C, for 3, 6, 24 h, and 3, 6 or 30 days. The curing time amounted to 60, 80 and 100 s. The authors found an increase of Bis-GMA monomer up to 3 days. After that time, the release was maintained at a stable level. The amount of Bis-EMA monomer released was much higher than that of Bis-GMA monomer throughout the study period (up to 30 days). On the other hand, the release of TEGDMA and UDMA monomers was at a similar level, and much lower in comparison with the above-mentioned monomers. The authors found that the amount of eluted monomers depends upon the chemical structure of the examined compound and sample storage time.[24]

Carvalho et al. investigated the effect of light sources (LED and QTH curing units) and curing mode techniques on sorption, solubility and biaxial flexural strength of a composite resin.[31] In their opinion, tested curing units produced no influence on sorption, solubility or biaxial flexural strength of tested composite resins. They observed that ethanol storage media caused more damage on a composite resin than water storage media.[31]

Summarizing the above results, it should be stressed that the proper installation and polymerization of the composite material with a diode curing unit reduces the monomer concentration and its negative influence on the dental pulp, surrounding tissues as well as entire organism.

Conclusions

The amount of UDMA and TEGDMA monomers released from composite fillings differed significantly, depending on the source of polymerization applied as well as the pH of the solution and sample storage time. The degree of material polymerization depends on the lamp used; significantly more monomers are released from samples polymerized with halogen lamps in comparison with those cured using a diode lamp.

The use of soft start function in a diode lamp did not influence significantly the amount of monomers released from composite samples.

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The bond shear strength of methacrylate materials used to reduce dental and alveolar undercuts

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Abstract

Background. The reduction of dental and alveolar undercuts on plaster models is an important issue in the process of planning partial and complete prostheses. In recent years, new materials such as methacrylate resins that can be used to reduce undercuts have emerged. Their great advantage is high temperature insensitivity and relatively high ease of use.

Objectives. The study aimed at determining the factors that affect the shear bond strength, and which material can be better used at the laboratory stage of preparing the plaster model to facilitate the denture bearing area and reduce the traumatizing impact of the prosthesis.

Material and methods. In the study, 2 composite materials Block-Out Gel LC (VOCO GmbH, Cuxhaven, Germany) and LC Block-Out Resin (Ultradent Products Inc., South Jordan, USA) were used for tests on the Tewerock and Stodent plaster. Specimens consisted of 20 mm × 10 mm × 10 mm plaster blocks as a base, and composite cylinders of 3 mm diameter and 5 mm height, attached to the blocks. The base of the sample was combined with a composite cylinder in the Individo Light Box halogen lamp (VOCO GmbH, Cuxhaven, Germany). A total of 120 samples were studied. The shear bond strength (SBS) test was performed using the Hounsfield H5KS model HTE S/N D83281 fitted with a 5.000-N head using a cutting knife speed of 5 mm/min.

Results. LC Block-Out Resin and Block-Out Gel LC materials deposited on class III plaster and polymerized at temperatures of up to 100°C had the best SBS (5.59 MPa and 4.0 MPa, respectively). Samples made of LC Block-Out Resin and class IV plaster showed no statistically significant differences between all the groups. Additional polymerization under 2.4 bar was the most effective in improving SBS among Block-Out Gel LC and class IV plaster samples.

Conclusions. The results of the studies show that both the plaster type and the polymerization process have a significant effect on the SBS of light-cured methacrylate material to plaster.

Key words: shear bond strength, composite materials, dental and alveolar undercuts, undercuts reduction, methacrylate resins

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Introduction

One of the current problems in dental prosthetics is the reduction of dental and alveolar undercuts, leading to the facilitation of the denture bearing area.[1–4] It is a very important issue after a paralelometric analysis and affects the accuracy of the prosthesis.[5,6] Up to now, waxes, phosphate cements and silicone materials have been used to reduce undercuts. These materials did not provide sufficient precision of prosthetic devices, because they combined with the plaster model mechanically rather than chemically and, therefore, there was a need to replicate the plaster model.[7,8] Block-Out Gel LC and LC (VOCO GmbH, Cuxhaven, Germany) Block-Out Resin (Ultradent Products Inc., South Jordan, USA) are the most often used methacrylate undercuts reduction materials. Block-Out Gel LC is made mainly of urethaneacrylate oligomer, triethylene glycol dimethacrylate and catalyst. LC Block-Out Resin is made mainly of diurethane dimethacrylate and triethylene glycol dimethacrylate. Differences in the composition of both products may influence bonding with the plaster. Both of these materials are light-cured resins, colored blue for the ease of use. They combine strongly with the plaster model. Therefore, there is no need to duplicate it. This has a positive effect on the accuracy of the prosthesis and reduces the costs. In a laboratory, the abovementioned materials are used for the reduction of undercuts on plaster models. It should be emphasized that these materials have not been subjected to such an analysis before.

Objectives

The aim of the study was to determine the factors that affect the shear bond strength and to establish which material can be better used at the laboratory stage of preparing the plaster model for the treatment of patients in whom there is a need to reduce undercuts, facilitate the denture bearing area and decrease the traumatizing impact of the prosthesis.

Material and methods

Two composite materials Block-Out Gel LC and LC Block-Out Resin (Ultradent Products Inc., South Jordan, USA) were analyzed in order to determine which of them better meets the laboratory-clinical requirements. Shear bond strength (SBS) of these substrates from plaster surface was investigated. To compare SBS of both composite materials, they were attached to plaster blocks made of Tewerock class IV (Kettenbach GmbH & Co. KG, Eschenburg, Germany) and Stodent class III (Zhermack SpA, Badia Polesine, Italy) of different hardness. Experimental tests of SBS were carried out at the Department of Dental Techniques and Technologies of Poznan University of Medical Sciences using the Hounsfield H5KS test machine model HTE S / N D83281 fitted with a 5.000-N head using a cutting knife speed of 5 mm/min. Specimens consisted of 20 mm × 10 mm × 10 mm plaster blocks as a base, and composite cylinders of 3 mm diameter and 5 mm height, attached to the blocks. The base of the sample was combined with a composite cylinder in the Individo Light Box halogen lamp (VOCO GmbH, Cuxhaven, Germany). In general, 120 samples were prepared and tested to determine the SBS [MPa] – 60 of them were made from Block-Out Gel LC (30 on Tewerock plaster blocks and 30 on Stodent blocks). The next 60 samples were made of LC Block-Out Resin (30 on Stodent plaster blocks and 30 on Tewerock blocks). The sample types were then divided into 3 groups. The control group (group I) did not undergo any transformation (n = 10). Group II was subjected to polymerization under the pressure of 2.4 bar (n = 10). Group III was polymerized at 100°C (n = 10).

The statistical analysis was performed using the Kruskal-Wallis test and the Friedman test with STATISTICA v. 12 software (StatSoft Inc., Tulsa, USA). Post-hoc tests were used in order to decide which groups were significantly different from each other. Statistical significance was set at the $p \leq 0.05$ probability level. The Shapiro-Wilk test showed that the distributions of values of some groups were not normal, thus the non-parametric tests were used for all analyses.

Results

All results are presented in Table 1.

In the control group, the SBS of LC Block-Out Resin and Block-Out Gel LC attached to Stodent class III plaster was 2.67 MPa and 2.29 MPa, respectively. After polymerization under 2.4 bar pressure (group II), SBS significantly decreased (0.91 MPa and 0.88 MPa, respectively; $p \leq 0.05$). For samples made of LC Block-Out Resin after polymerization in 100°C (group III), SBS increased (5.59 MPa; $p \leq 0.05$). Also for samples made of Block-Out Gel LC, SBS increased after polymerization in 100°C, but it was statistically significant only when compared to group II.

In the control group, the SBS of the LC Block-Out Resin and Block-Out Gel LC attached to Tewerock class IV plaster was 4.00 MPa and 2.41 MPa, respectively. In group II, SBS was 2.21 MPa and 3.37 MPa, respectively, and in group III – 2.39 MPa and 2.41 MPa, respectively. For samples made of Block-Out Gel LC, SBS in group II after additional polymerization under 2.4 bar was higher when compared to group III ($p \leq 0.05$). However, there were no differences regarding SBS between the control group and other groups.

Comparing the studied materials in the control group, we found no statistical significance between samples regarding SBS. In group II, SBS was statistically the highest for Block-Out Gel LC attached to Tewerock class IV plaster (3.37 MPa). In group III, the highest SBS was proved for samples made of LC Block-Out Resin attached to Stodent class III plaster (5.59 MPa).

Table 1. Comparison of the shear bond strength values between different samples

Studied samples		Group I control				Group II polymerization under 2.4 bar				Group III polymerization at 100°C			
		Q1	median	mean	Q3	Q1	median	mean	Q3	Q1	median	mean	Q3
		[MPa]				[MPa]				[MPa]			
1.	LC Block-Out Resin + Stodent III	1.73	3.02 II, III	2.67 II, III	3.55	0.53	0.79# I, III	0.91# I, III	1.16	4.01	4.45$& I, II	5.59$& I, II	6.02
2.	Block-Out Gel LC + Stodent III	1.63	2.41 II	2.29 II	3.04	0.44	0.76* I,III	0.88* I,III	1.20	2.82	4.21 II	4.0 II	4.64
3.	LC Block-Out Resin + Tewerock IV	2.49	3.70	4.0	4.54	1.61	1.68	2.21	1.80	1.48	2.01$	2.39$	3.06
4.	Block-Out Gel LC + Tewerock IV	1.51	2.20	2.41	3.01	3.13	3.19#* III	3.37#* III	3.76	2.07	2.46& II	2.41& II	2.88

# $p < 0.05$ in comparison between LC Block-Out Resin + Stodent III and Block-Out Gel LC + Tewerock IV; * $p < 0.05$ in comparison between Block-Out Gel LC + Stodent III and Block-Out Gel LC + Tewerock IV; $ $p < 0.05$ in comparison between LC Block-Out Resin + Stodent III and LC Block-Out Resin + Tewerock IV; & $p < 0.05$ in comparison between LC Block-Out Resin + Stodent III and Block-Out Gel LC + Tewerock IV; I – $p < 0.05$ in comparison with group I (control); II – $p < 0.05$ in comparison with group II (polymerization under 2.4 bar); III – $p < 0.05$ in comparison with group III (polymerization at 100°C).

Discussion

As the following research topic is not common and studied materials have not been subjected to such an analysis before, there is not much research in the literature that could be used in the discussion.

The reduction of undercuts using methacrylate resin obtains higher precision of partial and complete prostheses, which exerts a less traumatic effect on the denture bearing area.[9–12] As a consequence, blocking out undercuts reduces the number of the patient's visits, since there is no need to make as many adjustments of the prosthesis.[13–16]

In group II and III, the class of the plaster had an impact on SBS. Additional polymerization at 2.4 bar caused an increase of SBS for class IV plaster when compared to class III plaster. Additional polymerization at 100°C increased SBS for class III plaster. It was the most effective method of increasing SBS for class III plaster. These results may be related to the structure determined by the plaster type. The class of plaster determines its increased hardness and applicability. Class III plaster requiring less precision is most often used for prosthetic treatment. Class IV plaster is more rigid and undergoes only slight deformations. The smaller diameter of the plaster grain determines a better quality of the model. At the same time, it is associated with its lower porosity.

The results of the study showed that not only the plaster type, but also additional polymerization processes had a significant effect on the bonding strength of light-cured methacrylate material to plaster. Generally, additional polymerization at 100°C increased or did not statistically affect SBS at all when compared to the control group. The best parameters presented specimens made of LC Block-Out Resin and Stodent class III plaster after polymerization at 100°C. On the other hand, polymerization under pressure in most cases significantly decreased or did not affect SBS.

Therefore, the analysis of the results may suggest that models with lower hardness and greater porosity, i.e., class III plaster should be used for the reduction of the undercuts with the composite material, but only after additional polymerization at 100°C. Polymerization under pressure seems to be inefficient in most cases.

Conclusions

It is important to underline that additional laboratory stages increase total costs and time of denture fabrication. Polymerization can improve SBS, however not always. The polymerization at 100°C was the most efficient among LC Block-Out Resin attached to class III plaster samples. LC Block-Out Resin and Block-Out Gel LC seem to have sufficient adherence to the surface of models made of Stodent class III and Tewerock class IV plaster in the process of blocking out the dental and alveolar undercuts. Both the composite material and the plaster type had a significant impact on SBS.

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A comparison of intraocular pressure values obtained using a Goldmann applanation tonometer and a handheld version of applanation resonance tonometer: A preliminary report

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Abstract

Background. Despite the development of various methods of intraocular pressure (IOP) measurement, Goldmann applanation tonometry (GAT) is still the most popular. The measurement using GAT depends on the biomechanical properties of the cornea, such as the thickness, the radius of curvature, as well as the amount of the fluorescein used.

Objectives. The aim of the study was to compare IOP values measured by GAT with those measured by applanation resonance tonometry (ART).

Material and methods. A total of 47 patients (94 eyes), including 28 patients with primary open-angle glaucoma (POAG) and 19 subjects from the control group, were examined at the Glaucoma Outpatient Clinic of the Department and Clinic of Ophthalmology at Wroclaw Medical University (Poland). The measurements of IOP were performed using GAT and a handheld version of ART. Also, the central corneal thickness (CCT) of all patients was measured.

Results. The study showed that the IOP values measured by both tonometers were comparable, but ART-acquired values were higher than GAT-obtained values both in the glaucomatous group and in the control group. CCT had little impact on mean IOP difference between GAT- and ART-obtained values.

Conclusions. Applanation resonance tonometry is a precise method of IOP measurement and is less affected by biomechanical properties of the cornea than GAT. Our results show that ART is a new, promising, comfortable for both patients and doctors method of IOP measurement, which, in the future, can replace GAT.

Key words: comparison, glaucoma, applanation resonance tonometry, Goldman applanation tonometry

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Introduction

Goldmann applanation tonometry (GAT), despite over 50 years of history, is still considered the gold standard for intraocular pressure (IOP) measurement and is recommended by the European Glaucoma Society (EGS) as the most precise method.[1] Invented by Hans Goldmann in 1957, this technique is based on the Imbert-Fick law, in which the force required to flatten the sphere is a measure of IOP. The rule assumes that the surface of the sphere is perfectly dry and smooth.[2] Furthermore, the measurement depends on the biomechanical properties of the cornea, including the thickness, the radius of curvature, as well as the amount of the fluorescein used. Goldmann applanation tonometry is a tonometer calibrated to the mean corneal thickness – 520 nm, thus, it is not accurate for every type of the cornea.[3]

The studies show that the values of IOP measured by GAT are underestimated in patients after refractive eye surgery, and the number of those patients is still increasing. It was proven that ablation of the center of the cornea results in a constant decrease of IOP values by approx. 1.6 mm Hg in myopic eyes, as well as in hyperopic eyes. Additionally, extra reduction of IOP was observed in myopic patients (0.029 ±0.003 mm Hg per 1 µm of excised tissue), which is explained by the fact that the location of maximum ablation is in the center of the cornea. Assuming that 15 µm of tissue is excised for correction of 1 diopter, every corrected diopter decreases IOP by 0.5 mm Hg. The study confirms that GAT can show incorrect values of IOP after refractive surgery and in patients with corneal disorders.[4] Changes in the structure of the eye or the orbital cavity, various amount of fluorescein, astigmatism, eye clenching, as well as too narrow collar or tie, can also impact IOP measurement.[1]

Applanation resonance tonometry (ART) is one of the new methods of IOP measurement. Although the contact with the cornea resembles the Goldmann tonometer, the mechanism of measurement has changed. A convex, bakelite tip equipped with piezoelectric resonator is intended to measure the resonance frequency. That mechanism enables the simultaneous measurement of the applanation and the force required to applanate the cornea. Two versions of that tonometer are available – automatic (servo-controlled) and manual. It can be attached to a slit lamp, which is similar to GAT, or handheld. Applanation resonance tonometry has been known for many years and was described as a new, promising method. The correlation between GAT- and ART-acquired readings was analyzed in many studies.[5–7]

ART, as well as GAT, can be performed with the use of a slit lamp; it depends on the flattening of the desensitized cornea by an automatically exsertile tip. Three measurements of IOP are made, then averaged, but the contact time with the cornea is much shorter than during GAT, which greatly reduces the risk of cornea damage. There is no need to use fluorescein.[7,8]

Objectives

The aim of the study was to compare IOP values measured by GAT with those measured by ART. The handheld version of an automatic ART was used in this study.

Material and methods

Ninety-six eyes of 48 patients were enrolled in the study. Two eyes of 1 patient were excluded due to measurement failure. Finally, the study included 94 eyes of 47 patients – 56 eyes of 28 patients with primary open-angle glaucoma (POAG) treated pharmacologically, and 38 eyes of 19 healthy patients in the control group. The mean age of the patients was 59 years (range 23–83). The study included 64 female eyes and 30 male eyes. Pseudophakic patients after at least 6 months after cataract surgery were also qualified for the study. The Goldmann applanation tonometry results were corrected by CCT value (GAT_{CCT}), Goldmann applanation tonometry results without corrections by CCT value (GAT_{noCCT}) and applanation resonance tonometry results (ART) were compared. The measurements were made with at least a 10-min gap after each test, and the tests were performed in a random order. Central corneal thickness was measured by an ultrasound corneal pachymeter PIROP (ECHO-SON S.A., Puławy, Poland).

Statistical analysis was performed using STATISTICA v. 12 software. Distribution of the results was checked using the Shapiro-Wilk normality test. Differences and variance between GAT and ART were assessed using linear regression. The p-value <0.05 was considered statistically significant. Correlation analysis was performed using Pearson correlation coefficient.

Results

Mean GAT_{CCT} was 16.46 mm Hg, while mean GAT_{noCCT} was 17.27 mm Hg. Mean IOP measured with ART was 17.72 mm Hg (Table 1). The mean absolute value of IOP difference $|(ART - GAT_{CCT})|$ was 1.87 mm Hg with SD of 1.24 mm Hg. When compared to other differences, the calculated value was low with the lowest variance. The mean IOP difference ($ART - GAT_{CCT}$) was 1.40 mm Hg with SD of 1.76 mm Hg. The mean absolute value of IOP difference ($ART - GAT_{noCCT}$) was 2.07 mm Hg with SD of 1.70 mm Hg. The mean IOP difference ($ART - GAT_{noCCT}$) was 0.48 mm Hg, however, with a comparably higher SD of 2.64 mm Hg (Table 2).

Intracular pressure, measured by both ART and GAT, was higher in the glaucoma group than in the control group ($p < 0.05$). A statistical analysis showed the most significant correlation between the ART values and the GAT_{CCT} values (correlation test $p < 0.05$, correlation coefficient = 0.66). When compared to GAT, ART overstated IOP by about 2 mm Hg.

Table 1. Mean values for ART, GAT_{CCT} and GAT_{noCCT} with standard deviation (SD) in mm Hg

Method	Mean value [mm Hg]	SD [mm Hg]
ART	17.72	3.36
GAT_{CCT}	16.46	3.39
GAT_{noCCT}	17.27	3.48

Table 2. Mean differences between IOP measurements with standard deviation (SD)

Mean difference	value [mm Hg]	SD [mm Hg]
ART – GAT_{CCT}	1.4	1.76
|ART – GAT_{CCT}| (absolute values)	1.87	1.24
ART – GAT_{noCCT}	0.48	2.64
|ART–- GAT_{noCCT}| (absolute values)	2.07	1.70

The mean IOP difference between ART and GAT_{CCT} was 2.12 ±1.49 mm Hg (values > 12 mm Hg); 1.83 ±1.18 mm Hg (in the range of 12–18 mm Hg); and 1.75 ±1.15 mm Hg (values >18 mm Hg). According to ISO criteria for new tonometers, only in 5% of measurements the difference may be higher than 5 mm Hg. Tables 3 and 4 show the percentage of measurement values in which the differences between ART and GAT_{noCCT}, and between ART and GAT_{CCT} exceeded ±5 mm Hg. Unlike in the previous study with ART attached to a slit lamp, overstated high IOP values in manual ART were not noticed.[9] Corneal thickness had a little impact on the mean ART and GAT difference in IOP. The attempt to correct ART values by CCT reduces the correlation between ART and GAT.

Discussion

Our study proved that IOP values measured by ART and GAT were comparable. Nonetheless, ART tends to overstate the higher values of the pressure. The correlation between a Goldmann tonometer and a resonance tonometer is more significant when using a handheld, automatic version in comparison with ART attached to a slit lamp.

One of the first studies that compared GAT and ART was conducted by Hallberg et al. on 24 healthy patients and 24 patients with elevated IOP.[6] Intracular pressure was measured with the use of ART, during the corneal indentation phase and the phase when the sensor was removed. Goldmann tonometry was used as a reference method. The study showed a significant correlation between IOP indentation and IOP GAT (R = 0.92; p < 0.001; SD = 3.6 mm Hg; n = 104), as well as between IOP removal and IOP GAT (R = 0.94; p < 0.001; SD = 3.1 mm Hg, n = 104).

In further studies on a porcine eye model evaluating the use of ART for IOP measurement, ART-acquired values

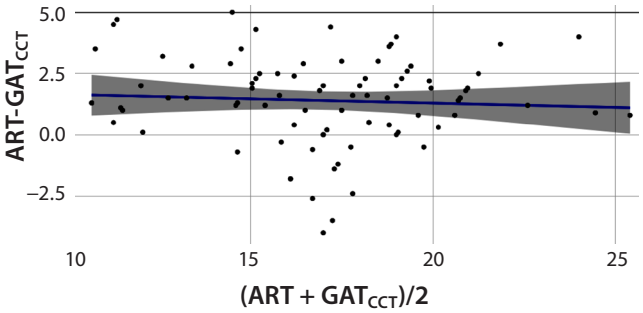

Fig. 1. The difference between Goldmann Applanation Tonometry corrected by CCT (GAT_{CCT}) and ART as a function of their mean IOP (n = 94 eyes). Solid lines show ±5 mm Hg. Correlation coefficient = 0.38. Correlation test p-value = 0.0001

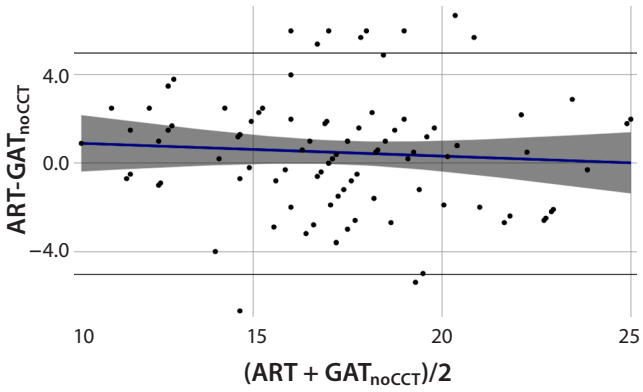

Fig. 2. The difference between Goldmann Applanation Tonometry (GAT_{noCCT}) and ART. As a function of their mean IOP (n = 94 eyes) Solid lines show +/– 5 mm Hg. Correlation coefficient = 0.09, with correlation test p = 0.38

Table 3. Number and percentage of measurements in which the difference between ART and GAT_{noCCT} was higher than 5 mm Hg (according to ISO standards for new tonometers)

IOP mm Hg	ART – GAT_{noCCT} > 5 mm Hg	% (n > 5 mm Hg)/(n)
9–16 mm Hg (n = 39)	n = 6	15.39
>16 mm Hg; <23 mm Hg (n = 47)	n = 7	14.8
≥23 mm Hg (n = 8)	n = 0	0
Total (n = 94)	n = 13	13.8

Table 4. Number and percentage of measurements in which the difference between ART and GAT_{CCT} was higher than 5 mm Hg (according to ISO standards for new tonometers)

IOP mm Hg	ART – GAT_{CCT} > 5 mm Hg	% (n > 5 mm Hg)/(n)
9–16 mm Hg (n = 37)	n = 0	0
>16 mm Hg; <23 mm Hg (n = 55)	n = 0	0
≥23 mm Hg (n = 2)	n = 0	0
Total (n = 94)	n = 0	0

were thought to be strongly operator-dependent.[10] However, more detailed studies regarding this topic showed that operator dependency did not significantly affect IOP values. The research by Hallberg et al. proved that off-center placement of the new designed device with a larger contact surface (7 mm) is not clinically significant. The precision of ART was well within the ISO standard requirements for a tonometer.[11] Precision of the 2 versions of ART – handheld and biomicroscope – was analyzed in a large, randomized, prospective study performed on 153 human eyes. The study presented that both ART attached to a slit lamp and handheld ART met the ISO criteria for tonometers. The biomicroscope setup demonstrated a marginally better precision when compared with handheld ART.[12]

The aim of the study by Jóhannesson et al. was to define the correlation between servo-controlled ART and manual ART with regard to GAT. Seventy-seven patients (152 eyes) were examined by 6 measurements using each method. Intracular pressure during the indentation phase (the dynamic state) and IOP during 2-second applanation (the static state) were analyzed. The study showed that manual ART met the ISO criteria of the standard deviation range for both phases, while the servo-controlled ART fulfilled the criteria only during the static stage.[7]

Elevated intraocular pressure is the main risk factor of glaucoma development; therefore, the majority of studies are focused on this group of patients. Ottobelli et al. examined 115 glaucomatous patients and 63 patients from the control group. Goldmann tonometry and double resonance tonometry were performed in a random order. The statistical analysis showed that ART overestimated IOP on average by 1.3–1.7 mm Hg compared to GAT, especially in patients with high IOP. The ART results were repeatable but the 2nd (ART 2) was lower than the 1st (ART 1), probably due to the increased tranquility of the patients during the 2nd test.[8,12] Also, Salvetat et al. investigated the repeatability and accuracy of servo-controlled ART. They obtained high repeatability of ART, but significantly lower than GAT. Common conclusion was drawn – ART overestimated GAT values, especially in patients with high IOP.[8,13]

Many papers compare new techniques of IOP measurement usually with a Goldmann tonometer as a reference point. Jóhannesson et al. in a prospective study of 53 subjects compared 3 tonometry methods – GAT, Pascal dynamic contour tonometry (DCT), and ART. The assessment of IOP was made prior to, directly after, 3, and 6 months after laser-assisted sub-epithelial keratectomy (LASEK), which is known to have a great impact on biomechanical properties of the cornea. Six measurements were obtained with a 5-min gap after each test in a randomly selected eye. The reduction of IOP was observed in the 3rd and 6th month after the laser eye surgery; however, the biggest decrease of IOP was noticed in the GAT group after 6 months (–1.7 ±1.8 mm Hg), then in the static ART group (–1.2 ±1.5 mm Hg), followed by DCT patients (–1.1 ±1.6 mm Hg) and the dynamic ART group (–1.0 ±1.5 mm Hg).[14] After 2 years of follow-up, dynamic ART showed no significant difference in the measured IOP. We concluded that corneal properties had the least impact on dynamic ART measurements, so we recommend it in patients after LASEK.[15]

Jóhannesson et al. analyzed the impact of ageing on eye proprieties in 2 healthy but age-differentiated groups. Intracular pressure was measured by ocular response analyzer (ORA), dynamic contour tonometry (DCT), applanation resonance tonometry (ART), and Goldmann applanation tonometry (GAT). Values obtained by ORA, DCT and GAT were higher in elderly patients than in the young population. Applanation resonance tonometry showed no difference of IOP between those groups. The authors suggest that age-independent results may be partially explained by the character of the ART method. The study also proved that ART does not depend on central corneal thickness (CCT) and corneal curvature (CC).[16]

Probable fluctuation of IOP after corneal surgeries was examined by Beckman et al. in a study from 2014. His research on 28 patients (29 eyes) after corneal cross-linking (CXL) and on a corresponding control group showed that ART can be a useful method for assessing corneal hysteresis (CH).[17]

In a recent study investigating the impact of multiple IOP measurements and the use of topical anesthetics on IOP, using GAT and ART, Jóhannesson et al. demonstrated the IOP reduction after repeated pressure measurements. Also, a reduction in IOP was observed after the use of topical anesthetics without multiple IOP measurements. Authors advise to take into account the reduction of IOP when topical anesthetics or repeated IOP measurements would be performed in a study.[18]

Conclusion

Applanation resonance tonometry, when compared to GAT, overstates IOP values by approx. 2 mm Hg. The difference between IOP measurements using ART and GAT is not significant in the range of tested values (8–25 mm Hg). The IOP level variance is comparable, which supports the statement that handheld ART is an equally predictable method as GAT. Its automatic, triple measurement along with the exam-quality indicator make ART a more objective method than GAT. Further technological improvement and large multi-center studies may improve ART position in the field of IOP tonometers.

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Does preemptive gabapentin modulate cytokine response in total knee arthroplasty? A placebo controlled study

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

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Abstract

Background. Gabapentin, as a structural analogue of γ-aminobutyric acid, has been investigated to provide pain relief in the early postoperative period following various surgical interventions.

Objectives. The objective of this study was to investigate whether preemptive oral administration of gabapentin 800 mg can reduce postoperative pain and modulate the inflammatory cytokine response in comparison to placebo in patients undergoing total knee arthroplasty under general anesthesia.

Material and methods. Fifty-two patients were randomly divided into 2 groups before surgery, either to receive peroral gabapentin 800 mg or placebo drug, 1 h before surgery. All patients had general anesthesia with endotracheal intubation, in a standardized fashion, by the same anesthetist. Thirty min before completion of surgery, intramuscular diclofenac sodium 75 mg was administered. Following extubation, visual analogue pain scale (VAS) scores and additional analgesic requirements were recorded at 15 min at post-anesthesia care unit (PACU), and at 4th and 24th h postoperatively. Plasma levels of interleukin 6 (IL-6), and tumor necrosis factor R (TNF-R) were measured at predetermined time points (T_0 1 h before administration of gabapentin, T_1 at postoperative the 4th h mark, and T_2 at postoperative at the 24th h mark).

Results. The VAS scores at postoperative 4th h were significantly higher in placebo and gabapentin groups compared with VAS scores at PACU and at 24th h. The groups did not differ in terms of additional analgesic requirements. In gabapentin group, IL-6 levels at T_1 and T_2 were significantly lower in comparison to values measured in placebo group at the same time points. This difference was not significant in TNF-R levels between the groups.

Conclusions. Though preemptive oral gabapentin administration did not reduce postoperative pain and analgesic requirements in total knee arthroplasty surgery, it attenuated IL-6 production on the first postoperative day.

Key words: preemptive, gabapentin, postoperative analgesia, serum IL-6, serum TNF

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Introduction

Osteoarthritis is the most commonly observed joint disorder affecting individuals aged 65 years and older. In these patients, total knee arthroplasty (TKA) is recommended due to joint destruction that results in severe daily pain. Although TKA is performed due to pain and the limitation of movement, joint replacement surgery itself is associated with significant postoperative pain that is also accompanied by increased levels of proinflammatory cytokines, leading to delay in postoperative rehabilitation and discharge from hospital.[1] Consequently, strategies intended for the modulation of perioperative inflammatory response have great clinical significance for functional recovery.[2] Recent studies suggest that surgical damage is correlated mainly with serum IL-6 levels as a sensitive indicator of the degree of surgical stress.[3] It has also been reported to be significantly increased with postoperative complications and mortality.[4] On the other hand, sufficient postoperative analgesia has been reported to decelerate proinflammatory activation by inhibiting the migration of cytokines and accelerating wound healing.[5]

Gabapentin, as a structural analogue of γ-aminobutyric acid, has been investigated to provide pain relief in the early postoperative period following various surgical interventions.[6] It has been reported to reduce pain and opioid consumption in the first 48 h following anterior cruciate ligament repair.[7] However, there is no clear data as to whether, in addition to its analgesic effects, gabapentin has an effect on acute phase response.

The aim of the present study was to evaluate the effects of a single dose of oral gabapentin (800 mg) administered before total knee arthroplasty, in preventing postoperative pain and reducing the effects of surgery on IL-6 and TNF-R.

The clinical study's registration was received from the Ethics Committee of Turgut Özal University Medical Faculty (No 08.11.2013/26-99950669/1135).

Material and methods

A total of 52 patients with American Society of Anesthesiologists physical status I–II (ASA I–II), who were to undergo elective total knee arthroplasty, were informed about the procedure and included in a prospective, randomized, double-blind, placebo-controlled study, and the approval from the Ethics Committee of Turgut Özal University Faculty of Medicine was obtained. The patients with drug or alcohol abuse, allergy to the study drug or non-steroid anti-inflammatory medications, patients with cardiac, respiratory, hepatic, and renal failure, patients with a history of peptic ulcer, and patients that had received steroids in the previous 7 days were excluded. During the preoperative visit, a 10-cm long visual analogue scale (VAS) was given to the patients (0 = no pain, 10 = intolerable pain). The patients were randomly divided into 2 groups before surgery, and another investigator administered peroral gabapentin (Neurontin®) (Pfizer, Istanbul, Turkey) 800 mg (group G) or saccharine (placebo) (group P) resembling the study drug 30 min before surgery. Following their arrival in the operating room, the patients underwent standard ASA monitorization (ECG, SpO_2, non-invasive blood pressure measurement), and a 20 G intravenous cannula was inserted on the dorsum of the hand to commence saline infusion at a rate of 5 $mL \cdot kg^{-1} \cdot h^{-1}$. Following preoxygenation, anesthesia was induced using propofol 2 $mg \cdot kg^{-1}$ and remifentanil 1 $\mu g \cdot kg^{-1}$ administered as intravenous (iv.) bolus. After the administration of rocuronium bromide 0.6 $mg \cdot kg^{-1}$, the patients were intubated using an endotracheal tube of an appropriate size. The anesthesia was maintained with sevoflurane 1–2%, 50% oxygen and 50% N_2O using controlled ventilation. Remifentanil infusion was continued at a rate of 0.25 $\mu g \cdot kg^{-1} \cdot min^{-1}$ during surgery. Remifentanil infusion rate was kept constant during surgery and sevoflurane concentration was titrated between 1 and 2% to maintain a mean arterial pressure (MAP) of 60–80 mm Hg. The administration of beta-blocker (bolus, iv.) was scheduled when the measured values were above target mean arterial pressure (MAP). Bradycardia was defined as a heart rate <50 beats per minute (bpm), and the administration of atropine was planned for treatment. Decreasing sevoflurane concentration and, should this fail, increasing fluid administration, were planned when MAP was below the target value.

All the patients were administered intramuscular diclofenac sodium 75 mg (Dikloron® 75 $mg \cdot 3\ mL^{-1}$, Deva, Istanbul, Turkey) 30 min before the completion of surgery. The inhalation agent and remifentanil infusion were stopped with the cessation of surgery. The neuromuscular block was reversed with the administration of iv. neostigmine 0.04 $mg \cdot kg^{-1}$ and atropine 0.02 $mg \cdot kg^{-1}$, and the patients were extubated. The Modified Aldrete Recovery Score was used as the criteria for the transfer of patients from the operating room to the post-anesthesia care unit (PACU) and in the follow-up of recovery. The patients with a Modified Aldrete Recovery Score ≥8 were transferred to the PACU from the operating room. The time to the first analgesic requirement was recorded. Another investigator, who was kept blind to the study protocol, repeated the pain assessment using visual analog scale (VAS) at 15 min in PACU, and at the 4^{th} and 24^{th} h marks. When the VAS pain score was >4, iv., tramadol 50 mg (Contramal® 100 mg) (Abdi İbrahim İlaç Sanayi ve Ticaret A.Ş. Istanbul, Turkey) was administered as iv. bolus. Any possible side effects (nausea, vomiting, respiratory depression) were evaluated.

Blood samples were obtained at predetermined time points for the measurement of serum TNF-R (sTNF-R) and IL-6 (T_0 1 h before oral administration of study drug, T_1 at postoperative 4^{th} h mark, and T_2 at postoperative 24^{th} h mark). The blood samples were stored at +4°C after collection and then sent to the laboratory with a cold chain for the measurement of biochemical parameters.

Blood sample collection

Venous blood samples (5 mL) were obtained from each patient via the antecubital vein 1 h before gabapentin administration and 4 and 24 h postoperatively. Blood was collected into a normal 10 mL Vacutainer® tubes (Becton, Dickinson & Co., Franklin Lakes, USA). For cytokine measurements, blood samples were centrifuged at 4000 g for 10 min at 4°C and the plasma was stored at –80°C until analysis. Serum concentrations of IL-6 and sTNF-R were measured by platinum enzyme-linked immunosorbent assays (ELISA) for quantitative detection (Bender MedSystems GmbH, Campus Vienna Biocenter 2, Vienna, Austria) according to the manufacturer's instructions. Sensitivity was 0.10 ng/mL for sTNF-R, and 0.92 pg/mL for IL-6.

Statistical analysis

The study data was uploaded for analysis to the SPSS (Statistical Package for Social Sciences) for Windows 22.0 (SPSS Inc., Chicago, USA) computer software. Descriptive statistics included mean ±standard deviation, median (min–max), frequency distribution and percentage. Pearson's χ^2 test and Fisher's exact test were used to evaluate the categorical variables. The applicability of the variables to normal distribution was evaluated using visual (histogram and probability graphs) and analytic methods (Shapiro-Wilk test). In case of a significant difference between 2 independent groups, the Student's t-test was used for normally distributed variables. For variables not distributed normally, the Mann-Whitney U test was used to compare 2 independent groups and the Friedman test was used to compare 3 dependent groups. When a significant difference was found between 3 dependent groups, Bonferroni correction was used to determine the source of the significant difference. The relationship between the variables was evaluated using Spearman's correlation coefficient. The level of statistical significance was set at $p < 0.05$.

Results

In terms of demographic features and surgery time, there was no statistically significant difference between the study patients (Table 1). Intraoperative MAP and heart rate values did not differ between the groups.

There was no statistically significant difference between the groups in terms of VAS scores ($p > 0.05$); however, VAS scores at the 4th h mark in group P and group G were significantly higher compared with VAS scores measured at PACU and at the 24th h mark ($p < 0.05$). The VAS score in group G at the 24th h mark was significantly higher than the VAS score at PACU ($p < 0.05$). The time to the first analgesic requirement was 78.6 ±35.2 min in group P and 87.2 ±20.8 min in group G ($p > 0.05$). There was no significant difference between the groups in terms of additional analgesic requirement ($p > 0.05$).

Serum IL-6 and sTNF-R values at T_0 in both groups were significantly lower compared with the values at T_1 and T_2. In group G, sTNF-R measured at T_1 was significantly lower than measured at T_2 ($p < 0.05$). In group G, serum IL-6 values at T_1 and T_2 were significantly lower than the values at the same time points in group P ($p = 0.048$; $p = 0.024$, respectively). In group P, sTNF-R values at T_0 were lower than those in group G ($p < 0.05$) (Fig. 1, 2).

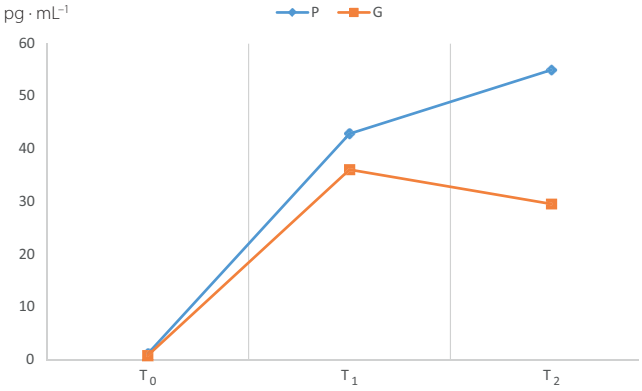

Fig. 1. Changes in serum concentration of IL-6 values

P – group P; G – group G; T_0 – 1 h before oral administration of study drug; T_1 – at postoperative 4th h; T_2 – at postoperative 24th h.

Table 1. Patients' characteristics, anesthesia and operative data

Variables		P (n = 26)	G (n = 26)	p-value
Age [year]		68.65 ±6.84	65.54 ±8.93	0.164[a]
Gender	male female	6 (23.1%) 20 (76.9%)	5 (19.2%) 21 (80.8%)	0.734
BMI [kg/m^2]		32.39 ±5.66	34.07 ±5.33	0.278[a]
ASA	I II	0 26 (100%)	3 (11.5%) 23 (88.5%)	0.235[b]
Anesthesia time [min]		108.08 ±18.77	113.85 ±17.91	0.262[a]
Operation time [min]		95.00 ±18.17	102.69 ±18.61	0.138[a]

Continuous variables were expressed as mean ±standard deviation and categorical variables were expressed as number (column percentage); [a] Student's t-test; [b] Fisher's exact test ; ASA – American Society of Anesthesiologists physical status; P – placebo group; G – gabapentin group.

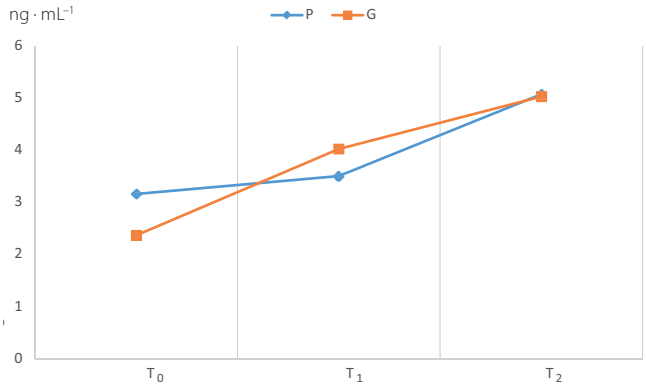

Fig. 2. Changes in serum concentration of TNF-R values

P – group P; G – group G; T_0 – 1 h before oral administration of study drug; T_1 – at postoperative 4th h; T_2 – at postoperative 24th h.

Discussion

The present study showed that preemptive oral gabapentin had no effect on providing postoperative pain relief after TKA, but it significantly attenuated IL-6 levels in the first 24 h after the postoperative period, most prominently in the early hours following surgery.

The preemptive use of gabapentin in different doses and in different surgical interventions has been studied in terms of decreasing postoperative pain scores and analgesic consumption.[8] The studies about oral gabapentin differ from each other either by the usage of different doses of gabapentin or the agents to which gabapentin was compared. Yet, there are contradictory reports about the ideal dose of preemptive gabapentin. Bang et al. performed a randomized, double-blind, placebo-controlled study on 46 patients undergoing arthroscopic rotator cuff repair, and evaluated oral gabapentin 300 mg vs placebo. Interestingly, the VAS scores in the gabapentin group 24 h after the postoperative period were significantly lower than in the control group.[9] In another study, the authors administered a higher dose of oral gabapentin, such as 1200 mg, 1 h before surgery, to evaluate postoperative pain following spinal surgery, and they reported that gabapentin reduced pain scores in the early postoperative period and morphine consumption, monitored by a patient-controlled analgesia (PCA) pump and, therefore, reduced the side effects associated with morphine use.[10] Similarly, this high dose of preoperative gabapentin administered 1 h before surgery reduced postoperative morphine consumption after lower extremity orthopedic surgery without affecting pain scores.[11] The difference in the results of the above studies might be due to the varying doses of gabapentin and dissimilar pain levels in the surgeries. Pandey et al. suggested that 600 mg and higher doses were better without side effects in patients undergoing lumbar discectomy procedure.[12] Although we used an intermediate dose (800 mg) consistent with the doses studied in the literature, in our study we could not show any benefits of gabapentin over the control group in total knee arthroplasties. Another randomized, double-blind, placebo-controlled study evaluated different doses of oral gabapentin (300 and 600 mg) vs placebo administered 1 h before surgery in patients undergoing caesarean section under spinal anesthesia. The evaluation of analgesic consumption at 6th, 12th, 24th and 48th h during the postoperative period revealed that gabapentin had no favorable effects on postoperative outcomes and did not reduce analgesic consumption.[13] On the contrary, Clarke et al. supported the use of gabapentin in the acute postoperative period, after total knee arthroplasty with different doses of oral gabapentin administered 2 h before surgery preoperatively and postoperatively more than once and continuing for 4 days. Besides, their patients also received celecoxib 2 h before surgery.[14] This study actually could not be compared with our study, because of the multiple use of gabapentin with another analgesic adjunct. As well as these studies, there are also other studies comparing gabapentin's effects with other analgesic agents resulting in contradictory ideas.[15]

It is known that the increased severity of surgery and magnitude of tissue injury are related with increases in the plasma levels of proinflammatory cytokines.[16] TNF-α and IL-6 are 2 early important features of acute injury that play a role in persistent postoperative pain syndromes.[17–19] Li et al. showed a positive correlation between dexmedetomidine administration, VAS scores and plasma TNF-α, IL-6 levels, in patients undergoing dental surgery.[20] This supported the finding that acute inflammation and, thus, inflammatory cytokine release caused postoperative pain, which provided an alternative treatment for inflammatory pain as well.[21] In another study, gabapentin was seen to play a role in the down-regulation of pro-inflammatory cytokine TNF-α, IL-1β and IL-6 expression and up-regulation of anti-inflammatory cytokine IL-10 expression in the rat spinal cord.[22] Apart from that, in a rat model of neuropathic pain, antinociception was observed as a result of inhibited expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 by gabapentin.[23] Unfortunately, we could not find many studies showing a one-to-one correspondence with our study to compare our results. Some other analgesic agents have been tried in this aspect in a few other studies. In one study, Pandazi et al. showed a decrease in serum levels of IL-6 in patients undergoing colorectal surgery, provided by parecoxib administered preincisionally vs postincisionally.[24] Similarly, Feng et al. observed a reduction in serum IL-6 levels by preincisional administration of rofecoxib in comparison with placebo.[25] However, Bao et al. could not show any change in the level of plasma TNF-α with either preincisional or postincisional parecoxib administration after total hip replacement.[26] This was the same in other studies with other types of surgical procedures.[27] Only one study showed a significant increase in the postoperative TNF-α levels, but it was in wound and peritoneal fluid supporting TNF-α's hypersensitivity for mechanical nociception.[28]

These examples are mainly about highly selective COX-2 inhibitors. Furthermore, Pirbudak et al. could not show any difference in either the inflammation markers, mainly C-reactive protein, or pain intensity in patients treated with either tramadol or tramadol plus gabapentin after lumbar disc herniation transacted with epidural steroid injection.[29] However, mainly in animal studies, gabapentin's antihyperalgesic effect in inflammatory pain models had been exerted.[30,31]

Although this paper is limited, as the sample size is small and postoperative analgesic consumption could not be standardized by PCA morphine, we still believe that this trial could be instrumental in demonstrating the importance of splitting the relationship between inflammatory response and acute postoperative pain for the patient's comfort.

In conclusion, the present study found that oral gabapentin administered in the preoperative period 1 h before surgery had no effect on providing postoperative pain relief after total knee arthroplasty; however, gabapentin significantly decreased IL-6 levels in the first postoperative 24 h, most prominently in the early period.

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Efficacy and safety of golimumab as add-on therapy to standard disease-modifying antirheumatic drugs: Results of the GO-MORE study in the Polish population

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Abstract

Background. The GO-MORE study was an open-label, multinational, prospective study that investigated the efficacy and safety of adding golimumab to synthetic disease-modifying antirheumatic drugs (sDMARDs) in patients with active rheumatoid arthritis (RA).

Objectives. The aim of this study was to assess the efficacy and safety of golimumab add-on therapy in the Polish subpopulation of the GO-MORE study.

Material and methods. Patients were administered 50 mg subcutaneous doses of golimumab once a month for 6 months, while continuing therapy with sDMARDs and/or glucocorticoids (GCS). The primary clinical endpoint was the proportion of patients with moderate or good European League Against Rheumatism (EULAR) response based on the 28-joint disease activity score (DAS28) erythrocyte sedimentation rate (ESR) after 6 months.

Results. The Polish subpopulation (129 patients) was similar to the overall study population (3,280 patients) with regard to age, sex, mean baseline DAS28, inflammatory markers, average methotrexate dose, and GCS use; however, they had a longer disease duration (median: 6.04 vs 4.9 years) and more Polish patients (85.9% vs 78.7%) had high disease activity (DAS28–ESR ≥3.2). At 6 months, 84.5% of Polish patients showed good or moderate EULAR response, 26.4% had low disease activity and 17.1% were in clinical remission, compared with 82.9%, 37.4% and 23.9%, respectively, in the overall study population. Golimumab safety profile was consistent with previous studies and comparable to the overall study population.

Conclusions. The addition of golimumab to sDMARD therapy in Polish RA patients showed good or moderate EULAR DAS28–ESR response in 84.5% of patients, mirroring the overall study population.

Key words: methotrexate, rheumatoid arthritis, golimumab, disease-modifying antirheumatic drugs

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Introduction

According to the current recommendations for the management of rheumatoid arthritis (RA), the main treatment goal is to attain remission of the disease symptoms, or alternatively to achieve low disease activity in patients who fail to attain remission with the available treatment methods.[1–3] The recommendations specify time periods in which this treatment goal should be achieved; i.e., improvement should be achieved within 3 months and the treatment goal (remission or low disease activity) should be met within 6 months.[1,2] To achieve this goal, synthetic disease-modifying antirheumatic drugs (sDMARDs) are recommended in the 1st phase of RA treatment. The first-line conventional sDMARD is methotrexate (MTX). If MTX is not tolerated or contraindicated, then leflunomide, sulfasalazine or hydroxychloroquine, alone or in combination with glucocorticoids (GCS), are recommended. In case of inefficacy or adverse reactions, other conventional sDMARDs or biologic agents are recommended. The recommended first-line biologics include tumor necrosis factor alpha (TNF-α) inhibitors, abatacept, tocilizumab, and, in certain conditions, rituximab.[2]

Table 1. Baseline clinical and demographic characteristics of the GO-MORE study patients, including the Polish population[9]

Characteristics		Overall study population	Polish patient group
Number of patients in the study		3280	129
Age [years]	mean (SD)	52.3 (12.8)	49.7 (11.06)
	median (min, max)	53.0 (18, 88)	52.0 (18, 78)
Females, n (%)		2716 (86.8)	111 (86)
Race, n (%)	Caucasian	2283 (69.6)	129 (100)
	other	997 (30.4)	0
BMI [kg/m^2], median (min, max)		26.2 (14.0, 54.5)	24.96 (16.7, 42.6)
Disease duration [years]	mean (SD)	7.6 (7.9)	8.78 (8.69)
	median (min, max)	4.9 (0.01, 56.6)	6.04 (0.12, 44.6)
DAS28–ESR	n	3270	129
	mean (SD)	5.97 (1.1)	5.96 (0.94)
	high disease activity (>5.1), n (%)	2572 (78.7)	110 (86)
DAS28–CRP	mean (SD)	5.41 (1)	5.34 (0.9)
CRP [mg/L]	mean (SD)	14.48 (20.38)	13.46 (18.62)
ESR [mm/h]	mean (SD)	34.9 (24.64)	32.8 (18.98)
Anti-CCP	n	3225	129
	positive (≥20 U/mL), n (%)	2318 (71.9)	102 (79)
Rheumatoid factor	n	3234	129
	positive (≥15 U/mL), n (%)	2344 (72.5)	127 (98)
HAQ-DI	mean (SD)	1.44 (0.67)	1.51 (0.57)
sDMARD	n	3270	129
	methotrexate monotherapy, n (%)	1681 (51.4)	87 (67)
	methotrexate + leflunomide, n (%)	216 (6.6)	1 (1)
	methotrexate + sulfasalazine, n (%)	150 (4.6)	8 (6)
	methotrexate + hydroxychloroquine, chloroquine, n (%)	433 (13.2)	10 (8)
	methotrexate + hydroxychloroquine, chloroquine + sulfasalazine, n (%)	106 (3.2)	1 (1)
	leflunomide monotherapy, n (%)	303 (9.3)	8 (6)
Glucocorticoids	n	3280	129
	GCS treatment (%)	2078 (63.4)	85 (66)
Number of previously failed sDMARDs	n	3279	129
	1, n (%)	1129 (34.4)	28 (22)
	2, n (%)	1176 (35.9)	48 (37)
	≥3, n (%)	974 (29.7)	53 (41)

anti-CCP – anti-cyclic citrullinated peptide antibody; BMI – body mass index; CRP – C-reactive protein; DAS28 – 28-joint disease activity score; DMARD – disease-modifying antirheumatic drugs; ESR – erythrocyte sedimentation rate; GCS – glucocorticoids; HAQ-DI – health assessment questionnaire disability index.

A new generation of TNF-α inhibitor is golimumab, an anti-TNF-α monoclonal antibody that is administered subcutaneously (SC) once a month. Golimumab has been extensively evaluated in placebo-controlled RA clinical studies; it has shown efficacy in MTX-naïve patients, in patients with inadequate response to MTX, in patients previously treated with at least one TNF-α inhibitor. Furthermore, golimumab can inhibit radiographic progression in the joints of MTX-naïve patients and of those with inadequate MTX response.[4–8] GO-MORE study was the first to evaluate the efficacy and safety of golimumab as an add-on therapy to different sDMARDs and to low doses (<15 mg/week) of MTX.[9]

The GO-MORE study evaluated golimumab as an add-on therapy (alone or in combination) to MTX, leflunomide, sulfasalazine, hydroxychloroquine, and chloroquine. This mimics the real-life clinical situation, when golimumab is typically administered in conjunction with different DMARDs.

The GO-MORE study included 129 Caucasian Polish patients from 13 centers across Poland. As the response to and safety of synthetic and biological DMARDs including golimumab may be genetically determined, and could be affected by different standards of treatment in various countries, the results of the GO-MORE study may vary across populations.[10,11] Therefore, we set out to investigate whether the efficacy of golimumab in a Polish subpopulation differed from the international study population, which combined ethnic groups and standards of treatment.

Objectives

The objective of the paper was to evaluate the efficacy and safety of golimumab as an add-on therapy to standard DMARDs in the Polish patient subgroup (129 patients) from the 1st part of the GO-MORE study, and to compare the efficacy results with worldwide study population.

Material and methods

This was a post-hoc analysis of the GO-MORE study (P06129; NCT00975130) which evaluated the efficacy and safety of golimumab in RA patients under conditions closely resembling routine clinical practice.[9] The GO-MORE study was an open-label, prospective, multicenter clinical trial that was conducted in 40 countries across Europe, Asia, North America, South America, and Africa, and involved 475 centers. It was approved by Bioethics Committees and conducted according to Good Clinical Practice and Declaration of Helsinki.

The study enrolled 3,280 patients with RA, aged over 18 years, diagnosed according to the revised 1987 criteria of the American College of Rheumatology.[12] Disease activity was evaluated using the 28-joint disease activity score (DAS28). The study patients had active form of the disease (DAS28–ESR ≥ 3.2) despite treatment with 1 or more of the following sDMARDs at stable doses for at least 1 month: MTX, sulfasalazine, hydroxychloroquine, chloroquine, chloroquine phosphate, leflunomide, gold salts, azathioprine, or cyclosporine. The exclusion criterion was prior use of biologics and any contraindications for TNF-α inhibitor use.

The study consisted of 2 parts. In part 1, the patients received 50 mg of SC golimumab, once a month for 6 months. Throughout the study the patients continued taking their regular doses of sDMARDs and/or GCS. Part 2 of the study included patients who did not attain remission after 6 months, but achieved good or moderate response according to the EULAR criteria.[13] The patients were randomly assigned (1:1) to one of 2 treatment groups receiving for the following 6 months 50 mg of SC golimumab once a month or combination regimen of SC and IV golimumab. Patients who did not participate in the study part 2 could continue golimumab therapy until week 48 as an extension of part 1.

The primary endpoint of part 1 was the percentage of patients who achieved good or moderate EULAR response (defined as DAS28–ESR improvement of >1.2 from a baseline score or an improvement of 0.6–1.2 in the case of a baseline score of ≤5.1). The key secondary endpoints included percentage of remissions and low disease activity according to DAS28–ESR, DAS28 calculated with C-reactive protein (CRP), simplified disease activity index (SDAI) and percentage of patients who developed minimal or no functional impairment (health assessment questionnaire disability index, HAQ-DI, ≤0.5). The essential efficacy evaluation criterion for both therapeutic regimens in part 2 was the percentage of patients who attained DAS28–ESR remission at the start of month 11 and at the end of month 12. Part 2 of the study included 505 patients with only a small number of Polish subjects (13), and therefore, this paper presents an analysis of results obtained with the Polish population participating in part 1 of the study.

Results

Baseline characteristics of the Polish patient group

The overall results of the GO-MORE study have been previously reported.[9]

The Polish GO-MORE clinical trial included 129 patients (Table 1). The majority (83%) of the patients enrolled in the study used MTX, and most took high doses of MTX (≥15 mg/week). A significantly lower proportion of patients (33%) took a sDMARD other than MTX, and only 16% of these patients received a sDMARD in combination with

MTX. The second most frequently used sDMARD was leflunomide (6% of patients in the Polish subpopulation and 9.3% of patients in the overall study population), and the remaining patients received a combination treatment, mostly MTX with chloroquine or hydroxychloroquine (13%). Tritherapy (MTX, hydroxychloroquine/chloroquine and sulfasalazine) was administered to only 1% of patients in the Polish subpopulation compared to 3.2% of patients in the overall population. The baseline characteristics of the Polish group of patients were similar to the overall study population in terms of age, sex, the mean baseline DAS28, HAQ-DI value, number of painful and swollen joints, laboratory inflammation markers, MTX dose, and percentage of patients treated with GCS (Table 1). Compared to the overall study population, the Polish patient population was characterized by a longer disease duration (median: 6.04 vs 4.9 years), a higher percentage of patients with high disease activity according to DAS28–ESR (86% vs 78.7% of patients), and a higher number of previous failures with at least 3 sDMARDs (41% vs 29.7% of patients).

Efficacy of golimumab

After 6 months of golimumab treatment, 85% (109/129) of the patients in the Polish subpopulation achieved good or moderate EULAR response, which was the primary part 1 endpoint. Low disease activity (DAS28–ESR < 3.2) after 6 months of treatment was achieved by 26% (34/129) and remission (DAS28–ESR < 2.6) was achieved by 17% (22/129) of patients. The percentage of patients who met the criterion of good or moderate EULAR response after 6 months (85%) was similar to the overall study population mean of 82.1%. The percentage of low disease activity and remission achieved after 6 months in the Polish patient population was lower than in the overall study population, i.e., 26% vs 37.4% and 17% vs 23.9%, respectively (Table 2).

The percentage of Polish patients who met the criteria of good or moderate EULAR response increased over time (i.e., 58% at the beginning of month 2, 78% at the beginning of month 4, and 85% at the end of month 6), which was similar to the overall population data (i.e., 64.9%, 76.9% and 82.1%, respectively). The percentage of Polish patients who attained remission over time also increased (i.e., to 3%, 12% and 17%, respectively), but these percentages were lower than in the overall population (i.e., 7.7%, 16.1% and 17.1%, respectively) (Table 2). There was a mean decrease in DAS28–ESR within 6 months of golimumab therapy from 5.96 (baseline) to 2.19 (±1.19).

The mean baseline SDAI score decreased from 35.16 at baseline to 21.96 (±12.23) at the end of treatment month 6. A total of 45% of patients (58/129) achieved low disease activity (SDAI < 11) and 10% of patients (13/129) achieved remission (SDAI < 3.3) after 6 months. Similar to the results seen with DAS28–ESR, the percentage of Polish patients achieving low disease activity according to SDAI increased over time, reaching 16%, 32% and 45% in months 2, 4 and 6, respectively. The percentage of Polish patients achieving remission according to SDAI also increased over time to 2%, 8% and 10% in months 2, 4 and 6, respectively. In the Polish group, these percentages based

Table 2. Percentage of patients who achieved good or moderate response, low disease activity or remission, according to DAS28–ESR (EULAR criteria) at subsequent measurement points. Results obtained in the Polish cohort (n = 129) and in the overall study population (n = 3280) are presented for comparison[9]

DAS28–ESR (EULAR)	Beginning of month 2		Beginning of month 4		End of month 6	
	Overall population	Polish patient group	Overall population	Polish patient group	Overall population	Polish patient group
Patients who achieved good or moderate response to the treatment (%)	64.9	58	76.9	78	82.1	85
Patients who achieved low disease activity (DAS28–ESR < 3.2) (%)	16.6	9	28.1	21	37.4	26
Patients who achieved remission (DAS28–ESR < 2.6) (%)	7.7	3	16.1	12	24	17

Table 3. Evaluation of Polish patients' functional improvement during golimumab treatment (n = 129)

	Before treatment (baseline)	Beginning of month 2	Beginning of month 4	End of month 6
No or minimal functional impairment(HAQ-DI ≤ 0.5), n (%)	8 (6)	18 (14)	26 (20)	25 (19)
Minimal (acceptable) disease symptoms(PASS), n (%)	14 (11)	59 (46)	74 (57)	94 (73)

HAQ-DI – health assessment questionnaire disability index; PASS – patient acceptable symptom state.

Table 4. The effect of golimumab on Polish patients' quality of life as determined by the EQ-5D (n = 129)

	Before treatment (baseline)	Beginning of month 2	Beginning of month 4	End of month 6
EQ-5D (mean ±SD)	0.44 ±0.28	0.15 ±0.22	0.19 ±0.26	0.22 ±0.26

on SDAI were lower than the percentages of low disease activity and remission determined by the DAS28–ESR in the overall study population.

Subgroup analysis of efficacy of golimumab

A subgroup analysis of the Polish patients showed no significant differences in the percentages of patients who achieved good or moderate EULAR response at the end of month 6 due to the dose of MTX, the use of DMARDs other than MTX, the number of previously failed DMARDs, and the use or non-use of GCS.

Effect of golimumab on quality of life

The effect of golimumab treatment on the patients' physical function was measured in all patients using the HAQ-DI score. Prior to the treatment, the patients' general physical function measured by HAQ-DI was 1.51 ±0.568, and disease activity evaluation by the patient using visual analogue scale (VAS) 0–100 mm was 63.56 ±17.357. Golimumab treatment significantly improved the patients' functional condition and more patients reported symptoms that were acceptable and did not disturb their everyday life (Table 3). In addition to the functional condition improvement, the patients treated with golimumab also reported an improved quality of life, as measured by the EQ-5D questionnaire (Table 4). The percentage of Polish patients without a disability or with minimal impairment of function (HAQ-DI ≤ 0 5) increased from 6% at baseline to 14%, 20% and 19% at the beginning of month 2, 4 and the end of month 6, respectively. In addition, the percentage of Polish patients exhibiting minimal (acceptable) disease symptoms (patient acceptable symptom state, PASS) increased from 11% at baseline to 46%, 57% and 73% at the beginning of month 2, 4 and the end of month 6, respectively. Therefore, treatment with golimumab significantly improved patients' physical function as assessed by HAQ-DI, and significantly improved health-related quality of life.

Safety and tolerability of golimumab

Golimumab safety was evaluated in all patients in the study in relation to adverse events observed during treatment (treatment emergent adverse event, TEAE). The percentage of Polish patients with at least 1 TEAE was 45% in part 1 of the study, and 15% of patients had a drug-related TEAE (Tables 5, 6). Abnormal laboratory results were observed in 3% of the patients, with the most common being elevated serum aminotransferase, elevated potassium, decreased thyroid stimulating hormone, or decreased fibrinogen. No deaths occurred during part 1 of the study or within 30 days after the last dose. Therefore, golimumab was well-tolerated in the Polish cohort, and the safety profile was consistent with previous studies on the drug.

Discussion

In this analysis of the GO-MORE study in the Polish subpopulation (129 patients), we found that SC treatment with 50 mg of golimumab once a month for 6 months, in combination with different sDMARDs, resulted in good or moderate EULAR response in the majority of patients (85%). By the 2nd month of treatment, more than half of the Polish patients (58%) had already achieved good or moderate EULAR response, and after 4 months, this proportion had increased to 78% of the patients. The data is similar to that observed in the overall GO-MORE study population.[9] On the other hand, remission rates in the Polish subpopulation improved more gradually; 3%, 12% and 17% of patients had achieved remission at the beginning

Table 5. Adverse events in the Polish patient subpopulation (n = 129) during golimumab treatment

Categories of adverse events	n (%)
All adverse events	59 (45)
Drug-related adverse events	20 (15)
Serious adverse events	4 (3)
Adverse events leading to early withdrawal	3 (2)
Deaths	0
Adverse injection site reactions	0
Clinically significant abnormal laboratory results	5 (3)

Table 6. Adverse events in the Polish patient subpopulation (n = 129) during 6 months of golimumab treatment according to various organ system manifestations

Organ system manifestations	Adverse events, n (%)
Infections	31 (24)
Musculoskeletal system	8 (6)
Gastrointestinal system	7 (5)
Respiratory system	6 (5)
Skin	6 (5)
General symptoms	5 (4)
Abnormal laboratory results	5 (3)
Metabolic disorders	4 (3)
Arterial hypertension	4 (3)
Hematologic disorders	3 (2)
Neoplasms: benign, malignant and unspecified	3 (2)
Nervous system	3 (2)
Dysrhythmias	2 (2)
	Serious adverse events (SAE), n (%)
Discopathy	1 (1)
Skin melanoma	1 (1)
Dysrhythmias	1 (1)
Endometrial hyperplasia	1 (1)

of month 2, 4 and the end of month 6, respectively. Therefore, although the clinical response with a combined golimumab therapy is achieved relatively early, it may take more time to achieve the main RA treatment goals, such as remission or low disease activity.

The remission rate (17%) observed in the Polish subpopulation using golimumab is similar to that observed previously using other anti-TNF-α drugs. For example, Hyrich et al. found that the remission rates (measured by DAS28) for 4000 RA patients, monitored after the 1st year, and subsequently from 2001 to 2008, after treatment with different anti-TNFα drugs (i.e., etanercept, infliximab and adalimumab) ranged from 8% to 19.4%.[10] On the other hand, in the CORRONA study, remission rates measured by DAS28 were slightly higher; after 6 months of adalimumab (874 patients), etanercept (640 patients) or infliximab (728 patients) therapy, remission rates were 25.2%, 28.4% and 28.2% of patients, respectively.[14] However, this difference in remission rates may have occurred as the patients in the CORRONA study had lower mean baseline disease activity (DAS28 = 4.4) compared to the Polish subpopulation of the GO-MORE study (DAS28 = 5.96). Moreover, the remission rate was lower after 6 months of treatment in the Polish subpopulation (17%) compared to the overall GO-MORE study population (23.9%). This difference may be due to the fact that the Polish patients had a longer disease duration compared to the overall study population (6.04 vs 4.9 years) and, therefore, they were most likely to be in a more advanced stage of the disease. Indeed, there were more Polish patients with high disease activity (i.e., DAS28–ESR > 5.1) compared to the overall study population (86% vs 78.7%). There was also a larger proportion of Polish patients with the presence of rheumatoid factor and/or anti-cyclic citrullinated peptide compared to the overall study population (99% vs 72.5% and 79% vs 71.9%, respectively), which usually indicates poorer outcomes in RA patients. In addition, the Polish subpopulation had a higher proportion of patients who had been previously unsuccessfully treated with at least 3 sDMARDs compared to the overall study population (41% vs 29.7%). Finally, there are differences in access to drugs and in treatment standards between Poland and other countries examined in the GO-MORE study, which could explain the minor differences in remission rates to golimumab treatment in the Polish subpopulation compared to the entire study population.

We also found that the proportion of Polish patients who achieved remission or had low disease activity was lower when measured using SDAI compared to DAS28–ESR. Using SDAI, only 10% of patients achieved remission and 45% of patients showed low disease activity after 6 months of treatment. This discrepancy between the DAS28 and SDAI results is probably due to the fact that SDAI includes the assessment of the patient's general condition by the physician. The physician may evaluate the patient's symptoms more rigorously than the patient themselves, who have become accustomed to the chronic ailments. Moreover, as the mean DAS28, CRP and ESR values were comparable between the Polish subpopulation and the overall study population, the lower remission and low disease activity rates (according to both DAS28–ESR and SDAI) in Polish patients compared to the overall study population should be interpreted with caution. Indeed, this may simply be due to the fact that the Polish subpopulation had a larger proportion of patients with higher baseline disease activity (DAS28–ESR > 5.1), as mentioned above.

A larger group of patients received MTX in the Polish subpopulation (83%) compared to the overall study population (79.0%); 67% of the Polish subpopulation and 51.4% in the overall study population were in MTX monotherapy. In addition, a larger proportion of Polish patients received MTX at high doses exceeding 15 mg/week. A significantly lower proportion of patients (33%) took a sDMARD other than MTX and only 16% of these patients received a sDMARD in combination with MTX.

The Polish subpopulation of the GO-MORE study showed a significant improvement in terms of physical function and quality of life, and golimumab was effective in patients who had shown insufficient response to previous therapies with 1 or more sDMARDs. The adverse events profile in the Polish subpopulation was similar to the overall study population and to that described in previous studies using golimumab, and was also similar to that observed using other anti-TNF-α drugs.[9,11,15,16] The most commonly observed adverse events were infections, and serious events were reported in only 3% of the Polish patients. In addition, no deaths or injection site reactions occurred in the Polish subpopulation.

The basic limitation of this analysis of the Polish subpopulation of the GO-MORE study was the small group of participants (129 patients), which makes it difficult to conduct a statistical analysis of golimumab efficacy among individual subgroups. The subgroup analysis showed no significant differences in the percentages of patients who achieved good or moderate EULAR response at the end of month 6 depending on MTX dose, use of sDMARDs other than MTX, number of failed sDMARDs, and use or non-use of GCS. Despite this, due to the small size of the Polish patient group, and consequently very small subgroups, the data should be interpreted with caution. Nevertheless, taking into account that the overall study population showed no efficacy differences among the abovementioned subgroups, the results obtained in the Polish patients seem to be reliable.[9] Another potential limitation of part 1 of the GO-MORE study may be its open-label character, which can have an inherent bias. However, results of the observational phase of the study may be most representative for the RA patient population, which is common in rheumatological practice.

In conclusion, the results of the GO-MORE study in the Polish population show the efficacy and safety of golimumab as an add-on treatment to different sDMARDs,

with or without GCS therapy, in patients with RA in whom previous DMARD therapies had failed, demonstrating good or moderate EULAR DAS28–ESR response in a large proportion of patients (85%). The onset of action was rapid, with good tolerability, and a safety profile consistent with the data described for golimumab in other clinical trials and in the Summary of Product Characteristics.

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Electrocardiographic T-wave parameters in families with long QT syndrome

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

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Abstract

Background. T-wave parameters, especially the Tpeak-Tend interval (TpTe), reflect the total dispersion of repolarization, whose amplification may lead to the development of life-threatening ventricular arrhythmias observed in the long QT syndrome (LQTS).

Objectives. The study attempted to evaluate QT, QTp (Q-Tpeak) and TpTe (Tpeak-Tend) intervals in unaffected and affected blood relatives of children with clinically confirmed LQTS as well as to determine whether the values of these repolarization parameters may be used in clinical practice.

Material and methods. The study group included 47 affected blood relatives (27 LQTS1 and 20 LQTS2) and 68 unaffected family members without clinically confirmed LQTS symptoms. The TpTe, QT and QTp intervals were measured manually in the lead V5 of standard ECGs and corrected using Bazett's and Fridericia's formulas.

Results. The RR, QT, QTp and TpTe intervals with their corrected values were significantly longer ($p < 0.0001$) in the affected subjects than in the unaffected subjects and, similarly, in LQTS1 and LQTS2 patients compared with the unaffected family members. The TpTe interval in LQTS2 showed only a tendency to be longer compared to LQTS1, but did not reach statistical significance ($p = 0.0933$). For affected blood relatives, only the TpTe interval ($p < 0.0409$) and QT interval, corrected with Bazett's ($p < 0.0393$) and Fridericia's ($p < 0.0495$) formulas, enabled differentiation between LQTS1 (mean TpTe = 103 ±15) and LQTS2 women (mean TpTe = 106 ±17). Moreover, there were statistically significant differences ($p < 0.05$) in the TpTe interval between the 6 sex subgroups: unaffected women and men as well as women and men with LQTS1 and LQTS2.

Conclusions. The electrocardiographic Tpeak-Tend parameter, in addition to the QT interval, is helpful in identifying affected blood relatives of children with LQTS, particularly for the group of LQTS1 and LQTS2 women. Further studies are required to assess the clinical importance of the TpTe interval in families with long QT syndrome.

Key words: repolarization, long QT syndrome, QT, Q-Tpeak, Tpeak-Tend intervals

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Introduction

Congenital long QT syndrome (LQTS) is a disease manifested by electrocardiographic repolarization abnormalities with the QT interval prolongation and predisposition for malignant ventricular tachyarrhythmias (torsade de pointes), potentially leading to recurrent syncope and sudden cardiac death (SCD).[1]

Among 15 types of LQTS mutations thus far identified, the most common LQTS1 (KCNQ1) and LQTS2 (KCNH2) genotypes differ in the clinical course, symptom-related triggers, duration and morphology of the repolarization wave in the ECG, determined by various action potential durations in cardiac myocytes, dependent on malfunctioning ion channels: slowly repolarizing cardiac potassium current I_{Ks} in LQTS1 and rapidly repolarizing cardiac potassium current I_{Kr} in LQTS2.[2–5]

The criteria for LQTS diagnosis, valid since 1993 (improved in 2012) and comprising a point scale (the Schwartz score ≥4), based on clinical presentation, family history and the electrocardiographic analysis of the QT interval, do not include the classification of the most common types of LQTS: LQTS1 and LQTS2.[1,6] Moreover, the criteria do not consider the diagnostic importance of the QTp (Q-Tpeak) and TpTe (Tpeak-Tend) intervals in the resting and exercise ECG that help differentiate between LQTS1 and LQTS2 and assess the risk of malignant ventricular arrhythmias.[7–12]

T-wave parameters, especially the Tpeak-Tend (TpTe) interval, may provide a more accurate electrophysiological marker of ventricular arrhythmia risk than the QT interval.[12,13] The question of whether TpTe reflects transmural repolarization heterogeneity or total dispersion of repolarization is still a matter of debate.[5,14–16]

The study attempted to evaluate the QT, QTp and TpTe intervals in unaffected and affected blood relatives of children with long QT syndrome as well as to determine whether the values of these repolarization parameters may be used in clinical practice as a possible method of identifying affected and unaffected subjects, and whether they may help differentiate between LQTS1 and LQTS2 types.

Material and methods

Thirty-five unrelated families with long QT syndrome (each with 2 generations of members) were enrolled into the study. This group consisted of 115 adult blood relatives of 62 children with clinically diagnosed LQTS (35 children with LQTS1 and 27 with LQTS2) who were admitted to the Department of Pediatric Cardiology of the Medical University of Silesia in Katowice (Poland). Based on the ECG analysis of the repolarization period as well as clinical presentation, personal and family history of syncope and arrhythmia and/or aborted SCD, the study group included 47 adult blood relatives with LQTS and 68 unaffected family members without clinically confirmed LQTS symptoms. All the affected patients had score values ≥4 according to the Schwartz and Moss criteria.[1]

The affected family members were 27 patients with LQTS1 and 20 patients with LQTS2. Identification of the most common LQTS1 or LQTS2 in the patients with long QT syndrome was based on the analysis of the repolarization period: morphology, amplitude and duration of the T-wave in the standard ECG (in LQTS1, T-wave: broad-based, smooth, with a higher amplitude; in LQTS2, T-wave: bifid, notched, flat, with long duration) (Fig. 1, 2), and a history of genotype-specific triggers (sympathetic stimulation in LQTS1: mainly exertion – particularly swimming, emotional stress, cardiac events – usually during rest; in LQTS2: sudden arousal from rest or sleep, auditory stimuli).[5]

In order to search for mutations, genomic DNA was obtained from all patients, using mSSCP and sequencing. Furthermore, the transcriptional activity of the encoding genes *KCNQ1* and *KCNH2 (HERG)* was determined in the study, using the quantitive real-time polymerase chain-reaction (QRT-PCR). The expression of the investigated genes was inferred from the analysis of the number of mRNA copies per 1 μg total mRNA isolated from whole blood. The genetic data was presented in earlier papers.[17,18]

The study exclusion criteria were bundle branch block or any other intraventricular conduction defect, atrial fibrillation, lack of sinus rhythm, and use of medications known to prolong the QT interval.

In the study, digital 12-lead resting electrocardiograms of 115 blood relatives were recorded at a paper speed of 50 mm/s with an amplification of 10 mm/mV (AT2 plus Schiller AG, Baar, Switzerland).

The RR, QT, QTp, and TpTe intervals were measured manually in the lead V5 of standard ECG in 3 consecutive cardiac cycles, and then averaged. The QT interval was measured from the onset of Q-wave to the end of T-wave at the point of its return to the isoelectric line. The end of T-wave was defined as the intersection between the line tangent to the descending arm of T-wave and the isoelectric line. The QTp was the interval between the Q onset and T-wave peak. The TpTe was the interval from the peak of T-wave to the end of T-wave at the point of its return to the isoelectric line. If T-wave was inverted, the TpTe interval was measured from the lowest point of the inverted T-wave to the end of T-wave at the point of its return to the isoelectric line. The U-wave was not taken into account.[19] The RR interval was measured on the basis of an average of 3 cardiac cycles (the same cycles in which the QTp and TpTe intervals were measured). To correct for possible heart rate effects on QT and QTp, we applied Bazett's (QT, QTp/$\sqrt{RR}$) and Fridericia's (QT, QTp/$RR^{1/3}$) formulas. All measurements of the end of T-wave were analyzed blindly by another independent investigator, without access to the obtained results and clinical data.

Statistical analysis

The data was exported from an Excel v. 2010 datasheet to the STATISTICA v. 7.1 data analysis software system (StatSoft Inc. 2006, Kraków, Poland). Due to the lack of normal distribution of the investigated parameters, examined with the Shapiro-Wilk test, we used the Mann-Whitney U test. Variables that followed normal distribution were compared using the Student's t-test. In addition, the ANOVA test (Kruskal-Wallis test) and post hoc test (Student-Newman-Keuls) were used for the comparison of sex subgroups (medians and ranges). The level for statistical significance was set at a p-value <0.05. The ROC curve receiver operating characteristic (ROC) (area under curve [AUC]) analysis was also performed to find the cut-off point for the TpTe intervals with the highest sensitivity and specificity.

The study was approved by the Bioethics Committee at the Medical University of Silesia in Katowice (No. NN-6501-182/05).

Results

One hundred fifteen adult blood relatives of 62 children with clinically diagnosed LQTS were enrolled into the study: 47 adult blood relatives with LQTS (36 women and 11 men; mean age 41 ±16 years) and 68 unaffected family members (26 women and 42 men; mean age 40 ±14 years) without clinically confirmed LQTS symptoms (Table 1).

Among 27 family members with LQTS1 (22 women and 5 men; mean age 44 ±19 years), 10 subjects (5 mothers, 1 father, 1 sister, 1 brother, 1 father's sister, and 1 mother's sister) were symptomatic with a history of exercise-related syncope. Two mothers had a positive family history of sudden cardiac death by the age of 30 years. Among 20 family members with LQTS2 (14 women and 6 men; mean age 36 ±11 years), 3 mothers with cardiac arrest (TdP) and an implantable cardioverter-defibrillator reported recurrent syncope during pregnancy and puerperium. Twelve subjects (7 mothers, 2 fathers, 1 sister, 1 maternal grandmother, and 1 paternal grandmother) reported syncope, often occurring during rest and sleep, triggered by stress or auditory stimuli. In the past history of 3 LQTS2 families, cases of sudden cardiac death at a young age were reported. All subjects with cardiac events (10 with LQTS1 and 15 with LQTS2) received beta-blockers. Moreover, antiepileptic drugs were administered to 2 LQTS2 family members.

In the whole study group of 115 blood relatives, there were no significant differences (p = 0.822) in age between the affected and unaffected family members (Table 2). However, the unaffected men (mean age 42.88 ±13.03 years) were older (p = 0.0494) than the unaffected women (mean age 36.92 ±15.36) (Table 3).

Table 1. Clinical characteristics of affected and unaffected subjects

Study group	Affected (n = 47)		Unaffected (n = 68)
	LQTS1 (n = 27)	LQTS2 (n = 20)	
Age [years] (mean ±SD)	44 ±19	37 ±12	40 ±15
Range	20–83	21–54	18–77
Men/women	5/27	6/14	42/26
RR (mean ±SD)	810 ±128	790 ±111	902 ±135
Range	550–1120	610–1000	600–1400
QTcB (mean ±SD)	468 ±15	485 ±37	412 ±26
Range	450–500	450–550	320–450
Symptomatic/asymptomatic	10/17	15/5	0/68

values (ms) are shown as a mean ±standard deviation (SD) and a range; QTcB – QT intervals corrected for heart rate (HR) using Bazett's formula.

Table 2. Electrocardiographic characteristics of unaffected and affected subjects

ECG parameters	Unaffected (n = 68)		Affected (n = 47)		p-value
	mean	SD	mean	SD	
AGE [years]	40.60	14.15	41.19	16.78	0.8222
HR [beats/min]	67.95	10.13	76.30	11.44	0.0001
RR	902	135	802	120	0.0001
QT	389	25	424	39	<0.0001
QTcB	412	26	475	27	<0.0001
QTcF	404	22	457	28	<0.0001
QTp	304	21	316	31	0.0080
QTpcB	322	22	353	24	<0.0001
QTpcF	315	19	340	25	<0.0001
TpTe	85	15	109	17	<0.0001

values [ms] are shown as a mean ±standard deviation (SD); QTcB, QTpcB – QT, QTp intervals corrected for heart rate (HR) using Bazett's formula; QTcF, QTpcF – QT, QTp intervals corrected for heart rate (HR) using Fridericia's formula.

Table 3. Electrocardiographic characteristics of unaffected women and unaffected men

ECG parameters	Unaffected women (n = 26)		Unaffected men (n = 42)		p-value
	mean	SD	mean	SD	
Age [years]	36.92	15.36	42.88	13.03	0.0494
HR [beats/min]	70.32	9.88	66.48	10.13	0.1074
RR	870	123	923	140	0.1074
QT	388	27	390	25	0.7932
QTcB	418	33	408	21	0.0945
QTcF	408	28	402	17	0.1915
QTp	305	22	303	21	0.8518
QTpcB	329	26	317	18	0.0227
QTpcF	321	22	312	16	0.0486
TpTe	83	17	87	13	0.1446

values [ms] are shown as a mean ±standard deviation (SD); QTcB, QTpcB – QT, QTp intervals corrected for heart rate (HR) using Bazett's formula; QTcF, QTpcF – QT, QTp intervals corrected for heart rate (HR) using Fridericia's formula.

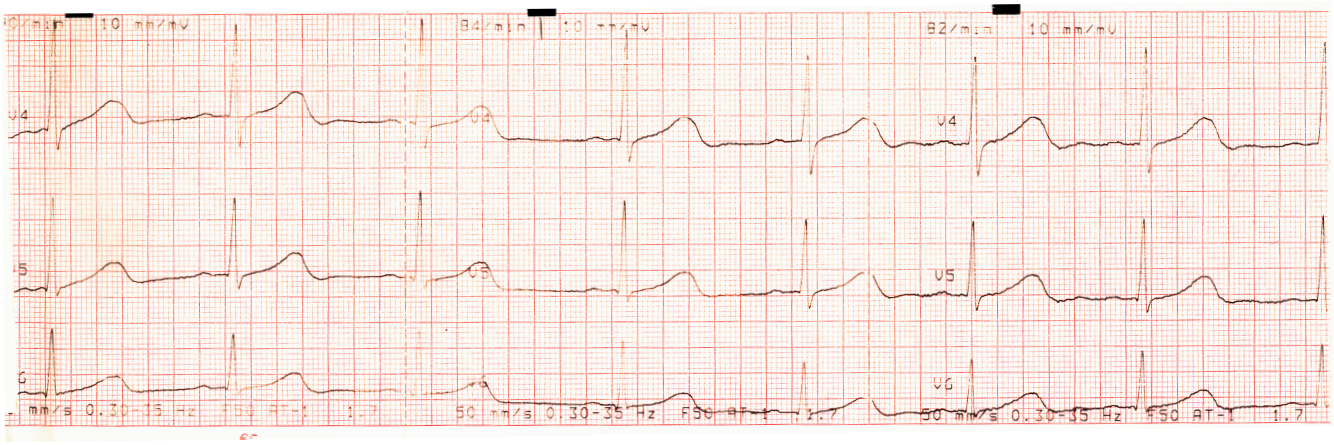


Fig. 1. Precordial leads (V4–V6) in the ECG of LQTS1 patient. Broad-based T-wave. TpTe:100 ms, QTcB: 456 ms

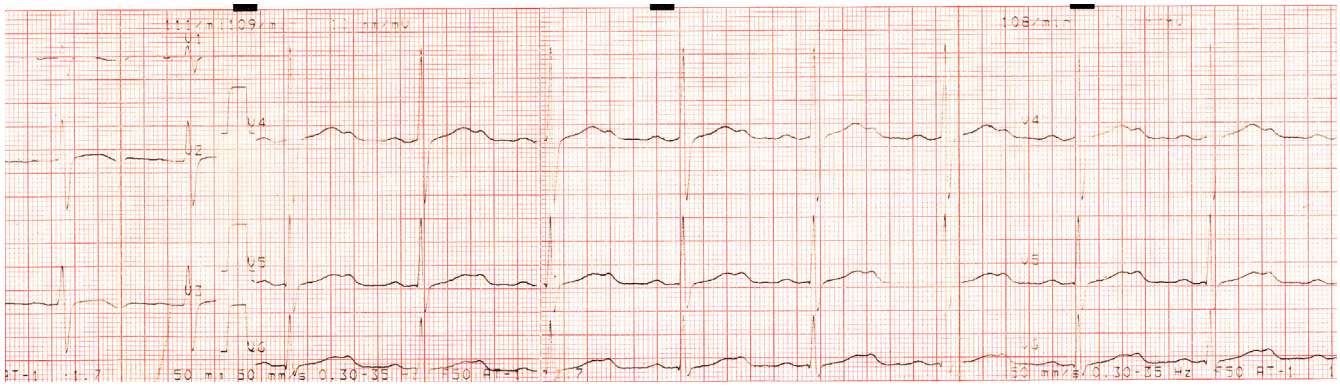


Fig. 2. Precordial leads (V4–V6) in the ECG of LQTS2 patient. Bifid T-wave. TpTe: 140 ms, QTcB: 481 ms

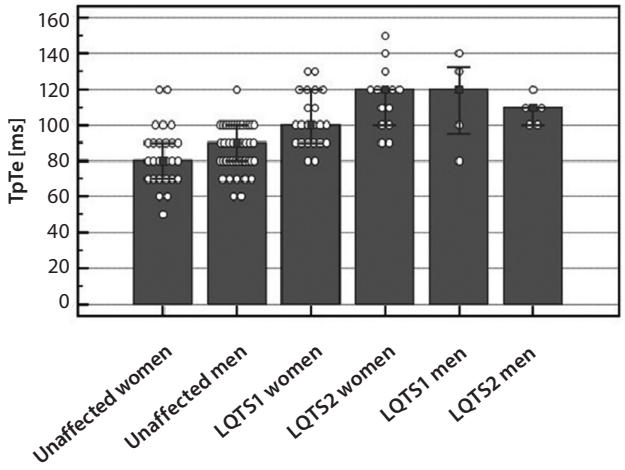


Fig. 3. Statistically significant differences (p < 0.05) in the TpTe intervals between the 6 sex subgroups: 1 – unaffected women; 2 – unaffected men; 3 – LQTS1 women; 4 – LQTS2 women; 5 – LQTS1 men; 6 – LQTS2 men. Values [ms] are shown as a median and a range

All the subjects presented sinus rhythm. Heart rates in the unaffected blood relatives varied from 42 to 100 beats/min, while in the affected subjects, the values ranged from 53 to 109 beats/min (Table 1). The RR intervals were significantly longer (p = 0.0001) in the unaffected subjects than in the affected subjects (Table 2).

The baseline TpTe intervals and the QT, QTp intervals (ms) with their values corrected using Bazett's (QTcB, QTpcB) and Fridericia's (QTcF, QTpcF) formulas were significantly greater in the affected than in the unaffected subjects (p < 0.0001) (Table 2).

There were no sex differences in the TpTe intervals and QT, QTp intervals with their corrected values between the unaffected women and unaffected men, except the QTpcB (p = 0.0227) and QTpcF (p = 0.0486), and in the affected women compared with the affected men, except the QTcB (p = 0.0394) and QTpcB (p = 0.0108) (Tables 3, 4).

The LQTS1 and LQTS2 patients showed significantly longer (p < 0.0001) mean TpTe, QT, QTp intervals and higher corrected values than the unaffected family members, except the QTp in LQTS1 (p = 0.0714).

The RR, QT and QTp intervals with their corrected values showed no statistically significant differences between the LQTS1 and LQTS2 types (Table 5). The TpTe interval in LQTS2 showed a tendency to be longer compared to LQTS1, but did not reach statistical significance (p = 0.0933). However, the TpTe interval showed statistically significant sex differences between the LQTS1 and LQTS2 women (p = 0.0409) (Table 6). In addition, QTcB (p = 0.0393) and QTcF (p = 0.0495) values were significantly greater in the women with LQTS2 than in LQTS1 women (Table 6).

The age of 5 men with LQTS1 varied from 20 to 74 years, TpTe – from 80 to 140 ms; the age of 6 men with LQTS2 varied from 28 to 54 years, TpTe – from 100 to 120 ms.There were no significant differences between the LQTS1 and LQTS2 men in the RR, TpTe intervals and the QT and QTp intervals with their corrected values. Moreover, there were statistically significant differences ($p < 0.05$) in the TpTe interval between the 6 sex subgroups: unaffected women and men as well as women and men with LQTS1 and LQTS2 (Fig. 3). The area under the ROC curve (AUC) for the TpTe intervals of the LQTS1 and LQTS2 women was 0.705, indicating that this variable is a relatively good discriminator. The cut-off point for the TpTe intervals that optimizes the values of sensitivity and specificity is for values ≥100 ms (Fig. 4).

Discussion

The ventricular repolarization analysis has been shown to be effective in identifying electrical myocardial instability that leads to the development of ventricular arrhythmia (TdP) observed in the long QT syndrome. In clinical evaluation, the last part of total T-wave (the TpTe interval) has been found to be amplified in patients with congenital long QT syndrome and acquired LQTS.[13,20–23] The TpTe interval has been suggested to reflect repolarization heterogeneity that can be easily demonstrated in a standard electrocardiogram (ECG).[24]

Therefore, in clinical practice, the TpTe interval is considered a more sensitive marker of arrhythmogenesis compared with the QT interval.[13,20,23]

In families of LQTS patients, measurements of the Tpeak-Tend interval (TpTe) seemed to be important for the identification of affected relatives, but this parameter did not distinguish symptomatic from asymptomatic subjects.[8,12,25]

The present study showed that the LQTS-affected patients (LQTS1 and LQTS2) had significantly longer QT, QTp and TpTe intervals than the unaffected family members, which may possibly contribute to the increased risk of cardiac events, although we did not evaluate the association of repolarization parameters, such as TpTe, with cardiac events.

Similarly, Viitasalo et al. (revision Holter recordings) and Kanters et al. (ECG analysis) described increased TpTe intervals in patients with LQTS2 compared with LQTS1 subjects or unaffected family members, but they could not find significant differences between symptomatic and asymptomatic patients in any group.[25]

Table 4. Electrocardiographic characteristics of affected women and affected men

ECG parameters	Affected women (n = 36)		Affected men (n = 11)		p-value
	mean	SD	mean	SD	
Age [years]	42.06	16.63	38.36	17.78	0.4739
HR [beats/min]	76.51	9.16	75.63	17.55	0.4435
RR	794	96	825	183	0.4662
QT	426	34	416	52	0.5977
QTcB	479	29	461	14	0.0394
QTcF	461	28	445	24	0.1254
QTp	319	27	305	43	0.5718
QTpcB	358	24	337	16	0.0108
QTpcF	344	24	326	24	0.0808
TpTe	108	17	111	16	0.5299

values [ms] are shown as a mean ±standard deviation (SD); QTcB, QTpcB – QT, QTp intervals corrected for heart rate (HR) using Bazett's formula; QTcF, QTpcF – QT, QTp intervals corrected for heart rate (HR) using Fridericia's formula.

Table 5. Electrocardiographic characteristics of LQTS1 and LQTS2 subjects

ECG parameters	LQTS1 (n = 27)		LQTS2 (n = 20)		p-value
	mean	SD	mean	SD	
Age [years]	44.41	19.33	36.85	11.65	0.3276
HR [beats/min]	75.73	12.13	77.07	10.71	0.6827
RR	810	128	790	111	0.6748
QT	420	37	430	41	0.3662
QTcB	468	15	485	37	0.2917
QTcF	451	19	465	35	0.2917
QTp	315	30	316	34	0.5186
QTpcB	351	20	356	29	0.6436
QTpcF	339	21	342	30	0.4322
TpTe	105	17	114	15	0.0933

values [ms] are shown as a mean ±standard deviation (SD); QTcB, QTpcB – QT, QTp intervals corrected for heart rate (HR) using Bazett's formula; QTcF, QTpcF – QT, QTp intervals corrected for heart rate (HR) using Fridericia's formula.

Table 6. Electrocardiographic characteristics of LQTS1 and LQTS2 women

ECG parameters	LQTS1 women (n = 22)		LQTS2 women (n = 14)		p-value
	mean	SD	mean	SD	
Age [years]	46.14	18.28	35.64	11.50	0.1047
HR [beats/min]	75.98	9.40	77.33	9.05	0.7090
RR	800	101	786	91	0.7333
QT	418	30	439	38	0.1118
QTcB	468	15	497	37	0.0393
QTcF	451	17	477	35	0.0495
QTp	315	26	324	29	0.1888
QTpcB	353	21	366	29	0.1487
QTpcF	340	21	351	27	0.1398
TpTe	103	15	116	17	0.0409

values [ms] are shown as a mean ±standard deviation (SD); QTcB, QTpcB – QT, QTp intervals corrected for heart rate (HR) using Bazett's formula; QTcF, QTpcF – QT, QTp intervals corrected for heart rate (HR) using Fridericia's formula.

The TpTe interval seems to be a useful electrocardiographic parameter that helps differentiate between LQTS1 and LQTS2 types in resting or exercise ECG.[7–9,12,25]

Our previous study suggests that in diagnostic classification of long QT syndrome as LQTS1 or LQTS2 type in affected children, the most valuable electrocardiographic parameter is the TpTe interval assessed during rest and during the recovery phase after exercise.[7] In the present study of blood relatives of these children, heart rates were comparable in LQTS1 and LQTS2 subjects as well as in LQTS1 and LQTS2 women. We observed that the measures of the TpTe and QT intervals did not provide differentiation between LQTS1 and LQTS2 types; however, the TpTe interval in LQTS2 showed a tendency to be longer compared to LQTS1, still not reaching statistical significance.

In addition, there were statistically significant differences in the TpTe interval between the 6 sex subgroups: unaffected women and men as well as women and men with LQTS1 or LQTS2.

Moreover, the TpTe interval and the corrected QT (QTcB and QTcF) values were differential parameters regarding LQTS1 and LQTS2 women. These are novel findings and similar data has not been found in the available literature to date.

The long QT syndrome is a disorder of diverse phenotypic presentation with some affected patients and their families.

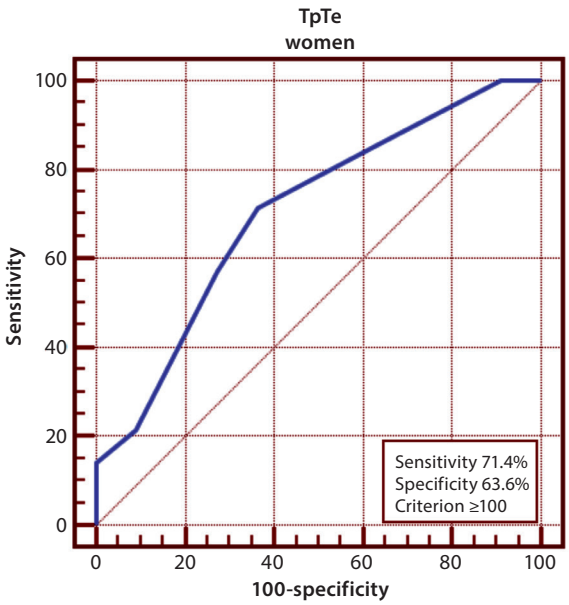
The clinical Schwartz criteria, demonstrating sex-related differences in QTc prolongation, are still valid and the designation of QTc values ≥450 ms in men and ≥460 ms in women as prolonged QTc values comes from the standpoint of AHA/ACCF/HRS guidelines.[19]

The Schwartz score, with QT rate correction, after adding other electrocardiographic T-wave parameters (particularly TpTe and QTp), might provide the most useful clinical information with a potential for identifying patients affected by LQTS, as well as it may help differentiate between the LQTS types (LQTS1 and LQTS2 women in particular).

In addition, abnormal values of these parameters correlate well with increased arrhythmic risk.

Twelve-lead standard ECG remains the primary, cheap and most commonly used cardiac diagnostic tool that can help identify affected blood relatives and differentiate between LQTS1 and LQTS2 types, which may have implications for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death, aimed at modifying a lifestyle and avoiding specific triggers, according to 2015 ESC guidelines.[6,26,27]

Clinical usefulness of the TpTe interval with regard to identifying affected blood relatives of LQTS patients and differentiating between LQTS1 and LQTS2 types, particularly in terms of sex-related differences, seems to be an interesting issue, but needs further studies, particularly for larger groups of patients.



Area under the ROC curve (AUC)	0.705
Standard error[a]	0.0883
95% confidence interval[b]	0.529–0.844
z statistic	2.317
Significance level p (area = 0.5)	0.0205

Fig. 4. The ROC curve (AUC) analysis for the TpTe intervals of LQTS1 and LQTS2 women. The cut-off point for the TpTe intervals that optimizes the values of sensitivity and specificity is for values ≥100 ms

Based on these results, the authors conclude that the electrocardiographic Tpeak-Tend parameter, in addition to the QT interval, is helpful in identifying affected blood relatives of children with LQTS, particularly for the group of LQTS1 and LQTS2 women.

Further studies are required to assess the clinical importance of the TpTe interval in families with long QT syndrome.

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Clinicopathological features of metaplastic breast carcinoma

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Abstract

Background. Metaplastic carcinoma of the breast (MpBC) is defined as a group of heterogeneous malignant neoplasms that contain glandular and non-glandular components with mixed epithelial and mesenchymal differentiations.

Objectives. The aim of this study was to research the clinical and pathological characteristics of MpBC determining its rank among all breast cancers.

Material and methods. Metaplastic carcinoma of the breast was found in 7 out of 1,164 patients who had been diagnosed with breast cancer within the period of 12 years in our hospital. Demographic and clinical characteristics of the patients were retrieved from the patient files, and their final status was verified by a phone call. Diagnoses of the patients were confirmed by examining hematohylen and eosin (H&E) preparations. They were stained immunohistochemically for estrogen receptor (ER), progesterone receptor (PR), C-erbB-2, CK5/6 (Sitokeratin5/6), and EGFR (epidermal growth factor receptor), and the subgroups were determined according to the WHO classification.

Results. All patients were female with a median age of 61 years (41–87 years). Three of them were diagnosed with stage IIB, 2 with IIIB and 1 with IV. Four patients had squamous type of metaplastic cell differentiation, 1 spindle, 1 adenosquamous, and 1 osteosarcomatous. In 6 out of 7 patients, ER, PR and C-erbB-2 expressions were negative immunohistochemically. In the case of squamous metaplasia, estrogen receptor was 10% and progesterone receptor was 5% positive. CK5/6 was positive in 5 cases. Epidermal growth factor was positive in all cases.

Conclusions. Metaplastic carcinoma of the breast is relatively rare and, in our series, its incidence was 0.6%. According to its immunohistochemical characteristics, MpBC can be interpreted as a subgroup of triple-negative breast cancers (TNBC). Five of the presented patients resembled the subgroup of TNBC with a basaloid phenotype. The chemotherapy regimens suggested in the treatment of MpBC are platin in the epithelial subgroup and high-dose anthracycline in the mesenchymal subgroup. There is a need of new studies that evaluate different choices of treatment as MpBC has a bad prognosis and an aggressive nature.

Key words: metaplastic breast cancer, breast cancer, triple-negative breast cancer

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Introduction

Breast cancer is the most frequent cancer in women. Metaplastic carcinoma of the breast (MpBC) is a very rare and aggressive subgroup with a bad prognosis within the breast cancers.[1–3] Metaplastic carcinoma of the breast is defined as a group of heterogeneous malignant neoplasms that contain glandular and non-glandular components with mixed epithelial and mesenchymal differentiations.[4] Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu, c-erbB-2) are negative in more than 90% of MpBC, while cytokeratin 5/6 (CK5/6), cytokeratin 14, and epidermal growth factor receptor (EGFR) show expressions immunohistochemically.[5] This subgroup, for which standard treatment protocols have different responses due to its heterogeneous nature, needs new and tumor-specific treatment methods.

In this study, the research of the clinical and pathologic characteristics of the MpBC that determine its rank among all breast cancers was aimed.

Methods

Metaplastic carcinoma of the breast was found in 7 out of 1,164 patients that had a diagnosis of breast cancer within the period of 12 years in our hospital. Demographic and clinical characteristics of the patients were retrieved from the patient files, and their final status was verified by a phone call. Histopathological findings of the patients were evaluated by examining the preparations fixed in formalin and stained with hematoxylen and eosin (H&E).

They were immunochistochemically re-evaluated for ER (1/100, clone EP-1), PR (1/400, SP2), C-erbB-2 (1/800, e2-4001+3B5), CK5/6 (1/100, D5/16B4), and EGFR (1/25, EP38Y). Hormone receptors, nuclear staining, and the reaction intensity of the tumor cells were evaluated immunohistochemically. The staining percentage of the tumor cell nuclei was noted. Estrogen receptors and PR were considered positive if the nuclear staining was more than 10%. Regarding C-erbB-2 staining, the lack of staining on tumor cells or incomplete membrane staining <10% was considered as 0. Score 1 – weak and incomplete membrane staining >10%. Score 2 – weak to moderate complete membrane staining >10%. Score 3 – strong complete membrane staining >10%.[6] Subgroups were determined according to the WHO classification (Table 1).[4]

Results

All patients were female with a median age of 61 years (41–87 years). Two of them were premenopausal and 1 patient had 2 cases of breast cancer at her pedigree. Clinical and histological findings of the patients with metaplastic cancer are summarized in Table 2.

Table 1. WHO classification of metaplastic carcinoma of the breast[2]

Metaplastic carcinoma low-grade adenosquamous carcinoma
Fibromatosis-like metaplastic carcinoma squamous cell carcinoma spindle cell carcinoma carcinoma associated with mesenchymal differentiation
Chondroid differentiation osseous differentiation other types of mesenchymal differentiation
Myoepithelial carcinoma

Table 2. Clinical and histologic findings in patients with metaplastic cancer

Characteristics	Metaplastic breast cancer (n = 7)
Median age	61 (41–87)
Tumor size T1 T2 T3 T4	 1 4 – 2
Multicentric	2
Lymph node N0 N1 N2	 1 5 1
Metastasis M0 M1	 6 1
Stage stage IIA stage IIB stage IIIB stage IV	 1 3 2 1
Dominant metaplastic component epithelial type metaplasia squamous spindle mixed type metaplasia osseous	 5 1 1

Two patients had draining wounds due to the tumor. Five patients had tumors with diameters <5 cm. The tumors were multi-centric in 2 patients and were associated with foci of carcinoma in situ. All patients had palpable and multiple axillary lymph nodes. Lymph nodes formed a conglomerate in 1 patient. This patient also had lung, bone and brain metastases at diagnosis.

Two patients were diagnosed with stage IIIB and 1 patient had 3 cycles of neoadjuvant chemotherapy. Modified radical mastectomy was performed in the cases with stages IIA, IIB, and those that had neoadjuvant chemotherapy. Toilet mastectomy was performed in the case of stage IIIB with a draining wound. The patient with stage IV was diagnosed by a core biopsy, but was not operated.

The patient with metastatic disease refused chemotherapy. The 87-year-old patient with stage IV could not have any treatment due to her other health problems.

Two patients did not keep their follow-up and no information could be obtained about their final status. Four patients had CA (doxorubicin and cyclophosphamide) chemotherapy regimen. Two of these patients had paclitaxel treatment for 12 weeks. The patient that was ER and PR positive had 4 cycles of CA and tamoxifen (2 × 10 mg) treatment later. A locally advanced patient had 3 cycles of neoadjuvant and 3 cycles of postsurgical CAF (cyclophosphamide, doxorubicin and 5-florourasil) treatment. The median follow-up duration of the patients was 34 (10–58) months. Three out of 5 followed-up cases were disease-free, while 2 of them died due to metastases (Table 3).

Histological and immunohistochemical analysis

After the examination of the H&E preparations, 4 patients were demonstrated to have a squamous type of metaplastic cell differentiations, 1 spindle, 1 adenosquamous, and 1 osseous. Six of 7 patients were found to be estrogen and progesterone receptors and C-erbB-2 expression negative. In 1 patient with squamous (adenosquamous) metaplasia, ER was 10% and PR was 5% positive. Conventional fluorescent in situ hybrilization (FISH) method did not show any *HER2/neu* gene amplification upon (+2) *C-erB-2* expression score in the same patient. CK5/6 was positive in 5 patients. CK5/6 could not be evaluated in 1 patient despite consequent stains. It was negative in 1 patient. Epidermal growth factor receptor was positive in all patients (Table 4).

Discussion

DNA microarray and immunohistochemical methods have been quite popular recently in determining the subgroups of breast cancer biologically and clinically. Considering gene expressions, breast cancer that is ER (+) is classified into 2 subgroups as luminal A and luminal B, and ER (–) is classified into 3 subgroups as *HER2* expressing, basal-like and null (similar to normal), although there have not been any certain criteria accepted yet.[7] With this classification, we aim to obtain data that would present patient follow-up and treatment choice, demonstrating the molecular basis of the heterogeneity that is observed in breast cancer.

Triple-negative breast cancer (TNBC) is a heterogeneous tumor group that makes 10–20% of all breast cancers and has an aggressive clinical course.[8,9] 55–80% of TNBC has a basaloid phenotype.[8,10] Cytokeratin (CK) 14, CK5/6, CK 17,epithelial growth factor receptor (EGFR), p63, CD10, laminin, KIT, nestin, caveolin 1, and NGFR can be used as basal or myoepithelial cell determinant. Basal-like breast cancers show expressions of basal cytokeratin with a high molecular weight (CK5/6, CK14 and CK17), p-kadherin and fascin. In determining basal-like breast cancers, specificity of being ER (–), *HER2* (–), CK5/6 and EGFR (+) is 100%, and sensitivity is 76%.[11] In more than 90% of MpBC, CK5/6, CK14 and EGFR expressions are positive, while ER, PR and HER2 are negative immunohistochemically. Estrogen receptor or PR expression frequency is 0–17% in MpBC.[5] All our cases are triple-negative, except the patient in the grey zone. Considering this, all the presented patients can be

Table 3. Follow-up (7 patients)

Follow-up period			58 months	56 months	34 months	10 months	11 months
Disease-free survival	–	–	58 months	56 months	34 months	T4N1M0 at diagnosis	metastatic
Tamoxifen	–	–	+	–	–	–	–
Chemotherapy	4 cycles of CA	4 cycles of CA	4 cycles of CA	3 + 3 cycles CAF(*)	4 cycles of CA + Paclitaxel	Paclitaxel	refused treatment
Survival			alive, disease-free	alive, disease-free	alive, disease-free	10 months metastatic	
Differentiation	osseous	squamous	squamous (#)	squamous	spindle	squamous	squamous

* 3 cycles of neoadjuvant, 3 cycles of adjuvant chemotherapy; # adenosquamous type.

Table 4. Results of differentiation and immunohistochemical analyses

	1	2	3	4	5	6	7
	osseous	squamous	squamous (*)	squamous	spindle	squamous	squamous
ER	–	–	++/+++	–	–	–	–
PR	–	–	+/+++	–	–	–	–
C-erbB-2	–	–	++/++++	–	–	–	–
CK5/6	–	+	?	+	+	+	+
EGFR	+	+	+	+	+	+	+

* adenosquamous type.

accepted as TNBC. CK5/6 expression could not be evaluated in the case with adenosquamous differentiation, while it was negative in the case with osseous differentiation. MpBC can be evaluated as a subgroup of TNBC considering its immunohistochemical characteristics. Five of the patients presented with squamous differentiation resembled the subgroup of TNBC with a basaloid phenotype. Positivity of the EGFR expression in all cases of MpBC suggests that antiangiogenic treatment alternatives may be helpful.[2]

Immunohistochemically, C-erbB-2 scoring is a subjective evaluation. C-erbB-2 score was +2 in 1 patient of the group presented. However, there was no HER2/neu gene amplification with the FISH method. ER and PR positivity were within the grey zone limits in this patient. The patient, whose cancer was classified as T1N1M0 (stage IIA), had 4 cycles of AC chemotherapy. The patient used tamoxifen (2 × 10 mg) for 4 years and is disease-free at present.

The incidence of MpBC within breast cancers is reported as 0.02–0.5%.[1,2,12] It made 0.6% of the cases that had been diagnosed with breast cancer at our pathology unit during 12 years. Metaplastic cell differentiation with squamous cells was the most frequent. However, 1 patient had sarcomatous changes that showed osseous differentiation.

Metoplastic carcinoma of the breast is mostly seen in the 5th decade. Compared to classical breast carcinoma, tumor size is bigger, axillary lymph node involvement is less frequent and axillary lymph node involvement at the diagnosis is 8–40% in MpBC.[5,13–15] Six of the 7 patients presented had a metastatic lymph node. Metoplastic carcinoma of the breast is observed to metastasize to the bones and lungs with hematogeneous spread rather than lymphatic spread.

The majority of MpBC tumors is estrogen receptor (ER), progesterone receptor (PR), and HER2 negative, triple-negative breast cancer (TNBC), and usually carries a worse prognosis compared to non-metaplastic TNBC.[3,5] Metoplastic carcinoma of the breast is usually more aggressive than pure invasive ductal or invasive lobular cancers often presenting with larger staging (T2, T3); however, lymph node involvement is less likely to be noted.[2] The prognosis of MpBC was poorer than that of invasive ductal carcinoma and TNBC; the 5-year overall survival rate was 54.5% in MpBC vs 85.1% in invasive ductal carcinoma and 73.3% in TNBC ($p < 0.001$).[16] In a multicentric study of 405 patients, it was differently reported that there was no difference between ductal breast cancers and MpBC; however, spindle cell type shows aggressive biologic behavior and chemotherapy provides a longer survival in an early stage.[17]

Clinical findings are like in other breast cancers. MpBC can show benign characteristics at sonography, mammography and MRI as well as findings of invasive carcinoma. It has no specific radiologic findings.[13,18] Radiologic findings can change according to the component the tumor contains.[19] Metoplastic carcinoma of the breast is seen as a spiculated mass with irregular margins that often has intermediate to high signal intensity in T2W images and is low or isointense in T1W images in MRI.[13]

Metaplastic carcinoma of the breast may be treated with breast-conserving surgery in suitable cases. It is reported that there is no difference in the survival rate between breast-conserving surgery and a radical mastectomy.[1,20] However, a modified radical mastectomy is the preferred method of treatment if the tumor is big at diagnosis and local recurrence rate is 35–62% in the first 2–5 years compared to the recurrence rate of 17–20% for invasive ductal carcinoma of similar tumor size.[1]

There is no specific guideline for the treatment of MpBC. It is known that long-term adjuvant chemotherapy is not useful in such cases.[5,21] In 42 out of 47 cases of breast cancer, no survival benefit from adjuvant chemotherapy.[5] The heterogeneous nature and sarcoma-like characteristics of MpBC are blamed for the failure of standard chemotherapy schemes.[21] Today, for the treatment of MpBC, a high-dose anthracycline is suggested as a chemotherapy regimen in the case of epithelial subgroups. There is a need for new studies that would evaluate different choices of treatment as MpBC has a bad prognosis and an aggressive nature.

In conclusion, MpBC is relatively rare and, in our series, its incidence was 0.6%. According to its immunohistochemical characteristics, MpBC can be interpreted as a subgroup of triple-negative breast cancers (TNBC). Most of the presented patients resembled the subgroup of TNBC with a basaloid phenotype.

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The influence of gender on selected risk factors for chronic non-communicable diseases in patients hospitalized in surgical wards: A cross-sectional study

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Abstract

Background. Chronic non-communicable diseases (CNCDs) are the leading cause of mortality in the world. Identification of risk factors and the implementation of preventive measures can effectively reduce the chance of disease and death due to CNCDs.

Objectives. The aim of this study was to analyze selected risk factors of CNCDs in women and men hospitalized in surgical wards.

Material and methods. The study group included 420 patients aged 18–84 years who were hospitalized in surgical wards. All participants were interviewed prior to anthropometric measurements, blood pressure, and fasting blood tests. A statistical analysis of the material was performed with the use of Student's t-test, χ^2 test, Fisher's exact test, Mann-Whitney U test, and analysis of variance (ANOVA).

Results. The analysis of the study material showed abdominal obesity in 63% of patients, more likely in women ($p < 0.001$); increased total cholesterol values in 30% of patients, more frequently in women ($p = 0.025$); blood pressure values ≥140/90 mm Hg in 28% of patients, more frequently in men ($p < 0.001$); alcohol abuse (≥5 points in the Michigan Alcoholism Screening Test, MAST) in 12.6% of patients, more frequently in men ($p < 0.001$).

Conclusions. Both women and men are at risk of developing CNCDs; however, women should pay more attention to psychological counseling and the prevention of obesity and hypercholesterolemia, while men should be educated on how to prevent hypertension and alcohol abuse.

Key words: obesity, risk factors, cardiovascular diseases, smoking, diabetes mellitus type 2

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Introduction

As a result of globalization, demographic changes and unfavorable changes in lifestyle, i.e., excessive consumption of highly processed foods and sugary drinks and low levels of physical activity that result in an increased number of overweight and obese population, chronic non-communicable diseases (CNCDs) constitute a serious problem for the entire health care sector all over the world. It is estimated that 38 million people died in 2012 due to CNCDs – mainly cardiovascular diseases, diabetes, cancers, and chronic respiratory diseases. In developed countries, they account for 70% of all deaths.[1] In Europe, the percentage of deaths caused by CNCDs is even higher (80%).[2]

In Poland, the vast majority of the population dies due to chronic non-communicable diseases. In 2012, 46.1 per 10,000 persons died due to cardiovascular diseases, 25.6 per 10,000 persons due to cancer, 5.2 per 10,000 persons due to respiratory diseases, and 1.9 per 10,000 persons due to diabetes.[3]

Therefore, in 2012, the state members of the World Health Assembly decided to implement the program "25×25" which calls for reducing the number of premature deaths due to CNCDs by 25%.[4] To accomplish that, the therapeutic team members (doctors, nurses, nutritionists) should take action in the field of primary prevention, i.e., diagnose the incidence and then implement educational activities aimed at reducing or eliminating these factors. This will contribute to a lower number of new cases – and thus deaths – due to chronic non-communicable diseases.

The major modifiable (lifestyle-related) risk factors for CNCDs include: improper diet, smoking, alcohol abuse, low physical activity, elevated levels of blood pressure (BP), total cholesterol (TC), and glucose (GL).

The aim of the study was to analyze the prevalence of selected risk factors (e.g., low level of activity, alcohol abuse, improper diet, obesity, depression, smoking, elevated levels of blood pressure, total cholesterol and glucose) of CNCDs in women and men hospitalized in surgical wards, and identify the target group of patients who should be included in educational program covering the primary prevention of CNCDs during hospitalization.

Material and methods

This study was a descriptive, cross-sectional survey conducted among 420 patients in the age group 18–84 years, hospitalized in Department of Urology, Department of General Surgery, Department of Orthopedics and Traumatology of the Locomotor System, and Department of Neurosurgery at the University Clinical Hospital in Wrocław, Poland. Persons eligible for the study were patients who, on the examination day, did not have surgery, and their diagnosed condition did not significantly affect their lifestyle in the period of 3 months prior to hospitalization. The exclusion criteria disqualifying prospective participants covered conditions such as cancers, active infectious diseases, and the cases when persons were subjected to surgery within the 3 months prior to the survey. All participants were informed of the purpose and course of the study, and also possibility of withdrawal at any stage. They also signed a written consent to participate in the study.

The research project was approved by the Bioethics Committee (No. KB 566/2013).

The data collected for the study was obtained by the diagnostic survey method implementing tools such as the author's questionnaire and standardized questionnaires, i.e., International Physical Activity Questionnaire (IPAQ), Beck Depression Inventory (BDI), Menu Scoring Method by Starzyńska, Fagerstrom Test for Nicotine Dependence, Michigan Alcoholism Screening Test (MAST), Inventory to Measure Coping Strategies with Stress (Mini-COPE), Chronic Obstructive Pulmonary Disease Questionnaire (COPD Questionnaire), and Systematic Coronary Risk Evaluation (SCORE).[5–12]

Our questionnaire was a survey of own authorship, which included questions about sociodemographic data, i.e., age, sex, marital status, place of residence, education, income per person in the family, socioeconomic status.

Blood pressure (BP) was measured with the use of OMROM M6 Comfort blood pressure monitor (Omron Healthcare, Kyoto, Japan) in accordance with current guidelines recommended by the Polish Society of Hypertension.[13] The analysis included the mean value of 2 blood pressure recordings.

The BP values over 140/90 mm Hg on the examination day were regarded as elevated, whereas the group of patients with hypertension (HTN) included persons with documented diagnosis of hypertension or being treated for HTN.

The glucose (GL) and total cholesterol (TC) levels were measured from first morning blood samples taken after a minimum 8-h fast, using CardioChec Brand Analyzers (PTS Panels Co., PTS Diagnostics, USA). The abnormal value of TC was established at the level of >190 mg/dL according to the European Guidelines on cardiovascular disease prevention in clinical practice (v. 2012).[14] Patients with lipid disorders were defined as those with documented disease or taking medications to lower the TC levels in the blood. The established value for abnormal level of glucose was >100 mg/dL following the Guidelines on diabetes management in clinical practice (v. 2014).[15] Patients defined as suffering from diabetes (DM) were those with a documented disease or those taking medication to lower their blood glucose levels.

The body mass index (BMI) was calculated as kg/m^2 from the weight and height, measured with the use of medical scales ZMP RADWAG T 6496 (ZMP RADWAG Co., Radom, Poland), in accordance with current guidelines

announced by the World Health Organization (WHO).[16] Participants were weighed only in their pyjamas (without shoes). Obesity was defined as a BMI of 30 kg/m^2 or greater.

Waist circumference (WC) in cm was measured with an insertion tape. Measurements were taken from the midpoint between the iliac crest and the lower ribs measured at the sides. Abdominal obesity was established at waist circumference ≥94 cm in men and ≥80 cm in women.[17]

The Menu Scoring method by Starzyńska was applied to evaluate a diet. A score lower than 12 is indicative of an improper diet.

The International Physical Activity Questionnaire short form was used to evaluate participants' physical activity during the 7 days prior to hospitalization by the frequency (day/week), duration (min/day or h/day), and intensity (sedentary, light, moderate, or vigorous) of physical activity. According to the IPAQ scoring protocol, the data collected was converted to Metabolic Equivalent Task minutes per week (MET-min/wk): total minutes over last 7 days spent on light, moderate, and vigorous activity were multiplied by 3.3, 4.0, and 8.0, respectively, to create MET scores for each activity level. Physical activity levels were also classified into 3 categories: low, moderate and high, according to the scoring system provided by IPAQ. The physical activity of people aged 18–69 years was evaluated according to the guidelines.

The Fagerstrom Test for Nicotine Dependence is a standard instrument in the form of a questionnaire to assess the nicotine addiction. The degree of nicotine addiction is evaluated in 3 categories as low (0–4 points), moderate (5 points), and high (6–10 points).

The Michigan Alcoholism Screening Test is a fast screening tool consisting of 25 questions designed to identify alcoholism in subjects. Obtaining ≥5 points shows the compliance with the examination criteria for alcoholism, 4 points – the examined person is probably an alcoholic, ≤3 points – the examined person is probably not an alcoholic.

The Beck Depression Inventory is used for self-report inventory to assess the level of depression. The result of 0–11 points indicates a lack of depression, 12–26 points – mild depression, 27–49 points – moderately severe depression, 50–63 points – very severe depression.

The Chronic Obstructive Pulmonary Disease Questionnaire is a tool which identifies patients who may suffer from COPD, and who have not been diagnosed with other respiratory diseases. It is designed to be used in people over 40 years of age who smoked or smoke cigarettes, or were/still are exposed to other risk factors for COPD, such as air pollution with dust or chemicals at home or work. The questionnaire consists of 8 questions, and each answer is assigned a number of points. By adding up the points scored in questions 1–8, the obtained result allows evaluating the risk of COPD. The score of: ≤16 points – suggests the diagnosis of other respiratory diseases, e.g., asthma; ≥17 points – suggests diagnosis of COPD.

The SCORE was computed using all the variables required for its calculation (i.e., sex, age, cigarette addiction, TC level, and systolic BP). Based on the obtained results, the patients were assigned to one of the risk groups: significantly increased (≥10%), increased (5–9%), moderate (1–4%), and low (0%). People with diabetes and previously diagnosed cardiovascular disease belong to the group of increased risk.

The Polish version of the inventory Mini COPE was implemented in the study to define strategies for coping with stress (adopted by Zygfryd Juczyński and Nina Ogińska-Bulik). Inventory Mini-COPE consists of 28 statements, which are divided into 14 strategies of coping with stress: active coping, planning, positive reevaluation, acceptance, sense of humor, turn to religion, seeking emotional support, seeking instrumental support, dealing with something different, denial, venting, use of psychoactive substances, cessation, and self-blame.

All the data received from surveys was entered in Microsoft Office Excel 2010 and the statistical analysis of the parameters studied in the paper was performed with the use of STATISTICA software v. 10.0 along with the Student's t-test, χ^2 test, Fisher's exact test, the Mann-Whitney U test, and ANOVA. The level of significance was established at $p < 0.05$.

Results

The study involved 420 patients hospitalized in a general surgery ward (35.3%), urology (17.9%), orthopedics traumatology (20.1%), and neurosurgery (26%) wards. Among the patients, women accounted for 48.8% and men for 51.2% of the studied population. The youngest participant in the study was 18 years old, and the oldest was 84 years old. Table 1 shows some characteristics among the patients.

The analysis of the material showed that significantly more men than women on the examination day were characterized with higher values of BP (>140/90 mm Hg) ($p < 0.001$). However, prior to admission, women were more often diagnosed with HTN (43.4% vs 40.9%). In the group of people who on the examination day had BP ≥ 140/90 mm Hg, 63% were suffering from HTN. In contrast, 37% of the studied population were people with BP ≥ 140/90 mm Hg, thus requiring further examination for the diagnosis of hypertension.

On the examination day, impaired fasting glucose (IFG) (≥100 mg%) was diagnosed in 18.0% of women and 20.9% of men, while increased levels of TC (>190 mg/dL) occurred in 30% of patients, significantly more often among women than men ($p = 0.025$). Prior to admission to the hospital, 11.6% of patients were diagnosed with DM and 20% were treated for lipid disorders (Table 2).

Among the patients, 20% smoke actively, and 21% were exposed to passive smoking. The vast majority of smokers (84.5%) declared that they smoke a pack of cigarettes a day or less. Women significantly prevail among patients

Table 1. Characteristics of the studied group

Variables	Total n = 420	Gender		p-value
		women n = 205	men n = 215	
Age (M ±SD)	52.4 ±16.2	52.4 ±16.1	52.3 ±16.3	0.929[a]
Place of residence: rural area town with ≤10,000 inhabitants city with 10,000–100,000 inhabitants city with >100,000 inhabitants	 108 (25.7%) 23 (5.5%) 89 (21.2%) 200 (47.6%)	 46 (22.4%) 13 (6.3%) 52 (25.4%) 94 (45.9%)	 62 (28.8%) 10 (4.6%) 37 (17.2%) 106 (49.3%)	0.123[b]
Maritial status: single married widow/widower	 106 (25.3%) 271 (64.7%) 42 (10%)	 47 (22.9%) 127 (62.0%) 31 (15.1%)	 59 (27.6%) 144 (67.3%) 11 (5.1%)	0.003[#b]
Education: basic basic vocational secondary vocational high school post-secondary bachelor's degree master's degree	 35 (8.4%) 76 (18.1%) 111 (26.5%) 74 (17.7%) 12 (2.9%) 9 (2.2%) 102 (24.3%)	 25 (12.3%) 34 (16.7%) 39 (19.1%) 43 (21.1%) 8 (3.9%) 6 (2.9%) 49 (24%)	 10 (4.6%) 42 (19.5%) 72 (33.5%) 31 (14.4%) 4 (1.9%) 3 (1.4%) 53 (24.7%)	0.002[#b]
Professional activity: student (up to 26 years) labourer white-collar worker disability retiree pensioner unemployed	 22 (5.2%) 44 (10.5%) 122 (29.1%) 48 (11.4%) 141 (33.6%) 43 (10.2%)	 12 (5.9%) 15 (7.3%) 60 (29.3%) 17 (8.3%) 80 (39%) 21 (10.2%)	 10 (4.7%) 29 (13.5%) 62 (28.8%) 31 (14.4%) 61 (28.4%) 22 (10.2%)	0.049[#b]
Socioeconomic status*: high medium low	n = 325 82 (25.5%) 184 (56.6%) 59 (18.2%)	n = 162 43 (26.5%) 89 (54.9%) 30 (18.5%)	n = 163 39 (23.9%) 95 (58.3%) 29 (17.8%)	0.817[b]

M – mean; SD – standard deviation; [a] – Student's t-distribution test; [b] – χ^2 test; [#] significant differences ($p < 0.05$); * respondents subjectively assessed their economic status.

Table 2. Prevalence of individual CVD risk factors in patients

Variable	Gender		p-value
	women n = 205	men n = 215	
BP > 140/90 mm Hg*	20%	36.6%	<0.001
Hypertension	43.4%	40.9%	NS
GL > 100 mg/dL*	18%	20.9%	NS
Diabetes	11.7%	11.6%	NS
TC > 190 mg/dL*	35.1%	25.1%	0.033
Hypercholesterolemia	22.4%	19.1%	NS
Excessive weight	58.5%	65.1%	NS
Overweight	35.1%	40.9%	NS
Obesity	23.4%	24.2%	NS
Abdominal obesity	74.6%	62.9%	<0.001
Current smoking	18.5%	21.4%	NS
Abuse alcohol	2.2%	19.2	<0.001
Improper diet	93.7%	95.3	NS
Low level of activity	22.9%	24.2%	NS
Symptoms of depression	13.2%	5.2%	0.016

* on the examination day; NS – not statistically significant; BP – blood pressure; GL – glucose; TC – total cholesterol.

who are occasional smokers ($p < 0.001$), whereas men prevail among patients smoking 2 packs a day or more ($p < 0.001$). The mean number of points obtained by smokers in the Fagerstrom test evaluating the degree of dependence on nicotine was 4.09 ±2.41. A significantly higher number of points was obtained by men than women ($p = 0.002$). In 1/3 of all smokers (including 2/3 of smokers over 40 years) appears the risk of developing COPD. This group represents 7.3% of the study population. Gender does not affect the risk of developing COPD. It was also revealed that 7% of patients think that they are exposed to inhalation of air contaminated by exhaust fumes and gases (e.g., CO_2, SO_2) in the workplace – men more often than women ($p < 0.01$).

The analysis of the study material concerning the risk of death from cardiovascular diseases (CVD) showed that:

- 1/4 of patients belongs to the low-risk group, more women than men ($p < 0.001$);
- over 1/3 of patients belong to a group of moderate risk, more men than women ($p < 0.001$);
- every 10th patient belongs to a high-risk group;
- over 1/3 of patients belong to a group of significantly increased risk, more men than women ($p < 0.001$).

It was also shown that the average risk of death according to the SCORE scale was 6.3% for men; however, it was much lower for women (2.6%).

The analysis of the study material showed no statistically significant difference in the number of people with abnormal body weight in terms of gender. However, women (74.6%) significantly more often than men (62.9%) are characterized by abdominal obesity (Table 2).

Evaluation of the diet showed that 64.4% of patients eat only 3 meals a day. Men consume fewer meals per day than women ($p < 0.01$) and their menu also less often includes milk, cheese, fruit and vegetables ($p < 0.05$). In the studied group of patients, almost half of them declared consuming fish once a week, and 30% do not eat fish at all (or they do very rarely). In contrast, 1/3 of patients consume sweets every day or a few times a week, and about 30% claim that they consume more than 5–6 g (a teaspoon) of table salt daily (more men than women; $p = 0.007$). The average total number of points obtained by patients was 8.92 ±4.53. This means that the diet of patients is improper. Only slightly more than 5% of patients reached a score ranging from 12 to 27 points (their menu is just sufficient or satisfactory). Women more often obtained a greater mean number of points in the menu survey (9.4) than men (8.5) ($p = 0.035$).

In the study group, 43.1% of patients do not drink alcohol, and women are more likely to be abstinent than men ($p < 0.001$). In contrast, men are overrepresented both among social drinkers ($p < 0.01$) and people who drink regularly 1 ($p = 0.024$) or 2 ($p = 0.048$) drinks a day. The analysis of the data obtained through the MAST questionnaire showed that:

- 12.6% of patients met the criteria for alcoholism, more men than women ($p < 0.001$);
- 7.1% of alcohol drinkers probably have a problem with alcohol abuse, more men than women ($p = 0.001$);
- women are characterized by better culture of drinking alcohol (obtained lower mean number of points – 0.3 vs 3.2; $p < 0.001$) than men.

The analysis of the study material obtained through IPAQ survey showed that women gained an average of 3815 ±5069 MET (min/week), and men 3901 ±4574 MET (min/week) ($p > 0.05$). The analysis also showed that high physical activity is characteristic for every 3rd patient; 44% declared moderate activity, and the remaining 23.5% of patients assess their activity as light. There was no significant difference in activity levels in terms of gender.

At the time of examination, 91% of patients had no symptoms suggestive of depression, whereas symptoms of mild depression were observed in 8.3% of the patients, more frequently in women ($p = 0.005$). The analysis of BDI showed that women significantly more often received more points than men (4.0 vs 2.7; $p = 0.013$).

Patients, most often in situations of severe stress, apply the method of finding emotional support (2.41 ±0.66 points) and actively coping with stress (2.38 ±0.59 points). In contrast, hardly ever, in difficult moments do they turn to psychoactive substances (0.13 ±0.41 points) or apply the method of denial (0.23 ±0.47 points). When going through rough patches, women more often than men try to occupy themselves with something else to not think about the problem ($p < 0.001$); their attention is directed towards religion ($p < 0.001$), discharging emotions ($p < 0.001$) or ceasing all activities ($p = 0030$). In contrast, men are more likely to actively cope with stress ($p = 0.014$) or plan on how to solve the problem ($p = 0.016$).

Among the immediate family members of patients (parents, siblings, grandparents), there have been reported a number of chronic non-communicable diseases, i.e.,:

- hypertension was diagnosed in immediate family members of 50% of patients;
- immediate family members of every 3rd patient suffer from coronary heart disease or had a myocardial infarction;
- immediate family members in 1/5 of patients had a stroke;
- 29.3% of patients' family members suffer from diabetes.

Nearly 2/3 of the patients have a positive family history of cardiovascular diseases, i.e., meaning that the immediate family members were diagnosed with hypertension, ischemic heart disease, heart attack or stroke, while almost 1/3 of patients had a family history of developing diabetes. In families of female patients, immediate members of family were significantly more frequently diagnosed with cardiovascular diseases ($p < 0.001$) and diabetes ($p = 0.033$).

The analysis of the study material revealed the following correlations at the significance level of $p < 0.005$:

- weak positive correlation between age and SBP values ($r = +0.258$), values of DBP ($r = +0.121$), glucose levels ($r = +0.304$), total cholesterol levels ($r = +0.161$), and Beck Depression Inventory ($r = +0.267$);
- weak negative correlation between age and the level of physical activity ($r = 0.168$);
- weak positive correlation between BMI and SBP values ($r = +0.368$), values of DBP ($r = +0.322$), glucose levels ($r = +0.274$), serum TC ($r = +0.143$), and the result of the SCORE scale ($r = 0301$);
- weak positive correlation between waist circumference and the values of DBP ($r = +0.287$), glucose level ($r = +0.319$), TC level ($r = +0.105$), the Beck Depression Inventory result ($r = +0.106$), and the MAST test result ($r = +0.252$);
- moderate positive correlation between waist circumference and SBP values ($r = +0.403$);
- high positive correlation between waist circumference and the SCORE scale result ($r = +0.500$).

The studies showed that, with age, the value of SBP increases and the growth rate is approx. 0.26 mm Hg/year. It was also shown that the relationship between the age factor and SBP is stronger in women than in men. The value of SBP also increases with increasing values of BMI and waist circumference. This dependence is stronger in women than in men.

Discussion

Due to the global spread and a high share in overall mortality, CNCDs pose a serious problem in both medical and economic terms for the entire health sector. By identifying risk factors for these diseases, we can effectively reduce the number of new cases and premature deaths caused by them, as well as decrease the costs associated with the treatment and loss of full capacity for active independent life. In the present study, the attempt was made to identify selected risk factors for CNCDs among patients hospitalized in surgical wards with a special focus on gender as a variable affecting prevalence of the risk factors.

Hypertension is one of the major risk factors for CVD incidence such as stroke, and, as a consequence, has a significant impact on the prevalence and mortality.[18–20] Studies carried out in China and USA showed that HNT is a predictor for the occurrence of higher TC values.[21,22] Own study confirmed that HTN is slightly more frequently diagnosed in women than in men;[23] however, many authors indicated that HTN is more common in men.[24–28] In Bosnia and Herzegovina and Brazil, approx. 40% of men are treated for HTN, which is the same result as the one obtained in own study.[27,29] The research carried out in Lithuania showed, however, that over 70% of local men suffer from HTN.[25]

The increased level of TC is one of the most important risk factors contributing to the development and death from CVD.[30,31] The study carried out in the USA showed, however, that hypercholesterolemia is a predictor for hypertension and diabetes.[22] In our study, increased levels of TC (>190 mg/dL) occurred on examination day more frequently in women (57.1%) than in men (42.9%), which confirmed the findings of other authors.[21,25] In Bahrain, Malaysia and Norway increased levels of TC were, on the other hand, more frequently observed in men.[23,28,32]

Studies in many countries have shown that patients with increased glucose levels or diabetes more often suffer from CVD, stroke and obesity.[18–20,33–35] The study by Sun et al. showed that the presence of higher TC values correlates with diabetes.[21] In the USA, approx. 25% of women suffer from DM.[22] In the present study, the percentage was much lower, and gender did not affect significantly the number of people with diabetes, as was confirmed by the results of other authors.[24,25] In Malaysia and Lebanon, women were significantly more likely to develop DM;[23,36] however, other studies showed that men often had a significantly higher values of GL.[21,37] On examination day, the IFG (≥100 mg%) were diagnosed in 18.0% of women and 20.9% of men. A similar proportion of men with IFG was reported by other authors.[29,32,38]

Smokers are predisposed to developing HTN, stroke and COPD.[18,19,26,39,40] Smokers with diabetes or the ones having increased blood glucose levels are exposed to a higher risk of complications and premature death.[41] In 2005, the smoking addiction affected 42% of Polish males and 25% of Polish females.[42] Although, in recent years, there has been a decrease in the number of active smokers, still about 15–20% of the population in developed countries are active smokers.[28,29,32,43–47] This was confirmed by the results of own study.

Most likely, the reduction in the percentage of smokers in the European Union was influenced by the introduction of laws prohibiting smoking in public places. In our study, there was no statistically significant difference between women and men in terms of the number of smokers. In contrast, mainly in developing countries, men are more often active smokers.[23,25,27]

In the present study, 11.2% of the respondents were characterized by an increased risk of death due to CVD within the next 10 years (from 5–9%) and 36.6% had a significantly increased risk (>10%). Similar results were also obtained in Finland, where 27% of women and 63% of men were assigned to a group of high 10-year risk of death from cardiovascular disease (>5%).[48] In Romania, the percentage was slightly lower and amounted to 25%.[49] In contrast, the study in France showed that only 1% of respondents was characterized by a 5% or greater 10-year risk of CVD death.[44] This is probably related to the effective prevention of risk factors for CVD (decrease in the number of smokers and those suffering from HTN). Therefore, over 6 years (2007–2012), a decrease in the 10-year risk of CVD death in both men and women was observed. The test results of MONICA and SEPHAR survey showed that among men the average risk of death, according to the SCORE scale, is around 5%, whereas for women it was much lower (around 2%).[49,50] In the present study, similar results were obtained. However, the results from the HAPIEE study (conducted in the Czech Republic, Poland and Russia) showed that the mean value representing the risk of death for men was approx. 7.5% (the lowest rate was recorded in Poland – 7.37%, the highest in Russia – 9.07%), while the risk of death from CVD among surveyed women was 3 times lower.[50]

Meta-analyzes of several studies demonstrated a significant influence of overweight and obesity on the development of many chronic diseases. People with excess body weight are more likely to develop DM, CVD (including HTN and stroke), dyslipidemias, chronic respiratory disease, i.e., COPD, as well as mood disorders, i.e., depression.[20–22,26,29,38,51–54] In the present study, up to 58.5% of women and 66% of men had excessive body weight (BMI >25). Among government officials in Bahrain and professional drivers in Brazil, this percentage was even higher, which may be associated with sedentary type of work.[29,32] The inhabitants of developing countries were also characterized by a higher percentage of excessive body weight both in women and in men.[24,25] In contrast, residents of low-income countries were characterized by a lower percentage of overweight and obese population than our study showed.[26,55] In the present study, 23.4% of women and 24.2% of men had varying degrees of obesity (BMI >30). However, in many countries, the proportion of obese women was much higher than in our study.[22,23,25]

It was also shown that WC >80 cm for women and >94 cm for men is a strong predictor for the development of HTN, DM and dyslipidemia, and strongly correlated with increased mortality due to CVD and chronic respiratory diseases in particular.[33,56–60] In the present study, 74.6% of women and 62.9% of men were characterized by abdominal obesity. Similar results were also obtained by other authors.[32] In Brazil, the percentage of men with abdominal obesity was a bit smaller, but it is due to different criteria for obesity (WC >102 cm).[29] However, in Nigeria, only 9% of men are characterized by abdominal obesity.[55] This is most likely related to the different measurement criteria (WC >102 cm) and the large energy expenditure during agricultural work. In contrast, a large percentage of Nigerian women (over 50%), despite the different criteria (WC >88 cm) were characterized by abdominal obesity, which is associated with sedentary work (mainly trade).[55]

Poor eating habits play an important role in the development of CVD and DM.[14,18,61,62] The WOBASZ study showed that Poles consume too little cereal, milk and fish.[63] This is confirmed by results of our study. Only 31.9% of women and 17.7% of men consumed 4–5 meals a day, while only 11% of patients consumed 5 servings of vegetables or fruit each day. In the USA, a similar percentage of the population eat fruit and vegetables in the recommended amounts, while in Bahrain or Nepal an even smaller percentage of those surveyed eat 5 servings of vegetables or fruit, which is definitely associated with lower socio-economic status of Nepalese.[26,32,46] Studies demonstrated that every additional gram of salt in the diet increased the chance of developing HTN by 14%.[26] In the present study, every 3rd patient overuses salt when preparing food, while in the USA only 0.6% of Americans consume less than recommended 5–6 g of salt per day.[46]

The authors of numerous studies have shown that alcohol increases the risk of HTN, stroke, triglyceridemia, and DM.[26,64–66] The EZOP Poland study showed that approx. 10.9% of respondents (18.6% of men and 3.3% of women) abuse alcohol;[67] these results were confirmed in present study. In Germany, approx. 7% of the population consumed "dangerous" amounts of alcohol – men more frequently than women.[43] However, in Nepal, people with low socio-economic status (approx. 25% of respondents) consumed 5 or more doses of alcohol each day.[26]

Low level of physical activity (PA) is strongly correlated with the incidence of CVD, hemorrhagic stroke, and diabetes.[19,34,61,68] Smokers characterized by low levels of PA are more likely to develop COPD.[39] In contrast, people leading a sedentary lifestyle often exhibit symptoms of depression.[69] The NATPOL 2011 showed that the number of physically inactive Poles was 28.2% of the population.[70] The IPAQ results obtained in the survey conducted among residents of Warszawa showed that 32% of them were characterized by low levels of PA, while our study results showed that 23.5% of respondents were characterized by low levels of PA (by IPAQ).[71] The studies conducted in Mexico and Norway showed that men are more physically active than women.[24,28] In Lithuania, the opposite tendency was observed.[25] Our findings did not confirm the gender dependence.

Many studies have reported the influence of depression on the risk of developing CVD, stroke and obesity.[18,72–76] The EZOP Poland study showed that women more often show symptoms of depression.[67] The present study confirmed this dependence. The study conducted by Cooper et al. among African Americans showed that the greater the abdominal obesity, the more intensive depressive symptoms are.[76] Our study confirmed a positive correlation between WC and the BDI.

Our study findings showed that actively coping and seeking emotional support are the most commonly used strategies in tackling stress among surgical patients. Moreover, the patients who have been subjected to neurological rehabilitation because of a history of stroke, most often cope with stress with the abovementioned strategies.[77] Our study showed that a higher intensity of depressive symptoms in the study group resulted in more frequent use of dysfunctional strategies in stressful situations, i.e., self-blame, which was confirmed by Klein et al.[78] This is probably the consequence of the fact that people who are not able to cope with stress actively experience the feeling of hopelessness resulting in bad mood.

Conclusions

Gender does not differentiate patients in terms of the number of diagnosed risk factors.

Women were significantly more frequently characterized by increased values of TC and diagnosed with depression and abdominal obesity, while men more often were characterized by the values of BP ≥ 140/90 mm Hg and alcohol abuse.

Men's diet is less balanced and varied when compared to women's diet.

Abdominal obesity significantly increases the risk of HTN, and this dependence is stronger in women than in men.

In women, the average risk of death from CVD according to the SCORE scale is 2.5 times lower than in men.

Members of a therapeutic team should implement measures to identify modifiable risk factors for CNCDs in patients hospitalized in surgical wards, especially among men, and apply proper educational activities for prevention purposes in the abovementioned groups.

Strengths and limitations

This paper is a pioneer study carried out among patients who were treated in hospital for surgical reasons and additionally agreed to participate in the study aiming to determine the prevalence of CNCDs in the Polish population. The results confirmed the need for developing preventive

measures against increasing prevalence of CNCD among Polish population. The interdisciplinary team has a chance to diagnose the first symptoms of CNCDs or their risk factors often only during hospitalization for other reasons (i.e., surgical reason) when the direct contact with a patient allows for thorough examination. Next interdisciplinary team should select a group of patients who will be subjected to additional educational activities. These activities could be carried out by nurses as one of their responsibilities is patient education.

However, there were a few limitations to the study. The first one concerned the questions which focused on physical activity within the last 7 days prior to hospitalization, and patients usually change their routine before surgery. The second limitation refers to a higher BP level resulting probably from the stress connected with surgery. The last limitation is connected with self-report instruments used in this study which provide subjective assessment of the current reality.

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Awareness and attitudes towards clinical trials among Polish oncological patients who had never participated in a clinical trial

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Abstract

Background. Participation in a clinical trial significantly shortens waiting time associated with receiving specialist care. Furthermore, it may be the case that, through clinical trials, subjects can access medicines that are not typically available in Poland.

Objectives. The aim of this study was to determine the opinions of oncological patients about clinical trials.

Material and methods. The research has been carried out during the years 2014–2016. A proprietary questionnaire consisting of 10 closed, single and multiple choice questions about awareness and perceptions of clinical trials, and 5 questions concerning demographic information was used. A group of 256 patients with cancer (54% women, 46% men), aged 21–77 years, was surveyed.

Results. Respondents were statistically more likely to decide to participate in a clinical trial as oncological patients than the healthy volunteers (Pearson's χ^2 test $p = 0.00006$). The desire to qualify for clinical trials in no way depends on the knowledge of side effects (Pearson's χ^2 test $p = 0.16796$).

Conclusions. Our study found that the patients' awareness about clinical trials varied. However, a positive attitude towards research was visible. The main identified barriers to clinical trial participation were fear of possible side effects. Most patients regarded clinical trials as useful, and considered that they are conducted to introduce new treatment/new drug.

Key words: clinical trials, patient attitudes, awareness, perception, patients with cancer

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Introduction

The era of randomized cancer clinical trials began in 1958 with the first use of systemic therapy following a radical mastectomy in the treatment of breast cancer.[1] In 2014 in Poland, there were 396 new clinical trials recorded. Approximately, 1/4 of patients in clinical trials in Poland are enrolled in oncology studies (23% in 2014).[2,3] Oncology is a frequent area of clinical research in Poland due to many factors. Firstly, in Poland, access to the national healthcare system is limited, and medications are expensive; hence, with the offer of better medical care, free drugs and diagnostic procedures, patient recruitment in clinical trials is very high. This factor is particularly important when treating patients in areas where the availability of effective drugs is limited at this stage of the development of medicine (i.e., oncology). Secondly, the high motivation to participate in clinical trials may also result from the relatively long time patients have to wait to see a specialist in Poland. Participation in a clinical trial, therefore, significantly shortens the average time associated with receiving specialist care. Furthermore, it may be the case that, through clinical trials, subjects can access medicines that are not typically available in Poland.

Material and methods

Sample and place of study

The prospective study was conducted among 284 patients of Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warszawa, Poland in 2014–2016. Patients were recruited to collect a mixture of tumor types (soft tissue sarcoma, bone sarcoma, malignant melanoma, gastrointestinal stromal tumor, breast cancer, and lung cancer) and cancer stages; study included patients who had never participated in a clinical trial. Exclusion criteria included age <18 years. We selected our sample on the basis of respondents' availability. We conducted the study with patients available at a given time and place at the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warszawa, based on the randomness of their visits to the Institute. The sample included patients of different sociodemographic data: age and gender, place of residence, marital status, and educational stage. Participation in the survey was voluntary and anonymous. Out of 284 selected patients, 256 (90%) agreed to participate in the study and completed the questionnaire. Analysis was based on responses from these 256 respondents. We analyzed all subjects as a group, regardless of the type of cancer.

Instruments

We applied the "Paper and Pencil Interview" (PAPI) technique. This survey-based study was performed using the authors' own questionnaire; it included 10 closed questions about the awareness and perceptions of clinical trials as well as single- and multiple-choice questions; 5 questions concerning demographic information. The majority of questions in our questionnaire were adapted from a literature review and previous similar studies. All the possible answers are shown in Tables 1–4 and Fig. 1.

Ethics

The Ethical Committee consent for the presented research is not required. According to the statement of the Ethical Committee of the Medical University of Warsaw: "The Committee does not provide opinions on surveys, retrospective studies, or other non-invasive research."[4]

Data analysis

The data was collected in a Microsoft Excel database. Survey responses were aggregated into frequencies and percentages. Statistical analysis was performed using STATISTICA v. 10 software (StatSoft, Tulsa, USA). Descriptive statistics of respondent demographics, awareness of clinical trials, and a willingness to participate in clinical trials were analyzed. Associations among the variables were evaluated by χ^2 test. A value of $p < 0.05$ was considered statistically significant.

Results

Socio-demographic characteristics of patients

The study was performed in 256 patients with cancer, including 138 women (54%) and 118 men (46%). The mean age of respondents was 46 (range: 21–77 years). The main part of the population taking part in the study comprised married persons (44%); with regard to other previously mentioned sociodemographic data, 38% of patients had secondary education, and about 75% lived in an urban area. Table 1 presents the socio-demographic characteristics of the patient population.

Patients' attitudes towards clinical trials

In terms of knowledge and awareness of clinical trials, 69.9% of the participants had previously heard about clinical trials, but 64.8% had an interest in participating in cancer clinical trials, and only 53.9% had an interest in participating in clinical trials as healthy volunteers. Respondents were statistically more likely to decide to participate in a clinical trial as oncological patients than as healthy volunteers (Pearson's χ^2 test $p = 0.00006$). Other factors, including age, gender, educational level, and resident area were not significantly associated with willingness to participate in clinical trials ($p > 0.05$). Most

respondents (60.2%) said their doctors had not bgought up the option of taking part in a clinical trial during the treatment planning phase. In the overall group (n = 256), 94.1% of the patients regarded clinical trials as useful. Most patients (89.1%) were aware that during the clinical trial they might experience side effects – Table 2.

Table 1. Sociodemographic characteristics of patients participating in the survey

Variables	n	%
Total	256	100
Gender		
female	138	54
male	118	46
Age		
mean (SD)	46 (12) years	
range	21–77 years	
Marital status		
single	105	41
married	113	44
widowed	38	15
Education		
primary	46	18
vocational	46	18
secondary	97	38
university	67	26
Place of residence		
urban area	192	75
rural area	54	25

The desire to qualify for clinical trials in no way depends on the knowledge of the side effects (Pearson's χ^2 test p = 0.16796).

The most frequent source of information about clinical trials was mass media (94%), other patients who took part in clinical trials before (46%), and physicians (37%) – Fig. 1.

The survey group recognized the benefits of clinical trials, such as treatment that may be more effective than the standard approach (85.2%), and regular and careful attention from some of the best cancer doctors (10.2%). Among the more frequently cited barriers were the fear of possible side effects (69.1%) and frequent hospital visits (12.9%) – Table 3.

Clinical trials were more often associated with positive factors; they were most often associated with the introduction of new therapy (62.1%) and the advancement of medical knowledge (46.1%). However, 46.1% of respondents indicated that patients in clinical trials are "treated like guinea pigs" – Table 4.

Discussion

In our study, only 69.9% of respondents had previously heard about clinical trials. Similar results were obtained among rural Latinos, where that percentage was 68%.[5] However, a previous public opinion study conducted in Poland in 2004 (n = 1003) showed that only 28%

Table 2. Attitudes about cancer clinical trials among respondents

Question	Answers		
	yes n (%)	no n (%)	do not know n (%)
Have you ever heard of cancer clinical research studies?	179 (69.9)	18 (7.0)	59 (23.1)
Would you be interested in participating in a cancer clinical research study?	166 (64.8)	90 (35.2)	–
Would you be interested in participating in a clinical trial as a healthy volunteer?	138 (53.9)	90 (35.2)	28 (10.9)
Have you ever talked to your doctor about participating in clinical trials?	102 (39.8)	154 (60.2)	–
Do you think clinical trials are useful?	241 (94.1)	5 (1.9)	10 (4.0)
During the clinical trial, would you experience side effects?	228 (89.1)	28 (10.9)	–

Table 3. Benefits and barriers for participation in a clinical trial

Question	Answer	n (%)
Benefits of clinical trial participation	treatment that may be more effective than the standard approach	218 (85.2)
	regular and careful attention from some of the best cancer doctors	26 (10.2)
	economic benefit	7 (2.7)
	other	5 (1.9)
Barriers to participation in clinical trials	fear of possible side effects	177 (69.1)
	frequent hospital visits	33 (12.9)
	inconvenient follow-up location	14 (5.5)
	lack of trust in doctor/pharmaceutical companies	7 (2.7)
	other	25 (9.8)

Table 4. Factors associated with clinical trials

Variables	Answers	
	yes n (%)	no n (%)
Contribution to medicine	118 (46.1)	138 (53.9)
Feeling like a guinea pig/being experimented on	118 (46.1)	138 (54.9)
Introduction of new drugs/therapy	159 (62.1)	97 (37.9)
Improved health for the clinical trial participants	105 (41.0)	231 (59.0)
Risk, danger for clinical trial participants	74 (28.9)	182 (71.1)
Financial benefit for patients	38 (14.8)	218 (85.2)
Novelty	23 (9.0)	233 (91.0)
Unfair effect of pharmaceutical companies	18 (7.0)	238 (93.0)
Financial benefit for physician	7 (2.7)	249 (97.3)
Deterioration in health for the clinical trial participants	7 (2.7)	249 (97.3)

of respondents, at any time, had heard about the clinical trials.[6] The differences in the results obtained in our country can be explained by the fact that our study was conducted 10 years later, which may indicate that clinical trials in Poland are more popularized now.

In this survey, 64.8% of the interviewed subjects indicated willingness to participate in cancer clinical trials; however, only 53.9% indicated willingness to participate in clinical trials as a healthy volunteer. In a study conducted in Saudi Arabia, 61% of the 117 interviewed subjects who were aware of clinical trials expressed their willingness to participate in them.[7] Nevertheless, in the study conducted in India, 58.9% of the participants expressed willingness to participate in clinical trials.[8] In another study in the United States, 44% of patients demonstrated willingness to participate or have already enrolled in clinical trials, whereas in the study in Great Britain (n = 1040), only 30.4% of the respondents conveyed their enthusiasm to participate in clinical trials.[9,10] In a previously cited Polish study, the number of people willing to participate in a clinical trial with regard to disease increases about 5-fold compared with a desire to participate as a healthy volunteer (from 15% to 71%).[6] The potential benefit of participating in a clinical trial for an oncological patient is the access to promising new treatments often not available outside the clinical trial setting – this may explain the difference. Possibly, the main reason people lack willingness to participate in a clinical trial is that they are not aware that the studies are an option for them. Our research has shown that 60.2% of the patients had not discussed participating in clinical trials with their doctor. This is consistent with the results of another study indicating that 73% of patients did not recall discussing clinical trial participation with their doctor.[11]

In our study, 84% of all respondents pointed to mass media as the most common source of information on clinical trials. For 46%, it were other patients who, prior to participation in clinical trials, were the primary source

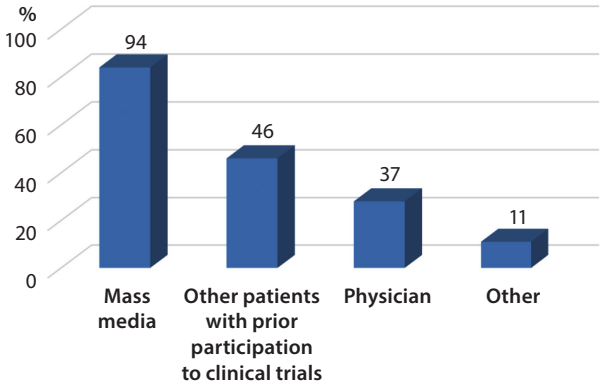

Fig. 1. Source of knowledge about clinical trials among respondents (The data does not give a total of 100%, because the respondents could choose multiple answers)

of that information. An American study from 2015 showed that people aware of clinical trials, most learned about clinical trials online (58%).[12] The Center for Information and Study on Clinical Research Participation (CISCRP), founded in 2013, showed that the Internet is the most common way of finding out about clinical trials.[13]

With regard to the recognition of clinical trials as important and necessary, 94.1% of the patients regarded clinical trials as useful, and admitted that participating in them can bring benefits; the respondents in a public opinion study also highly appreciated the need for clinical trials (71%).[6]

The decision about whether to participate in clinical trial is very personal and depends on many factors, both positive and negative. In our study, one such factor is the fear of being treated like a guinea pig (46.1%); on the other hand, equal number of respondents considered that participating in a clinical study contributes to medical knowledge. In another Polish study, 88% of the public opinion established the fact that there could be advances in medicine as a result.[6] In addition, 62.1% of our study population stated that a clinical trial is conducted for the introduction

of a new treatment/new drug. In turn, in the OMNIBUS study conducted in May 2010, most respondents (35%) associated clinical trials with advances in medicine, and in a study by Shapiro et al., they also believed that clinical trials contribute to the development of medicine (63%).[14,15]

Clinical trials can offer benefits for many people with cancer. The patients in our study said that clinical trials are an alternative treatment for the disease – clinical trial treatment was thought to be better than the standard treatment (85.2%). Other studies have established that the most important benefits that encourage patients to participate in clinical trials are: the possibility of reducing one's chance of getting cancer and the possibility of preventing others from getting cancer in the future, the psychosocial benefits of trial participation, and also the possible benefit of treatment effectiveness.[16,17]

On the one hand, clinical trials are the basis for improvements in oncologic patient care, but on the other they have significant barriers. In our study, 69.1% of patients identified the fear of side effects as the greatest barrier to clinical trial participation, and 89.1% said that during clinical trials they may experience unexpected side effects. As many as 76% of the survey respondents of public opinion study claimed that participating in tests of a new drug may harm their health.[6] Other barriers that discouraged patients from participating in clinical trials are fear, mistrust of the medical community, discouragement from oncologist or family physician, financial burden, difficulties in commuting, and lack of information.[8] However, a survey done by the CISCRP in 2013 found that 94% of respondents believe clinical research is safe for those who participate in it.[13]

Conclusions

In conclusion, our study found that patient's awareness about clinical trials varied. However, a positive attitude towards research was visible. The main identified barrier was the fear of possible side effects. Most patients regarded clinical trials as useful, conducted to introduce a new treatment or drug. These results might be helpful for improving clinical researchers' understanding about clinical trial participants and useful when developing effective outreach strategies of recruitment for clinical trials.

Study limitations

This study had limitations due to a single group and lack of a control group. Further research should be performed to compare the results of:

a) oncological patients who had never participated in a clinical trial vs oncological patients who have previously participated in a clinical trial;
b) oncological patients who had never participated in a clinical trial vs healthy individuals.

This study is the first research on the awareness and attitudes towards clinical trials among oncological patients in Poland.

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A pilot data analysis of a metabolomic HPLC-MS/MS study of patients with COPD

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Abstract

Background. Chronic obstructive pulmonary disease (COPD) is a heterogeneous condition with multiple clinical faces. Metabolomic profiling studies small molecules present in biological samples by combined use of chromatography with mass spectrometry.

Objectives. The goal of our work was to perform a high performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) metabolomic study to compare the concentrations of metabolites in COPD patients and in controls.

Material and methods. Participants were recruited at the University Hospital, Hradec Králové, Czech Republic, with the approval of the ethics committee. The analysis of blood samples was performed at Health Sciences Center (HSC) in Kuwait. The blood samples were analyzed for concentrations of acylcarnitines and amino acids by high performance liquid chromatography (Waters 2690 HPLC; Waters, Milford, USA) and a triple-quadruple tandem mass spectrometer (Quattro LC, Micromass, Manchester, United Kingdom).

Results. Groups of 10 subjects with COPD and 10 healthy controls were analyzed. Carnitine analysis showed that the free carnitine to acylcarnitine ratio (C0/AC ratio) was significantly lower in COPD (0.58 μM/L) compared to the controls (0.73 μM/L; $p = 0.002$). The mean C8/C2 ratio in the COPD group was significantly higher (0.03 μM/L) – in the control group it was 0 μM/L ($p = 0.03$). Amino acid analysis showed lower levels of phenylalanine in the COPD group (22.05 μM/L) compared to the controls (30.05 μM/L; $p = 0.008$). The alanine concentrations were significantly lower in the COPD group (173 μM/L) than in the control group (253 μM/L; $p = 0.001$). The pyroglutamate levels were higher in COPD (1.58 μM/L) than in the controls (1 μM/L; $p = 0.040$).

Conclusions. The carnitine and acylcarnitine levels in COPD subjects in this study possibly indicate a predisposition to atherosclerosis as a result of inadequate β-oxidation of fatty acids and show the presence of oxidative stress. Furthermore, the high sensitivity to changes in circulating amino acid levels may allow us to detect subclinical malnutrition and take early preventative interventions such as nutritional supplementation and patient education.

Key words: amino acids, chronic obstructive pulmonary disease, liquid chromatography-tandem mass spectrometry, carnitine, metabolomics

Introduction

Chronic obstructive pulmonary disease (COPD) is a term referring to a heterogeneous group of chronic lung diseases marked by restricted airflow. Lung damage in COPD is nonreversible and progressive, but may be halted through proper treatment. The two most prevalent conditions characteristic for COPD are emphysema and chronic bronchitis, but new, not yet fully elucidated phenotypes such as asthma-COPD overlap syndrome (ACOS), pulmonary cachexia and frequent exacerbators are emerging.[1–3] COPD has a severe impact on patients' health and quality of life.[4,5]

Metabolomic studies represent a modern trend in biological and clinical scientific work.[6] Metabolomic profiling studies small molecules (molecular weight <900 Da) present in biological samples. Originally, mainly nuclear magnetic resonance (NMR) was applied for this purpose. Presently, the combination of chromatographic methods with mass spectrometry (combinations of gas or liquid chromatography coupled to mass spectrometry – GC-MS or LC-MS) has become a widely applied analytical method for global profiling of metabolites.[7]

Only a limited number of metabolomic profiling studies was performed in patients with COPD. A limited number of papers are devoted to analyzing concentrated samples of exhaled air in patients with COPD using chromatographic methods or NMR spectroscopy.[8–11]

A metabolomic study based on the use of NMR spectroscopy was able to pinpoint the differences between serum of healthy individuals and COPD patients; particularly, decreased concentrations of lipoproteins and amino acids and increased concentrations of glycerolphosphocholine, acetate, ketone bodies, carnosine, m-hydroxyphenylacetate, phenylacetyglycine, pyruvate, and α-ketoglutarate.[12] Another NMR metabolomic study demonstrated the method by investigating plasma and urine samples from 197 COPD patients and 195 adults without COPD.[13] The findings were not conclusive and experiments need to be repeated and validated. On the other hand, the authors found this approach suitable for the identification of novel biomarkers, useful for determining COPD therapeutic outcomes.[13] A metabolomic approach for studying COPD patients was applied to a defined cohort from the Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE) study.[14] Serum from healthy subjects and Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II, III and IV patients with COPD was investigated using NMR spectrometry. The findings were confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and correlated with other data. NMR spectrometry helped in identifying decreased concentrations of lipoproteins and N,N-dimethylglycine. On the other hand, increased concentrations of glutamine, phenylalanine, 3-methylhistidine, and ketone bodies were shown in GOLD stage IV COPD patients who also had decreased branched-chain amino acids (BCAAs) levels. There were found negative correlations between BCAAs, their degradation products, 3-methylhistidine, ketone bodies, and triglycerides on one side and cachexia on the other side. These concentrations positively correlated with systemic inflammation.[14]

In the ECLIPSE study, the concentrations of amino acids and dipeptides were measured by LC-MS/MS in groups of patients divided according to the GOLD characteristics. Differences in the levels of several amino acids and other small molecules were confirmed in the groups of COPD patients.[15] The study showed differences among various groups of COPD patients (emphysema and no emphysema; cachectic and non-cachectic). Additionally, COPD patients were distinguished from 'healthy' smokers used as a control group. These results are in agreement with the findings of another study that concentrated on the differentiation of subjects with or without emphysema.[16]

High performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) may be used for the identification of subtle differences in biomarkers between COPD patients, healthy controls, smokers and non-smokers, etc. This method proved to be useful not only in metabolomic studies, but also in various proteomic investigations identifying proteins that could help differentiate healthy subjects from diseased groups of patients.[17–19] Some proteins present at very low concentrations show different expression in various COPD patients, reflecting the possible pathogenic mechanisms and the extent and severity of lung remodeling. This has a potential in COPD diagnosis and disease activity tracking by biomarkers for the benefit of patients by improving the disease management.

The goal of our work was to perform a HPLC-MS/MS metabolomic study to compare the abundance of several small molecules in COPD patients and healthy volunteers.

Material and methods

Diagnostic criteria for chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease was diagnosed on the basis of an evaluation of patients' lung functions, symptoms and history of exacerbations. All patients in this study performed a spirometry test and met the COPD diagnostic criteria of post-bronchodilator values of forced expiratory volume in 1 s/forced vital capacity (FEV1/FVC) <0.70.[1,2]

Patients

Blood samples from COPD patients for this study were obtained from the participants registered in the Czech Multicenter Research Database of Chronic Obstructive Pulmonary Disease (ClinicalTrials.gov identifier NCT01923051). Blood samples from patients with coronary artery disease and healthy controls were obtained

from the Department of Cardioangiology and the Transfusion Department of the University Hospital in Hradec Králové (Czech Republic), respectively. The ethics committee of the hospital approved the study. All participants signed an informed consent form. The analysis of blood samples was performed at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University (Kuwait).

The inclusion criteria for this study were: non-smokers or ex-smokers of at least 6 months, patients without acute exposition to carbon monoxide (CO) due to smoke or pollution inhalation (measured by smokerlyzer) and COPD patients with post-bronchodilator values of FEV_1 < 60%.

Patients were excluded upon the following criteria: current smoker or ex-smokers of less than 6 months (CO levels > 10 ppm), a known metabolic disease including diabetes type I and II, kidney disease with moderate to severe creatinine clearance, and the presence of coronary artery disease.

Venous blood was collected by standard venipuncture into BD Vacutainer® Heparin Tubes (Becton Dickinson, Mississauga, Canada). Heparinized blood was spotted onto a filter paper cards and left to dry at room temperature for at least 24 h.

We analyzed 2 groups of subjects (Table 1). They included 10 subjects with COPD and 10 healthy controls. The mean age of the COPD group was 61.5 years with a male to female ratio (m:f) of 1. The mean age of the control group was 55 years with m:f of 1. Although the healthy control group was younger, this difference was not significant (p = 0.052). The mean body mass index (BMI) was matched in the COPD group (25.3 kg/m^2) and in the control group (27.10 kg/m^2), with p = 0.496. The COPD patients had an average fat free mass index (FFMI) of 18.79 kg/m^2; FEV_1 of 33 %; and FEV_1/FVC of 0.52. Spirometric data and FFMI were not available for the control group.

HPLC-MS/MS instrumentation

The blood samples were analyzed for acylcarnitines and amino acids by a triple-quadruple tandem mass spectrometer (Quattro LC, Micromass, Manchester, UK) with a positive electrospray ionization probe. High performance liquid chromatography (Waters 2690 HPLC) with an autosampler (Waters, Milford, USA) was used for automatic injection. A designed tandem mass spectrometry (MS/MS) program for automatic profiling of acylcarnitines and amino acids was used. The NeoLynx program (Quattro LC, Micromass, Manchester, United Kingdom) was used to determine the concentration of the diagnostic metabolites.

HPLC-MS/MS method

Using a BSD 400 Puncher (BSD Biosample punchers, Brendale, Australia), 2–3-mm disks were punched from the center of dried blood spots. The disks were placed in a capped glass for extraction of the blood with 200 µL

Table 1. Descriptive statistics

	COPD (n = 10)	Control (n = 10)	p-value
Sex (male), n (%)	5 (50.0%)	5 (50.0%)	0.999
Age [years]	61.50 (28.00; 70.00)	55.00 (53.00; 61.00)	0.052
BMI [kg/m^2]	25.30 (19.47; 32.95)	27.10 (24.40; 29.40)	0.496
FFMI [kg/m^2]	18.79 (14.96; 25.79)	–	–
FEV_1 [%]	33.00 (26.00; 56.00)	–	–
FEV_1/FVC	0.52 (0.30; 0.74)	–	–
C0	24.75 (15.40; 44.20)	33.40 (19.10; 47.60)	0.140
C2	12.45 (8.10; 35.30)	10.95 (6.20; 14.10)	0.076
C3	0.43 (0.20; 0.67)	0.26 (0.00; 0.61)	0.140
C4	0.00 (0.00; 0.22)	0.00 (0.00; 0.19)	0.351
C5	0.00 (0.00; 0.22)	0.04 (0.00; 0.23)	0.525
C5-OH	0.04 (0.00; 0.39)	0.02 (0.00; 0.36)	0.716
C8	0.07 (0.00; 1.34)	0.00 (0.00; 0.16)	0.264
C14	0.00 (0.00; 0.09)	0.00 (0.00; 0.10)	0.564
C14:1	0.00 (0.00; 0.18)	0.00 (0.00; 0.31)	0.551
C16	0.56 (0.00; 5.56)	0.16 (0.00; 2.85)	0.412
C16-OH	0.00 (0.00; 0.04)	0.00 (0.00; 0.12)	0.842
C18:1	0.42 (0.00; 0.61)	0.00 (0.00; 0.74)	0.090
AC total	37.67 (26.99; 81.04)	45.44 (26.90; 61.48)	0.450
C0/AC	0.58 (0.53; 0.68)	0.73 (0.57; 0.86)	0.002
C3/C16	0.22 (0.00; 0.61)	0.07 (0.00; 0.84)	0.589
C3/C2	0.10 (0.00; 0.16)	0.22 (0.00; 0.82)	0.074
C5/C2	0.00 (0.00; 0.06)	0.01 (0.00; 0.09)	0.648
C8/C2	0.03 (0.00; 0.50)	0.00 (0.00; 0.06)	0.030
C14:1/C16	0.01 (0.00; 0.44)	0.00 (0.00; 0.34)	0.183
C16-OH/C16	0.00 (0.00; 0.06)	0.00 (0.00; 0.20)	0.829
Glutaryl	0.00 (0.00; 0.36)	0.00 (0.00; 0.29)	0.402
Valine	85.95 (57.20; 166.00)	97.40 (2.80; 134.00)	0.910
Pyg	1.58 (0.00; 3.43)	1.00 (0.00; 1.96)	0.040
Met	0.00 (0.00; 41.30)	0.00 (0.00; 0.00)	0.317
Phe	22.05 (17.20; 34.20)	30.05 (21.10; 43.70)	0.008
Tyr	21.75 (0.80; 171.00)	27.85 (22.20; 50.00)	0.130
Cit1	13.20 (0.00; 25.70)	12.40 (0.00; 26.40)	0.940
Glyc	90.40 (77.20; 163.00)	104.00 (67.00; 146.00)	0.496
Alanine	173.00 (141.00; 215.00)	253.00 (173.00; 346.00)	0.001
Leu/Ile	58.85 (37.30; 69.40)	64.65 (51.40; 96.00)	0.104
Phe/Tyr	0.00 (0.00; 3.43)	0.00 (0.00; 2.69)	0.487

BMI – body mass index; FFMI – fat free mass index; FEV_1 – forced expiratory volume in 1 s; FVC – forced vital capacity; C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.
The concentrations of metabolites are in units µM/L; categorical variable is described by absolute (relative) frequency and tested by Fisher's exact test; continuous variable is described by median (5th and 95th percentiles) and tested by the Mann-Whitney test.

of methanol, containing known concentrations of a mixture of isotopically labeled internal standards of diagnostic acylcarnitines and amino acids. After 20 min of shaking, the solvent was gently evaporated, and the residue was mixed with 80 µL of butanolic HCl and heated at 65°C for 15 min in a capped glass tube. The solvent was evaporated again and the residue was finally reconstituted in 80 µL of the mobile phase acetonitrile/water (80/20 v/v). A 20 µL aliquot of each sample was directly injected into the mobile phase flowing to the ionization probe of the tandem mass spectrometer at a flow rate of 0.1 mL/min. A Waters 2690 HPLC with an autosampler was used for automatic injection. The run cycle time for each sample was 2–3 min from injection to injection. A designed MS/MS program for automatic profiling of acylcarnitines and amino acids was used. The NeoLynx program for Neonatal Screening (Quattro LC, Micromass, Manchester, United Kingdom) was used for automatic detection of abnormal samples. The values obtained for COPD patients and patients with coronary heart disease were compared to those of healthy controls.

Statistical methods

Standard descriptive statistics was applied for the analysis: absolute and relative frequencies for categorical

Table 2. Influence of sex on the results

Carnitines and amino acids	COPD			Control		
	female (n = 5)	male (n = 5)	p-value	female (n = 5)	male (n = 5)	p-value
C0	24.60 (18.70; 44.20)	24.90 (15.40; 25.50)	0.602	24.60 (19.10; 35.70)	42.70 (20.10; 47.60)	0.117
C2	12.10 (9.70; 35.30)	12.90 (8.10; 19.50)	0.917	11.00 (7.69; 14.10)	10.90 (6.20; 12.70)	0.754
C3	0.47 (0.20; 0.67)	0.41 (0.34; 0.64)	0.917	0.16 (0.00; 0.61)	0.36 (0.00; 0.54)	0.916
C4	0.00 (0.00; 0.20)	0.07 (0.00; 0.22)	0.290	0.00 (0.00; 0.10)	0.00 (0.00; 0.19)	0.881
C5	0.00 (0.00; 0.22)	0.00 (0.00; 0.20)	0.999	0.03 (0.00; 0.09)	0.04 (0.00; 0.23)	0.832
C5OH C5-OH	0.00 (0.00; 0.18)	0.07 (0.00; 0.39)	0.577	0.00 (0.00; 0.04)	0.11 (0.00; 0.36)	0.034
C8	0.06 (0.00; 1.34)	0.10 (0.00; 0.51)	0.746	0.00 (0.00; 0.14)	0.00 (0.00; 0.16)	0.368
C14	0.00 (0.00; 0.09)	0.00 (0.00; 0.05)	0.638	0.00 (0.00; 0.10)	0.00 (0.00; 0.04)	0.521
C14:1	0.00 (0.00; 0.18)	0.00 (0.00; 0.01)	0.881	0.00 (0.00; 0.03)	0.00 (0.00; 0.31)	0.368
C16	0.29 (0.00; 1.56)	0.89 (0.00; 5.56)	0.245	0.00 (0.00; 0.31)	0.81 (0.00; 2.85)	0.034
C16-OH	0.00 (0.00; 0.03)	0.00 (0.00; 0.04)	0.366	0.00 (0.00; 0.06)	0.00 (0.00; 0.12)	0.881
C18:1	0.40 (0.00; 0.52)	0.44 (0.00; 0.61)	0.916	0.00 (0.00; 0.40)	0.00 (0.00; 0.74)	0.521
ACTotal	36.53 (33.42; 81.04)	38.81 (26.99; 46.62)	0.917	35.01 (26.90; 50.33)	49.39 (35.56; 61.48)	0.076
C0/AC	0.59 (0.55; 0.68)	0.57 (0.53; 0.66)	0.347	0.71 (0.65; 0.74)	0.77 (0.57; 0.86)	0.117
C3C16	0.15 (0.00; 0.61)	0.28 (0.00; 0.34)	0.675	0.00 (0.00; 0.65)	0.23 (0.00; 0.84)	0.147
C3C2	0.09 (0.00; 0.15)	0.11 (0.00; 0.16)	0.287	0.07 (0.00; 0.67)	0.25 (0.21; 0.82)	0.075
C5C2	0.00 (0.00; 0.06)	0.00 (0.00; 0.06)	0.906	0.01 (0.00; 0.05)	0.00 (0.00; 0.09)	0.911
C8C2	0.02 (0.00; 0.50)	0.04 (0.00; 0.19)	0.829	0.00 (0.00; 0.00)	0.00 (0.00; 0.06)	0.317
C14:1/C16	0.00 (0.00; 0.44)	0.01 (0.00; 0.12)	0.737	0.00 (0.00; 0.34)	0.00 (0.00; 0.00)	0.136
C16-OH/C16	0.00 (0.00; 0.00)	0.00 (0.00; 0.06)	0.136	0.00 (0.00; 0.20)	0.00 (0.00; 0.15)	0.881
Glutaryl	0.00 (0.00; 0.36)	0.00 (0.00; 0.00)	0.136	0.00 (0.00; 0.14)	0.03 (0.00; 0.29)	0.290
Valine	67.40 (57.20; 103.00)	95.30 (80.90; 166.00)	0.076	80.70 (2.80; 110.00)	103.00 (77.10; 134.00)	0.117
Pyg	1.23 (0.00; 3.43)	2.27 (1.18; 2.50)	0.251	1.18 (0.00; 1.96)	0.00 (0.00; 1.40)	0.281
Met	0.00 (0.00; 41.30)	0.00 (0.00; 0.00)	0.317	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.999
Phe	21.80 (17.20; 34.20)	24.00 (19.70; 24.50)	0.463	29.60 (21.10; 36.20)	30.50 (23.10; 43.70)	0.754
Tyr	26.30 (13.40; 171.00)	19.50 (0.80; 31.50)	0.251	28.00 (22.20; 50.00)	27.80 (22.20; 44.60)	0.675
Cit1	8.61 (0.00; 14.30)	18.90 (5.96; 25.70)	0.172	12.60 (0.00; 26.40)	9.26 (0.00; 20.40)	0.530
Glyc	88.40 (77.20; 163.00)	92.40 (78.30; 120.00)	0.754	113.00 (82.00; 146.00)	101.00 (67.00; 119.00)	0.465
Alanine	147.00 (141.00; 193.00)	176.00 (157.00; 215.00)	0.251	276.00 (197.00; 307.00)	230.00 (173.00; 346.00)	0.917
Leulle Leu/Ile	57.10 (37.30; 65.50)	59.50 (51.40; 69.40)	0.175	55.50 (51.40; 91.60)	77.50 (62.40; 96.00)	0.175
PheTyr Phe/Tyr	1.09 (0.00; 3.43)	0.00 (0.00; 0.00)	0.054	0.00 (0.00; 0.92)	0.00 (0.00; 2.69)	0.881

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.
The concentrations of metabolites are in units µM/L; continuous variable is described by median (5^{th} and 95^{th} percentiles) and tested by the Mann-Whitney test.

variables, and median supplemented by 5th–95th percentile range for continuous variables. Statistical significance of differences was tested using Fisher's exact test for categorical variables and the Mann-Whitney test for continuous variables. Spearman's correlation coefficient was used for the analysis of the relationship between continuous variables. Statistical analysis was computed using SPSS 22 (IBM Corporation, New York, USA; 2013).

Results

In the analysis of the concentrations of free carnitine and acylcarnitines, small variations between the 2 groups were noted. Most notably, the free carnitine to acylcarnitine ratio (C0/AC ratio) was significantly lower in the COPD group compared to the control group – COPD 0.58 μM/L (0.53; 0.68) and control group 0.73 μM/L (0.57; 0.86) with p = 0.002. The mean C8/C2 ratio in the COPD group was significantly higher, 0.03 μM/L (0; 0.50), whilst it was 0 μM/L in the control group (p = 0.03).

The analysis of amino acids showed significantly lower levels of phenylalanine in the COPD group compared to the controls. The phenylalanine level in the COPD group was 22.05 μM/L (17.20; 34.20) and in the control group, it was 30.05 μM/L (21.10; 43.70) with p = 0.008; the alanine levels were significantly lower in the COPD group, 173 μM/L (141; 215), than in the control group – 253 μM/L (173; 346); p = 0.001. The pyroglutamate levels were significantly higher in COPD patients, 1.58 μM/L (0; 3.43), than in the control group – 1 μM/L (0; 1.96); p = 0.040.

Gender had no influence on the metabolomic profile of COPD patients, and only a small effect in the control group (Table 2). In the control group, males had significantly higher levels of C5OH (0.11 μM/L) compared to females (0 μM/L); p = 0.034. Also, the levels of C16 were significantly higher in males (0.81 μM/L) than in females (0 μM/L); p = 0.034.

Increased age (Table 3) positively correlated with increased levels of free carnitine (Spearman's coefficient 0.695; p = 0.026) and the phenylalanine/tyrosine ratio (Spearman's coefficient 0.818; p = 0.004) in COPD patients. In the COPD group, a negative correlation with age was found in C5-OH (Spearman's coefficient –0.644; p = 0.044) and C14:1 (Spearman's coefficient –0.688; p = 0.028). In the control group, age positively correlated with the levels of C14 (Spearman's coefficient 0.765; p = 0.010) as well as the phenylalanine/tyrosine ratio (Spearman's coefficient 0.694; p = 0.026) and the phenylalanine level (Spearman's coefficient 0.671; p = 0.034).

In the COPD group, BMI (Table 4) positively correlated with C0/AC ratios (Spearman's coefficient 0.758; p = 0.011), whilst there was a negative correlation with C5/C2 ratios (Spearman's coefficient –0.809; p = 0.005). In the control group, the leucine/isoleucine ratio positively correlated with BMI (Spearman's coefficient 0.721; p = 0.019).

Table 3. Influence of age on the results

Carnitines and amino acids	COPD (n = 10)		Control (n = 10)	
	Spearman's coefficient	p-value	Spearman's coefficient	p-value
C0	0.695	0.026	0.419	0.229
C2	0.494	0.147	0.345	0.329
C3	0.335	0.343	0.287	0.422
C4	0.052	0.888	0.501	0.140
C5	–0.017	0.962	–0.069	0.850
C5-OH	–0.644	0.044	0.033	0.928
C8	–0.157	0.664	–0.174	0.630
C14	0.498	0.143	0.765	0.010
C14:1	–0.688	0.028	0.182	0.615
C16	–0.642	0.045	–0.210	0.560
C16-OH	–0.328	0.355	–0.092	0.800
C18:1	0.136	0.707	0.008	0.983
AC total	0.591	0.072	0.443	0.200
C0/AC	0.134	0.712	0.086	0.813
C3/C16	0.294	0.410	0.223	0.535
C3/C2	–0.205	0.570	–0.052	0.886
C5/C2	–0.224	0.534	–0.118	0.745
C8/C2	0.237	0.510	–0.530	0.115
C14:1/C16	–0.202	0.575	0.004	0.990
C16-OH/C16	–0.544	0.104	0.004	0.990
Glutaryl	0.017	0.962	0.180	0.618
Valine	–0.305	0.392	–0.203	0.574
Pyg	0.146	0.687	0.083	0.821
Met	0.292	0.413	–	–
Phe	–0.260	0.468	0.671	0.034
Tyr	0.250	0.486	0.364	0.301
Cit1	–0.310	0.384	0.321	0.366
Glyc	–0.280	0.432	0.499	0.142
Alanine	–0.104	0.776	0.437	0.207
Leu/Ile	–0.470	0.171	0.443	0.200
Phe/Tyr	0.818	0.004	0.694	0.026

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

Fat free mass index had no effect on the metabolomic profile of COPD patients (Table 5). In the COPD group, there was a negative correlation of FEV_1 with C3 (Spearman's coefficient –0.634; p = 0.049), C5 (Spearman's coefficient –0.676; p = 0.032) and leucine/isoleucine ratios (Spearman's coefficient –0.702; p = 0.024) (Table 6). There was a negative correlation between FEV_1/FVC and the C4 (Spearman's coefficient –0.635; p = 0.049) and

tyrosine levels (Spearman's coefficient –0.770; p = 0.009) in the COPD group (Table 7).

Discussion

The severity of COPD in patients is normally graded based on the degree of obstruction as measured by FEV_1. Today, it is accepted that COPD is a complex disease with systemic effects. Transcriptomics, proteomic and metabolomic investigation have the potential to contribute to a better understanding of COPD and its natural course. This is paramount, as COPD has been recently recognized as a condition affecting the whole organism. For example, it was shown that COPD patients are very likely to suffer from metabolic syndrome with increased pro-inflammatory markers from the lungs and adipose tissue.[20]

Table 4. Influence of BMI on the results

Carnitines and amino acids	COPD (n = 10)		Control (n = 10)	
	Spearman's coefficient	p-value	Spearman's coefficient	p-value
C0	0.467	0.174	0.624	0.054
C2	–0.370	0.293	0.285	0.425
C3	0.273	0.446	–0.043	0.906
C4	–0.546	0.102	–0.545	0.103
C5	0.027	0.940	0.437	0.207
C5-OH	–0.407	0.243	–0.032	0.929
C8	-0.069	0.850	0.067	0.854
C14	–0.341	0.334	0.470	0.171
C14:1	–0.294	0.409	–0.231	0.521
C16	–0.362	0.304	0.252	0.482
C16-OH	–0.479	0.161	0.528	0.117
C18:1	–0.548	0.101	0.231	0.521
AC total	0.273	0.446	0.612	0.060
C0/AC	0.758	0.011	0.455	0.187
C3/C16	–0.170	0.638	0.614	0.059
C3/C2	–0.278	0.437	0.292	0.413
C5/C2	–0.809	0.005	0.149	0.682
C8/C2	0.038	0.918	0.058	0.873
C14:1/C16	–0.292	0.413	0.199	0.582
C16-OH/C16	–0.389	0.266	0.510	0.132
Glutaryl	–0.450	0.192	–0.191	0.597
Valine	0.624	0.054	0.370	0.293
Pyg	0.394	0.260	0.194	0.592
Met	0.290	0.416	–	–
Phe	–0.498	0.143	0.612	0.060
Tyr	–0.442	0.200	0.511	0.132
Cit1	–0.006	0.987	0.389	0.266
Glyc	–0.152	0.676	0.539	0.108
Alanine	0.176	0.627	0.576	0.082
Leu/Ile	0.018	0.960	0.721	0.019
Phe/Tyr	–0.172	0.636	0.311	0.381

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

The development of modern instrumental analytical techniques contributes to the investigation of many pathological states and helps in the elucidation of metabolic and other processes inside the body of a patient. In our work, we investigated the concentrations of various small molecules, mainly various amino acids and free and acylated carnitines with a different length of the alkyl chain, using the HPLC-MS/MS method.

Carnitine is a quaternary ammonium compound, synthesized by the kidneys and liver from the amino acids lysine and methionine. It is also obtained through a diet, particularly through red meat and nuts. Carnitine has a major role in the citric acid cycle by binding and transporting various acyl groups to be metabolized through β-oxidation. Consequently, the disturbance of these processes leads to various pathological states.[21,22] Genetic disorders, such as primary carnitine deficiency, carnitine palmitoyltransferase deficiency type I and II among others, affect different steps in carnitine biotransformations.[23] The significantly lower ratio of free carnitine/acylated carnitine (C0/AC) in COPD patients, despite the lack of distinct differences in the concentration of free carnitine, and the total and individual acylcarnitine levels, possibly indicates a predisposition to atherosclerosis as a result of inadequate β-oxidation of fatty acids, as well as of being at risk for carnitine deficiency.[24,25]

Evidence suggests that impaired fatty acid oxidation has some effect on the development of type-2 diabetes.[26,27] The prevalence of diabetes in COPD patients is significant, and may be one of the factors which cause the progression and worsen the prognosis of COPD.[28–30] Acylcarnitines have a potential to activate proinflammatory signaling pathways as part of the shared mechanism of COPD and cardiovascular disease.[27,31] Metabolic heritability may be implicated in aberrant levels of some acylcarnitines, as it was shown that some metabolite levels are inheritable at birth and that some genes are associated with acylcarnitines.[32]

The role of inherited fatty acid oxidation deficiency and genetics in COPD may be further implicated by the higher levels of the acylated C8/C2 carnitines ratio in COPD patients. The C8/C2 ratio and the C8 levels are the most accurate markers of medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most common inherited defect in the fatty acid oxidation pathway marked by an inability to break down medium-chain fatty acids during periods of fasting.[33]

Additional findings relate to alanine, a nonessential amino acid found in a variety of foods. It may be synthesized by the body itself from pyruvate and a branched-chain

Table 5. Influence of FFMI on the results

Carnitines and amino acids	COPD (n = 10)	
	Spearman's coefficient	p-value
C0	0.176	0.627
C2	–0.103	0.777
C3	0.030	0.934
C4	–0.171	0.637
C5	–0.191	0.597
C5-OH	–0.226	0.530
C8	–0.144	0.692
C14	–0.178	0.624
C14:1	–0.156	0.668
C16	0.018	0.960
C16-OH	–0.045	0.902
C18:1	–0.363	0.302
AC total	0.188	0.603
C0/AC	0.212	0.556
C3/C16	–0.055	0.881
C3/C2	0.222	0.537
C5/C2	–0.507	0.135
C8/C2	–0.063	0.863
C14:1/C16	0.019	0.957
C16-OH/C16	0.130	0.721
Glutaryl	–0.623	0.054
Valine	0.661	0.038
Pyg	0.624	0.054
Met	0.058	0.873
Phe	–0.140	0.700
Tyr	–0.539	0.108
Cit1	0.299	0.402
Glyc	0.006	0.987
Alanine	0.430	0.214
Leu/Ile	0.152	0.676
Phe/Tyr	–0.455	0.187

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

Table 6. Influence of FEV_1 on the results

Carnitines and amino acids	COPD (n = 10)	
	Spearman's coefficient	p-value
C0	–0.098	0.787
C2	–0.055	0.879
C3	–0.634	0.049
C4	–0.170	0.639
C5	–0.676	0.032
C5-OH	0.158	0.664
C8	–0.410	0.240
C14	0.132	0.717
C14:1	0.439	0.204
C16	0.305	0.391
C16-OH	–0.053	0.884
C18:1	0.194	0.592
AC total	–0.154	0.671
C0/AC	0.037	0.919
C3/C16	0.120	0.740
C3/C2	0.392	0.263
C5/C2	0.097	0.789
C8/C2	–0.628	0.052
C14:1/C16	0.573	0.083
C16-OH/C16	–0.141	0.699
Glutaryl	0.044	0.904
Valine	–0.406	0.244
Pyg	0.579	0.080
Met	0.530	0.115
Phe	–0.096	0.793
Tyr	–0.474	0.166
Cit1	–0.180	0.620
Glyc	–0.031	0.933
Alanine	–0.369	0.294
Leu/Ile	–0.702	0.024
Phe/Tyr	0.083	0.819

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

Table 7. Influence of FEV_1/FVC on the results

Carnitines and amino acids	COPD (n = 10)	
	Spearman's coefficient	p-value
C0	0.248	0.489
C2	–0.285	0.425
C3	–0.248	0.489
C4	–0.635	0.049
C5	–0.478	0.162
C5-OH	0.175	0.630
C8	–0.344	0.331
C14	–0.417	0.231
C14:1	0.372	0.290
C16	0.288	0.419
C16-OH	–0.539	0.108
C18:1	–0.006	0.987
AC total	0.067	0.855
C0/AC	0.358	0.310
C3/C16	–0.261	0.466
C3/C2	–0.198	0.584
C5/C2	–0.343	0.332
C8/C2	–0.496	0.145
C14:1/C16	–0.091	0.803
C16-OH/C16	–0.389	0.266
Glutaryl	0.294	0.409
Valine	0.127	0.726
Pyg	0.370	0.293
Met	0.174	0.631
Phe	0.030	0.934
Tyr	–0.770	0.009
Cit1	–0.439	0.204
Glyc	0.176	0.627
Alanine	–0.406	0.244
Leu/Ile	–0.103	0.777
Phe/Tyr	–0.216	0.548

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

amino acid. Dietary sources include meat, seafood, dairy, eggs, beans, and nuts. It is an important source of muscle energy. Alanine is an amino acid linked to energy metabolism-related pathways of glycolysis, gluconeogenesis, the alanine cycle, and the citric acid cycle.[34] Decreased levels of alanine were reported earlier in relation to COPD and muscle wasting because of disturbed metabolism of proteins.[35] Our finding of decreased levels of alanine in patients with COPD compared to the control group may indicate a risk of protein malnutrition as do significantly lower levels of phenylalanine in COPD patients compared to the control group.

Higher levels of pyroglutamate in COPD patients are of unknown significance and should be researched further.

These are important findings, as natural physiological concentrations of substrates related to energy metabolism (alanine, phenylalanine and carnitines), and consequently to malnutrition, are disturbed in COPD patients, while the concentrations of substrates not linked directly

to energy metabolism (other amino acids) are not significantly compromised.

Chronic malnutrition is a common problem in a myriad of chronic diseases, COPD and heart disease being amongst them.[36,37] It is connected to complications during hospitalizations, a higher rate of readmissions, increased costs of care, and the loss of quality of life.[36,38] Malnutrition in COPD is a negative predictive factor, being strongly associated with in-hospital mortality and readmittance 30 days after discharge.[39,40] Skeletal muscles are affected by inactivity, unbalanced nutrition, age, and inflammation, leading to sarcopenia, which involves both muscle loss and muscle dysfunction with contractile impairment and metabolic anomalies. These responses are poorly understood (and are beyond the scope of this article), but 2 groups of patients may be identified: cachectic and with normal or increased BMI. Malnutrition may be present in cachectic patients as well as in patients with normal or increased BMI, i.e., sarcopenia and sarcopenic obesity.[41] Nutritional intervention is beneficial for malnourished patients, combined with exercise or a rehabilitation program, which, in the long term, may be associated with lower health care costs.[36,38,42] This is already being echoed by some national guidelines.[2]

Metabolomic profiling using the HPLC-MS/MS method is a new application in the investigation of COPD, and as such it serves as a source of essential information on processes that occur in COPD patients' bodies. Our results were obtained in the limited number of patients. We intend to continue our research in this direction, as it is highly desirable that future studies involve a higher number of subjects to investigate a varying role of individual comorbidities and physiological COPD parameters in the long term to elucidate further COPD pathology.

Limitations

This was a pilot study conducted on a small number of patients. The control group was younger than the COPD test subjects, although not statistically significantly. This was due to the strict exclusion criteria, namely smokers or ex-smokers of less than 6 months, and the presence of metabolic diseases including diabetes mellitus types 1 and 2. There is a high prevalence of diabetes mellitus in the Czech Republic, which is 8%, compared to the estimated worldwide prevalence of 2.8–4.4%.[43,44] As the incidence of diabetes mellitus increases with age, the set criteria excluded most of our older control group candidates from the study.[44] Further studies on significantly larger cohorts are needed.

Patients with chronic obstructive pulmonary disease are commonly afflicted with comorbidities, particularly coronary artery disease. As there is no data available on the metabolomic effects of cardiovascular diseases on the metabolism of COPD patients, future investigation on whether a cardiovascular disease concomitant with COPD further contributes to metabolomic changes may be beneficial.

As this pilot study already shows, there are metabolic differences between COPD patients and the control group, which makes future investigations of this kind rational.

Conclusions

Our study shows that the HPLC-MS/MS method is able to show minute changes in the metabolism of patients shown on a molecular level. The significance of many of these differences is not yet understood. The carnitine and acylcarnitine levels in COPD subjects in this study possibly indicate a predisposition to atherosclerosis as a result of inadequate β-oxidation of fatty acids and show the presence of oxidative stress. Furthermore, the high sensitivity to changes in circulating amino acid levels may allow us to detect subclinical malnutrition and take early preventative interventions such as nutritional supplementation and patient education.

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Reliability of computed tomography scans in the diagnosis of chronic rhinosinusitis

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Abstract

Background. Paranasal computed tomography (CT) has become the investigation method of choice to confirm or exclude the diagnosis of chronic rhinosinusitis (CRS) on the basis of its ability to deliver objective data regarding the presence of inflamed mucosa or polyps.

Objectives. The aim of the study was to assess the reliability of CT scan findings among untreated CRS patients without the presence of polyps in a nasal endoscopy.

Material and methods. Among patients with clinically demonstrated CRS considered for surgery, 93 subjects who had had 2 CT scans performed at different time points in the diagnostic process were enrolled into the study. Paranasal sinus involvement on both CT scans was scored using the Lund-Mackay (L-M) and modified Lund-Mackay scales. Both CT exams served to assess the extent of the potential endoscopic sinus surgery.

Results. The time interval between CT scans ranged from 31 to 1,162 days (mean: 338 days). The L-M scores from the 1st CT examination correlated statistically with the results of the 2nd CT ($r = 0.86$; $p < 0.05$). When compared to the 1st scan, the L-M score in the 2nd CT scan remained the same in 36 patients (39%), increased in 23 patients (25%) and decreased in 34 patients (36%). There was no statistically significant correlation between the change in the L-M scores and the time interval between CT examinations.

Conclusions. The present study indicates that mucosal thickening within paranasal sinuses among untreated patients with CRS is stable over short- and middle-time intervals, regardless of the initial intensity of the disease. The time delay between the CT examination and qualification for surgery does not influence the decision regarding the performance of the operation. The results suggest the conclusion that repeating CT scans in symptomatic, untreated patients with CRS should be seriously considered.

Key words: surgery, diagnosis, reliability, tomography, sinusitis

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Introduction

For many years, chronic rhinosinusitis (CRS) was commonly identified solely on the basis on the patient's subjective symptoms.[1] Later, in order to confirm a diagnosis, the symptom-based definition was reevaluated by the use of either radiological imaging or nasal endoscopy.[2] Computerized tomography (CT) provided detailed images of the sinuses and gave the examiner a clear view of those areas that are of key importance in the pathogenesis of CRS.[3] Thus, due to the relatively unsatisfactory specificity and positive predictive value of symptom-based diagnostic criteria, many physicians rely extensively on CT findings.[4] It is often the case among patients with mild disease severity or isolated symptoms, but also among those with CRS without polyps when the doctor lacks the confidence to treat without further imaging.

The growing availability of CT scanners means that such an examination is requested frequently, often at very distinct stages of the diagnostic or therapeutic process. Increasingly easy access and the promotion of low-dose devices may further encourage the performance of subsequent examinations. As a result of such a tendency, doctors might encounter new patients with already performed CT, and must decide whether to repeat the scan or to rely on the old one. The question becomes particularly important when surgical intervention is being considered. At the same time, data regarding the durability of CT scan findings, especially in respect to the severity of CRS, is still rare, and the issue is practically not discussed and questioned in the literature.[5]

Besides extra costs, repeated paranasal CT examinations are associated with additional radiation exposure, which is principally important in respect to the eye lens and thyroid gland.[6] The dose of radiation related to paranasal CT study is comparable to natural background radiation for about 8 months.[7] This is the same length of time the human body needs to repair the radiation injury caused by such an examination. Furthermore, the overall lower mean age of CRS patients and the fact that repeated CT examinations are performed for benign disease should raise serious concerns.

The aim of the present paper was to assess the reliability of CT scan findings among untreated CRS patients without polyps apparent in nasal endoscopy. Additionally, the evaluation of distinct paranasal CT scans performed in the same untreated patients allowed an analysis of the natural history of the condition.

Material and methods

Patients with CRS consulting or admitted to the Department of Otolaryngology at Wroclaw Medical University for surgery were considered to be enrolled into the study. Conditions for inclusion were a lack of polyps in nasal endoscopy and a previously performed CT scan. The 1st CT examination was conducted at the request of otolaryngologists from the outpatient department, or general practitioners. In the study group there were no patients with bronchial asthma or allergic rhinitis. Symptomatic patients for whom thorough conservative management had not been successful and who still met the diagnostic criteria for CRS were qualified for functional endoscopic sinus surgery (ESS).

The 2nd CT scan was considered after admission only in the following clinical situations: when the time interval between scans was longer than 6 months; when the 1st CT was not appropriate for an image guided system if required; in the case of problems with the examination on CD-ROM or when symptoms clearly differed from the intensity of changes on the 1st CT scan. The CT examination was performed on a GE Discovery 750 HD scanner (General Electric Healthcare, Milwaukee, USA) using the low-dose protocol: tube potential 120 kVp, 45 mAs; detector configuration 64 × 0.625 mm; pitch 1.3; section thickness 0.625; and gantry rotation time 0.4 s. Patients were scanned with a scanning range from the top of the frontal sinuses to the level of hard palate and from the tip of the nose to the region posterior to the clivus. The images were reconstructed with an adaptive statistical iterative reconstruction (ASIR) algorithm using 50% ASIR.

Only symptomatic patients who had not been treated pharmacologically between CT studies were finally accepted. Each patient gave written consent for the CT examination to be performed and to participate in the study. The study protocol was approved by our institutional review board.

Computed tomography scoring systems

In each case, both paranasal sinus CT scans were reviewed in random order and scored according to 2 staging systems: the Lund-Mackay (L-M) and the modified L-M. CT scans were subjected to an independent double-blind review by 2 physicians, and the results were compared.

Lund-Mackay scoring system

The L-M staging system is a measure of the degree of opacification in the paranasal sinuses.[8,9] When inflammation occupied 0% of the CT image, a score of 0 was assigned; a score of 2 was assigned when the changes occupied 100% of the sinus. All other degrees of inflammation were scored as 1. For the ostiomeatal complex (OMC): 0 = not occluded; 2 = occluded. The total score might range from 0 to 24.

Modified Lund-Mackay scoring system

It classifies the volume of inflammation in each sinus into 4 strata using intervals of 33%.[10] For the sinuses with no inflammation, a score of 0 was assigned; a score of 1 for inflammation occupying 1–33%; a score of 2 for inflammation in 34–66% of the sinus; a score of 3 for sinuses

occupied in 67–99%; and a score of 4 in the cases with total sinus opacification. For the OMC: 0 = not occluded; 2 = occluded. The total score might range from 0 to 44.

Patients were excluded if any of the elements of the scoring systems could not be properly evaluated.

Extent of the potential endoscopic sinus surgery according to CT examinations

The volume of sinus opacification determines the extent of the endoscopic surgery. However, a small change in the L-M score resulting from the time flow may have no effect on the extent of operation. Additionally, patients with equal L-M scores may require surgery of different extents.

In order to clarify the above ambiguities, the 1st and 2nd CT examinations served to estimate the range of potential ESS in patients with L-M scores ≥1. The surgery score was evaluated according to the modified classification proposed by Lund and Mackay, which included: uncinectomy, middle meal antrostomy, anterior and posterior ethmoidectomy, sphenoidectomy, and frontal recess surgery (0 = no procedure; 1 = procedure done).[11] Total scores range from 0 to 12.

Statistics

Statistical analysis was performed with STATISTICA software v. 12 (StatSoft, Tulsa, USA). The correlation between the extent of disease on the 1st and 2nd CT scan assessed by the L-M and modified L-M scales was effected using Pearson's r correlation analysis. Spearman's rho was used to measure the correlation between ranked variables. Statistical significance of correlation coefficients r and rho was tested with the t-test. All tests were verified at the significance level $\alpha = 0.05$. A p-value <0.05 was considered statistically significant.

Results

A total of 93 adult patients met the inclusion criteria and were enrolled into the study. There were 41 (44%) female and 52 (56%) male patients. The average patient age at the time of the 1st examination was 43.7 years (18–76 years). The time interval between CT scans ranged from 31 to 1,162 days (mean: 338 days; SD: 238 days).

The L-M scores ranged from 1 to 16 (mean: 7.48; SD: 4.26) in the 1st CT scan and from 0 to 18 (mean: 7.43; SD: 4.57) in the 2nd CT examination (Fig. 1). The modified L-M scores ranged from 1 to 34 (mean: 11.33; SD: 8.20) in the 1st CT examination and from 0 to 36 (mean: 11.45; SD: 8.29) in the 2nd CT scan.

Changes in L-M and modified L-M scores between CT scans were not statistically correlated with the patients' sex and age.

The correlation between the scores from the 1st and 2nd CT scans

The severity of sinus involvement on the 1st CT scan correlated statistically with sinus opacification on the 2nd scan as scored by the L-M system ($r = 0.86$; $p < 0.05$) (Fig. 2). Similarly, a statistically significant association was found for the modified L-M scale ($r = 0.83$; $p < 0.05$). The comparison disclosed that both scales are reliable, but L-M insignificantly more so.

Does the change in L-M score correlate with the change in modified L-M score?

The difference in sinus opacification between the 1st and 2nd CT scans assessed by the L-M scale correlated significantly with the same change scored by the modified L-M system (Spearman's rho = 0.87; $p < 0.001$).

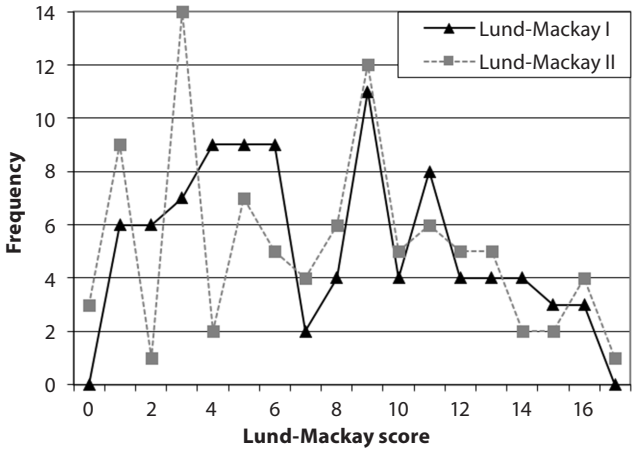

Fig. 1. The distribution of Lund-Mackay scores for the 1st and the 2nd computed tomography scans

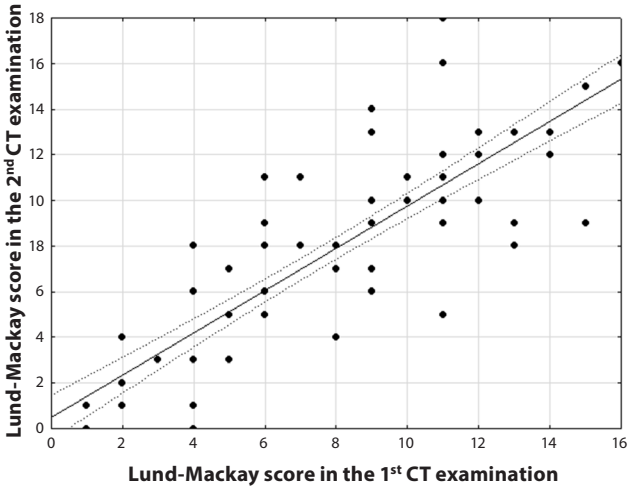

Fig. 2. Scatter-plot of the correlation between the 1st and the 2nd computed tomography scans

Change in L-M score between scans as a function of time interval between scans

The L-M score for 36 (39%) patients remained the same over time, increased in 23 (25%) and decreased in 34 (36%) cases. There was no statistically significant correlation between the change in the L-M scores and the time intervals between the 1st and 2nd CT examination (Fig. 3, 4).

In addition, patients were divided into 2 groups depending on the length of time intervals between CT examinations – above and below 1 year. In patients with a longer interval between scans, the change in the L-M scores was more pronounced, although the difference was not statistically significant.

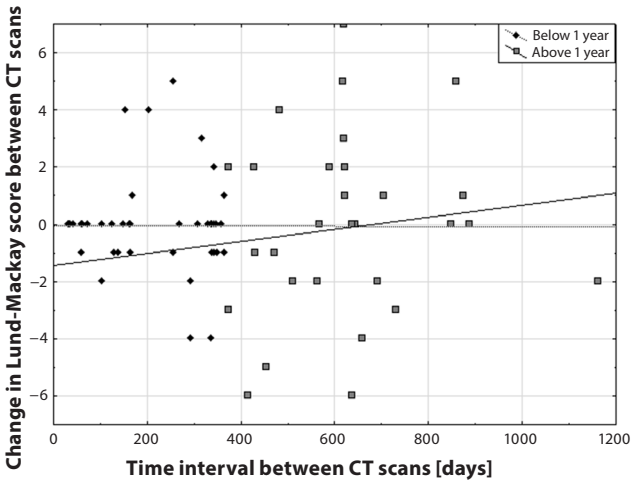

Fig. 3. Change in Lund-Mackay scores between scans as a function of time intervals between scans

Does the initial sinus opacification correlate with subsequent changes in mucosal inflammation?

There was no correlation between the initial sinus opacification and the subsequent change in inflammation intensity measured by the L-M and modified L-M systems. The initial severity of inflammation within the sinuses cannot determine how epithelial changes will evolve over time. There was no difference in the change in inflammation severity among patients with low-stage (L-M ≤ 3; n = 19) and high-stage CRS (L-M > 3; n = 74).

Does the change in L-M score between scans influence the extent of potential sinus surgery?

The mean extent of potential endoscopic sinus surgery assessed by modified L-M classification on the basis of the 1st and 2nd CT examination was 6 (SD: 3.22) and 5.96 (SD: 3.31), respectively. Two (2%) patients with L-M = 1 did not qualify for surgery on the basis of the 1st CT scan. According to the 2nd CT scan, surgical treatment was cancelled in 8 (8.6%) patients including: the 2 mentioned above with unchanged L-M = 1, 3 consecutive patients who had no inflammatory changes in CT (L-M = 0) and 3 patients with the L-M score decreased to 1. The scope of surgery based on the 2nd CT did not change compared to that performed on the basis of the 1st CT in 45 patients (48%), while it increased in 18 (19%) and in 30 (32%) decreased.

Changes in sinus opacification between 1st and 2nd CT examinations correlated significantly with a change in the extent of potential sinus surgery (Spearman's rho = 0.73; $p < 0.001$ for L-M, and Spearman's rho = 0.68; $p < 0.001$ for modified L-M scale).

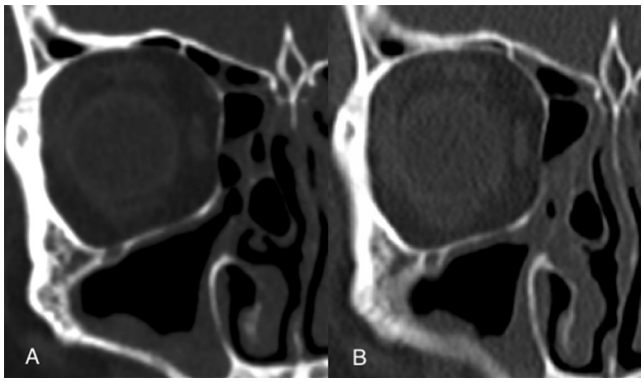

Fig. 4. Coronal images of CT scans obtained in untreated patient with chronic rhinosinusitis in 1-year interval; slight change in sinus opacification between the 1st (A) and the 2nd (B) CT examination

Discussion

The diagnosis and management of chronic rhinosinusitis still continue to provide many difficulties. One of the current questions is how stable over time is mucosal thickening within sinuses. Our results allow us to state that the CT scan findings in untreated CRS without polyps generally remain at the same level over short- and middle-time intervals. Contrary to the common expectation, mucosal thickening was not demonstrated to have a clear tendency to grow over time. Inflammatory changes may either slightly increase or decrease, and the direction in which this will evolve is unpredictable. Herein, the chance for a larger change grew insignificantly with the length of the follow-up. Second CT scans performed after 12 months or longer revealed more pronounced mucosal thickening compared to the scans repeated after a shorter time. Such observations also mean that mucosal disease among CRS subjects increases over a longer period than that considered in this study.

It was proved herein that CT scans in CRS possess good test-retest reproducibility. In contrast to Bhattacharyya, who analyzed a similar issue, we included only subjects without polyps in nasal endoscopy.[5] The essential advantage of the present study is the higher number of patients and a much longer follow-up period.

These results allow us to conclude that repeating CT scans in symptomatic, untreated patients with CRS should be seriously considered. Spontaneous withdrawal of the mucosal thickening to the extent that disqualifies patients from surgery is very unlikely. One of the goals of pre-operative CT is to delineate the extent of disease to omit unnecessary intervention in disease-free sinuses. The outcomes of the study indicate that the time delay between CT examination and qualification for surgery should, however, not influence decisions regarding the surgery, but it might slightly change its extent.

In the adult population, a quantified L-M scale can be used to distinguish between patients with and without CRS.[12] Typically, L-M scores >3 are highly likely to represent true CRS. L-M scores ≤3, categorized as low-stage CRS, are unclear, and additional clinical judgment or corroborating data are required to establish diagnoses. Although recalcitrant, low-stage CRS forms a relatively small subset, patients with minimal mucosal disease on CT scans but having symptoms suggestive of CRS can cause some therapeutic dilemmas. In these patients in particular, the fact that the clinical course of CRS is characterized by variations in severity should be taken into consideration. It seems to be also possible that CT can identify "incidental" mucosal thickening, which does not represent true disease.[13] Such incidental opacification of sinuses on CT scans is identified in 27–45% of asymptomatic individuals.[14] However, according to the same data, as much as 40% of patients who met the criteria for the diagnosis of CRS do not have radiological evidence of disease.[15,16] Thus, among subjects with minimally affected CT scans, despite the failure of medical therapy, there is often a reluctance to perform surgery. Repeating CT examinations might hypothetically guard against subjecting healthy patients to invasive procedures or extended therapy. In the present study, changes in sinus opacification over time among patients with low-stage CRS did not differ significantly from those in patients with high-stage CRS. The results indicate that even a limited mucosal disease is stable over time. This supports the current approach that the presence of a minimally affected CT scan should not exclude the diagnosis of CRS.[17] Moreover, the results encourage considering the implementation of ESS among symptomatic low-stage CRS patients resistant to medical management.

The major drawback of the Lund-Mackay system is its inability to subgrade the volume of inflammatory disease in sinuses.[10] For this reason, the new staging system was proposed by 5 American societies, called the modified L-M scale.[10] Although the scale has a more efficient ability to subgrade the level of inflammation, the application of modified L-M in the present study did not give any benefits. Furthermore, the L-M scoring system showed a higher level of reliability, which is consistent with earlier data published by Okushi et al.[18]

Despite the propagation of the symptom-based diagnosis of CRS and the good specificity of endoscopic examination, in many situations physicians are willing to repeat CT, thus forgetting the principle of radiation protection: as low as reasonably achievable (ALARA). This raises the danger that CT will be over-prescribed as a diagnostic tool in CRS. The results of the present study indicate that inflammatory changes within paranasal sinuses in symptomatic, untreated patients with CRS are stable over time, regardless of the initial intensity. Thus, in those cases when complaints persist, repeating CT scans for reasons other than the planning of surgical intervention is not justified. Such a conclusion is also important in terms of the increasing public awareness of the dose burden related to that examination.

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Liver dysfunction in sepsis

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Abstract

Despite continuous progress in medicine, sepsis remains the main cause of deaths in the intensive care unit. Liver failure complicating sepsis/septic shock has a significant impact on mortality in this group of patients. The pathophysiology of sepsis-associated liver dysfunction is very complicated and still not well understood. According to the Surviving Sepsis Campaign (SSC) Guidelines, the diagnosis of liver dysfunction during sepsis is based on the increase in bilirubin concentration >2 mg/dL and the occurrence of coagulation disorders with INR > 1.5. The lack of specificity and ability to distinguish acute liver failure from previous liver dysfunction disqualifies bilirubin as a single parameter reflecting the complex liver function. Clinical manifestations of sepsis-associated liver dysfunction include hypoxic hepatitis, sepsis-induced cholestasis and dysfunction of protein synthesis manifesting with, e.g., coagulopathies. Detoxifying liver dysfunction, which is associated with an increase in serum ammonia concentration, manifesting with e.g., confusion, loss of consciousness and hepatic encephalopathy, may be disguised by analgosedation used in the intensive care unit. To determine a liver dysfunction in a critically ill patient, the concept of shock liver may be used. It is a complex syndrome of hemodynamic, cellular, molecular and immunologic changes leading to severe liver hypoxia. In clinical practice, there is no standardized diagnostic panel that would allow for an early, clear diagnosis of acute liver dysfunction, and there is no therapeutic panel enabling the full restoration of damaged liver function. The aim of the article is to present the pathophysiology and clinical manifestations of sepsis-associated liver dysfunction.

Key words: sepsis, MODS, liver dysfunction, shock liver

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Introduction

Despite the continuous progress in medicine, sepsis remains the leading cause of deaths in the intensive care units (ICUs). American data estimate the incidence of sepsis to be about 300 cases/100,000 people, and the mortality rate ranges between 30% and 50%.[1–3] That is more than the number of deaths caused by prostate cancer, breast cancer and AIDS all together.[4]

The prevalence of sepsis in Poland according to recently published data is estimated to be about 25% of patients hospitalized in the ICUs. Among patients diagnosed with severe sepsis, almost half of them (44%) developed septic shock.[5]

The development of multiple organ dysfunction syndrome (MODS) is one of the complications of sepsis. A study conducted by Kubler et al, which analyzed the course and outcome of severe sepsis in Poland, revealed that patients admitted to ICUs were severely ill. Dysfunction of 1 or 2 organs was diagnosed in 9–12% of patients at the time of admission, whereas most of the patients (89%) developed dysfunction of 3 or more organs during their stay in ICUs.[6]

The level of organ dysfunction, including liver failure, may vary from a mild organ dysfunction to life-threatening fulminant organ failure.

During sepsis, not only infection itself, but also hyperactivity of the inflammatory response, microcirculatory failure, and side effects of the therapy are responsible for liver injury.

The liver plays a pivotal role in maintaining homeostasis. Its functions include: metabolism of carbohydrates, lipids, proteins and hormones; biosynthesis of blood components, enzymes and clotting factors; production of bile; detoxification; metabolism of nitrogen compounds – synthesis of urea; storage of glycogen, cholesterol, vitamins (A, D, B_{12}), iron, and many more.

The incidence of sepsis-associated liver dysfunction (SALD) is hard to establish due to the lack of a homogeneous definition, and consequently lack of a thorough registry. What is more, the incidence of SALD varies, as currently there are no specific diagnostic tools available, especially ones that could detect liver injury in the early stages.

In a study performed by Birrer R. et al., the presence of hepatic injury was identified in 1.1% of admissions.[7] The causes of hepatic injury in the group of patients were: hypotension, congestive heart failure (secondary liver hypoperfusion), sepsis, respiratory failure resulting in hypoxia, and other causes resulting in hypoxemia. Sepsis was diagnosed in 16.1% of patients who developed hypoxic hepatitis.

Another study performed by Kobashi H. et al. observed SALD in 34.7% of the patients.[8] Among those who developed SALD, the authors distinguished 3 groups: "hepatocellular" (21.8%), "cholestatic" (48.1%) and "shock liver" (30.1%). In each group, jaundice as a complication was observed in 17.6%, 33% and 8.5% of cases, respectively.

The aim of this article is to explain the pathophysiology and review the clinical manifestations of sepsis-associated liver dysfunction.

The liver in sepsis

The liver is a parenchymatous organ composed of 3 types of cells: hepatocytes (HCs), Kupffer cells (KCs), and liver sinusoidal endothelial cells (LSECs).

In the course of sepsis/septic shock, the metabolism of HCs is modified towards the inflammatory response. The main cytokine of the liver inflammatory response is interleukin-6 (IL-6), which is responsible for synthesizing acute phase proteins such as C-reactive protein (CRP), α-1 antitrypsin, fibrinogen, prothrombin, and haptoglobin.[9] The increase in acute phase protein concentrations leads to inhibition of the protein C pathway, and thus it is responsible for the increase of coagulation factor activity. Secretion of IL-6 is induced by endotoxin (lipopolysaccharide – LPS) and tumor necrosis factor-α (TNF-α).

Lipopolysaccharide also stimulates secretion of TNF-α, interleukin-1β(IL-1β), interleukin-12 (IL-12) and interleukin-18 (IL-18) by KCs.[10] Il-18 is the main factor responsible for LPS-induced liver damage. IL-18 leads to interferon-γ (IFNγ) secretion, which results in hepatocyte apoptosis, an increase in TNF-α concentration and upregulation of CD14 expression. CD14 is a monocyte/macrophage surface receptor responsible for binding the lipopolysaccharide binding protein (LPS/LBP) complex.

Kupffer cells are macrophages of the liver, which are responsible for scavenging portal vein blood from bacteria and endotoxins. As a response to LPS stimulation, KCs release TNF-α, IL-1β, IL-6, IL-12 and IL-18, reactive oxygen species (ROS), and nitric oxide (NO), which induce endothelial cell and hepatocyte injury. During the early stages of sepsis, as a response to KCs' release of TNF-α and leukotriene B4, neutrophils are recruited to the liver.[11] Cytokines produced by neutrophils are responsible for further damage of hepatocytes.

Endothelial cell (EC) dysfunction contributes to the development of MODS.[12] Liver sinusoidal endotheliac cells are also involved in cytokine release in response to LPS stimulation, and they are the main hepatic source of endothelin-1 (ET-1), which is a strong vasoconstrictor.[13,14] During sepsis, ET-1 is secreted in response to NO release from inducible nitric oxide synthase (iNOS).[15]

Endothelin-1 has also been found to have a strong correlation with the inflammatory response. It involves the expression of cytokines such as TNF-α, IL-1 and IL-6, as well as the activation of transcription factors such as nuclear factor kappa B (NF-κB).[16] Endothelin-1 also increases synthesis of TNF-α in monocytes and macrophages.[17] In their study, Brauner et al. found ET-1 concentrations to be an early and sensitive predictor of mortality in patients with septic shock.[18]

In order to maintain a proper hepatic perfusion, a balance of vasoactive effects of ET-1 and NO is needed.

Nitric oxide is responsible for the relaxation of vascular smooth muscle cells, the regulation of hepatic blood flow and the inhibition of platelet aggregation and the adhesion of leukocytes to endothelium.[19] The impact of NO on liver depends on its source. The endogenous NO released from endothelial nitric oxide synthase (eNOS) helps protect the liver cells from damage caused by vasoconstriction induced by endothelin-1 (ET-1) release, whereas iNOS promotes microvascular dysfunction and thereby SALD.[15]

The other components that regulate the vasculature tone are carbon monoxide (CO) and hydrogen sulfide (H_2S).

One of the products of cysteine metabolism is H_2S, which is synthesized in the brain, in the liver, and in vessels.[20] H_2S may also by synthesized by microflora of the gastrointestinal tract and transferred to the liver via the portal circulation.[21] Synthesis of H_2S is increased during sepsis.[22] Hydrogen sulfide relaxes vascular smooth muscle cells and inhibits their proliferation and platelet aggregation.[23] Finally, H_2S oxidation may contribute to exacerbation of sepsis-associated tissue hypoxia.[24]

Carbon monoxide (CO) is one of the products of heme degradation by heme oxygenases (HO) (Fig. 1). Carbon monoxide is responsible for maintaining the liver's regional perfusion, resulting in the activation of leucocytes.[25,26] Furthermore, HO-1/CO prevents EC apoptosis via suppressing inflammatory reactions contributing to EC apoptosis. CO generated through heme catabolism by HO has an antiapoptotic effect on ECs through activation of mitogen-activated protein kinases (MAPK).[27] It is still not clear which of the above-mentioned properties of CO has a hepatoprotective influence in sepsis.

In a study on rodent model ischemia-reperfusion induced systemic inflammation, exogenous CO was shown to have a hepatoprotective effect via improving liver cell integrity and the redox state as well as protecting the liver microcirculation.[28]

Clinical manifestations of SALD

In septic patients, the spectrum of liver dysfunction may vary from subclinical to symptomatic liver failure. In critically ill patients the concept of "shock liver" may be used. "Shock liver" is a syndrome of hemodynamic, cellular, immunologic and molecular disorders.[29] SALD can manifest in 2 clinical forms – jaundice/sepsis-induced cholestasis, and hypoxic hepatitis (HH).[19] Coagulopathy may be another symptom of SALD. These processes are still not well understood due to the complexity of their pathomechanisms.

Jaundice/sepsis-induced cholestasis

The synthesis of bile is a complex process, requiring proper energy input and normal function of transmembrane proteins.[30] Energy shortage caused by hypoxemia/liver hypoperfusion may impair most bile synthesis steps.[31]

Liver histological examinations in patients with jaundice occurring during bacterial infection revealed the presence of intrahepatic cholestasis. The impairment of bile transport is a result of alterations in genes activating transcription and modifying posttranslational treatment of bile acids transporting proteins caused by LPS and proinflammatory cytokines.[32]

Laboratory test abnormalities include an increase of total bilirubin (>2 mg/dL), alkaline phosphatase, ALT, and AST.[33]

Hypoxic hepatitis

Hypoxic hepatitis (HH) may be the cause of fulminant hepatitis. In septic shock, an increase in blood flow and cardiac output is not enough to compensate increased hepatic oxygen demand.[34] Decreased hepatic blood flow in shock does not always cause HH; it may occur in patients with normal blood pressure.

Other risk factors causing HH are LPS and inflammatory cytokines. Hypoxic hepatitis may also be a result of the reoxygenation phase in the course of the ischemia/reperfusion phenomenon.

In the course of HH, except for a rapid (24 h from the onset of shock) substantial increase in aminotransferases and lactate dehydrogenase activity, an early decrease in serum prothrombin concentration is observed.[35]

Coagulopathy

A wide range of coagulopathy may be observed in sepsis, starting with mild deviation in laboratory results (prolonged clotting time, decreased number of platelets) to severe coagulopathy and/or disseminated intravascular coagulation (DIC).

The main cause of coagulopathy in sepsis is microvascular endothelial injury resulting in an imbalance between fibrinolysis and coagulation.[36] The changes seen in endothelial injury include loss of vascular tone, capillary obstruction by platelet or fibrin clots, as well as the degradation of heparan sulfate leading to a pro-coagulant state.[37]

Coagulopathy may be another symptom of liver disease. Several factors may contribute to hemostatic changes in liver disease (Table 1).[38]

Assessment of liver function

Diagnosis of liver dysfunction in sepsis, according to the Surviving Sepsis Campaign (SSC) Guidelines, is based on an increase in serum bilirubin concentration >2 mg/dL (34.2 μmol/L) and occurrence of coagulopathy (INR > 1.5).[39]

Currently, there are no specific biomarkers available that would allow for an early diagnosis of acute liver

damage in the course of sepsis/septic shock and distinguishing it from a pre-existing liver pathology. Liver function can be assessed using static and dynamic parameters.

Static parameters include:

– secretory capacity – bilirubin;
– parameters of cholestasis – alkaline phosphatase, γ-glutamyltransferase;
– intracellular enzymes activity – alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase;
– synthesizing capacity – albumin, clotting factors V and VII.[40]

Table 1. Factors contributing to hemostatic changes in liver dysfunction

Hemostatic changes in liver dysfunction	
promoting hemostasis	impairing hemostasis
• low plasminogen activity • decreased protein S, protein C and antithrombin activity • increased VWF activity • increased factor VIII serum concentration	• reduced hematocrit • thrombocytopenia • production of nitric oxide and prostacyclin • low serum concentration of coagulation factors II, V, VII, IX, X, and XI • dysfibrinogenemia • vitamin K deficiency • elevated activity of tPA • low serum concentrations of plasmin inhibitor, factor XII and TAFI

VWF – von Willebrand factor; TAFI – thrombin activatable fibrinolysis inhibitor; tPA – tissue plasminogen activator.

Table 2. Causes of hyperbilirubinemia in sepsis

1. Cholestasis
2. Hemolysis – drug-induced/infection-related • in normal RBCs • in RBCs with enzyme defects
3. Hepatic dysfunction • ischemia: hypotension, hypoxia • hepatocellular damage • bilirubin transport dysfunction: decreased uptake, canalicular transport, clearance

RBCs – red blood cells.

However, these parameters cannot be used for continuous and rapid monitoring of liver function in patients treated in ICU, nor are they diagnostic or prognostic in this group of patients.[40,41] As mentioned above, according to the SSC Guidelines, serum bilirubin concentration (>2 mg/dL or >34.2 μmol/L) is used as a single marker guideline to diagnose liver dysfunction.[39] Due to a number of drawbacks limiting its application, serum bilirubin is not an appropriate marker to reflect complex liver function. Increase in serum bilirubin concentration is neither specific nor does it allow acute liver dysfunction to be distinguished from a pre-existing liver pathology.[41] Table 2 shows causes of hyperbilirubinemia in sepsis.[33]

Dynamic parameters assessing liver function include:

– indocyanine green (ICG), caffeine and bromosulfophthalein clearance;
– liver detoxification capacity – measuring the concentration of $^{14}CO_2$ in exhaled air (measuring the concentration of [^{14}C]aminopyrine, [^{14}C]methacetin, [^{14}C]erythromycin metabolites) and measuring the concentration of lidocaine/midazolam serum metabolites;
– ability to eliminate galactose.[40]

Maximal liver function capacity

Kaffarnik et al., in their study published in Critical Care in 2013, attempted to assess the maximal liver function capacity (LiMax) test as a useful tool for early diagnosis of sepsis-associated liver dysfunction.[42] LiMax is a non-invasive breath test using ^{13}C-labeled methacetin, which is exclusively metabolized by cytochrome P450 (1A2) to $^{13}CO_2$ and acetaminophen. The idea of the test is to measure the amount of exhaled $^{13}CO_2$. The result is given as the LiMax value in μg per kg of body weight per hour. It shows the speed of substrate metabolism, thus allowing one to evaluate the metabolic capacity of the liver.

Table 3. Types of drug-induced liver injury. Examples of hepatotoxic drugs

Cellular (↑alanine aminotransferase)	Cholestatic (↑total bilirubin, ↑alkaline phosphatase)	Mixed
• Acetaminophen • Allopurinol • Amiodarone • Baclofen • Isoniazid • Ketoconazole • Lisinopril • Losartan • Methotrexate • NSAIDs • Omeprazole • Pyrazinamide • Rifampin • Statins • Tetracyclines • Valproate	• Amoxicillin/clavulanate • Anabolic steroids • Chlorpromazine • Clopidogrel • Oral contraceptives • Erythromycins • Irbesartan • Phenothiazines • Tricyclic antidepressants	• Amitriptyline • Azathioprine • Captopril • Carbamazepine • Clindamycin • Enalapril • Nitrofurantoin • Phenobarbital • Phenytoin • Sulfonamides • Trimethoprim/sulfamethoxazole • Verapamil

In the study, a pathologic deterioration of LiMax values in patients with septic shock was observed within 2 days after the onset of sepsis. Among patients with LiMax, <100 μg/kg/h the mortality rate was 55%, and with LiMax >100 μg/kg/h the mortality rate was 0%. The authors' conclusion was that LiMax values <100 μg/kg/h could be a good predictor of morbidity and mortality.

Indocyanine green clearance

Indocyanine green (ICG) is a non-toxic, water-soluble fluorescent dye. Its spectrophotometric evaluation does not depend on oxygen saturation and serum bilirubin concentration. Indocyanine green clearance (ICG PDR) can be used to reflect the liver function. Because it is not metabolized, it is secreted almost exclusively by the liver and it is not subject to enterohepatic circulation.[43]

A significant limitation in using ICG PDR is the hemodynamic condition of the patient, as ICG PDR depends on hepatic blood flow.[44] Additional parameters limiting the use of this test are the serum bilirubin concentration, serum albumin concentration, body weight, and patient's age.[45] Studies have shown that ICG PDR may be a diagnostic and prognostic tool in monitoring acute liver failure in critically ill patients in the ICU, but there are still no randomized control trials clearly confirming the utility of ICG PDR in daily clinical practice.[43]

It is worth underlining that in clinical practice there are no standardized diagnostic panels allowing for an early, clear diagnosis of acute liver dysfunction. Until now only a few studies have been published, and their results remain equivocal.

Therapeutic considerations

Currently, there is no specific therapeutic treatment available for the full restoration of damaged liver function. The therapy, according to the SSC Guidelines, should focus on eradicating infection and treating sepsis and its complications.[39] Furthermore, there are tools available that could reduce the risk of further damage to this organ.

These include:

1. avoiding potentially hepatotoxic drugs;
2. early enteral feeding of hemodynamically stable patients;
3. glucose concentration monitoring and adequate glucose supply if necessary;
4. extracorporeal liver support – Molecular Adsorbent Recirculating System (MARS) albumin dialysis, single-pass albumin dialysis (SPAD).[19,46,47]

Drugs are an important cause of liver injury. Examples of potentially hepatotoxic drugs are given in Table 3.[48]

La Mura proposed simvastatin administration for the prevention of LPS-induced intrahepatic endothelial dysfunction. He found that prophylactic simvastatin prevents endotoxemia-induced liver injury, reduces liver inflammation, and prevents microvascular dysfunction in rodents.[49] In other studies, prophylactic simvastatin has also been reported as being able to correct endothelial dysfunction.[50]

Summary

The incidence of sepsis-associated liver failure is hard to estimate, but it is incontestable that liver failure as a complication of sepsis dramatically worsens the outcome of the patients. It is important to remember that during sepsis not only the infection itself is responsible for liver dysfunction, but also hyperreactivity of the inflammatory response, microcirculatory failure, and side effects of the therapy. Only an early diagnosis of sepsis and its complications as well as quick implementation of therapeutic bundles allow reducing the incidence of severe organ complications, to shorten the hospitalization time and improve patients' quality of life.

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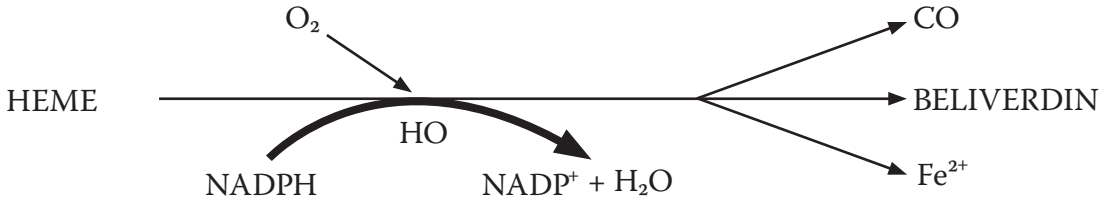

Fig. 1. Heme degradation products

HO – heme oxygenase; CO – carbon monoxide; NADPH – r educed form of nicotinamide adenine dinucleotide phosphate; NADP+ – nicotinamide adenine dinucleotide phosphate.

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Injectional anthrax in human: A new face of the old disease

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Abstract

Unusual human behavior leads to the emergence of new forms of infectious diseases and new routes of infection. In recent years, a new form of anthrax, called injectional anthrax, emerged and was related to 2 human anthrax outbreaks in Europe. The infection was caused by heroin contaminated with anthrax spores. The new form of anthrax differs from the earlier known "natural" forms of the disease in symptoms, length of the incubation period and recommended treatment. Despite medical treatment, the mortality rate in injectional anthrax is about 35%. This article presents an overview of the forms of anthrax infection in humans, with focus on injectional anthrax syndrome, as well as actual recommendations for treatment, including antibiotic therapy, surgery and possibilities of administering anthrax antitoxin. As a source of contamination of heroin have not been identified and new cases of injectional anthrax might occur again in any country in the future.

Key words: treatment, anthrax, drug users, soft tissue infection

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Anthrax is an animal and human disease caused by *Bacillus anthracis* – a Gram-positive bacterium that produces spores extremely resistant to many (broad spectrum) physical and chemical factors, such as drying, heat, gamma radiation, ultraviolet light, various pH, and many disinfectants.[1,2] Thus, the spores may remain viable and infectious in the environment for decades. Wilson and Russell proved that anthrax spores have been still able to germinate after 60 years of storage in soil samples under laboratory conditions.[3] In the Kruger National Park, *B. anthracis* has been recovered from bones estimated to be approx. 200 years old.[4] Germination of anthrax spores occurs usually within the infected host producing the vegetative forms of the bacteria. However, Saile and Koehler revealed that anthrax spores may germinate in the rhizosphere and around grass plants roots.[5] Favorable conditions for anthrax spore germination might include soils rich in calcium and organic matter with a pH above 6.0 and temperature of soil above 15°C.[6]

Vegetative *B. anthracis* cells within the infected host multiply rapidly, eventually killing the host.[1] Concentration of anthrax cells in blood from carcasses can reach 10^9 CFU/mL. The microorganisms enter soil and water during terminal hemorrhaging from the rectum, nostrils or mouth of the animal carcass or upon carcass destruction by scavenging carnivores.

The low CO_2 level in open air, when compared with the level in tissue, induces sporulation that enables transformation of *B. anthracis* vegetative cells into the highly resistant spores. The spores are an infective form of the bacterium. Consequently, human can be infected via contact with contaminated soil, infected animals and infected or contaminated animal products.[1,2]

Apart from natural outbreaks, anthrax is considered to be "a bioterrorism agent." Although natural anthrax outbreaks are rare in developed countries, bioterrorism events may currently occur at any time, in any form (e.g., anthrax letters in 2001). The rarity of the disease makes it difficult to diagnose anthrax cases by clinicians and in laboratory diagnostics conducted by clinical microbiologists.[7]

The detection of *B. anthracis* in clinical and environmental samples can be conducted using conventional and molecular tests. In blood or other body fluid samples (including fluid from cutaneous lesions), or tissue specimens, *B. anthracis* is readily visualized in capsule-stained smears and readily isolated in pure cultures. Cultured microorganisms are identified based on Gram stain, colony morphology, a motility test, a capsule production test, and a γ phage plaque assay. Also, matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) might be used to identify cultured *B. anthracis* provided that a database dedicated to bioterrorism agents is available. Molecular tests, such as polymerase chain reaction (PCR) and real-time PCR, can be used for anthrax detection in clinical and environmental samples as well as for cultured bacteria.[1,7] Moreover, rapid tests based on specific antibodies, such as lateral flow immunochromatographic (LFI) assay for anthrax detection, have been developed. But the sensitivity and specificity of the rapid tests are not satisfying.[8] Recently, Cox et al. described modified LFI combined with γ phage amplification that might increase the sensitivity and specificity of the assay.[9]

Forms of anthrax infection in human

Dependent upon the route of infection, 3 primary forms of human anthrax are recognized: cutaneous, pulmonary (inhalational) and gastrointestinal. Septicaemia and hemorrhagic meningitis may complicate all 3 forms. Cutaneous anthrax developed when anthrax spores get into the skin, usually through a scrape, cut, abrasion or insect bite. More than 90% of the lesions occur on exposed areas, such as the face, neck, arms or hands. Following the infection, the incubation period can range from 1 to 19 days but is usually 2–7 days. Lesion begins as a pruritic papule which enlarges and is surrounded by a ring of vesicles 2–4 days post infection. The vesicles may contain a hemorrhagic exudate. This area is also surrounded by a small ring of erythema and marked edema develops which can extend to some distance from the lesion. The lesion is usually 1–3 cm in diameter and remains round and regular. Rarely, a lesion may be larger and irregularly shaped. Unless a secondary infection occurs, there is no pus or local pain, although painful lymphadenitis may occur in the regional lymph nodes. Eventually, the vesicle or vesicular ring ruptures, discharging a clear fluid, and a central depressed black necrotic lesion, known as an eschar, is formed. The eschar begins to resolve about 10 days after the appearance of the initial papule. Resolution is slow (2–6 weeks), regardless of treatment.[10–15] Cutaneous anthrax is the most common form of *B. anthracis* infection. The mortality rate of this form without medical treatment is estimated to be 20%. When appropriate treatment is applied, the mortality rate is reduced to 1%.[1] However, when the lesion is located on the face, neck or chest, clinical symptoms may be severe, toxic and fatal.[1,2,10]

Inhalational anthrax occurs when a person inhales anthrax spores. This form is regarded as the most deadly form of anthrax. Only about 10–15% of patients with pulmonary anthrax survive without treatment. However, about 55% of patients survive with aggressive treatment.[16] The incubation period of this form of the disease is generally considered to be 1–6 days. However, during the largest outbreak of inhalational anthrax in Sverdlovsk in 1979, a mean incubation period of approx. 10 days was reported with some cases taking up to 43 days.[17] The initial symptoms are nonspecific and with a clinical picture similar to that of a typical pneumonia from other causes and cardiovascular collapse with noninfectious causes. The symptoms begin with a mild fever, fatigue, malaise, myalgia, a non-productive

cough, and some chest or abdominal pain. The disease progresses rapidly, and within 2–3 days the second phase is characterized by high fever, toxemia, dyspnea, and cyanosis. Hypothermia and shock are the ultimate causes of death. In up to half of the patients, meningitis develops as a complication. In radiographic examination, mediastinal widening is a characteristic finding but is nonspecific for inhalation anthrax. Less specific findings include pleural effusions and parenchymal infiltrates.[10,12–15]

Gastrointestinal form of anthrax is a result of consumption of contaminated food (usually meat) or drinking contaminated water. It can present clinically as either oropharyngeal or intestinal infection. Typically, the incubation period is 1–6 days. Oropharyngeal anthrax is characterized by sore throat, mucosal ulcerations, soft tissue edema, enlargement of cervical lymph nodes, and dysphagia. Intestinal anthrax is caused by infection of the bowel or stomach. The symptoms of intestinal anthrax are initially non-specific and include nausea, vomiting, anorexia, mild diarrhea, and fever. In some cases, the clinical picture may become more severe 24 h after the initiation of symptoms, and may include acute diarrhea, nausea, vomiting, and abdominal pain. With the progression of the illness, abdominal pain, hematemesis, bloody diarrhea, and massive ascites occur, and signs suggestive of acute abdomen appear. Toxemia, sepsis and shock then develop, followed by death.[10,12–15] Without treatment, the mortality rate in this form is about 50%.[15]

All the above anthrax forms have been known to exist since ancient centuries and could be a result of natural infections as well as a bioterrorism related events. In the 21st century, a new form of anthrax appeared in humans – it has been called injectional anthrax.

Injectional anthrax syndrome

The term 'injectional anthrax' has been proposed by Ringertz et al., when the first case of heroin related anthrax was described.[18] Injectional anthrax is presented by a severe soft tissue infection at the injection site, such as cellulitis, abscess or necrotizing fasciitis. The infection is very often complicated by septic shock and meningitis.[18–20] Besides antibiotic treatment, a surgical debridement is often necessary in treating the infection.[21,22] Despite medical treatment, the mortality rate of this form of anthrax is about 35%.[19,23,24]

Important differences between injectional and cutaneous anthrax have included lack of black eschar formation typical for cutaneous form and an increased risk of shock along with a significantly higher mortality rate.[16,20,24]

Injectional anthrax is transmitted by intravenous, subcutaneous or intramuscular injection of contaminated heroin. The incubation periods are estimated from a day (or less) to 10 days or more. But the incubation periods are only approximations because of the uncertainty about which dose of the drugs was contaminated. This was estimated based on the timing of the last injection at an infected site. It must be kept in mind that the actual incubation periods might be longer by an unknown margin. The illness duration ranged from less then a day to over 28 days, and was calculated as the interval between the initial hospital admission and patients discharge home (or death). The longest duration of illness was related to necessity of expensive debridement of damaged tissue and its consequent reconstructive plastic surgery.[25]

According to Interim Clinical Guidance for the Management of Suspected Anthrax in Drug Users, published by National Services Scotland and Health Protection Scotland, any drug user who presents with severe infection of soft tissue, signs of meningitis or severe sepsis (even without evidence of soft tissue infection), signs and symptoms of gastrointestinal or inhalational anthrax, should be considered as a possible case of anthrax.[26]

Injectional anthrax cases in Europe

The first case of injectional anthrax was described in 2000 in Norway.[19] The biggest outbreak of this new anthrax form started in December 2009 in Scotland. Between December 2009 and December 2010, a total of 119 cases were reported in Scotland, 5 cases in England and 2 cases in Germany. No further cases were documented until June 2012, when cases reemerged in Scotland, England, Germany, and were diagnosed for the first time in France, Wales and Denmark. Between June 2012 and April 2013, a total of 15 cases were recorded.[23,25]

Non-injectional anthrax in heroin users

During the anthrax epidemic among heroin users in 2009–2013, cases of systemic anthrax were also noticed as a consequence of inhaling heroin. Snorting or smoking contaminated heroin would allow viable spores of *B. anthracis* to penetrate into the upper and lower respiratory tracts and into the gastrointestinal tract. Inhalation or ingestion of anthrax spores might result in systemic illness. Systemic anthrax had fulminant course and usually results in death.[25]

Genetic relatedness of *B. anthracis* from heroin users

Genetic relatedness of *B. anthracis* from heroin users were investigated using high resolution genotypic methods: single nucleotide polymorphism (SNP) analysis and multilocus variable-number tandem repeat analysis (MLVA) for analysis of 22 and 31 markers, respectively. All strains isolated between 2000 and 2012 shared the same 22 SNPs.

Moreover, all the strains possessed the 2 highly distinctive „heroin-specific" SNPs, which differentiate the isolates from other investigated strains belonging to the same genetic group.[19,27] MLVA typing of the isolates from heroine users revealed differences only in 2 markers: Bams30 and pXO2, which are highly mutable. No one of the isolates was different in both of the markers.[19] Deviation in only 1 highly mutable marker are frequently observed among isolates originated from a defined endemic region.[28] In the putative evolutionary analysis such isolates are clustered into a single complexes. Such complexes of highly related genotypes can be regarded as the same outbreak strain based on the investigation of outbreak scenarios and analysis of epidemiological data.[19,27] In the investigated outbreak, it was concluded that all the isolates from heroin users are of a single strain, originating from a single source of the infection. Moreover, this single source could be even a single infected animal.[25]

However, recently published results of whole genome analysis of *B. anthracis* isolates associated with injectional anthrax revealed 2 tight genetic clusters: one group (G-I) was exclusively associated with the 2009–2010 outbreak and located primary in Scotland, whereas the other (G-II) comprised more recent (2012–2013) cases but also a single Norwegian case from 2000. The level of genome variation between these 2 groups is frequently seen within a single country and could be indicative of a close geographic relationship for the 2 sources of contamination. Whether each group represents a single contamination or multiple contaminations from a single source is hard to determine. But the 10-year lapse in G-II injectional anthrax cases suggests that there is a single contaminating source for the G-II cases but that there have been 2 or more contamination events.[29]

Source of contamination

It is highly speculative how the heroin was contaminated. At least 3 possibilities are mentioned: via addition of cutting agent derived from an animal, such as bone meal, via wrapping in animal hide contaminated with anthrax spores, or via contamination with soil during drug manufacturing and trafficking.[25,29,30] At the beginning of the anthrax epidemic in Europe, it was suspected that the heroin contaminated with anthrax spores came from Afghanistan, where it was contaminated at the primary source, for example during raw heroin production, as Afghanistan produces 90% of the world's heroin.[29] However, the isolates from heroin users were excluded by genotyping from the Vollum branches strains commonly found in Afghanistan and Pakistan.[25] It was also shown that the heroin was not contaminated in the final destination (Scotland/UK/Europe). The investigation conducted by Price et al. revealed that *B. anthracis* strain from infected heroin users in different European countries is closely related to anthrax isolates previously found in infected animals (goats) in Turkey.[29] Turkey is located on the Balkan Route, which is commonly used for trafficking heroin from Afghanistan into European countries. It is believed that Turkish laboratories play a significant role in the conversion of the morphine base into usable form of heroin.[29] Moreover, it is known that for transport of illegal heroin, animal skins (particularly goat skins) are frequently used. Contamination with *B. anthracis* spores from goat skin is, therefore, one of the possible explanations for the origin of the anthrax spores in the heroin.[25,30] Thus, it was hypothesized that Turkey is a point of origin of the heroin contamination. But it must be kept in mind that isolates from several countries located on the major routes for trafficking heroin were not included in the conducted investigation. There is also a second route for trafficking heroin, called the Silk Route, which passes through many anthrax endemic countries where isolates, belonging to the same genetic group as *B. anthracis* from heroin users, have been found.[29] Nevertheless, molecular analyses provided evidence that a similar source of heroin contamination, resulting in injectional anthrax in heroin users, could have been active at least since the year 2000.[19,30]

Treatment

Initiation of appropriate treatment, particularly administration of a combination of antimicrobial drugs, as soon as possible, is critical to improving the survival of the patient. Treatment recommendations differ depending on the clinical form of anthrax.

Uncomplicated cutaneous anthrax should be treated with fluoroquinolones (ciprofloxacin, levofloxacin or moxifloxacin) or doxycycline orally. Clindamycin is an alternative option if fluoroquinolones and doxycycline are contraindicated or unavailable. Also, treatment with penicillin or amoxicillin is an option, but only if the isolate is known to be susceptible to penicillin. Moreover, adequate dosages must be used because of the potential for developing drug resistance during treatment with subtherapeutic dosing. Typically, if naturally acquired, cutaneous anthrax is treated for 7–10 days. However, if bioterrorism-related or an aerosol exposure is suspected, patients should be treated for 60 days. This is because the patients are likely to have also inhaled spores and a potential for reactivation of latent infection may exist. It must be kept in mind that cutaneous anthrax with extensive edema, lesions on the head or neck, or signs of systemic involvement require intravenous therapy, a multidrug approach is recommended.[13,31,32]

In severe soft tissue infections in injectional form of anthrax, timely surgical debridement, to remove dead or devitalized tissue, is the most important treatment because it would enable the removal of the primary source of toxin production. When extravascular fluid collections

are present, drainage may also be important. Empiric antibiotic treatment should be started to cover *B. anthracis*, as well as other microbiological agents commonly causing soft tissue infection. This treatment includes clindamycin and ciprofloxacin intravenously in combination with other antibiotics, such as metronidazole, penicillin and flucloxacillin (i.e., a 5 drug combination).[13,26]

It is worth underlining that surgery might be contraindicated or indicated, depending on the form of anthrax. For example, in cutaneous anthrax, surgery can lead to the dissemination and poor outcome. On the other hand, surgery may be indicated for gastrointestinal anthrax to identify and address potentially fatal complications, such as bowel ischemia, necrosis, and perforation.[32]

Disseminated anthrax, such as inhalational anthrax with meningitis, should be treated with clindamycin and ciprofloxacin intravenously in combination with at least 1 other active drug with adequate central nervous system (CNS) penetration, e.g., penicillin, vancomycin, rifampicin, imipenem, meropenem, chloramphenicol or gentamycin.[25] Moxifloxacin and levofloxacin are considered equivalent alternatives to ciprofloxacin. Clindamycin is recommended because of its potential ability to inhibit exotoxin production. Another protein synthesis inhibitor is linezolid. If clindamycin or linezolid are unavailable, rifampin has been widely used. Although rifampin is not a protein synthesis inhibitor, it reveals synergistic effect with a primary drug. Chloramphenicol is also a protein synthesis inhibitor that penetrates CNS and has historically been used to treat anthrax successfully. If meningitis is suspected, doxycycline should not be used because it does not adequately penetrate the CNS. Center for Disease Control and Prevention (CDC) recommends that intravenous combination treatment for systemic anthrax with possible meningitis should be provided for 2–3 weeks or until the patient is clinically stable, whichever is longer.[32] During the anthrax outbreak among injecting drug users in Scotland in 2009–2010, patients who have survived severe/systemic illness have been on appropriate antimicrobials for 3–4 weeks, initially intravenously (with 3 agents) and then orally with ciprofloxacin and clindamycin.[26]

Because toxin-mediated morbidity is a major complication of anthrax systemic infections, for inhalational anthrax associated with respiratory compromise, extensive edema, and meningitis, corticosteroids have been suggested as adjunct therapy.[31] Also, the role of anthrax antitoxin in treatment for systemic anthrax is worth mentioning. However, the therapy is still considered investigational. CDC recommends adding an antitoxin to the combination antimicrobial drug treatment for any patient for whom systemic anthrax is highly clinically suspicious. Whereas antimicrobials kill anthrax bacteria, the antitoxin neutralizes anthrax toxin circulating in the body, responsible for the severe illness. There are currently 2 antitoxins in the CDC Strategic National Stockpile: raxibacumab (GlaxoSmithKlaine, London, UK) and Anthrax Immune Globulin Intravenous (AIGIV) (Cangene Corporation, Winnipeg, Manitoba, Canada). Raxibacumab is approved by Food and Drug Administration (FDA) for postexposure prophylaxis and treatment for anthrax under the Animal Rule Summary. AIGIV is not FDA-approved and could be made available under an Investigational New Drug protocol or an Emergency Use Authorization during a declared emergency.[32]

Conclusions

Unusual human behavior enabled the development of a new form of the disease known from the ancient times. Interestingly, cases of injectional anthrax occurred at time intervals in 2000, 2009–2010 and 2012–2013. Molecular investigation suggested a similar source of anthrax contamination of heroin in all cases. Nevertheless, the source was not identified and destroyed. For these reasons, a new case of injectional anthrax might occur in the future in any country. Heroin exposure routes which are risk factors of injectional anthrax include: injection intravenously, injection intramuscularly, injection subcutaneously, smoking, snorting. However, people with anthrax infection brought on by smoking or snoring contaminated heroin developed generalized symptoms and not the local symptoms typical for injectional anthrax. Moreover, some cases exposed to contaminated heroin by injection presented with little or no localized signs of infection but with generalized symptoms indicating toxemia and disseminated infection.[25] For these reasons, in any drug user who presents severe soft tissue infection or signs of systemic disease, anthrax infection should be considered.

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Telocytes in the female reproductive system: An overview of up-to-date knowledge

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Abstract

Telocytes are emerging cell population localized in the stroma of numerous organs, characterized by a distinctive morphology – small cell body with very long, slender prolongations, termed telopodes. Those cells can be found in the whole female reproductive system: in the vagina, uterus, oviducts and ovaries, mammary glands and also in the placenta. In our review, we aim at complete and transparent revision of the current knowledge of telocytes' localization and function, enriched by the analysis of the possible future direction of development of their clinical applications. The function of telocytes in the reproductive system has not been fully elucidated yet; however, many researchers point at their role in the regulation of local microenvironment, myogenic contractile mechanism, bioelectrical signaling, immunomodulation and regulation of blood flow. Additionally, previous research suggests that telocytes might act as sex hormone level sensors and are connected with pregnancy maintenance. As the morphology and number of those cells change under pathological conditions, such as pre-eclampsia, endometriosis and ovarian failure, there is a chance that they may contribute to therapy of abovementioned conditions. The impact of telocytes on stem cells and angiogenesis has been proven in many organs, and may be useful in regenerative medicine of the female reproductive system. A recently found connection between the proliferation rate of breast cancer cells and stromal cells like telocytes might be a step forward to the management of mammary gland neoplasms.

Key words: ovary, uterus, placenta, telocytes, telopodes

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Introduction

Definition

Telocytes (TCs) are a new type of cells located in the stroma of several organs. Their most characteristic feature is the presence of extraordinarily long, slight prolongations called telopodes (Tps).[1] As far as the female reproductive system is concerned, TCs were described in the mammary gland, vagina, uterus, uterine tube, and placenta.[2–6]

A brief history

The history of TCs derives from Interstitial Cajal Cells (ICCs) discovered by the Spanish neuroanatomist and Nobel Prize winner Santiago Ramon y Cajal. In 1889, Cajal found a new type of cells located within the muscle layer of the intestine, between nerve ganglia and smooth muscle cells.[1]

About a half a century later, Faussone-Pellegrini et al. proved the existence of cells similar to ICCs in the gastrointestinal tract with the use of electron microscopy.[7] In 2005, Popescu et al. found interstitial cells resembling ICCs in the specimens from a mammary gland.[2] They called them Interstitial Cajal-like Cells (ICLCs). A re-examination of the muscular coat of the gut performed by Pieri et al. showed that ICLCs are functionally and morphologically different from ICCs.[8] As a result of these studies, in 2010 Popescu suggested that the name "Interstitial Cajal-like Cells" should be changed to "Telocytes" to clearly distinguish these 2 cell populations.

In the female reproductive system, TCs were first described in 2005, and they were found within the wall of the uterus.[4,9] Further research proved their existence also in the vagina, uterine tubes and placenta (Fig. 1).[3,6,10]

The name "Telocytes" derives from ancient Greek – the word "telos" refers the object of huge potential.[11] Nowadays, thanks to TCs' morphology and functions, they arouse huge interest among researchers as potential contributors to intercellular communication, cancerogenesis and a target for regenerative medicine purposes.[12–14] In our review, we are trying to summarize the current knowledge of tissue distribution, identification methods and potential functions of TCs in female reproductive system.

General aspects of telocytes

Cell phenotype

TCs are small cells with 1–5 long and thin prolongations named telopodes (Tps).[11] The cell body is rather small, its average length oscillates around 9.39 ±3.26 μm. It contains big, heterochromatic nucleus and a thin, perinuclear rim of cytoplasm including few organelles, mainly mitochondria.[11,15] The shape of the cell body is influenced by the actual number of Tps, ranging from piriform, through spindle and triangular to stellate.[16] Tps are up to 1000 μm long, which makes them one of the longest structures in the body, except for some axons.[15,17] As they are composed of dilated segments, named podoms, and thin podomeres, their shape is termed as moniliform.[15,16] Podoms contain organelles responsible for protein synthesis and intercellular signaling, namely rough and smooth endoplasmic reticulum, Golgi apparatus, mitochondria, and caveolae.[12,16] Considering the small size of TCs' body and even more narrow Tps (around 0.5 μm wide podoms), it would be utterly difficult to distinguish them from other cells in light microscopy, thus the golden standard for identifying TCs is transmission electron microscopy (TEM).[16,18]

Localization

Until now, TCs have been identified in the female reproductive system, placenta, mammary gland, cardiovascular system, urinary system, gastrointestinal tract, liver and pancreas, trachea, lungs and pleura, spleen, bone marrow, dura mater, choroid plexus, meninges, trigeminal ganglion, testicles and prostate, skin, skeletal muscles, and eyes.[16,19]

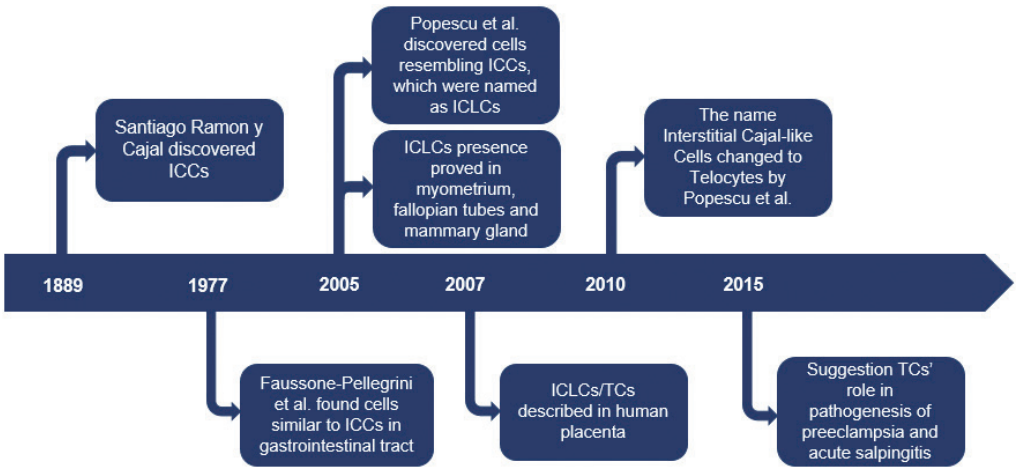

Fig. 1. A timeline of key discoveries in telocytes' research

Cell immunophenotype

Since numerous cells, for instance fibroblasts, neurons or pericytes, have a morphology closely resembling that of telocyte, it became apparent that there is a need for finding specific markers for TCs. Until now, TCs have been confirmed for expression of CD34, c-Kit, vimentin, PDGFR-α and -β, caveolin-1, CD44, Sca-1, Nanog and Oct4.[16,17,20]

Nevertheless, those markers are not perfect. There are a lot of discrepancies between studies in terms of CD34 and c-Kit expression in TCs in different tissues. From all abovementioned markers, CD34 is considered as the most reliable for TCs, although Suciu et al. reported that only 70% of the cells in the placenta, which have TC's morphology, express CD34.[20,21] Moreover, the second most frequently used marker, c-Kit, is not expressed by TCs in gastrointestinal tract.[22] That inconsistency might be caused by imperfections in technical procedures or the existence of tissue-specific subtypes of TCs.[18,21] Another difficulty is connected with the expression of those markers in cells with similar morphology – CD34 is expressed by endoneurial fibroblasts, while vimentin together with PDGFR-β are present in fibroblasts, pericytes and neurons.[18] In order to minimize the risk of misidentification, at least double immunofluorescence staining should be used in order to detect TCs. All things considered, a universal marker for TCs should be found – expressed in every tissue and characteristic only for them.

Intercellular communication

TCs establish both homo- and heterocellular junctions together with connections with extracellular matrix.[20] Moreover, they also communicate through extracellular vesicles, which is a form of juxtacrine/paracrine signaling.[23]

Homocellular junctions are formed between 2 Tps or between Tps and TC's body.[24] In most cases, they are connected by simple apposition of 2 plasma membranes, but there also exist more complex forms of linkage – puncta adhaerentia minima, processus adhaerens, recessus adhaerens, and manubria adhaerentiva. Their primary function is to maintain the integrity of 3-dimensional network of TCs during changes of shape of the tissue. Second presumptive function is linked with catenins, which are important components of adherens junctions, as they might play role in mechanosensing. The last type of homocellular contact is gap junction (nexus), which allows the exchange of ions and small molecules between adjacent cells.[20]

Heterocellular junctions typically have the morphology of point contacts, nanocontacts, planar contacts or simple apposition of plasma membranes.[20] TCs are connected through them with fibroblasts, myofibroblasts, pericytes, endothelial cell, neurons, stem cells, macrophages, mast cells, lymphocytes, plasma cells, Schwann cells, cardiomyocytes and smooth muscle cells.[16,21,24,25] Distinctive type of heterocellular junctions is stromal synapse, which links TCs with immune cells and cardiac stem cells.[24,26]

Extracellular vesicles shedded by TCs can be divided into 3 groups: exosomes (from endosomes), ectosomes (from plasma membrane) and multivesicular cargo (multiple, tightly packed endomembrane-derived vesicles).[23] Vesicles mainly contain proteins, lipids, miRNAs, mRNAs, and mtDNA.[12] Hence, they are crucial for intercellular signaling and they even might be connected with a modification of the post-transcriptional activity of recipient cells.[27] Fibronexuses and focal adhesions were found between TCs and components of extracellular matrix.[20]

Electrophysiological properties of telocytes

Since TCs form an abundant network interconnecting numerous types of cells in the interstitium, a hypothesis was postulated that TCs may be involved in bioelectrical signaling. Thus far, it has been confirmed that TCs express: T-type calcium channels ($Ca_v3.1$ and $Ca_v3.2$), small-conductance calcium-activated potassium channels (SK3), large-conductance calcium-activated potassium channels (BK_{Ca}), inwardly rectifying potassium channels (IK_{ir}) and calcium-dependent hyperpolarisation-activated chloride inward channels.[28,29,30,31]

L-type calcium channels, transient outward potassium channels (I_{to}) and ATP-sensitive potassium channels (K_{ATP}) were not detected in TCs in previous studies.[30,31]

Physiological functions of telocytes

At the present moment, TCs are considered as cells with a rather mysterious function, as there is still a lack of direct evidence for their actual properties. The most frequently proposed one is connected with their role in intercellular signaling – they might be responsible for the integration of signals from numerous systems (for instance nervous, vascular, and immune), regulation of tissue homeostasis and long distance communication including bioelectrical signaling.[16,32] Secondly, since it is proven that TCs are located in close proximity to stem cells, they may be crucial for tissue regeneration and repair.[33] Moreover, TCs build a scaffold, which probably enables the maintenance of proper organization of extracellular matrix and cell migration, also during organogenesis process.[34,35] Additionally, TCs presumably have angiogenic properties thanks to vascular endothelial growth factor (VEGF) expression and they may be vital to anti-oxidative protection, as they are abundant in mitochondrial superoxide dismutase (SOD2).[36,37]

Telocytes in female reproductive system

Telocytes in vagina

Proto-oncogene c-Kit positive cells with long processes, which nowadays might be classified as TCs, were found in muscular layer of the human vagina. Shafik et al. suggested their potential role in generating slow waves resulting in the contractility of smooth muscle cells in the vagina.[3] However, their presence and role in that organ should be thoroughly assessed in the future.

Telocytes in uterus

In the human uterus, TCs were observed in the interstitial space of the endometrium and myometrium.[38,39] Endometrial TCs were detected in the stroma of the stratum functionalis and stratum basalis around the endometrial glands, while myometrial TCs form a 3-dimensional network intermingling with smooth muscle bundles.[1,38] That location in the myometrium may suggest that TCs might be involved in myogenic contractile mechanism during sperm transport prior to fertilization, embryo implantation and delivery.[1]

The quantity of TCs in the endometrium and the myometrium correlates with the reproductive state.[40] Immature rat uteruses present the lowest density of TCs compared to pregnant and postpartum ones.[41] The number of endometrial TCs in pregnant state increases, while there is a reduction of their number in the myometrium.[40] Hence, changes in the quantity of TCs in the myometrium may be associated with the prevention of preterm delivery.[25] Moreover, the highest number of myometrial TCs were found in the postpartum uteri, which may be related with its involution.[40]

Additionally, the morphology of Tps is different in pregnant and non-pregnant myometrium – in non-pregnant uterus podomers are wider and podoms are thinner compared to pregnant ones (Table 1).[31]

The most useful markers used for the identification of TCs in the uterus are CD34 and PDGFR-α/PDGFR-β in double immunolabelling.[25] PDGFR-α/β is mostly expressed at TC's cell body, while Tps are intensely positive for CD34.[28] TCs in uterus are also positive for α-SMA, CD44, vimentin, Sca-1, and c-Kit.[41] Nevertheless, the expression of c-Kit here is questionable. Yang et al. detected various types of TCs in uterine samples: c-Kit(–)/vimentin(+), c-Kit(+)/vimentin(+) and c-Kit(+)/CD34(+). As a consequence, they suggested the presence of various subpopulations of TCs in uterus which may perform disparate functions.[42]

The comparison of markers applicable to the detection of TCs in female reproductive system is shown in Table 2. TCs also express connexin43, a gap junction protein which might play an essential role in decidua maturation. A decrease in the expression of that protein is linked with recurrent pregnancy loss.[41,43]

Furthermore, the expression of estrogen receptor alpha (ERα) and progesterone receptor A (PR-A) were confirmed on the surface of uterine TCs.[44] This suggests that TCs may be the sensors of sex hormone levels. TCs also express these receptors in uterine tubes and upper lamina propria of the human urinary tract.[45,46]

Additionally, human uterine TCs express 2 types of channels at their cell membrane: T-type calcium channels ($Ca_v3.1$, $Ca_v3.2$) and small-conductance calcium-activated potassium channels (SK3).[25]

The presence of $Ca_v3.1$ and $Ca_v3.2$ channels were confirmed both in cell body and Tps of TCs. Expression of $Ca_v3.1$ and $Ca_v3.2$ channels correlates with the reproductive state: $Ca_v3.1$ expression in pregnant and non-pregnant

Table 1. The variation in morphology of Tps and extracellular vesicles between non-pregnant and pregnant myometrium[31]

Parameter	Non-pregnant myometrium	Pregnant myometrium
Podom thickness [nm]	268.6 ±8.27	316.38 ±17.56
Podomer gauge [nm]	81.94 ±1.77	75.53 ±1.81
Number of exosomes/ shedded microvesicles	26/89	20/168
Diameter of extracellular vesicles [nm]	58–405 average 151	65–362 average 170

Table 2. Expression of markers on TCs in individual parts of female reproductive system

Organ	Marker							
	CD34	c-kit	vimentin	PDGFR-α	PDGFR-β	ERα	PR-A	Additional markers
Vagina	n/d	+	n/d	n/d	n/d	n/d	n/d	n/d
Uterus	+	+/–	+	+	+	+	+	α-SMA CD44 Sca-1
Uterine tubes	+	–	+	n/d	n/d	+	+	n/d
Ovaries	+	n/d	+	+	+	n/d	n/d	n/d
Mammary gland	+	+/–	+	n/d	n/d	n/d	n/d	CD10
Placenta	+	+	+	n/d	n/d	n/d	n/d	caveolin1

ERα – estrogen receptor α; PR-A – progesterone receptor A; n/d – no data.

state is equal, while $Ca_v3.2$ channels are mostly detected in pregnant myometrium.[25] Moreover, in non-pregnant state $Ca_v3.1$ were strongly expressed in Tps, while $Ca_v3.2$ were observed only in cell body. The differences in expression of these channels suggest that TCs may play role in detection of mechanical stretching of the pregnant uterus.[28]

SK3 channels were observed in the uterus of several species (for instance in human, mice or rat). Their expression is also correlated with pregnant or non-pregnant state. The density of SK3 channels in non-pregnant myometrium is elevated where they are present on TCs and vascular endothelium, in contrast to pregnant state with decreased number of channels, which are only located on vascular endothelium.[47] Downregulation of SK3 channels during pregnancy probably reduce contractility of the uterus. Changes in the expression of SK3 channels could also be caused by different stage of the hormonal cycle (expression of channels influenced by sex hormones) or diseases connected with hormonal imbalance.[29]

Uterine TCs create connections with other cells and components of extracellular matrix (for instance collagen or elastic fibres). Heterocellular junctions between TCs and smooth muscle cells, nerve endings and blood vessels were additionally observed.[48] TCs in uterus can also communicate by shedding membrane microvesicles: exosomes and ectosomes. No differences were noticed in number and diameter of shedded vesicles in pregnant myometrium compared with non-pregnant (Table 1).[25] Moreover, Diaz-Flores et al. proved that TCs have endocytic properties, which suggest existence of bidirectional information exchange between TCs and neighbouring cells.[49]

Additionally, TCs establish contacts with immune cells, namely lymphocytes, eosinophils, basophils, plasma cells, and macrophages.[38] Chi et al. confirmed that TCs are able to activate peritoneal macrophages, which may result in increased amount of IL-6, IL-10, IL1R1, TNF-α and iNOS. Pathologically high levels of these proteins could lead to implantation failure, immunologically-mediated abortion or endometriosis.[50]

TCs may also be useful in uterine regenerative medicine. Recent studies revealed that TCs from pregnant and non-pregnant myometrium have different reactivity to low-level laser stimulation (LSSS). Tps from pregnant uterus are more susceptible to extension after using LSSS compared to Tps from non-pregnant uterus. These differences are probably caused by variations in TCs' cytoskeleton structure (up-regulation of integrins) in pregnant and non-pregnant state.[51]

Telocytes in uterine tubes

TCs are located in lamina propria and muscular layer of uterine tubes. Their Tps create a 3-dimensional network between smooth muscle cells (SMCs), nerve endings and blood vessels. Close relations between TCs and SMCs may suggest that TCs participate in uterine tube contractility.[52]

TCs in the uterine tube present a typical cell phenotype. They shed 3 types of extracellular vesicles: exosomes (diameters: 60–100 nm), ectosomes (100–250 nm) and microvesicles (250–350 nm).[52] Similarly to the uterus, they express on their surface PR-A and ERα receptors, which might be connected with the control of peristaltic movements in the uterine tube due to changes in estrogen (acceleration of contractions) and progesterone (deceleration of contractions) levels.[1]

TCs are connected with other cells located in the uterine tubes tissue. Heterocellular junctions with fibrocytes, pericytes, SMCs, nerve endings, mast cells and stem cells were observed.[42] Yang et al. claimed that Tps are located in close vicinity to lymphocytes and plasma cells, which, according to their study, may suggest that TCs are involved in the stimulation of plasma cells to antibodies synthesis. However, the aforementioned property requires further investigation.[53]

Yang et al. proved that TCs' quantity and ultrastructure dramatically change in rat model of acute salpingitis. TCs retrieved from salpingitis-affected uterine tubes presented numerous abnormalities, such as: loss of organelles, cytoplasmic vacuolization, dilatation of rough endoplasmic reticulum and loss of intercellular junctions. Additionally, the number of TCs was significantly decreased.[53] The declined quantity of TCs, which is probably caused by the overproduction of iNOS, COX-2, LPO and estradiol, damages those cells and were also observed in pelvic endometriosis and tubal ectopic pregnancy.[40,42,54] Nevertheless, TCs of nearly normal appearance can be found even in endometriosis-affected uterine tube. Presumably, that is the reason why some women in this state have only reduced fertility instead of complete infertility.[42]

Telocytes in ovary

TCs were detected in the stroma of mice ovaries and they were positive for CD34, vimentin, PDGFR-α and -β. Thus far, the function of TCs in ovaries has not been determined; however, there is an assumption that they might be responsible for maintaining the local microenvironment.[55]

Liu et al. found statistically a significant decrease in the number of TCs in ovaries affected by cyclophosphamide-induced premature failure compared to healthy controls. As a consequence, they might be used as a marker of declining ovarian functions caused by the intake of cyclophosphamide.[55]

Telocytes in mammary gland

The existence of TCs in the mammary gland was confirmed in 2005 and they present morphology which is typical for them. They form a network of inactive mammary gland in the stroma, mainly in the interlobular space and also in the interlobular area, though in very small quantities. TCs closely encompass capillaries and mammary

gland ducts, predominantly by their Tps located perpendicularly to long axes of those structures.[56]

In mammary gland, TCs are positive for CD34 and vimentin.[57] However, there is an incongruity between the studies in terms of expression of c-kit – Gherghiceanu et al. along with Mou et al. confirmed the presence of that marker, while Petre et al. negated its expression.[56–58] Additionally, TCs in mammary gland might be partially positive for CD10.[58]

TCs are connected by stromal synapses with stromal immune cells, such as plasma cells, lymphocytes, mast cells and macrophages. Contacts with fibroblasts were also observed.[56] The direct link between TCs and endothelial cells or pericytes have not been found.[13]

Apart from the possible involvement of TCs in the organization of properly functioning structure of mammary gland, they may play an important role in the modulation of an immune system, thanks to their connections with immune cells.[56]

As accurate arrangement of stroma has a beneficial effect on maintaining local microenvironment, any alterations in the function of TCs' network may lead to an increase in the risk of neoplastic process in the tissue.[13] Mou et al. investigated the influence of TCs and other stromal cells on the growth dynamic of breast cancer.[57] They observed that TCs establish membrane-to-membrane connections with breast cancer cells in co-culture and presumably participate in the formation of neoplastic cell clusters. There was an increase in the proliferation index and a reduction in the apoptosis ratio among breast cancer cells accompanied by stromal cells, compared to isolated breast cancer cell culture.[57] Furthermore, the number of heterocellular junctions formed by TCs is diminished in neoplastic tissue.[13] Summing up, TCs along with other stromal cells may contribute to neoplasm development and survival.[57] As a consequence, TCs might emerge as a novel target in breast cancer therapy.

Telocytes in placenta

Placental TCs were detected by Suciu et al. in the large and peripheral stem villi, where they are located just beneath the trophoblast. Their Tps are orientated parallelly to the basement membrane and circularly or longitudinally to blood vessels.[21,40] They are positive here for: c-kit, CD34, vimentin, caveolin1, VEGF and iNOS.[25]

TCs create heterocellular contacts with mast cells, myofibroblasts, SMCs and specific placental macrophages, called Hofbauer cells (HBCs).[25] However, the function of TCs in human term placenta is still unknown. The presence of junctions between TCs and HBCs suggest their possible contribution to immune surveillance. Considering the fact that placenta is not an innervated organ, Bosco et al. suggested that TCs may be crucial for signal transduction resulting in blood flow in foetal vessels and aetiopathogenesis of pre-eclampsia.[59]

Conclusions

TCs are a unique cell population, located in the stroma of numerous organs, also in the female reproductive system. Their role specific for that system can be connected with their involvement in muscular layer contractility, pregnancy maintenance, immunomodulation and tissue regeneration. Alterations of their number in the female reproductive system might be connected with pre-eclampsia, endometriosis or acute salpingitis and further research on that subject may lead to a turning point in TCs-related treatment of those conditions.

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Gastrointestinal complications in patients with diabetes mellitus

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Abstract

Diabetes mellitus is a systemic disease which affects patients of various age. Hyperglycemia induces damage of vascular endothelium, development of chronic inflammation, organic and functional lesions in several systems and organs. The principal gastroenterological complaints linked to the manifestation of the disease include abdominal pain, diarrhea, nausea, flatulence, and vomiting. However, complications in the alimentary system may manifest exclusively by difficulties in reaching normoglycemia and numerous persistent episodes of hypoglycemia. The most frequent complication of diabetes mellitus affecting the alimentary tract involves gastroparesis and disturbances in pancreatic function. Diabetes may also aggravate other coexisting diseases, such as gastroesophageal reflux or periodontitis. Subject-based references accentuate also a significantly more frequent manifestation together with diabetes of other autoimmune diseases, such as celiac disease or autoimmune gastritis. Also, a hepatic microangiopathy and increased incidence of certain tumors, linked to the manifestation of insulin resistance, may be regarded to represent complications of long-term diabetes. Rapid diagnosis and adequate treatment may significantly improve a patient's quality of life and influence the prolonged control of glycemia. Nevertheless, this requires a rigorous analysis of the signs and clinical condition of a patient as well as individualization of recommendations and therapy.

Key words: diabetes mellitus, gastrointestinal disorders, insulin resistance

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Diabetes mellitus (DM) represents a disease affecting an increasingly large group of people. Around 415 million people worldwide are estimated to be affected and by 2040 the number of such patients is thought to reach around 642 million.[1] The principal hazard for the patient linked to the disease involves chronic complications of micro- and macropathy character: they induce severe organic dysfunctions and may lead to a sudden death of the patient. Troublesome, even if usually not linked to direct hazard of death, are alimentary disturbances. They used to develop in patients with long-lasting diabetes mellitus, frequently uncontrolled and metabolically unbalanced for many years. The abnormalities may affect practically any fragment of alimentary system. Signs/symptoms such as nausea, vomits, disturbed swallowing, abdominal pain, disturbed absorption, diarrhea, obstipation may represent prodromes of another, coexisting disease or they may develop in the course of metabolically uncontrolled diabetes mellitus.

The link with diabetes mellitus manifests also more frequently in developing infections, functional and organic disturbances and some tumors. Moreover, poorly controlled diabetes may result in lesions in central and peripheral nerve fibers, which renders relevant signs/symptoms variable and their diagnosis, therefore, poses a challenge for physicians-practitioners.

Diseases in oral cavity as an index of metabolically unbalanced diabetes mellitus

Diabetes mellitus results in the appearance of problems already in the preliminary fragment of alimentary tract. The main diseases in the oral cavity include fungal infections and periodontal pathology. Prodromes of such diseases include inconveniences, such as dry oral cavity, reddening and hyperemia of throat, and atrophic lesions on the tongue. The most frequent infections of the oral cavity include candidiasis, accounting for 40–60% infections of the oral cavity. Studies conducted by Shenoy et al. demonstrated significantly more frequent infections with *Candida* in groups of patients with diabetes type 1 (DM1) – 30% or diabetes type 2 (DM2) – 33% infections, as compared to diabetes-free patients – 7% infections.[2] The values of CFU/mL were also significantly higher in the groups of DM1 and DM2 than among healthy individuals. The study demonstrated also a significant positive relationship between CFU/mL on one hand and fasting glycemia and HbA_{1c} level, on the other.[2] The course and intensity of candidiasis reflect also the intensity of disturbances in carbohydrate metabolism. In patients with a pre-diabetic condition, candidiasis manifested a statistically more severe course (evaluated on the basis of standard laboratory techniques and by dental defects) than in healthy patients.[3] Another disease of the oral cavity which used to affect diabetic patients involves periodontitis. It develops 2.6 times more frequently in diabetic patients than in the healthy population, and in persons with a metabolically unbalanced diabetes this incidence continues to increase (up to 2.9-fold higher one).[4,5] In parallel, it can be concluded that the relationship is bilateral: diabetes aggravates periodontitis and makes it more difficult to cure, while the chronic inflammatory process intensifies disturbances in carbohydrate metabolism.[6] In the meta-analysis by Wang et al., conducted on the basis of studies including around 1,135 persons, it was confirmed that after 3 months of intense treatment targeting periodontal diseases, reduced values of HbA_{1c} were detected.[7] Similar results were obtained by Białecka et al., who recorded a significant reduction in HbA_{1c} 1 year following tonsillectomy.[8] The studies confirmed the need for systematic dental control and intense treatment of any inflammatory foci in the oral cavity in order to gain metabolic control of diabetes mellitus.

Diabetic disturbances in function of esophagus

One of the principal disturbances related to the esophagus in diabetic patients involves manifestation of a peristaltic wave (low amplitude of contractions and contractions of third order) with a delayed passage and a dysfunction of the lower sphincter (a decreased tension of LES). There exists data indicating that the complication develops in as many as 75% diabetic patients with a coexisting autonomic neuropathy. Principal symptoms manifested in patients with DM include dysphagia and esophageal burning. Obesity and insulin resistance which accompany DM2 represent recognized risk factors of Barrett's esophagus development, of progression of already manifested inflammatory lesions and intensification of dysphagia.[9] The relationship between obesity, diabetes and insulin resistance on one hand and an increased incidence of esophageal adenocarcinomas was confirmed in several reports.[10,11] In studies conducted on 2,836 veterans, diabetic patients were found to suffer more frequently from esophageal and cardiac adenocarcinomas. In a model of logistic regression, the relationship was documented between incidence of esophageal adenocarcinoma (EAC) on one hand and age, black race and tobacco smoking on the other. However, the increase in incidence of EAC in diabetic patients proved to be independent of coexisting obesity in this group of patients. The study confirmed also more frequent manifestation of EAC in persons with gastroesophageal reflux disease (GERD) at high values of glycosylated hemoglobin.[12] It should be mentioned also that other frequently developing complications of diabetes, such as gastroparesis, also promotes the development of gastro-esophageal reflux, which also represents an independent risk factor of adenocarcinoma development.[13]

Gastric disturbances in the course of diabetes mellitus

One of the more frequently developing complications of diabetes involves gastroparesis. It involves a syndrome of signs/symptoms related to upper fragment of alimentary tract reflecting disturbances in stomach emptying. Epidemiological data indicates that the complication (used to reflect disturbances of autonomic system) develops in 5–12% patients with diabetes and significantly more frequently in patients already affected by other complications. In the study by Bharucha et al., 47% of the patients, already affected by other complications, demonstrated delayed emptying of the stomach.[14] The first and most characteristic symptom of gastroparesis involves nausea, developing in 90% of patients. Other symptoms, such as early post-alimentary satiety, vomiting, flatulence and the sensation of gastric distension, may induce disturbances in digestion and absorption of food components and difficulties in gaining metabolic control in diabetes. Frequently, no relationship can be detected between the intensity of symptoms and the function of gastric emptying. Nevertheless, in the study by Gourcerol et al., the increasing resistance of pylorus in diabetic patients was found to correlate with the intensity of symptoms and quality of life, evaluated using the gastrointestinal quality of life (GIQLI) questionnaire.[15] The intensity of gastroparesis manifests a correlation with absence of metabolic balance of diabetes in patients with diabetes type 1, although it has not been finally proven in patients with type 2 diabetes.[16] Apart from the neuropathy of vagus nerve, disturbed emptying of the stomach may be induced by acute hyper- and hypoglycemia, hypo- or hyperinsulinemia, disturbed secretion of hormones in alimentary tract, and the more frequent in this group colonization with *H. pylori*.[17,18] Histopathological investigations confirmed that patients with gastroparesis, due to persisting chronic inflammatory process, manifest a decreased number of interstitial Cajal cells (ICCs), the number of NO-secreting neurons is reduced, atrophy of smooth muscles is accompanied by presence of lymphocyte infiltrates.[19] On the other hand, the studies by Choi et al. confirmed presence of macrophages $CD206^{+}$, which protected against gastroparesis.[20] The number of such macrophages manifested correlation with number of ICCs.

Apart from a disturbed emptying of stomach, diabetic patients may also manifest a modified contractility of duodenum. In studies by Barshop et al., the duodenal contractile activity was found to correlate more closely with signs/symptoms of gastroparesis than the motoric activity of cardia.[21] Diagnosis of gastroparesis used to be equivocal, as the reference approach involves isotopic scintigraphy and a patient exhausts the criteria when 2 h after the start of the test his/her stomach continues to contain at least 60% of original radioactivity and after 4 h it contains over 10% of original radioactivity. External electrogastrography represents a noninvasive and useful technique, but due to the costs involved, it is poorly accessible. The first-line drug in the treatment of the complication include pro-kinetic drugs, such as metoclopramide, itopride and the ghrelin receptor agonist, relamorelin. However, their efficacy is restricted or they are not generally accessible. In the treatment of gastroparesis, the role of frequent consumption of small meals, of a liquid diet, containing low amount of lipids, used to be stressed, as it significantly alleviates symptoms. In addition, proton pump inhibitors and antidepressants such as amitriptyline and escitalopram can be used. In experimental studies attempts were made to use irbesartan, as a drug which reduces leptin concentration, the hormone which significantly delays the process of gastric emptying. However, the obtained results remain equivocal and require further observations.[22]

Small intestine: Celiac disease and changes in intestinal microflora as diseases coexisting with diabetes mellitus

With increasing frequency, the coexistence of disturbances in absorption is seen to affect patients with type 1 diabetes. Depending on source data, celiac disease coexists in 6 to 15% of patients with diabetes. In most of the patients, it is diagnosed within 5 years after the diagnosis of diabetes (in 79% patients, celiac disease is diagnosed within the first 5 years, in 55% within 2 years and in 40% within a year after developing diabetes).[23] However, it is significant that just 10% of patients present typical clinical signs/symptoms of the disease. The only sign of the disease may involve relapsing hypoglycemias and a poor metabolic balancing of diabetes. Coeliac disease developing in persons with diabetes manifests also a quite distinct course than that developing in diabetes-free individuals. In diabetes, the incidence of celiac disease in men and women is almost the same, without the 3-fold prevalence of incidence among women, detected in the general population. In order to exclude celiac disease, detection of anti-TTG antibodies is employed as well as genetic studies, allowing for the identification of HLA-DQ2 antigen, present in 90% of patients with celiac disease and in 55% of patients with diabetes type 1. Apart from celiac disease, diabetic patients are also more frequently troubled by a disturbed motoric activity of alimentary tract in the form of periodic diarrheas, which frequently vanish spontaneously. Diarrhea used to manifest at night. Diabetic patients and, in particular, patients with type 2 diabetes frequently manifest the small intestine bacterial outgrowth (SIBO). However, data on the topic is equivocal: some studies documented lower incidence of the syndrome than that manifested in general population and the authors linked the fact with alimentary interventions in patients with diabetes.[24]

Diseases of large intestine connected with diabetes

The principal variables promoting the development of colorectal carcinoma involve a high fat and high carbohydrate diet, resulting in overweight and obesity, disturbances in carbohydrate metabolism and hyperinsulinemia. Several scientific reports accentuate also the role of diabetes as an independent risk factor of developing colorectal carcinoma.[25] Moreover, a positive relationship was documented between the value of HbA_{1c} and the incidence of intestinal polyps.[26] Metformin, as the drug of the first-line therapy of type 2 diabetes, induces the reduction in hyperinsulinemia and reduces insulin resistance. Nevertheless, apart from its anti-hyperglycemic effects, it exerts also other metabolically favorable sequels. In the mechanism of AMP kinase activation, metformin inhibits aberrant crypt foci (ACF), the marker of colorectal carcinoma (CRC).[27] Studies of Choe et al. in the group using metformin documented a significantly lower frequency of colorectal polyps, their significantly lower size, lower number of hyperplastic and villiform polyps ($p = 0.01$), and less advanced cases of CRC.[27] However, it should be mentioned that metformin administered for many years reduces the level of vitamin B_{12}. A similar effect is exerted by proton pump inhibitors (PPIs), commonly used in gastritis and, therefore, the control of the level and possible supplementation of vitamin B_{12} represent an indispensable element of coordinated treatment with the drugs.[28]

Liver and diabetes: Does diabetic microangiopathy of the liver exist?

The main hepatic pathologies diagnosed in diabetic patients include simple fatty degeneration of the liver, most frequently linked to type 2 diabetes, obesity and lipid disturbances and nonalcoholic fatty hepatitis, leading to fibrosis and cirrhosis of the liver. In patients with type 1 diabetes, the syndrome of Mauriac may develop, characterized by hepatomegaly. Diabetic fibrosis of the liver represents a form of hepatic microangiopathy in which sinusoidal fibrosis of the liver takes place with no traits of fatty degeneration. Deposits of collagen in liver sinusoids and remodeling of basement membrane with a normal ultrasonographic pattern are typical for the disease.[29] According to Hudacko et al., this type of complication is linked to diabetic microangiopathy, developing in patients with DM1 and DM2, particularly in those with prolonged diabetic anamnesis and coexisting complications, most frequently of diabetic nephropathy type.[30] Frequently, also chronic elevation of alkaline phosphatase activity is detected.[29]

Propensity for various types of infections also characterizes diabetic patients, including viral hepatitis. It was confirmed that DM2 develops more frequently in persons infected with HCV than in persons free of such an infection or in those with coexisting HBV infection.[31] This is liked to the presence of insulin resistance, higher secretion of tumor necrosis factor alpha (TNF-α), as well as to the direct effect of viral proteins on the effect of insulin.[32,33] Numerous studies also confirmed the relationship between HCV infection and type 1 diabetes, although the incidence of such an infection is much lower than in type 2 diabetes, and its manifestation is mainly linked to the induction of autoimmune process during therapy with interferon.[34,35]

Tumors in alimentary tract versus type of diabetes

For years it has been known that the risk of developing a tumor in the alimentary tract is markedly higher in diabetic patients than in normal individuals. The relationship is based (in the case of persons with type 2 diabetes) on the manifestation of overweight and obesity, hyperinsulinemia and insulin resistance. The phenomena lead to elevated concentrations of IGF-1 which, in turn, stimulate uncontrolled cell proliferation, while inhibiting the process of apoptosis. Similarly, in patients with type 1 diabetes, exogenous hyperinsulinemia may lead to pathological hypertrophy of cells and tissues. In studies by Mannucci et al., the teratogenic effect of high doses of exogenous insulin was confirmed.[36] Patients with type 2 diabetes significantly more frequently develop tumors of liver, pancreas, esophagus and large intestine (in the female population, rectal tumors are seen particularly frequently). They used to involve adenomas, in contrast to myomas, the incidence of which is comparable to that in general population. In patients with type 1 diabetes also more frequently manifestations of hepatic and pancreatic carcinoma are observed and any tumors in their in situ forms.[37] A significant problem for physicians is posed by the manifestation of pancreatic cancer in persons with freshly diagnosed diabetes. In such cases, diabetes presents frequently a principal sign of the concealed disease. Such a risk affects most frequently lean individuals of a mature age, with no pronounced insulin resistance, in whom clinical pattern is not typical for type 1 diabetes, while the signs/symptoms appeared suddenly and manifest a pronounced intensity. However, imaging studies in this group of patients are conducted exclusively in patients with a high risk of pancreatic tumor. A significant risk of developing a pancreatic tumor is present also in persons with class 3 diabetes. In persons with chronic pancreatitis, the presence of cysts of over 3 cm in diameter, with a dilation of pancreatic duct and with the presence of solid structures linked to cyst wall or in its lumen, provides an indication for oncological control.[38] In the year of 2009, in studies published by European Association for the Study of Diabetes (EASD), the suspicion was advanced that the application of long-term acting glargine analogue may augment the incidence of breast cancer. However, such results have not been confirmed in the Outcome Reduction

with an Initial Glargine Intervention (ORIGIN) study, published in 2014.[39] Neoplastic process was found to be inhibited by the principal drug used in therapy of type 2 diabetes, metformin. The drug, due to its involvement in the signaling pathway of LKB1 kinase (a controller of AMP-activated protein kinase – AMPK), exerts a confirmed anti-proliferative effect and forms a recognized neoplastic suppressor. Metformin effect is also linked to the ability of AMPK to maintain low levels of cell energy while the phosphorylation of p27KIP and tuberous sclerosis complex 2 (TSC2) proteins leads to the inhibition of signaling network proliferation.[40] In Zwolle Outpatient Diabetes project Integrating Available Care studies (ZODIAC16), the application of metformin from the beginning of the disease was found to be associated with a lower mortality of patients due to tumors and the effect seemed to be dependent on the dose.[41]

Summing up the above, the scope of the pathology related to alimentary system in diabetic patients depends on the duration of diabetes, the extent of its metabolic equilibration, on manifestation and intensity of accompanying diseases. Current therapy of diabetes is based on achieving normal metabolic control and on screening toward commonly known principal complications of diabetes, such as retinopathy, neuropathy and diabetic nephropathy. Complications of diabetes may affect many other systems and organs, including in particular alimentary tract. Recognition of the complications and implementation of appropriate treatment requires that the doctor will take a holistic view of the patient and will individualize respective recommendations and the therapy.

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