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# Contents

## Original papers

## Reviews

# Perforator artery response to tissue expansion: An experimental study in rabbits

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

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## Abstract

**Background.** The reconstruction of defects is a major area of interest in plastic surgery. Tissues are expanded to extend the tissue surface area and to prepare more reliable perforator flaps.

**Objectives.** Because expanded perforator flaps have become more popular, the aim of this study is to determine the response of the perforator artery to tissue expansion.

**Material and methods.** We used a rabbit S1 perforator (first perforator branch of the thoracodorsal arteries) flap model. In 12 New Zealand White rabbits, left flaps were used as the experimental group ($n = 12$) and right flaps were used as the control group ($n = 12$). Both flaps were constructed in the dorsal skin. The experimental group was further divided into 3 subgroups according to expansion volume: 150 mL ($n = 4$), 200 mL ($n = 4$) and 250 mL ($n = 4$). We evaluated the responses of the perforator arteries to tissue expansion using the resistivity index (RI), the pulsatility index (PI), vessel diameter (D), histopathological examinations, and angiography.

**Results.** After 3 weeks of expansion, the perforator artery diameter had increased ($p = 0.002$) and the RI had decreased ($p = 0.031$) in the experimental group. The perforator artery diameter ($p = 0.006$) and RI had increased ($p = 0.003$) in the control group. No significant changes were observed in the PI in either group ($p > 0.05$) and no significant differences in post-expansion measurements were observed between experimental subgroups ($p > 0.05$).

**Conclusions.** Suprafascial expansion of a perforator flap leads to an increase in diameter and a decrease in the RI of the perforator artery. The decrease in RI may indicate increased flap perfusion.

**Key words:** perforator artery, tissue expansion, experimental, rabbit, Doppler indices

**Cite as**
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## Introduction

Defect reconstruction is a major area of interest in plastic surgery. Tissues are expanded in this process in order to extend the surface area and to provide more reliable flaps. Although expanded free or pedicled perforator flaps have been successfully used, only a few studies have investigated the effects of tissue expansion on the perforator artery.

Our search for an economical experimental animal model with well-defined perforator vessels and a reliable anatomy that enables the use of commercially available tissue expanders led us to the use of rabbits. The cranial region of rabbit dorsal skin is supplied bilaterally by the first perforator branch of the thoracodorsal arteries (S1 perforator).[1] Guerra et al. emphasized that the fascia does not contribute to the blood supply of the S1 perforator flap, which is a true perforator flap.[2]

Color Doppler ultrasound imaging, which has been used in the planning of perforator flaps,[3–5] was recently employed for the postoperative assessment of perforator flap perfusion.[6] Vascular Doppler ultrasonography is used to determine blood flow velocity, which in turn is needed to calculate parameters such as the resistivity index (RI) and the pulsatility index (PI). These indices are used to assess vascular resistance.

The aim of this study is to determine the response of the perforator artery to various volumes of expansion of a rabbit S1 perforator flap. The perforator artery response was evaluated using Doppler ultrasonographic measurements, histopathological examination and angiography.

## Material and methods

The protocol for the use of rabbits in this study was approved by the local ethics committee. Twelve New Zealand White rabbits weighing 2,400–3,380 g were used. Each animal was housed in a single metal cage in a room with controlled temperature (25°C) and light (12 h day/12 h night). All animals were fed a commercial rabbit diet (23% protein) and drinking water ad libitum. Infection prophylaxis was ensured through the preoperative administration of 50 mg/kg of cephazoline sodium intramuscularly. Pain management was handled through the postoperative administration of 1 mg/kg of diclofenac sodium intramuscularly for 5 days.

The left S1 perforator flaps were used as the experimental group (n = 12), and the right S1 perforator flaps were used for the control group (n = 12). The experimental group was subdivided into 3 groups according to expansion volume: 150 mL (n = 4), 200 mL (n = 4) and 250 mL (n = 4). Anesthesia was provided as 40 mg/kg of ketamine and 10 mg/kg of xylazine, given intramuscularly. In the prone position, right and left S1 perforator arteries of each rabbit were examined with color Doppler ultrasonography using a Logiq P5 ultrasound system and an 11L transducer (General Electric Company, Boston, USA). The quantitative data obtained with vascular Doppler ultrasound include blood flow velocities (e.g., peak systolic flow velocity (PS), end diastolic flow velocity (ED) and mean flow velocity (TAMAX)). Semiquantitative Doppler data include those indices calculated on the basis of the blood flow velocities (e.g., RI and PI) using the following equation:

$$RI = [PS - ED]/PS \text{ and } PI = [PS - ED]/TAMAX$$

The PS, ED, TAMAX, RI, PI, and vessel diameter (D) of the S1 perforator arteries were recorded.

Under general anesthesia, after preparing the skin with povidone iodine and closing with sterile cloths, we reached the subpannicular area through a 4-cm horizontal incision at the level of the left superior iliac crest. By caudal to cranial dissection up to the left axillary area, we reached the point where the S1 perforator emerges from the latissimus dorsi muscle. We prepared a pouch (13 × 8 cm) with a cranial border leading to the S1 perforator artery, and then implanted a 150-milliliter elliptical smooth tissue expander (12 × 7 × 3.2 cm) with an external port (Mentor, Santa Barbara, USA) into the prepared pouch. The port was placed in a new subpannicular pouch that was prepared caudal to the incision. All of the expanders were inflated to 50 mL perioperatively. No surgical intervention was performed to the right side of the dorsum, with this side serving as a control. Serial expansions were made on postoperative days 7, 10, 13, 16, and 19 in increments of 20 mL, 30 mL or 40 mL, leading to final volumes of 150 mL, 200 mL and 250 mL, respectively (4 rabbits each). On postoperative day 21, under general anesthesia, we repeated Doppler ultrasound examination of the S1 perforator in the experimental and control groups. Afterwards, tissue expanders were extracted through the implantation incision. A monoblock flap consisting of the experimental and control flaps was harvested on the right and left S1 perforator arteries.

The borders of the flap were as follows (Fig. 1A,B): cranial border – horizontal line passing through the superior angles of the scapulae; caudal border – horizontal line passing through the posterior superior iliac crests; right lateral border – right anterior axillary line; left lateral border – left anterior axillary line.

By retrograde dissection of the right and left S1 perforator arteries through the latissimus dorsi muscles, we reached the thoracodorsal arteries. The thoracodorsal arteries were divided at the point where they branch out of the subscapular artery and the flaps were taken to another surgical table. At this stage, under general anesthesia, the animals were euthanized by exsanguination via cutting of the axillary artery. The right and left thoracodorsal arteries located in the monoblock flap were cannulated with 26 G intravenous catheters. Heparin (10 mL of 100 IU) was given through the right and left thoracodorsal arteries for flap exsanguination. Urografin

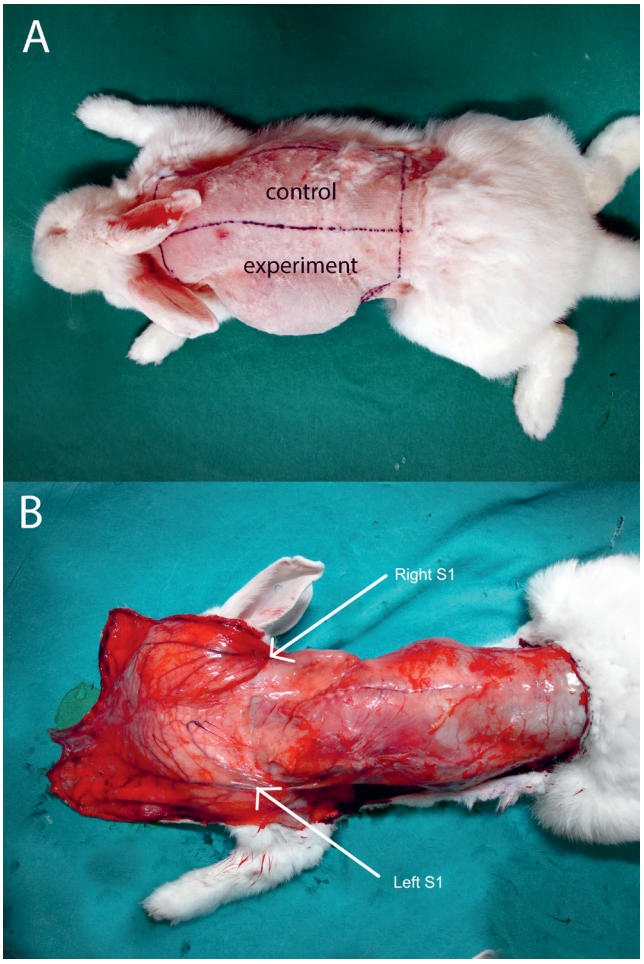


Fig. 1. A – borders of the flap (left – experimental group, right – control group). B – a monoblock flap harvested on right and left S1 perforators. Arrows show right and left S1 perforators

(5 mL; Bayer Schering Pharma, Berlin, Germany) was given slowly from the right and left catheters until radiopaque fluid was observed oozing from the distal end of the flap. Immediately after the Urografin injection, angiography was performed with X-ray (Optima XR200amx; General Electric Company) at a 68.5-centimeter source-to-image-receptor distance (50 kV, 2.00 mAs, 0°). After angiography, biopsies from the right and left S1 perforator arteries were taken from the point where they pierce the deep fascia. Specimens were Van Gieson-stained and examined under a light microscope at ×200 magnification. The internal elastic membrane that separates the tunica intima from the tunica media and the external elastic membrane that separates the tunica media from the tunica adventitia were defined. The thickness of the tunica media and tunica adventitia layers in the experimental and control groups were compared by measuring the thicknesses in the photographs taken at the same magnification (×200) using Adobe Photoshop CS6 v. 13.0 (Adobe Inc., San Jose, USA).

## Statistical analysis

All analyses were performed using the SPSS Statistics for Windows v. 17.0 software package (SPSS Inc., Chicago, USA). Categorical measurements are presented as numbers and percentages. Continuous measurements were summarized and are presented as means ± standard deviation (SD). The Mann–Whitney U test was used if the data did not exhibit a normal distribution. The Wilcoxon test was used to compare initial and post-expansion measurements. A p-value <0.05 was considered statistically significant.

# Results

No significant differences were observed in the initial Doppler measurements between the experimental and control groups (Table 1). Our comparison of the initial and post-expansion (3 weeks) measurements of the experimental group revealed that D increased with expansion while RI and PS had decreased. Changes in ED, TAMAX and PI were not statistically significant. Comparing the initial and post-expansion measurements of the control group, we noted that D and RI had increased with expansion while ED decreased. The changes in PS, TAMAX and PI were not statistically significant (Table 2). The median D of the experimental group increased by 31.3%, while that of the control group increased by 11.8% (Table 2) (Fig. 2). With expansion, RI decreased in the experimental group but increased in the control group (Table 2) (Fig. 3). The changes in PI were not significant in either group (Table 2).

Subgroup comparisons revealed no significant differences in post-expansion Doppler measurements between

Table 1. Initial Doppler measurements of experimental and control groups

| Variables | Experimental group | | Control group | | p-value |
|---|---|---|---|---|---|
| | mean | median (range) | mean | median (range) | |
| PS [cm/s] | 45.1 | 42.4 (26.4–81.0) | 39.9 | 36.7 (29.2–67.2) | 0.410 |
| ED [cm/s] | 11.1 | 11.7 (5.6–18.6) | 8.8 | 8.0 (6.2–16.1) | 0.060 |
| TAMAX [cm/s] | 17.4 | 17.4 (10.9–24.8) | 15.2 | 15.0 (8.2–22.2) | 0.319 |
| PI | 2.0 | 2.0 (1.4–3.0) | 2.1 | 1.9 (1.5–3.0) | 0.551 |
| RI | 0.8 | 0.8 (0.7–0.9) | 0.8 | 0.8 (0.7–0.9) | 0.319 |
| D [mm] | 1.7 | 1.6 (1.5–2.1) | 1.7 | 1.7 (1.5–1.8) | 0.347 |

PS – peak systolic flow velocity; ED – end diastolic flow velocity; TAMAX – mean flow velocity; PI – pulsatility index; RI – resistivity index; D – vessel diameter.

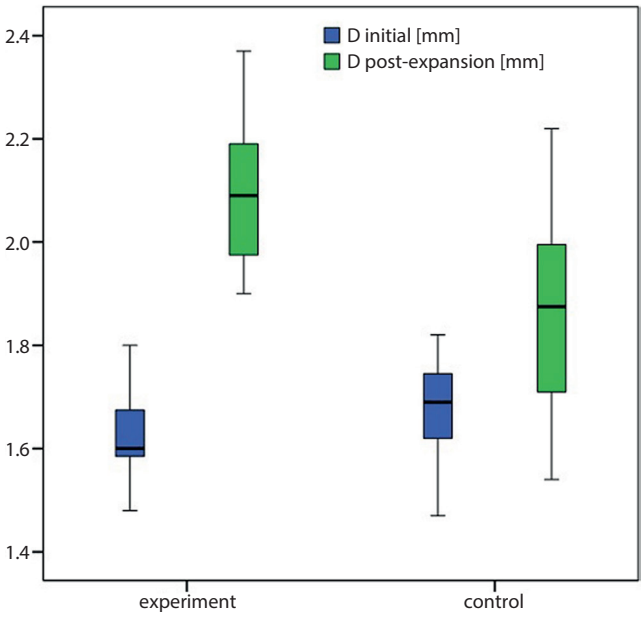


Fig. 2. Initial and post-expansion diameter distribution in experimental and control groups

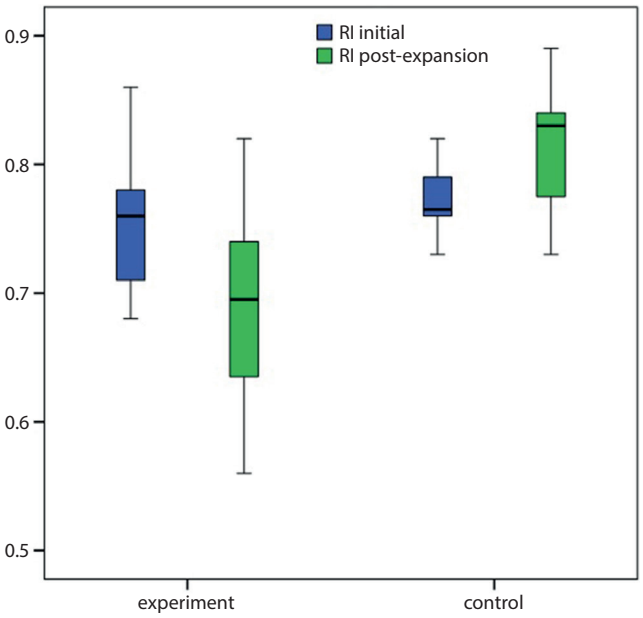


Fig. 3. Initial and post-expansion resistivity index distribution in experimental and control groups

Table 2. Comparison of initial and post-expansion (3 weeks) Doppler measurements of experimental and control groups

| Variables | Experimental initial | Experimental post-expansion | p-value | Control initial | Control post-expansion | p-value |
|---|---|---|---|---|---|---|
| | median (range) | median (range) | | median (range) | median (range) | |
| PS [cm/s] | 42.4 (26.4–81.0) | 35.7 (26.5–46.1) | 0.015 | 36.7 (29.2–67.2) | 33.3 (22.6–57.0) | 0.272 |
| ED [cm/s] | 11.7 (5.6–18.6) | 9.9 (6.9–19.8) | 0.937 | 8.0 (6.2–16.1) | 6.6 (3.6–10.0) | 0.041 |
| TAMAX [cm/s] | 17.4 (10.9–24.8) | 15.8 (12.6–24.0) | 0.388 | 15.0 (8.2–22.2) | 12.4 (8.8–23.8) | 0.169 |
| PI | 2.0 (1.4–3.0) | 1.5 (1.0–2.0) | 0.055 | 1.9 (1.5–3.0) | 2.0 (1.6–2.7) | 0.875 |
| RI | 0.8 (0.7–0.9) | 0.7 (0.6–0.8) | 0.031 | 0.7 (0.7–0.9) | 0.8 (0.7–0.9) | 0.003 |
| D [mm] | 1.6 (1.5–2.1) | 2.1 (1.9–2.4) | 0.002 | 1.7 (1.5–1.8) | 1.9 (1.5–2.2) | 0.006 |

PS – peak systolic flow velocity; ED – end diastolic flow velocity; TAMAX – mean flow velocity; PI – pulsatility index; RI – resistivity index; D – vessel diameter.

Table 3. Post-expansion measurements of the experimental group subdivided according to volume of expansion

| Variables | Expansion volume | | | p-value | | |
|---|---|---|---|---|---|---|
| | 150 mL | 200 mL | 250 mL | | | |
| | median (range) | median (range) | median (range) | 150 vs 200 | 150 vs 250 | 200 vs 250 |
| PS [cm/s] | 37.2 (29.0–41.2) | 32.9 (27–37.7) | 37.5 (26.5–46.1) | 0.486 | 1.000 | 0.886 |
| ED [cm/s] | 11.7 (10.2–17.5) | 8.5 (6.9–9.3) | 10.2 (7.1–19.8) | 0.059 | 0.486 | 0.200 |
| TAMAX [cm/s] | 19.1 (12.6–20.9) | 14.34 (12.8–16.3) | 16.4 (13.0–24.0) | 0.343 | 1.000 | 0.686 |
| PI | 1.6 (1.0–1.9) | 1.6 (1.5–2.1) | 1.4 (1.1–1.9) | 0.486 | 0.886 | 0.200 |
| RI | 0.7 (0.56–0.7) | 0.7 (0.7–0.8) | 0.7 (0.6–0.8) | 0.057 | 0.686 | 0.486 |
| D [mm] | 2.1 (2.0–2.2) | 2.1 (1.9–2.4) | 2.0 (1.9–2.4) | 0.686 | 0.486 | 0.686 |

PS – peak systolic flow velocity; ED – end diastolic flow velocity; TAMAX – mean flow velocity; PI – pulsatility index; RI – resistivity index; D – vessel diameter.

the expansions of 150 mL, 200 mL and 250 mL (Table 3). We compared the experimental and control groups according to the angiography of the monoblock flap, which consisted of the right and left sides of the rabbit dorsal skin. Vascular arborization was more complex in the experimental group (Fig. 4). Histopathological examination of the S1 perforators in the experimental and control groups at the end of the expansion period revealed no marked changes in the thickness of the tunica media or tunica adventitia (data not shown) (Fig. 5).

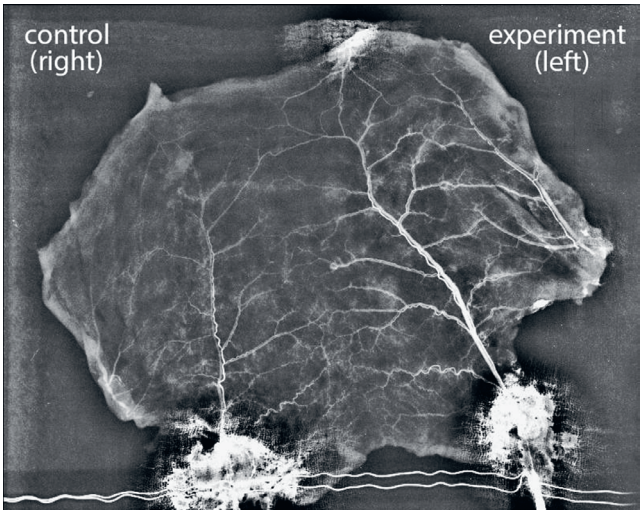


Fig. 4. Angiographic view of a monoblock flap. Note the more complex vascular arborization of the experimental side

## Discussion

According to the consensus regarding perforator flap terminology: "A muscle or myocutaneous perforator is a blood vessel that traverses through muscle to pierce the outer layer of the deep fascia and to supply the overlying skin. A skin flap that is vascularized by a muscle perforator is called a muscle perforator flap or musculocutaneous perforator flap."[7] Recently, perforator flaps have become more popular in the field of surgical reconstruction. The advantages of perforator flaps over muscle or musculocutaneous flaps include decreased donor-site morbidity, less postoperative pain, faster rehabilitation, absence of postoperative muscle atrophy, and the possibility of harvesting a sensate flap.[8]

Plastic surgery often involves the challenge of repairing a large defect with only a limited area of donor tissue. Methods such as tissue expansion and surgical or chemical delay have been employed in flap surgery. Although expanded

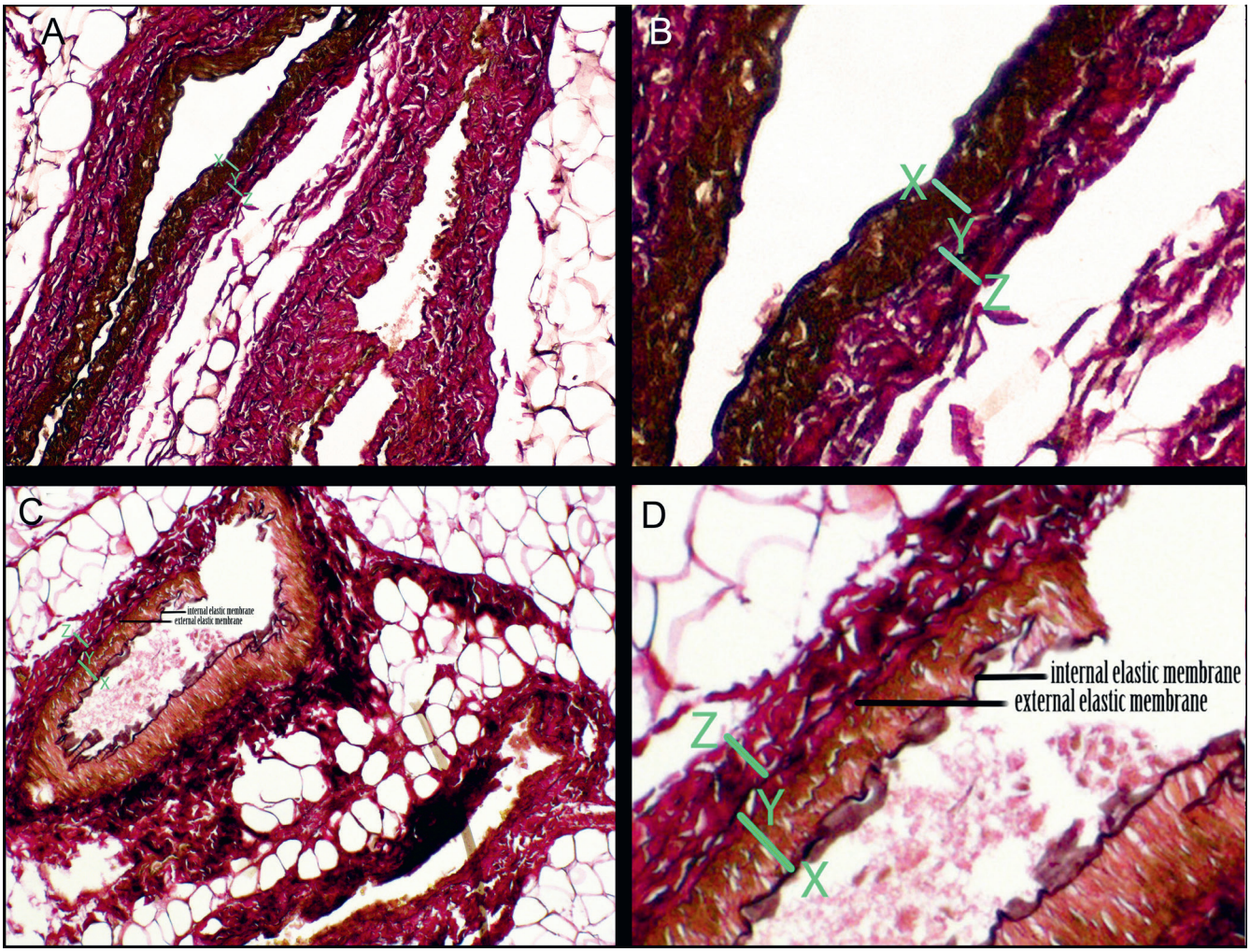


Fig. 5. A – histopathological view of an experimental S1 artery (Van Gieson's stain; ×200 magnification). B – ×2.5 digital magnification of the vessel wall of the S1 artery shown in Fig. 5A. C – histopathological view of a control S1 artery (Van Gieson's stain; ×200 magnification). D – ×2.5 digital magnification of the vessel wall of the S1 artery shown in Fig. 5C.

Internal and external elastic membranes are indicated in Fig. 5C,D. X-Y – thickness of tunica media; Y-Z – thickness of tunica adventitia.

free and pedicled perforator flaps have been used successfully, only a few clinical reports have studied the effects of tissue expansion on the perforator artery. These studies evaluated the response of the perforator artery as evidenced only by changes in D.[9,10] In this study, we evaluated changes in other parameters in addition to D. To the best of our knowledge, this is the first study to evaluate the response of the perforator artery to tissue expansion using blood flow velocity, RI, PI, histopathology, and angiography.

Angel et al. described the first perforator branch of the thoracodorsal artery, naming it the S1 perforator in rabbits. They showed that it is possible to elevate a large skin flap based solely on the S1 perforator.[1] Guerra et al.[2] classified the S1 perforator as a type 1 or indirect muscle perforator. We chose the rabbit S1 perforator flap in our study because it is a suitable experimental model for studying perforator artery changes.

The measurement of blood flow velocity is operator-dependent, while RI and PI measurements are taken independently of the probe angle.[6] High and low values of RI and PI indicate high and low peripheral resistance. The calculation accuracy of PI is inferior to that of RI because of technical errors in calculating TAMAX. The RI is simply calculated from PS and ED with a relatively small margin of error. The reproducibility of RI is superior to that of PI.[11]

## Effect of tissue expansion on the perforator artery diameter

Shang et al.[10] used pre-expanded, deep inferior epigastric artery flaps for reconstructing burn contractures of the hand and wrist. They measured the perforator artery diameter with computed tomographic angiography before and 3 months after implanting tissue expanders. They reported a 33% (±8%) increase in the mean diameter of the perforator vessels after expansion. Hocaoğlu et al.[9] used suprafascially pre-expanded perforator flaps, measuring the initial and post-expansion D with Doppler ultrasound. They reported a mean D increase from 0.48 (±0.08) mm to 0.65 (±0.1) mm, which means a 35% increase in D. We observed a 31.3% increase in the median diameter of the perforator artery after tissue expansion in the experiment group. Our finding is comparable to those of the other 2 studies. Apart from these reports, we did not find anything in the literature on the effect of tissue expansion on perforator artery diameter.

## Effect of tissue expansion on the RI and PI of the perforator artery

Radmehr et al.[12] reported that detecting a gradual increase in RI and PI is an effective means of diagnosing renal allograft dysfunction in post-transplant patients in the early postoperative period. They believe that this gradual increase in RI and PI is caused by peripheral vascular resistance. Arya et al.[6] reported that the PI of the perforator artery gradually increases when there is venous occlusion in the free deep inferior epigastric artery perforator flaps. They stated that the increase in PI is secondary to a rise of peripheral vascular resistance caused by venous occlusion. While their study reports no RI data, we believe that if it had been studied, they would have found an increase in RI resulting from venous occlusion.

Our study is the first to investigate the effect of tissue expansion on the RI and PI of a perforator artery. The comparison of the initial and post-expansion measurements of the experimental group revealed a decrease in RI with tissue expansion (Table 2). This decrease in RI indicates a decrease in the resistance of the capillary bed distal to the measurement location and an increase in tissue perfusion. The decrease in PI in the experimental group was not statistically significant in our study (Table 2). For a perforator flap model, we believe it is reasonable to use RI rather than PI to determine intra-flap resistance.

There was no statistical significance when post-expansion Doppler measurements of the 3 experimental subgroups (150 mL (n = 4), 200 mL (n = 4) and 250 mL (n = 4)) were compared (Table 3). Our results show that over-expansion does not lead to a greater decrease in RI or a greater increase in D than conventional expansion. After the tissue is adequately expanded for reconstruction, it does not seem reasonable to use over-expansion to increase flap perfusion and viability.

## Histopathological findings

After elongating the saphenous artery and veins of rats using tissue expanders under the leg adductor muscles, Stark et al.[13] reported that tissue expansion did not affect the vessel wall thickness. Our comparison of biopsies from the experimental and control groups also revealed no change in vessel wall thickness. Moreover, we observed no changes in the thickness of the tunica media or the tunica adventitia (Fig. 5A–D).

## Angiographic findings

Reports on angiography on a variety of expanded flaps can be found in the literature. Cherry et al.[14] performed angiography on expanded random-pattern skin flaps in pigs. Kim et al.[15] and Mutaf et al.[16] performed angiography on expanded gracilis muscles and expanded venous flaps in rats. In accordance with the angiographic findings of other types of expanded flaps, we observed that tissue expansion increases the complexity of vascular arborization in perforator flaps.

## Interpretation of unpredicted Doppler findings in the control group

When planning our study, we did not expect to find significant changes in the Doppler measurements of the control group. According to the angiosome concept of Taylor

and Palmer,[17] "choke" anastomoses between arteries and arterioles dilate when a skin flap is delayed, and blood flows across the choke vessels according to a pressure gradient. Tissue expansion is one type of delay. Subsequent expansion of an angiosome will likely cause changes at the source of the artery of the adjacent angiosome. As Taylor and Minabe[18] describe, the flaps used in our study are composed of 2 angiosomes (the experimental and control segments) of rabbit dorsal skin that are linked via choke vessels at the vertebral line. In the experimental segment of the flap, the choke vessels dilated in response to expansion. We believe that the increased RI observed in the control segment of the flap is an adaptation that occurred to direct the blood flow from the control segment to the experimental segment through the choke vessels, thereby increasing perfusion of the expanded tissue.

Taylor and Palmer[17] also stated that the intelligent use of a delay will allow for the safe capture of adjacent vascular territory. The survival length of a skin flap depends on the diameter and length of the dominant vessel upon which the flap is based and on the diameter and span of the adjacent captured artery or arteries. In light of this information, we hypothesize that the increase in perforator artery diameter of the control segments of the flaps contributed to the survival of the experimental segments of the flaps. The unforeseen increases in D and RI in the control group may result from the systemic effects of vasoactive substances that were released within the experimental segment secondary to expansion.[16]

## Limitations

The placement of expanders in loose-skinned animals such as rabbits and rats may differ from their use in humans, who have fixed skin. Thus, a comparison of results between such animals and humans may lead to incorrect conclusions.[18]

# Conclusions

Suprafascial expansion of a perforator flap leads to increased diameter and decreased RI of the perforator artery. The decrease in the RI may indicate increased flap perfusion. Further studies are needed to confirm our perforator artery findings in the expanded and unexpanded adjacent tissue.

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# Renal response to tunicamycin-induced endoplasmic reticulum stress in BDNF heterozygous mice

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## Abstract

**Background.** The protective effects of brain-derived neurotrophic factor (BDNF) against endoplasmic reticulum (ER) stress in neuronal tissue and endometrial cells have been reported.

**Objectives.** The aim of this study was to determine whether endogenously produced BDNF protects the kidneys against tunicamycin-induced (Tm) ER stress.

**Material and methods.** Brain-derived neurotrophic factor heterozygous knockout mice ($BDNF^{(+/-)}$) and their wild-type (WT) littermates were used. The animals were divided into 4 groups: WT, $BDNF^{(+/-)}$, WT+Tm, and $BDNF^{(+/-)}$+Tm ($n = 7$ in each group). After 3 days of saline or Tm injection (0.5 mg/kg; intraperitoneally (i.p.)), renal BDNF, glucose-regulated protein 78 (GRP78), and caspase-12 levels as well as serum BDNF concentration were measured with enzyme-linked immunosorbent assay (ELISA). In the kidney sections, hematoxylin & eosin (H&E) staining, GADD153 immunostaining and TUNEL staining were performed. Serum creatinine levels were measured as an indicator of renal function.

**Results.** Circulating and tissue BDNF levels were significantly lower in the $BDNF^{(+/-)}$ and $BDNF^{(+/-)}$+Tm groups. Renal levels of GRP78 and caspase-12, apoptotic index, and GADD153 staining were significantly higher in the WT+Tm and $BDNF^{(+/-)}$+Tm groups. However, apoptosis was more pronounced in the $BDNF^{(+/-)}$+Tm group than in the WT+Tm group ($p < 0.01$). Similarly, GADD153 staining was more pronounced in the $BDNF^{(+/-)}$+Tm group than in the WT+Tm group ($p < 0.05$). Tm caused a mild deterioration in the kidney tissue of the WT+Tm group, while general deterioration, pyknotic nuclei and swollen cells were observed in the $BDNF^{(+/-)}$+Tm group. Serum creatinine concentrations were significantly higher in the WT+Tm ($p < 0.05$) and $BDNF^{(+/-)}$+Tm ($p < 0.05$) groups.

**Conclusions.** This study showed that endogenous BDNF may play a protective role in kidneys against ER stress-induced apoptosis via the suppression of GADD153. As a result, BDNF and related signaling pathways could be considered for therapeutic/protective approaches in kidney disorders.

**Key words:** apoptosis, endoplasmic reticulum stress, kidney, BDNF heterozygous mice, GADD153

# Introduction

The endoplasmic reticulum (ER) is a multifunctional organelle necessary for the folding and processing of nascent proteins, the synthesis of cholesterol, steroids and other lipids, as well as for calcium storage. The protein folding capacity of the ER is impaired under different physiological and pathological conditions which induce ER stress.[1,2] During ER stress, unfolded proteins which accumulate in the ER lumen trigger an adaptive cell response, called unfolded protein response (UPR), in order to maintain cell homeostasis.[2,3] As an ER resident chaperone, glucose-regulated protein 78 (GRP78) recognizes unfolded proteins and plays an important role in the regulation of UPR through the activation of 3 pathways: PKR-like ER kinase (PERK), activated transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). Activation of UPR pathways first results in the global attenuation of protein synthesis and prevents any further accumulation of unfolded proteins. Upregulation of chaperones such as GRP78 and GRP94, which improve ER folding capacity, and upregulation of the ER-associated degradation system, which activates the degradation of misfolded proteins, are 2 other major adaptive responses.[1–3] Despite these defense mechanisms, ER stress can trigger apoptosis, depending on the severity and duration of stress. Transcriptional activation of the C/EBP homologous protein (CHOP) – also known as a growth arrest or the DNA damage-inducible gene 153 (*GADD153*) – as well as activation of the cJUN NH2-terminal kinase (JNK) pathway and activation of ER-associated caspase-12, are involved in ER stress-mediated apoptosis.[4,5] Endoplasmic reticulum stress-induced apoptosis and tissue injury have been implicated in various diseases, including neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases, ischemia-reperfusion injury, diabetes, and atherosclerosis.[6–9]

As a trophic factor, brain-derived neurotrophic factor (BDNF) promotes neuronal development, differentiation and survival in the central and peripheral nervous systems.[10] Although it was originally discovered in brain tissue, the expression of BDNF and its main receptor – tropomyosin-related kinase B (TrkB) – have been found in different non-neuronal tissues such as the heart, kidney, liver, lungs, skeletal muscle, blood vessels, adipose tissue, and prostate.[11–13] In addition to its well-known neuroprotective effects, BDNF participates in the regulation of energy metabolism, mood, blood pressure, and angiogenesis. Plasma levels of BDNF have been shown to be reduced in patients with type 2 diabetes, acute coronary syndrome, major depressive syndrome, and chronic kidney disease.[14–17] The protective effect of BDNF against ER stress-induced apoptosis has been reported in neuronal cells as well as in endometrial epithelial cells.[18–20] It has been suggested that the suppression of CHOP and/or caspase-12 activation may play important role in BDNF-mediated neuroprotection during ER stress.[18,19]

Though the expression of BDNF and the Trk receptor has been shown in kidneys,[13,21,22] there is little knowledge available about the physiological roles of BDNF on glomerular or tubular functions. However, in vitro and in vivo studies have shown that exogenous administration of BDNF inhibits podocyte damage and prevents the glomerular lesions and proteinuria associated with adriamycin nephropathy.[21] Recently, Kurajoh et al. have demonstrated that patients with chronic kidney disease have reduced plasma BDNF concentration and suggested that plasma BDNF level is a predictor of chronic kidney disease.[17] These findings indicate the protective role of BDNF in normal kidney physiology. However, it remains unclear whether BDNF protects the kidneys against ER stress. Since ER stress is associated with various renal pathologies, such as diabetic nephropathy, idiopathic focal segmental glomerulosclerosis, membranous nephropathy, and minimal change disorders, we believe that answering this question will be valuable in elucidating the mechanisms of these renal pathophysiological states.[23–27] For this purpose, the effects of reduced endogenous concentrations of BDNF on ER stress-induced kidney damage was examined both in physiological and tunicamycin-induced conditions. Brain-derived neurotrophic factor heterozygous mice were used because they are characterized by reduced BDNF expression. The results of the BDNF heterozygous mice and their wild-type littermates were compared.

# Material and methods

## Animals

In the present study, we used a transgenic mouse model originally established by Korte et al.[28] In heterozygous knockout mice, 1 allele of the BDNF coding region is replaced by a neomycine-resistance gene ($BDNF^{(+/-)}$), which results in completely viable, fertile and BDNF-deficient animals. Male $BDNF^{+/-}$ mice aged 6 to 8 months and their wild-type littermates (as control) were used in current study. The presence of the transgene in each subject was confirmed with polymerase chain reaction (PCR) analysis of tail tissue as described in earlier studies.[29] All experiments were approved by the Local Institutional Animal Care and Use Committee of the Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey.

## Groups

The animals were divided into 4 groups (n = 7 in each group): wild-type (WT), wild-type mice injected with tunicamycin (WT+Tm), BDNF heterozygous ($BDNF^{(+/-)}$), and BDNF heterozygous mice injected with tunicamycin ($BDNF^{(+/-)}$ +Tm).

## Tunicamycin injection

The endoplasmic reticulum stress was induced with tunicamycin (Tm, Sigma-Aldrich T7765; Sigma-Aldrich, St. Louis, USA), which inhibits N-glycosylation in ER.[18,20,30,31] A single dose of Tm (0.5 mg/kg) was injected intraperitoneally (i.p.) in the WT+Tm and BDNF$^{(+/-)}$+Tm groups, while the same volume of saline solution was given i.p. as a vehicle in the WT and BDNF$^{(+/-)}$ groups. Three days after Tm or saline injection, the animals were sacrificed and blood and tissue samples were harvested. The blood samples were allowed to clot for 1 h at room temperature and then centrifuged at 1,000×g for 15 min. Serum samples were used for creatinine and BDNF measurements. For histological and immunohistochemical analysis, the right kidney was fixed in 10% neutral formalin. The left kidneys taken from the animals were homogenized in ice-cold PBS and then centrifuged at 5,000×g for 5 min. The supernatants were used for enzyme-linked immunosorbent assay (ELISA) measurements.

## Serum creatinine measurement

Serum creatinine levels were measured spectrophotometrically as described in previous research.[32]

## Measurements of serum BDNF levels

The circulating BDNF levels in the serum samples were measured with a commercial ELISA kit (E-EL-M0203; Elabscience Biotechnology, Wuhan, China), according to the manufacturer's instructions. Prior to analysis, the samples were diluted (1:2) and added to the appropriate micro ELISA plate wells which were pre-coated with an antibody specific to mouse BDNF. The absorbance of the resulting yellow product was measured at a wavelength of 450 nm with a microplate reader (BioTek ELx800 brand REF ELX508 SN1310149; Thermo Fisher, Waltham, USA). The concentrations of BDNF in the samples were calculated by comparing the optical density (OD) of the samples to the standard curve and multiplying by the dilution factor.

## Measurements of renal BDNF, GRP78 and caspase-12 levels

In tissue homogenates, BDNF, GRP78 and caspase-12 levels were assayed using the appropriate ELISA kits (E-EL-M0203, Elabscience Biotechnology; E-EL-M2696, Elabscience Biotechnology; and E0781Mo, Bioassay Technology Laboratory, Shanghai, China, respectively). The analyses were performed according to the manufacturer's instructions and read using an absorbance microplate reader (BioTek ELx800). BDNF, GRP78 and caspase-12 levels were expressed as the proportion of total protein, which was determined with the Breadford method (ThermoFisher; 23200).

## Histological evaluations

The kidney tissues were quickly fixed in 10% neutral formalin. Routine hematoxylin & eosin (H&E) staining was applied to sections 5 μm in thickness from paraffin blocks, following tissue attachment and paraffin embedding procedures, in order to reveal the structure of the tissues. GADD153 and TUNEL staining were also performed.

## TUNEL assay

The detection of apoptotic cells in renal tissue slides was performed using an In Situ Apoptosis Detection Kit (ab206386; Abcam, Cambridge, UK), according to the manufacturer's instructions. First, deparaffinization and rehydration procedures were performed on the paraffin tissue sections. Then, the proteinase K digestion method was used to unmask the antigens. After treatment with TdT Equilibration Buffer, the TdT labeling reaction mix was applied at 37°C. Following treatment with stop buffer and blocking buffer, 1X Conjugate and DAB solutions were used. All sections were contrasted with methyl green. The TUNEL-positive cells in the tissue sections were counted under a light microscope at a ×40 magnification by randomly selecting 5 areas from each section. Cell death was reported as the percentage of TUNEL-positive cells vs total cells (apoptotic index).

## GADD153 immunuhistochemistry

GADD153 was detected in paraffin-embedded tissues using an immunoperoxidase technique. The kidney sections were deparaffinized in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed with citrate buffer (pH 6.0) in a microwave oven. All subsequent staining incubation steps were done at room temperature, and Tris-buffered saline (pH 7.4) was used for all washes and diluents. The slides were thoroughly washed after antibody incubation and were blocked with protein blocking solution for 15 min. Then, primary antibody for mouse GADD153 (Santa Cruz Biotechnology, Santa Cruz, USA) was added to the slides at 1:100 dilution and they were incubated overnight. A biotinylated link antibody plus streptavidin-horseradish peroxidase kit (ThermoFisher) was applied, along with a 3-amino-9-ethylcarbazole (AEC) chromogen and peroxide substrate to detect the labeled antibodies. The slides were counterstained with Mayer's hematoxylin and cover slipped with an aqueous mounting medium. The image analysis included the calculation of positive staining (intensity) area per section at ×40 magnification and was carried out with ImageJ software v. 1.46r. For each image, the immunolabeled tissue area was determined, normalized by the total tissue area, and presented as a percentage of total tissue cross-sectional area.

## Statistical analysis

All data is given as mean ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) and Tukey's post-hoc tests using Graphpad Prism v. 4.0 software (Graphpad Software, San Diego, USA). Correlations were analyzed using Pearson's correlation analysis. Values of $p < 0.05$ were considered statistically significant.

# Results

In animals injected with Tm, weight loss was observed 3 days after the injection. Body weight decreased from 23.1 ±2.3 g to 19.9 ±1.7 g and from 25.3 ±2.8 g to 20.7 ±2.0 g in the WT+Tm and BDNF$^{(+/-)}$+Tm groups, respectively. These changes in body weight were not statistically significant.

## BDNF level in serum and kidney

Serum BDNF levels were significantly lower among the BDNF$^{(+/-)}$ mice (0.14 ±0.04 ng/mL) than the wild-type group (0.22 ±0.02 ng/mL; $p < 0.01$). As seen in Fig. 1A, Tm injection did not cause a significant change in serum BDNF levels, and the difference between wild-type and heterozygous subjects was preserved (WT+Tm vs BDNF$^{(+/-)}$+Tm; $p < 0.05$). The BDNF levels in kidney tissue were markedly lower among the BDNF heterozygous animals, and were not affected by Tm injection (Fig. 1B).

## GRP78 level in kidney

The concentration of GRP78 as an ER stress marker was measured with ELISA in mice treated with saline solution or Tm. In the saline-injected animals, no difference in renal GRP78 levels was noted between the WT and BDNF$^{(+/-)}$ groups (3.08 ±1.8 and 2.94 ±1.1 ng/mg protein, respectively). As shown in Fig. 2, Tm caused a significant increase in the GRP78 kidney levels of both the wild-type (6.24 ±1.9 ng/mg protein; $p < 0.05$) and the BDNF heterozygous mice (6.14 ±2.1 ng/mg protein; $p < 0.05$). There was no significant difference between the WT+Tm and BDNF$^{(+/-)}$+Tm groups (Fig. 2).

## Histological analysis

The kidney tissue was examined histologically using H&E staining to evaluate the effects of ER stress and BDNF deficiency. In comparison to the WT group, a slight deterioration in tubular structures and cells was observed in the kidney sections of the BDNF$^{(+/-)}$ group. Tunicamycin injection caused a mild deterioration in the tubular structures of the kidneys of the WT+Tm group, while general tissue deterioration, pyknotic nucleus, and swollen cells were observed in the BDNF$^{(+/-)}$ +Tm group (Fig. 3A–D).

## Renal apoptosis

Apoptosis in kidney tissue was demonstrated with TUNEL immunohistochemical analysis; the results are presented in Fig. 4. The apoptotic index was 0.144 ±0.06 and 0.476 ±0.19 in the WT and BDNF$^{(+/-)}$ groups, respectively. Tunicamycin-induced ER stress caused a significant increase in apoptosis in both the WT+Tm ($p < 0.01$ vs WT) and BDNF$^{(+/-)}$+Tm ($p < 0.001$ vs WT) groups. Apoptotic index was calculated as 1.636 ±0.96 and 3.422 ±0.99 for

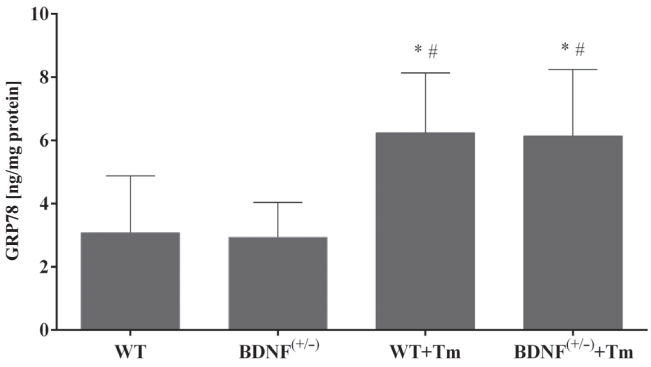


Fig. 2. GRP78 concentration in the kidneys of the WT, BDNF$^{(+/-)}$, WT+Tm, and BDNF$^{(+/-)}$+Tm groups. Data is presented as mean ±SD (n = 7 mice per group). *p < 0.05 vs the WT group; #p < 0.05 vs the BDNF$^{(+/-)}$ group

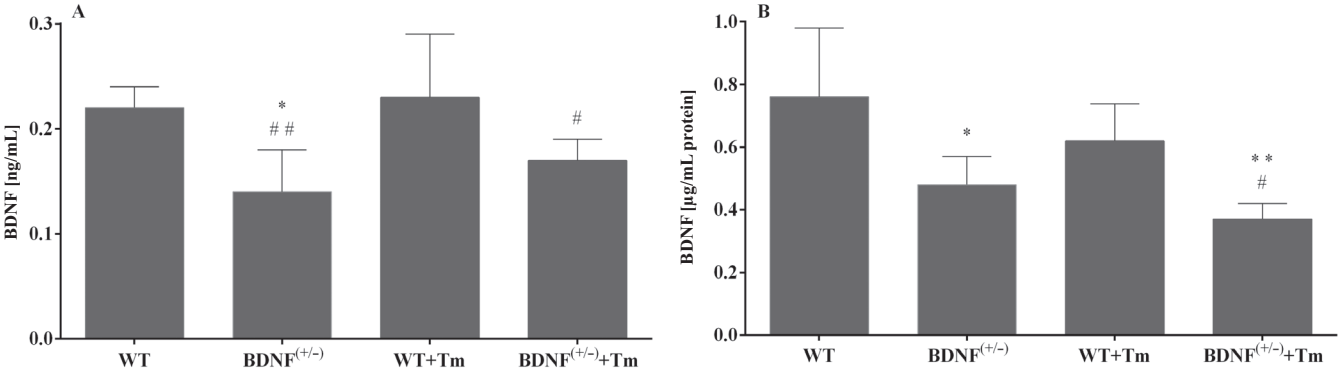


Fig. 1. BDNF level in serum (A) and kidney homogenates (B) in the WT, BDNF$^{(+/-)}$, WT+Tm, and BDNF$^{(+/-)}$+Tm groups. Data is presented as mean ±SE (n = 7 mice per group). *p < 0.05, **p < 0.01 vs the WT group; #p < 0.05, ##p < 0.01 vs the WT+Tm group

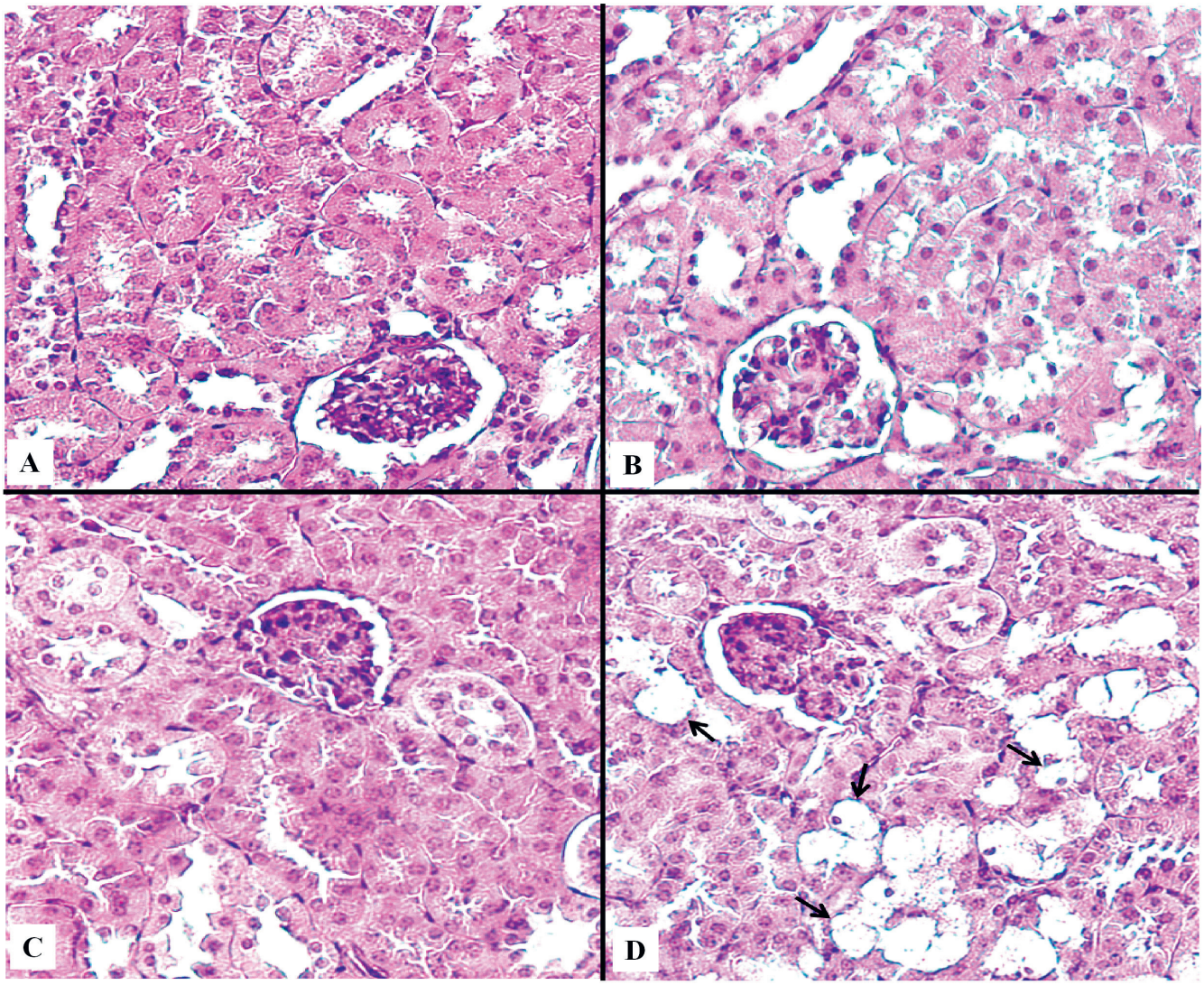


Fig. 3. Representative images of H&E analysis (×20 magnification) of WT kidney tissue (A), BDNF$^{(+/-)}$ kidney tissue (B), WT+Tm kidney tissue (C) and BDNF$^{(+/-)}$+Tm (D) kidney tissue. Arrows indicate swollen cells

the WT+Tm and BDNF$^{(+/-)}$+Tm groups, respectively. Tunicamycin-induced renal apoptosis was more pronounced in the BDNF$^{(+/-)}$+Tm group than in the WT+Tm group ($p < 0.01$).

## Caspase-12 level in kidney

The caspase-12 levels in the kidney tissue were similar between the WT (0.75 ±0.50 µg/mg of protein) and BDNF$^{(+/-)}$ groups (0.71 ±0.63 µg/mg of protein). A single dose of Tm caused a significant increase in both the WT+Tm (1.59 ±0.42 µg/mg of protein; $p < 0.05$) and BDNF$^{(+/-)}$+Tm groups (1.65 ±0.60 µg/mg of protein; $p < 0.05$). This value was not statistically significant in the WT+Tm group compared to the BDNF$^{(+/-)}$+Tm group.

## GADD153 immunohistochemistry

Positive GADD153 staining was detected in all groups with weak to moderate positive cytoplasmic staining (intensity) in the glomeruli and tubule cells (Fig. 5). The proportion of immunopositively stained area for GADD153 was 7.55 ±1.41% and 7.84 ±1.20% in the WT and BDNF$^{(+/-)}$ groups, respectively. Tm injection caused a significant increase in both the WT+Tm (10.44 ±1.12%; $p < 0.01$) and BDNF$^{(+/-)}$+Tm groups (12.35 ±1.30%; $p < 0.001$). GADD153 immunostaining was significantly higher in the BDNF$^{(+/-)}$+Tm group than in the WT+Tm group ($p < 0.05$).

## Serum creatinine

Serum creatinine concentration was at a similar level in the WT and BDNF$^{(+/-)}$ groups (0.29 ±0.07 mg/dL and 0.31 ±0.06 mg/dL, respectively). Creatinine levels were higher among Tm-injected animals: 0.40 ±0.06 mg/dL in the WT+Tm group ($p < 0.05$) and 0.42 ±0.07 mg/dL in the BDNF$^{(+/-)}$+Tm group ($p < 0.05$). There was no statistical difference between the WT+Tm and BDNF$^{(+/-)}$+Tm groups.

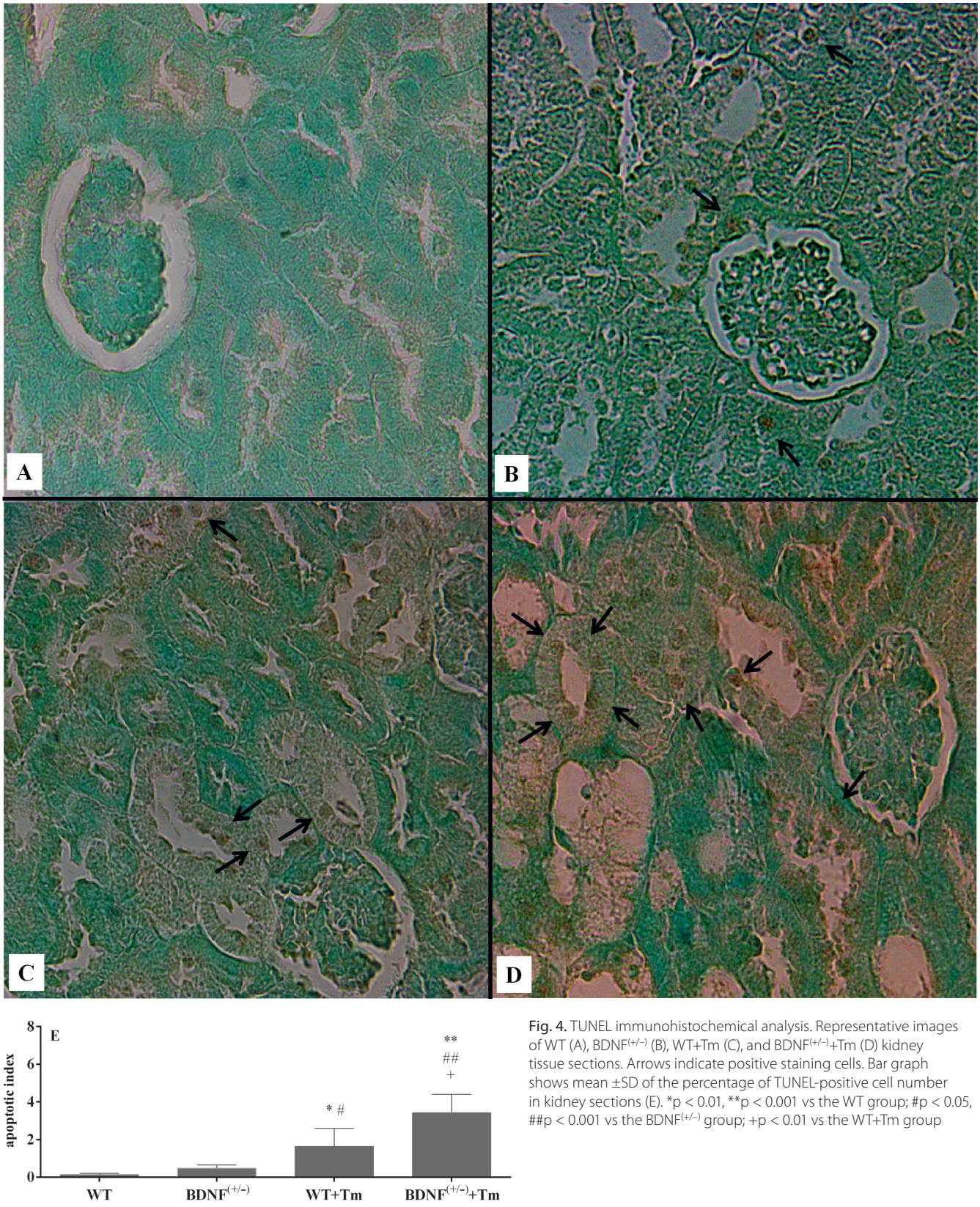


Fig. 4. TUNEL immunohistochemical analysis. Representative images of WT (A), BDNF$^{(+/-)}$ (B), WT+Tm (C), and BDNF$^{(+/-)}$+Tm (D) kidney tissue sections. Arrows indicate positive staining cells. Bar graph shows mean ±SD of the percentage of TUNEL-positive cell number in kidney sections (E). *p < 0.01, **p < 0.001 vs the WT group; #p < 0.05, ##p < 0.001 vs the BDNF$^{(+/-)}$ group; +p < 0.01 vs the WT+Tm group

## The results of correlation analysis

Correlation analysis showed a positive correlation between serum BDNF concentration and renal BDNF level ($r = 0.3909$; $p < 0.05$). However, there is no correlation between serum BDNF levels and the other parameters studied. As shown in Fig. 6, there was a negative correlation between renal BDNF concentration and serum creatinine level, apoptotic index, and GADD153 immunostaining.

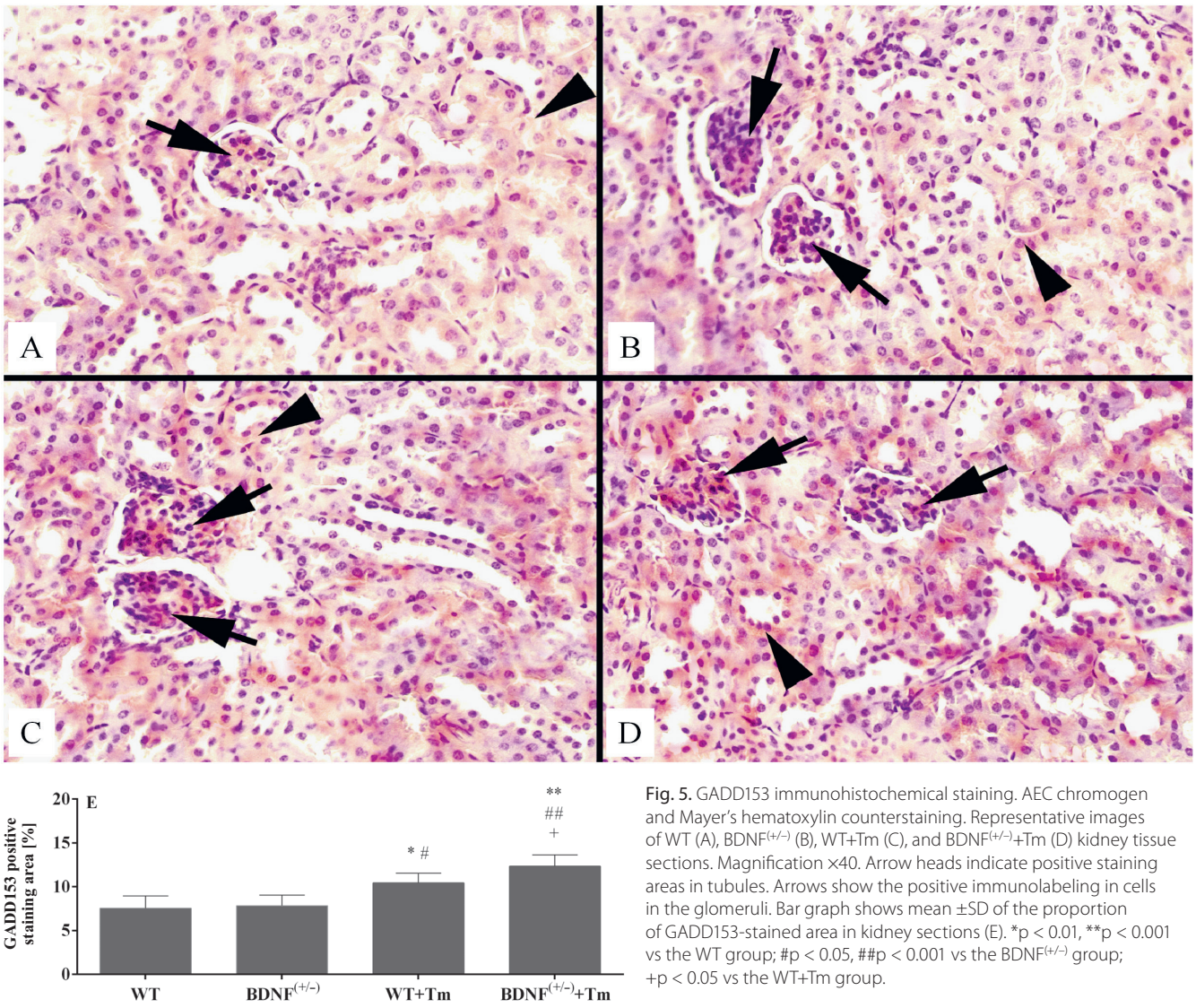


Fig. 5. GADD153 immunohistochemical staining. AEC chromogen and Mayer's hematoxylin counterstaining. Representative images of WT (A), BDNF$^{(+/-)}$ (B), WT+Tm (C), and BDNF$^{(+/-)}$+Tm (D) kidney tissue sections. Magnification ×40. Arrow heads indicate positive staining areas in tubules. Arrows show the positive immunolabeling in cells in the glomeruli. Bar graph shows mean ±SD of the proportion of GADD153-stained area in kidney sections (E). *p < 0.01, **p < 0.001 vs the WT group; #p < 0.05, ##p < 0.001 vs the BDNF$^{(+/-)}$ group; +p < 0.05 vs the WT+Tm group.

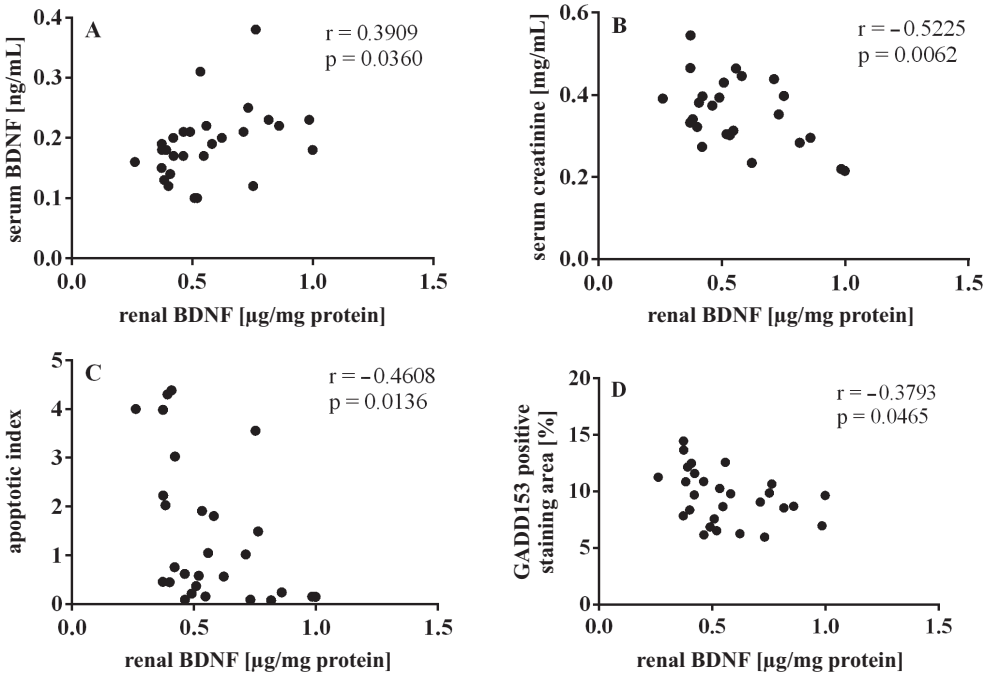


Fig. 6. Correlation between renal BDNF concentration and serum levels of BDNF (A), serum creatinine level (B), apoptotic index (C), and GADD153 immunostaining (D).

## Discussion

The present study demonstrated that 1) chronic BDNF deficiency did not affect parameters of ER stress such as GRP78, caspase-12 and CHOP/GADD153 or apoptosis under baseline conditions, 2) chronic BDNF deficiency made kidneys more susceptible to ER stress-induced apoptosis, and 3) endogenously produced BDNF may have a protective effect in kidneys by suppressing the CHOP/GADD153 pathway, which mediates ER stress-induced apoptosis.

The transgenic animal model used in this study is characterized by a lack of 1 of the 2 alleles which code for BDNF, which results in reduced BDNF expression. In previous studies, lower expression of BDNF in both the brain and peripheral tissues (such as the heart and intestines) has been reported in BDNF$^{(+/-)}$ mice, as well as lower circulating BDNF levels.[33–35] It is known that reduced BDNF levels induce hyperphagia, obesity, hyperglycemia, insulin resistance, and behavioral abnormalities (including increased aggression and hyperactivity) in these transgenic mice.[36,37] In the present study, the presence of a transgene in each animal was confirmed using PCR in the tail tissue and chronic BDNF deficiency was confirmed with measurements of BDNF, showing significantly lower serum and tissue levels among the BDNF$^{(+/-)}$ mice than in their wild-type littermates. Furthermore, increased food intake, body weight, aggression, and fat mass were observed in BDNF$^{(+/-)}$ mice during the experiments (data not shown).

The endoplasmic reticulum stress refers to physiological or pathological states such as oxidative stress, hypoxia and energy depletion which result in the accumulation of misfolded or unfolded proteins in the ER lumen.[1,2] As the ER stress-induced tissue injury has been implicated in a variety of diseases, ER research is attracting more and more attention. Tunicamycin, thapsigargin and brefeldin A are defined as ER stress-inducing agents with different mechanisms of action.[38] Tunicamycin induces ER stress by inhibiting N-linked glycosylation and can be used in both in vivo and in vitro studies.[18,20,30,31] Previous studies reported increased GRP78 levels in kidneys following Tm-induced renal ER stress, as well as reduced renal function after 1–5 days of Tm injection.[30,31] In the present study, comparing to the saline injected control groups, renal GRP78 levels found to be increased in both wild-type and BDNF heterozygous mice after 3 days of Tm injection. Furthermore, in accordance with previous studies, higher creatinine levels were detected in serum samples, signifying reduced renal function.

The results of our BDNF assays showed that Tm-induced ER stress did not alter serum or renal BDNF levels. Since BDNF is a secretory protein, a decrease in BDNF levels can be expected during ER dysfunction. Indeed, Wei et al. demonstrated that homocysteine-induced ER stress modeling was associated with a decrease in BDNF expression as well as increased ER stress in the hippocampus following 7 days of intracerebroventricular homocysteine administration.[39] Although there is no study in the literature to compare our results with, we propose that the short duration of the experiment (3 days) may have been insufficient for such an effect.

During ER stress, UPR can lead to cell repair and survival through the upregulation of ER chaperones and the global attenuation of protein synthesis, both of which result in a reduction of unfolded protein load in the ER lumen.[2–4] However, when ER functions are critically impaired, apoptosis can be triggered in order to protect the organism by eliminating the damaged cells. It has been reported that at least 3 pathways are involved in ER stress-induced apoptosis: transcriptional activation of CHOP/GADD153, activation of the JNK pathway and caspase-12.[5]

In this study, ER stress-induced apoptosis was proven by the increased apoptotic index in the Tm-injected mice. Although there were no differences in renal GRP78 levels between the WT+Tm and BDNF$^{(+/-)}$+Tm groups, apoptosis was more prominent in the BDNF-deficient animals. Furthermore, histological analysis of the renal sections revealed that the kidneys in the BDNF-deficient mice were more susceptible to Tm-induced tissue injury. These results may point to a protective role of endogenous BDNF against ER stress-induced apoptosis and renal injury. In previous studies, the protective effect of BNDF has been shown in neuronal tissue and endometrial epithelial cells during ER stress[18–20] as well as in adriamycin-induced nephropathy.[21] Although ER stress-induced apoptosis was greater in BDNF-deficient mice, the serum creatinine levels (an indicator for renal function) in these mice were not different from those of the WT+Tm group. Nevertheless, a negative correlation between renal BDNF expression and serum creatinine concentration suggests that BDNF may also be important in terms of renal function. The effect of BDNF would be important in preserving kidney function in renal diseases involving ER stress. It has been reported that baseline serum BDNF levels were significantly lower in hemodialysis patients than in the age-matched control group.[40] This finding is also consistent with the study by Kurajoh et al., in which serum BDNF levels were lower in patients with chronic kidney disease.[17] Considering the contribution of ER stress to deteriorating kidney function in chronic kidney disease,[23,24] it can be suggested that in patients with both chronic ER stress and BDNF deficiency, renal function would be more vulnerable.

The molecular mechanisms involved in the protective effects of BDNF against ER stress have been studied in neuronal cells. Shimoke et al. revealed that BDNF prevents ER stress-induced apoptosis by suppressing the activation of caspase-12 through a phosphatidylinositol 3-kinase (PI3-K) dependent mechanism, while Chen et al. suggested that the suppression of CHOP activation contributes to BDNF-mediated neuroprotection during ER stress.[18,19] In the present study, caspase-12 and GADD153 levels were measured in renal tissue and our results showed

that Tm administration significantly increased caspase-12 and GADD153 levels in both wild-type and BDNF heterozygous mice. Although the Tm-induced increase of caspase-12 levels were similar in both groups, the increase in GADD153 was greater in the BDNF heterozygous mice than the wild-type ones. These results suggest the possibility that endogenous BDNF could protect kidneys against ER stress-induced apoptosis by reducing the GADD153 increase.

The role of the CHOP/GADD153 pathway in Tm-induced renal damage has been demonstrated in both in vitro and in vivo studies. Carlisle et al. have shown that a siRNA-mediated inhibition of CHOP expression significantly reduced Tm-induced apoptosis in HK-2 cells (a cell model of human renal proximal epithelial cells).[41] The same study concluded that Tm-induced renal damage was less pronounced in CHOP$^{-/-}$ mice than in their wild-type littermates.[41] The positive correlation between GADD153 staining and renal apoptotic index in our study is consistent with previous findings. Additionally, the negative correlation between GADD153 and renal BDNF concentration and the higher expression of GADD153 during ER stress in the BDNF-deficient group both suggest the possibility that the protective effect of BDNF on ER stress-induced renal injury may be related to GADD153 expression.

As a result, this study showed that endogenous BDNF may play a protective role in kidneys against ER stress-induced apoptosis through the suppression of CHOP/GADD153. BDNF concentration could be associated with kidney function/kidney diseases. Patients with kidney disease might be examined for BDNF deficiency. Although further studies are needed, BDNF and its signaling pathways can be considered to have potential for protective/therapeutic strategies.

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# Melatonin protects against streptozotocin-induced diabetic cardiomyopathy through the mammalian target of rapamycin (mTOR) signaling pathway

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## Abstract

**Background.** Several studies demonstrated that the overexpression of mammalian target of rapamycin (mTOR) signaling protein is associated with cardiomyopathy. However, the effect of mTOR on the heart in hyperglycemic condition is still controversial.

**Objectives.** We aimed to investigate the expression of mTOR and antioxidant enzyme activity in cardiac hypertrophy in rats with streptozotocin-induced diabetes mellitus (DM), and the effects of the melatonin on diabetic cardiomyopathy (DCM).

**Material and methods.** Forty male Wistar rats were used as the experimental animals. The rats were divided into 4 groups (10 animals in each): group 1 (control group), group 2 (ethanol vehicle group), group 3 (iatrogenically DM-induced group), and group 4 (group given melatonin after iatrogenical DM induction). Streptozotocin was injected intraperitoneally to group 3 and 4 to induce experimental type 1 DM. Melatonin was injected intraperitoneally at a dose of 50 mg/kg/day for 56 days to group 4. We investigated expression of mTOR levels in heart muscle fibers of all groups. Laboratory analysis and transthoracic echocardiography were performed.

**Results.** Melatonin increased the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which were reduced due to hyperglycemia. The mTOR expression levels were significantly higher in group 3 (DM group) compared with controls, whereas melatonin treatment significantly decreased the levels of mTOR expression in group 4 (melatonin + DM group). Diabetic rats developed myocardial hypertrophy with preserved cardiac function.

**Conclusions.** Cardioprotective effect of melatonin may reduce damages caused by DM in the heart muscle fibers through the mTOR signaling pathway.

**Key words:** melatonin, diabetic cardiomyopathy, mammalian target of rapamycin

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# Introduction

Left ventricular hypertrophy (LVH) – an increase of left ventricular mass (LVM) – is an independent predictor of cardiovascular disease (CVD).[1] Diabetic cardiomyopathy (DCM) is one of the common diseases that cause LVH. High glucose-induced alterations in the myocardium structure, including cardiac fibrosis, cardiomyocyte hypertrophy and cardiac microvascular injury, play an important role in the pathophysiology of DCM and lead to the loss of systolic and diastolic function.[2–4] Intensive glycemic control can reduce microvascular and macrovascular complications of diabetes mellitus (DM), but it has not been fully demonstrated whether there is an obvious cardiovascular and all-cause mortality reduction in DCM.[5] Therefore, it is very important to carry out experimental studies on the prevention and treatment of DCM.

The mammalian target of rapamycin (mTOR), a member of the PI3K (phosphatidylinositol 3-kinase) related protein kinase family, is an important downstream molecule in the insulin and insulin-like growth factor 1 (IGF-1) signaling pathways, and plays a crucial role in cell growth, metabolism, and cell proliferation.[6] Previous reports have demonstrated the significant role of mTOR in cardiac function and metabolism in normal and diabetic hearts.[7,8] Release of free radicals caused by high glucose levels and consequent oxidative stress accelerate lipid peroxidation in muscle fibers, which results in the damage of their normal structure and the disruption of the cell membrane.[9,10] Oxidative stress and the mTOR signaling are 2 key factors within the pathological mechanisms of DCM.[11,12]

Melatonin (N-acetyl-5-methoxytryptamine) is a circadian endocrine molecule secreted by the pineal gland, which has important functions, such as being a powerful antioxidant, anti-apoptotic and free radical purifier.[13] As was shown in previous studies, melatonin is a highly effective antioxidant that can prevent the harmful effects of DM on the heart and greatly reduce oxidative damage in myocardial cells.[14,15] In light of the potential protective role of melatonin against myocardial damage, it is particularly noteworthy to explore whether melatonin interrupts the progression of DCM through modification of the mTOR signaling pathway and its downstream events. Therefore, this study was designed to discover the pathogenesis of the mTOR pathway in diabetes-induced cardiomyopathy and unveil the potential beneficial molecular basis of melatonin in DCM.

# Material and methods

## Animal model design

All the experimental procedures described in the study were performed in accordance with the Declaration of Helsinki and approved by Akdeniz University Institutional Animal Care and Use Committee Policies for Animal Use (Antalya, Turkey). As shown in Table 1, a total of 40 Wistar rats (8 weeks of age weighing 200–235 g) were randomly allocated into the following 4 groups; group 1 was the control group, group 2 was the vehicle group (50 cc/kg/day 10% ethanol injected group), group 3 was the DM group (iatrogenically DM-induced group), and group 4 was the melatonin + DM group (group given melatonin after iatrogenical DM induction) (Table 1).

## Induction of experimental diabetes mellitus type 1

To induce experimental type I DM (pancreatic insulin release was blocked iatrogenically by affecting Langerhans islet cells), rats in group 3 and group 4 were injected intraperitoneally with a single dose of streptozotocin (STZ, 130 mg/kg; Sigma-Aldrich, St. Louis, USA) in 0.1 mol/L citrate buffer with a pH of 4.5. As described previously, successful induction of DM was defined as a constant blood glucose >300 mg/dL in groups 3 and 4.[16,17]

## Transthoracic echocardiography and sample preparation

Transthoracic echocardiography was performed in rats from all groups using the ultrasound system iE33 (Philips Medical Systems, Andover, USA) with a 1–3 MHz broadband linear array transducer at the induction of DM or citrate buffer injection and 8 weeks later. After the rats were anesthetized (with 50 mg/kg ketamine hydrochloride), the heart was imaged in the 2-dimensional parasternal short-axis view 3 times over at least 30 cardiac cycles to assess reproducibility. Mid-ventricle was recorded at the level of the papillary muscle using the M-mode echocardiography, and interventricular septal and posterior wall thickness (IVS and LVPW), as well as left ventricular end-diastolic and end-systolic internal diameters (LVEDD and LVESD) were determined. According to a well-established method,

**Table 1.** Definition of animal groups and applied drugs

| Groups | Injected drug | Dose | Period | n |
|---|---|---|---|---|
| Group 1 (control) | none | none | none | 10 |
| Group 2 (vehicle) | 10% ethanol | 50 cc/kg/day | 1st day | 10 |
| Group 3 (DM) | streptozotocin | 130 mg/kg | for 56 days | 10 |
| Group 4 (melatonin + DM) | melatonin + streptozotocin | 50 mg/kg/day + 130 mg/kg | 1st day + for 56 days | 10 |

DM – diabetes mellitus.

the left ventricular mass (LVM) was calculated with the M-mode (cubic) using the formula: LVM = 1.04[(IVS + LVID + LVPW)$^3$ – (LVID)$^3$], where 1.04 is the specific gravity of muscle.[18] Left ventricle fractional shortening (FS) was used as an index of cardiac contractile function and was calculated from the inner diameters according to the formula: FS (%) = (LVEDD – LVESD)/LVEDD × 100.

## Melatonin administration

Melatonin (Sigma-Aldrich) was dissolved in absolute ethanol with a final concentration of 50 mg/kg. It was daily injected intraperitoneally in the melatonin + DM group (group 4) for 56 days.

## Tissue preparation

After the echocardiography animals were sacrificed. The hearts were carefully dissected and immersion-fixed in a fixative containing 4% paraformaldehyde and 0.2% picric acid for 24 h at 4°C. Then, the samples were washed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4°C, cryoprotected with 30% saccharose and frozen-embedded in Tissue-Tek.

## Immunohistochemistry

After incubating with rabbit antibodies against mTOR (1:500; Cell Signaling Tech., Beverly, USA) for 24 h at 4°C, biotin-conjugated goat anti-rabbit IgG was added to the 10-µm thick cryosections (1:300; Vector Lab., Peterborough, UK). Afterwards, the sections were exposed to avidin-biotin peroxidase complex (1:100; Elite ABC Kit, Vector Lab.) for 1 h. The reaction occurred with 0.05% 3.3-diaminobenzidine tetra-hydrochloride (Sigma-Aldrich) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01% $H_2O_2$ and 0.01% nickel ammonium sulfate, as described previously.[19] Incubations without the primary antisera served as controls.

## Western blot analysis

Fifty micrograms of protein was loaded into each line on 10% SDS-PAGE (sodium-dodecyl-sulfate polycrylamide) gels. After electrophoresis, the proteins were electro-transferred to the polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, USA). The cells were electro-transferred to Immobilon-P transfer membrane (Merck Millipore, Billerica, USA) and Tris-buffered saline (TBS) that included 5% non-fat milk for 1 h at room temperature. Then, the primary antibody was added and the samples were kept overnight at 4°C, washed; then, a secondary antibody was used. The same procedure was repeated for beta-actin which was used as internal control.

## Measurements of superoxide dismutase, catalase and glutathione peroxidase activity, and malondialdehyde levels

Heart samples were sonicated (Bandelin Sonopuls, HD 2070; Bandelin Electronic GmbH & Co. KG, Berlin, Germany) in 500 µL of ice-cold buffer (50 mM potassium phosphate, pH 7.0, 1 mM ethylenediaminetetraacetic acid – EDTA), centrifuged (glutathione peroxidase (GPx) – 15,000 g for 10 min at 4°C; catalase (CAT) – 10,000 g for 15 min at 4°C; superoxide dismutase (SOD) – 1500 g for 5 min at 4°C) and supernatants were collected and stored at –80°C for later biochemical analysis according to established methods.[20]

Superoxide dismutase activity was measured using a SOD activity assay kit (Cayman Chemical, Ann Arbor, USA) in accordance with the methods described by Kaya et al. and Misra et al.[20,21] An assay kit (Cayman-707002; Cayman Chemical) and spectrophotometric analysis were used to examine CAT enzymatic activity in the tissues in accordance with the methods described by Kaya et al. and Ziegler et al.[20,22] Glutathione peroxidase activity was estimated indirectly using the coupled reaction with glutathione reductase assessed with GPx kit (Sigma-Aldrich Chemia, Steinheim, Germany), in accordance with the methods previously by Kaya et al. and Paglia et al.[20,23] Malondialdehyde (MDA) levels were performed with a fluorometric method (as described in a previous paper) using 1.1.3.3-thtra-ethoxypropane as a standard.[24]

The protein concentrations were calculated spectrophotometrically (Shimadzu RF-5500; Shimadzu, Kyoto, Japan) using a protein assay reagent kit (Pierce, Rockford, USA), which is based on a modified Bradford method using bovine serine albumin as the standard.

## Quantification of staining intensities and statistical analysis

For the densitometry analysis, a Zeiss Axioscop-2 Plus microscope at ×40 magnification coupled with Image System Analysis, Axiovision v. 4.7 was used (Carl Zeiss, Jena, Germany). In a selected area, the average of the background color value was calculated. From the color measurement value of the heart muscle, this calculated average of the background gray value area was removed. Immunostaining intensity was presented as the mean of the measured heart muscle grey value minus the mean of the measured background grey value. Continuous variables were presented as mean + standard deviation (SD) for normal distributions. Normality of distribution was verified using the Shapiro-Wilk normality test. All continuous variables were distributing normally. All the data was presented as mean ±SD. Paired samples t-test was performed to compare glucose levels. One-way analysis of variation (ANOVA) was performed after the assessment of distributions for more than 2 independent groups and

a post hoc Dunnet test for unpaired data was applied to detect any differences between animal groups. SPSS software v. 21.0 for Windows (IBM Corp, Armonk, USA) was used for all statistical analyses. A p-value <0.05 was considered to indicate statistical significance.

# Results

## Blood glucose levels

The blood glucose levels are shown in Table 2. The blood glucose levels were found to be close to normal in all groups at the beginning of the study, but in animals that received STZ 130 mg/kg, the plasma glucose level was significantly increased in both DM groups (group 3 and group 4) at the end of the in vivo study ($p < 0.05$) (Table 2). Additionally, at the end of the study (56$^{th}$ day), there was a statistically significant weight loss in group 3 compared to 1$^{st}$ day (Table 3).

## Effect of hyperglycemia on cardiac phenotype

The hearts of animals in group 3 were hypertrophied and the ratio of the heart weight (W) and tibia length (L) as a marked of myocardial hypertrophy was significantly increased (W/L [mg/cm] from 72 ±3 to 90 ±8; $p < 0.05$). Echocardiography confirmed myocardial hypertrophy with protected systolic function in group 3, whereas echocardiographic data did not significantly change in group 4 (Table 3).

## The effect of melatonin treatment on expression of the mammalian target of rapamycin in heart muscle fibers shown with immunochemistry

The staining intensities of the expression of the mTOR levels in the diabetic group (group 3) were higher than in the control group (914.9 vs 510.7, $p < 0.05$). There was a difference of 79.2% between the control group (group 1) and the DM group (group 3). In the melatonin + DM group (group 4), the staining intensities of the expression of mTOR levels were significantly lower compared to the DM group (group 3), and this differences was 32.2% (620.3 vs 914.9, $p < 0.05$). No significant difference in the staining intensities of the expression of mTOR levels was found between the rats in groups 1 and 2 (510.7 vs 520.4, $p > 0.05$) (Fig. 1).

## The mammalian target of rapamycin in heart muscle fibers shown with western blot

The mTOR expression levels were measured in the rat heart muscle fibers in all groups and all data is shown in Fig. 2. The mTOR expression in rats treated with

**Table 2.** Blood glucose levels of group 3 (DM) and group 4 (melatonin + DM) post streptozotocin injection

| | 3$^{rd}$ day post injection (mg/dL) | 14$^{th}$ day post injection (mg/dL) | 28$^{th}$ day post injection (mg/dL) | 42$^{th}$ day post injection (mg/dL) | 56$^{th}$ day post injection (mg/dL) |
|---|---|---|---|---|---|
| Group 3 | 354 ±18 | 367 ±14 | 413 ±14 | 417 ±17 | 419 ±12* |
| Group 4 | 347 ±12 | 363 ±21 | 402 ±17 | 403 ±24 | 404 ±13* |

DM – diabetes mellitus. Data is presented as mean ± standard deviation (SD). * indicates a p-value <0.05 compared with the 3$^{rd}$ day of injection.

**Table 3.** Systolic and diastolic echocardiographic measurements of the groups

| Variables | | Group 1 n = 10 | Group 2 n = 10 | Group 3 n = 10 | Group 4 n = 10 |
|---|---|---|---|---|---|
| Systolic | septum [mm] | 1.43 ±0.03 | 1.45 ±0.06 | 1.97 ±0.03* | 1.39 ±0.13 |
| | LVESD [mm] | 1.50 ±0.06 | 1.63 ±0.30 | 1.43 ±0.15* | 1.57 ±0.10 |
| | LVPWD [mm] | 1.21 ±0.41 | 1.24 ±0.08 | 1.79 ±0.02* | 1.33 ±0.04 |
| Diastolic | septum [mm] | 0.68 ±0.40 | 0.71 ±0.01 | 1.05 ±0.05* | 0.79 ±0.05 |
| | LVEDD [mm] | 2.97 ±0.30 | 3.01 ±0.24 | 3.96 ±0.12* | 3.02 ±0.01 |
| | LVPWD [mm] | 0.82 ±0.03 | 0.80 ±0.04 | 1.17 ±0.06* | 0.79 ±0.02 |
| | LVM [mg] | 98 ±10 | 106 ±7 | 137 ±18* | 104 ±11 |
| | FS [%] | 54 ±1 | 44 ±3 | 49 ±3 | 51 ±7 |
| | heart rate [bpm/min] | 479 ±12 | 475 ±12 | 476 ±19 | 474 ±19 |
| Body weight | 1$^{st}$ day [g] | 213.70 ±8.32 | 219.50 ±6.36 | 223.80 ±8.39 | 217.60 ±7.87 |
| | 56$^{th}$ day [g] | 215.60 ±7.52 | 218.60 ±9.42 | 191.30 ±10.80* | 210.60 ±9.65 |

LVESD – left ventricular end systolic diameter; LVPWD – left ventricular posterior wall diameter; LVEDD – left ventricular end diastolic diameter; LVM – left ventricular mass; FS – fractional shortening. Data is presented as mean ± standard deviation (SD). * indicates a p-value <0.05 compared with control group.

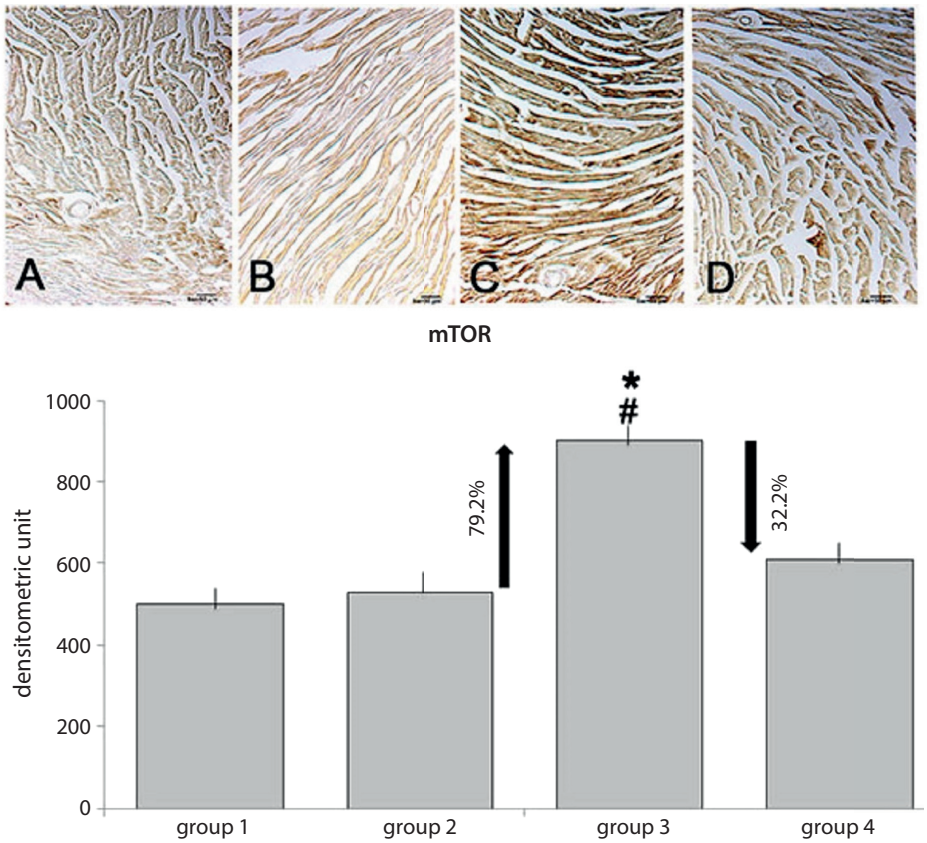


Fig. 1. Expression and densitometry analysis of the mammalian target of rapamycin (mTOR) in heart muscle fibers shown using immunohistochemistry. The staining intensities of the expression of the mTOR levels of the control group (group 1) (A), vehicle group (group 2) (B), diabetes mellitus (DM) group (group 3) (C), and melatonin + DM group (group 4) (D). Data was presented as mean ± standard deviation (SD); n = 10 for each group

* indicates statistically significant difference between DM group (group 3) and control group (group 1); # indicates statistically significant difference between the DM group (group 3) and melatonin + DM group (group 4); p < 0.05; bar: 50 µm.

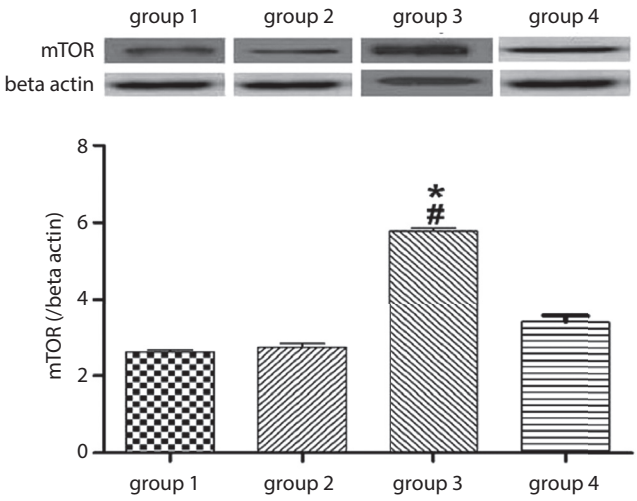


Fig. 2. The mammalian target of rapamycin (mTOR) levels in heart muscle fibers shown using western blot. The mTOR levels of the control (group 1), vehicle (group 2), diabetes mellitus (group 3), and melatonin + DM (group 4). Beta-actin was used for internal control. Data was presented as mean ± standard deviation (SD); n = 10 for each group

* indicates statistically significant difference between DM group (group 3) and control (group 1); # indicates statistically significant difference between DM group (group 3) and melatonin + DM group (group 4); p < 0.05; bar: 50 µm.

melatonin (group 4) was lower than in DM group (group 3) ($p < 0.05$). No difference was observed between the control group (group 1) and the vehicle group (group 2) (Fig. 2).

## Biochemical analysis

All 3 antioxidant enzymes (CAT, SOD and GPx) activities in heart fibers under diabetic conditions under melatonin treatment were measured. The serum antioxidant enzymes activities (SOD, CAT and GPx) were significantly lower in the DM group (group 3) than in the control group (group 1) (for all $p < 0.05$), whereas compared to the control, 50 mg/kg melatonin treatment resulted in a significant increase in the activities of antioxidant enzymes under hyperglycemic conditions. Additionally, it was shown that melatonin treatment protected the antioxidant activity in heart fibers under hyperglycemic conditions against the hypertrophy (Fig. 3).

# Discussion

In this study, 4 important results have been elicited. Firstly, the diabetic conditions triggered the development of cardiac hypertrophy. Secondly, mTOR expression increased in the diabetic setting is activated by high glucose and contributes to the development of DCM. Thirdly, melatonin treatment prevented the development of cardiac hypertrophy in hyperglycemia. Finally, melatonin treatment increased the activity of the antioxidant enzyme, which was reduced by the effect of DM.

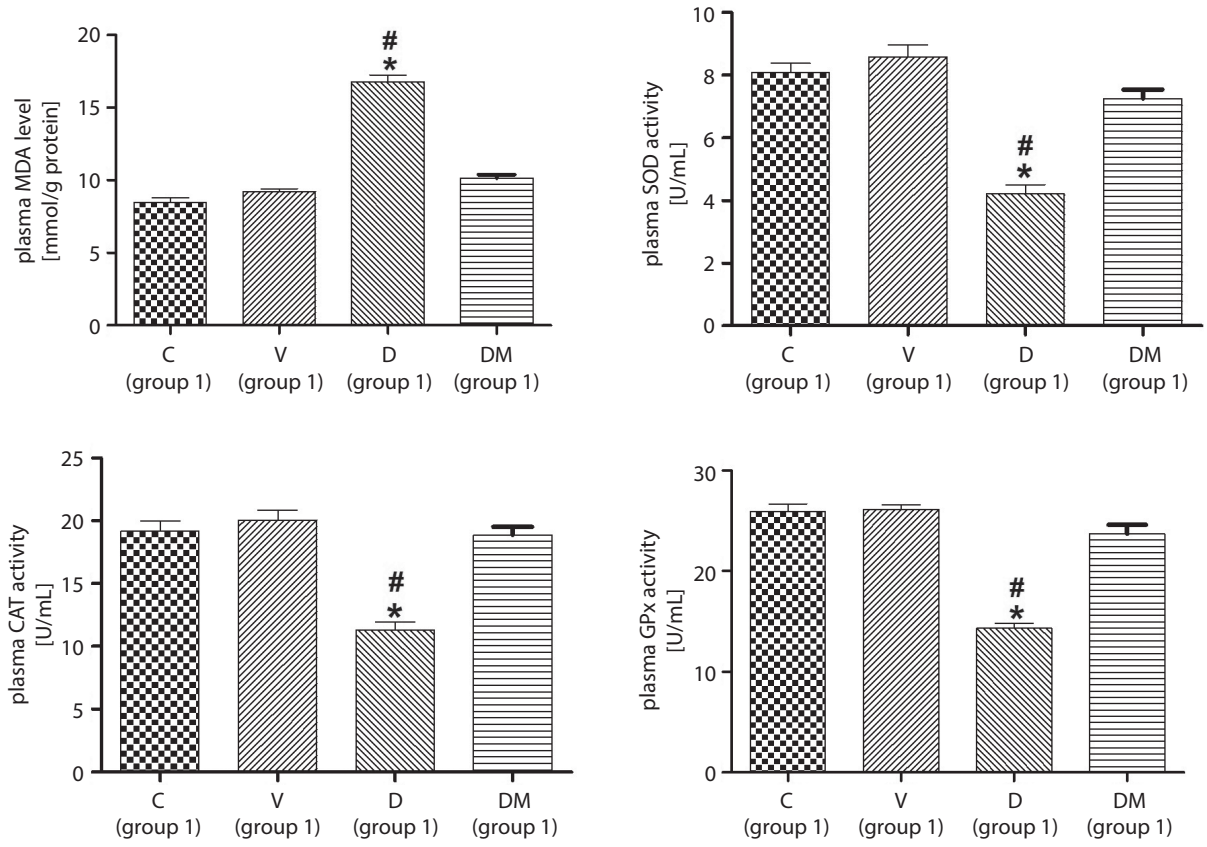


Fig. 3. Malondialdehyde (MDA) levels and antioxidant enzymes (glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD)) activities between groups. Diagram shows GPx, CAT and SOD enzymes activities and MDA levels in plasma samples of animal groups. Note that GPx, CAT and SOD activities were significantly lower in the DM group (group 3) than in the control group (group 1), and MDA levels were significantly higher in the DM group (group 3)

* indicates statistically significant difference between DM group (group 3) and control group (group 1); # indicates statistically significant difference between DM group (group 3) and melatonin + DM group (group 4); $p < 0.05$; bar: 50 µm.

Diabetic cardiomyopathy is a clinical condition diagnosed when ventricular dysfunction occurs in the absence of coronary atherosclerosis and hypertension according to the cardiology guidelines.[25] Diabetic cardiomyopathy observed in insulin-resistant or hyperinsulinemic states is characterized by impaired myocardial insulin signaling, mitochondrial dysfunction, endoplasmic reticulum stress, impaired calcium homeostasis, abnormal coronary microcirculation, activation of renin-angiotensin-aldosterone system, sympathetic nervous system, and maladaptive immune responses.[26] These pathophysiological changes lead to multiple toxic effects on cardiomyocytes. The mTOR is an important protein modulator of the insulin-signaling pathway. It was previously demonstrated that in DM, chronically activated mTOR induces multiple pathological events, including a negative feedback loop that suppresses insulin receptor substrate. The mTOR signaling pathway is correlated to the occurrence of both cardiomyopathy and cardiac hypertrophy. At the same time, researchers have shown that mTOR plays an important role in the cardiac hypertrophy that occurs in STZ-induced experimental DM. Moreover, short-term treatment with rapamycin, a mTOR inhibitor, was a promising strategy for cardiac diseases such as acute myocardial infarctions and cardiac hypertrophy in DM.[7,8,27–29]

In addition, it has been emphasized that melatonin treatment is the mediator of mTOR activation. For example, in one study, it was found that melatonin treatment reduces mTOR activation, which reduces cell viability and proliferation.[30] Our data supports the functional significance of mTOR in diabetic cardiac hypertrophy. In our study, we also provided an effective method of suppressing mTOR with melatonin treatment. This study is the first to demonstrate the molecule pathway of the effect of melatonin on diabetic cardiac hypertrophy by decreasing mTOR. In parallel with the literature, we propose a new pathway which higher glucose level stimulus induces diabetic cardiomyopathy through the activation of mTOR, and this pathway could be eradicated by melatonin treatment.

Anti-oxidative enzymes (SOD, CAT and GPx), which metabolize radicals or reactive oxygen species (ROS) into non-radical products, play a crucial role in antioxidant defense.[31] In different studies, it has been established that melatonin treatment increases the activity of antioxidant enzymes, such as SOD, CAT and GPx in different tissues in which oxidative stress was induced by various agents.[32,33]

It was proposed by several studies that high levels of oxidative stress are related to apoptosis of myocardial cells and the pathological process that causes cardiac hypertrophy.[34] It was shown in the current study that MDA levels were increased with DM, and this situation is indicating that high stress from ROS is involved in the pathological development of diabetic cardiomyopathy. Additionally, the melatonin + DM group (group 4) had remarkably increased antioxidant enzymes activities and significantly decreased MDA levels, which is a marker of oxidative stress compared to the DM group (group 3).

In conclusion, our data shows that the cardioprotective effect of melatonin caused by inhibiting mTOR expression may prevent cardiac hypertrophy. Demonstrating the underlying mechanism behind mTOR-mediated pathophysiological features in the heart will be important in improving therapies of DCM concerning melatonin.

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# Association between expression of HOTAIR and invasiveness of gliomas, and its predictive value

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

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## Abstract

**Background.** Hox transcript antisense intergenic RNA (HOTAIR) is upregulated and associated with a poor prognosis in many cancer types. Besides, it is involved in the invasion and metastasis of non-small-cell lung cancer and nasopharyngeal carcinoma.

**Objectives.** The aim of this study was to investigate the association between the expression of HOTAIR and the grades of gliomas, and to explore its possible mechanism, as well as to evaluate the value of HOTAIR applied in predicting the grades of gliomas.

**Material and methods.** A total of 123 patients undergoing glioma surgeries were enrolled. Patients with grade I and grade II–IV tumors were regarded as the control group (n = 36) and the case group (n = 87), respectively. The expression of HOTAIR, matrix metalloproteinase 7 (MMP-7), matrix metalloproteinase 9 (MMP-9), and vascular endothelial growth factor (VEGF) was detected with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in glioma tissues and then compared between grade I and grades II–IV. The correlation between the relative expression of HOTAIR and that of MMP-7, MMP-9 and VEGF was analyzed. Multivariate analysis was performed to identify independent risk factors. Receiver operating characteristic (ROC) curve was employed to evaluate the predictive value.

**Results.** The relative expression of HOTAIR, MMP-7, MMP-9, and VEGF was lower in glioma tissues of grade I than in the case of grades II–IV, and the relative expression of HOTAIR was positively correlated with the relative expression of MMP-7, MMP-9 and VEGF. Multivariate analysis showed that the relative expression of HOTAIR was independently associated with the grades of gliomas, but the relative expression of MMP-7, MMP-9 and VEGF was not. Besides, multivariate analysis showed that the expression level of HOTAIR >0.40 was an independent risk factor for grades II–IV after classifying the relative expression of HOTAIR, and ROC analysis showed that the expression level of HOTAIR >0.40 had a moderate value when applied in predicting grades II–IV.

**Conclusions.** Hox transcript antisense intergenic RNA might promote the invasion of gliomas through upregulating the expression of MMP-7, MMP-9 and VEGF, and the expression level of HOTAIR >0.40 had a moderate value when applied in predicting grades II–IV.

**Key words:** gliomas, hox transcript antisense intergenic RNA, grades, predictive value, invasiveness

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# Introduction

As the most common primary tumors in the central nervous system among adults,[1,2] gliomas are classified into 4 grades that reflect the degree of invasiveness/malignancy according to the current 2016 World Health Organization (WHO) Classification of Tumours of the Central Nervous System.[3] Grade I refers to benign and more circumscribed tumors with low proliferative potential. Grade II–IV tumors are malignant and diffusely infiltrative with increased cellular abnormalities, and are referred to as diffuse gliomas because of the characteristic of extensive, diffuse infiltration of glioma cells into the brain tissue.[4]

Hox transcript antisense intergenic RNA (HOTAIR) belongs to long non-coding RNAs (lncRNAs). Many studies have indicated that lncRNAs can be applied in predicting the prognosis in various cancer types.[5–8] Hox transcript antisense intergenic RNA is the first lncRNA discovered to have a trans-repressive role.[9,10] It is upregulated and associated with a poor prognosis in many cancer types.[11–15] Apart from that, it is involved in the invasion and metastasis of non-small-cell lung cancer and nasopharyngeal carcinoma through modulating the expression of matrix metalloproteinase 7 (MMP-7), matrix metalloproteinase 9 (MMP-9) and vascular endothelial growth factor (VEGF).[16,17] In this paper, the association between the relative expression of HOTAIR and the grades of gliomas was analyzed, and the possible mechanism was explored; moreover, the value of HOTAIR applied in predicting the grades of gliomas was evaluated. The aim of the study was to determine the potential of HOTAIR as a biomarker of invasiveness for gliomas.

# Material and methods

## Patients

A total of 123 patients undergoing glioma surgery were enrolled in Heze Municipal Hospital, China, from January 2013 to January 2017. There were 70 males and 53 females among them, and their average age was 44.76 ±15.82 years. A definite diagnosis of gliomas was made through a histopathologic examination in all patients. Neither radiotherapy nor chemotherapy were performed before operation. According to the current 2016 WHO Classification of Tumours of the Central Nervous System, grades I, II, III, and IV were found in 36, 32, 33, and 22 patients, respectively. The patients with grade I and grade II–IV tumors were regarded as the control group (n = 36) and the case group (n = 87), respectively. Immediately after excision, glioma tissue samples were placed in liquid nitrogen for 10 min, and then stored in a refrigerator at a temperature of –80°C. This study received the approval of the ethics committee of Heze Municipal Hospital (approval No. hz201216039). All patients provided informed consent.

## RNA extraction and quantitative reverse transcription-polymerase chain reaction

The total RNA from glioma tissues was extracted with the TRIzol™ reagent (Ambion, Cat. No. 15596-026; Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The complementary DNA (cDNA) was synthesized with the First Strand cDNA Synthesis kit (Novagen, Cat. No. 69001-3; Merck & Co., Inc., Kenilworth, USA). The primer sequences for HOTAIR, MMP-7, MMP-9 and VEGF were 5'-GAGAACGCTGGAAAAACCTG-3' (forward) and 5'-GTCAGAAAATGCTTCCCCAA-3' (reverse), 5'-TACAGGATCATTGGCTACACACC-3' (forward) and 5'-GGTCACATCGCTCCAGACT-3' (reverse), 5'-TGTACCGCTATGGTTACACTCG-3' (forward) and 5'-GGCAGGGACAGTTGCTTCT-3' (reverse), and 5'-AGGGCAGAATCATCACGAAGT-3' (forward) and 5'-AGGGTCTCGATTGGATGGCA-3' (reverse), respectively. *GADPH* was regarded as the reference gene (forward primer: 5'-AGGTCCACCACTGACACGTT-3', and reverse primer: 5'-GCCTCAAGATCATCAGCAAT-3'). The amplification conditions were as follows: 94°C for 3 min; 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; and 72°C for 7 min. The quantitative reverse transcription-polymerase chain reaction (qRT-PCR), data acquisition and analysis were performed with the ABI 7500 system (Life Technologies, Grand Island, USA). The relative expression of target genes was evaluated with the $2^{-\Delta\Delta Ct}$ method.

## Statistical analysis

Statistical analysis was carried out using the IBM SPSS Statistics v. 21.0 for Windows (IBM Inc., Armonk, USA), and significance was set at $p < 0.05$. The distribution of data was determined with the Kolmogorov–Smirnov test. Normally distributed data was expressed as mean ± standard deviation (SD) and compared with Student's t-test between glioma grade I and grades II–IV. Simple linear correlation analysis was employed to analyze the correlation between normally distributed data. In order to analyze the association between the expression levels of HOTAIR and the grades of gliomas, the relative expression of HOTAIR was classified into 4 levels based on the quartiles of grade I (level 1: ≤Q1; level 2: >Q1 and ≤Q2; level 3: >Q2 and ≤Q3; level 4: >Q3). Univariate analysis was performed with a simple $\chi^2$ test. Multivariate analysis was then performed with a backward stepwise logistic regression model. Receiver operating characteristic (ROC) curve was employed to evaluate the predictive value of independent risk factors.

# Results

## Relative expression of HOTAIR

The relative expression of HOTAIR in glioma tissues of grade I (n = 36) and grades II–IV (n = 87) was 0.41 ±0.20 and 0.80 ±0.31, respectively. The difference was statistically significant ($p < 0.001$).

## Relative expression of MMP-7, MMP-9 and VEGF

The relative expression of MMP-7, MMP-9 and VEGF in glioma tissues of grade I was 0.35 ±0.17, 0.31 ±0.19 and 0.39 ±0.21, respectively, all of which were significantly lower compared to grades II–IV (0.79 ±0.26, 0.70 ±0.25 and 0.83 ±0.35, respectively) (all $p < 0.001$).

## Simple linear correlation analysis between relative expression of HOTAIR and relative expression of MMP-7, MMP-9 and VEGF

Simple linear correlation analysis showed that the relative expression of HOTAIR was positively correlated with the relative expression of MMP-7, MMP-9 and VEGF ($r = 0.782$, 0.791 and 0.778, respectively; all $p < 0.05$) in all 123 glioma patients.

## Association between relative expression of HOTAIR, MMP-7, MMP-9 and VEGF, and grades of gliomas

Multivariate analysis showed that the relative expression of HOTAIR was independently associated with the grades of gliomas after adjusting sex and age, but the relative expression of MMP-7, MMP-9 and VEGF was not independently associated with the grades of gliomas (Table 1). High expression of HOTAIR was an independent risk factor for grades II–IV.

## Association between expression levels of HOTAIR and grades of gliomas

The classifying and univariate analysis results are shown in Table 2. Multivariate analysis showed that the expression level of HOTAIR >Q2 (0.40) was an independent risk factor for grades II–IV after adjusting sex and age (Table 3). The odds ratios (ORs) of level 3 and 4 were 5.061 and 9.016, respectively.

## Predictive value of HOTAIR expression level

The results of the expression level of HOTAIR >Q2 (0.40) applied in predicting grades II–IV are shown in Table 4. The area under ROC curve (AUC) was 0.716 (standard error: 0.056; $p < 0.001$), which indicated that the expression level of HOTAIR >Q2 (0.40) had a moderate value when applied in predicting grades II–IV.

# Discussion

Since HOTAIR is firstly discovered in human fibroblasts,[18] many studies have reported that HOTAIR is significantly upregulated in many cancer types and play an important role in their development. For gliomas, HOTAIR can act as a prognostic factor for the survival

**Table 1.** Association between relative expression of HOTAIR, MMP-7, MMP-9 and VEGF, and grades of gliomas after adjusting sex and age

| Genes | Regression coefficient | Standard error | Wald | OR | 95% CI | p-value |
|---|---|---|---|---|---|---|
| HOTAIR | 0.421 | 0.184 | 6.597 | 4.372 | 1.286–7.958 | 0.029 |
| MMP-7 | 0.283 | 0.122 | 1.674 | 1.984 | 0.783–4.141 | 0.256 |
| MMP-9 | 0.276 | 0.117 | 1.582 | 1.886 | 0.754–3.908 | 0.275 |
| VEGF | 0.291 | 0.124 | 1.785 | 2.094 | 0.791–4.247 | 0.244 |

HOTAIR – hox transcript antisense intergenic RNA; MMP-7 – matrix metalloproteinase-7; MMP-9 – matrix metalloproteinase-9; VEGF – vascular endothelial growth factor; OR – odds ratio; CI – confidence interval; Wald – the Wald $\chi^2$ test. Grading of gliomas was regarded as the dependent variable (binary variable: grade II–IV was assigned to 1, and grade I to 0), and the relative expression of HOTAIR, MMP-7, MMP-9, and VEGF (quantitative variables), age (quantitative variable) and sex (binary variable) were regarded as the independent variables.

**Table 2.** Classifying and univariate analysis (simple $\chi^2$ test) results of relative expression of HOTAIR

| Relative expression of HOTAIR | Grades II–IV (n = 87) | Grade I (n = 36) | $\chi^2$ | p-value |
|---|---|---|---|---|
| Level 1: ≤Q1 (0.19) | 2 | 9 | 32.379 | <0.001 |
| Level 2: >Q1 and ≤Q2 (0.40) | 4 | 9 | | |
| Level 3: >Q2 and ≤Q3 (0.58) | 19 | 9 | | |
| Level 4: >Q3 | 62 | 9 | | |

Q1–Q3 – quartiles of grade I.

Table 3. Association between expression levels of HOTAIR and grades of gliomas after adjusting sex and age

| Expression levels of HOTAIR | Regression coefficient | Standard error | Wald | OR | 95% CI | p-value |
|---|---|---|---|---|---|---|
| Level 1 | – | – | 7.325 | – | – | 0.009 |
| Level 2 | 0.412 | 0.283 | 1.584 | 1.575 | 0.332–3.658 | 0.274 |
| Level 3 | 0.476 | 0.315 | 6.278 | 5.061 | 1.158–7.029 | 0.032 |
| Level 4 | 0.511 | 0.367 | 18.213 | 9.016 | 1.392–12.164 | <0.001 |

Grading of gliomas was regarded as the dependent variable (binary variable: grade II–IV was assigned to 1, and grade I to 0), and the relative expression of HOTAIR (rank variable), age (quantitative variable) and sex (binary variable) were regarded as the independent variables. Level 1 was regarded as the reference.

Table 4. Results of the expression level of HOTAIR >Q2 (0.40) applied in predicting grade II–IV

| Cut-off value | Predicting criterion | Grade II–IV (n = 87) | Grade I (n = 36) |
|---|---|---|---|
| Q2 (0.40) | >0.40 (positive) | 81 | 18 |
| | ≤0.40 (negative) | 6 | 18 |

of glioma patients and a biomarker for the identification of glioma molecular subtypes.[11] In vitro, HOTAIR is associated with the progression of the cell cycle of glioma cells. Downregulation of HOTAIR can inhibit the proliferation, invasion and migration of tumor cells, and meanwhile promote the apoptosis of glioma cells.[2,19] In our study, the relative expression of HOTAIR was lower in glioma tissues of grade I than in the case of grades II–IV, and multivariate analysis showed that high expression of HOTAIR (>0.40) was an independent risk factor for grades II–IV. These results suggested that HOTAIR was associated with the grades of gliomas and could act as a biomarker reflecting the invasiveness of gliomas.

Hox transcript antisense intergenic RNA is involved in the Wnt/β-catenin, Akt and p53 signaling pathways through the combination with polycomb repressive complex 2 (PRC2) in tumor cells,[14] which may affect the invasion and migration of tumor cells.[20] The Wnt/β-catenin signaling pathway plays an important role in the development of tumors.[21] HOTAIR can activate the Wnt/β-catenin signaling pathway through downregulating the expression of Wnt inhibitory factor 1 (WIF-1),[22] which may reduce the degradation of β-catenin in the cytoplasm.[23] β-catenin can upregulate the expression of VEGF, MMP-7 and cylinD1 as a transcriptional factor after the entry into the nucleus. Vascular endothelial growth factor, MMP-7 and cylinD1 may induce the formation of tumor vessels, and promote the invasion and migration of tumor cells.

As one of important tumor suppressor genes in the Akt signaling pathway, the phosphatase and tensin homology deleted on chromosome 10 (PTEN) gene can inhibit the activation of the Akt signaling pathway. Hox transcript antisense intergenic RNA may silence PTEN through enhancing the promoter methylation of PTEN, which may lead to the activation of the Akt signaling pathway. The activation of the Akt signaling pathway can promote the invasion and migration of tumor cells, and meanwhile reduce the apoptosis of tumor cells by upregulating the expression of MMP-9 and downregulating the expression of BAX and FOXO1.[12] Besides, HOTAIR may induce the formation of tumor vessels by upregulating the expression of VEGF and downregulating the expression of transforming growth factor β (TGF-β) via the p53 signaling pathway.[24]

In this study, the relative expression of HOTAIR, MMP-7, MMP-9, and VEGF was lower in glioma tissues of grade I than in the case of grades II–IV, and the relative expression of HOTAIR was positively correlated with the relative expression of MMP-7, MMP-9 and VEGF. However, multivariate analysis showed that the relative expression of MMP-7, MMP-9 and VEGF was not independently associated with the grades of gliomas. These results suggested that high expression of MMP-7, MMP-9 and VEGF might be induced by HOTAIR in grade II–IV tumors. Therefore, HOTAIR might promote the invasion of gliomas through upregulating the expression of MMP-7, MMP-9 and VEGF. Next, the value of the expression level of HOTAIR >0.40 applied in predicting grades II–IV was evaluated with ROC curve. This result showed that the expression level of HOTAIR >0.40 had a moderate value when applied in predicting grades II–IV.

## Conclusions

Hox transcript antisense intergenic RNA might promote the invasion of gliomas through upregulating the expression of MMP-7, MMP-9 and VEGF, and the expression level of HOTAIR >0.40 had a moderate value when applied in predicting grades II–IV.


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# Premature cyclosporine cessation and TBI-containing conditioning regimen increase the risk of acute GvHD in children undergoing unrelated donor hematopoietic stem cell transplantation

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## Abstract

**Background.** Acute graft-versus-host disease (aGvHD) is a potentially fatal complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Identifying its risk factors would enable the proper prophylaxis and management, which may significantly improve the general outcome of children treated with HSCT.

**Objectives.** The aim of this single-center, retrospective cohort study was to assess the potential risk factors for grades II–IV of aGvHD in children after the 1st allo-HSCT from an unrelated donor (UD), performed as a result of an underlying malignant disease.

**Material and methods.** From among patients who received HSCT in our center in the years 2004–2015, 237 were included in the study cohort. All the patients received standard aGvHD prophylaxis consisting of cyclosporine (CsA) and a short course of methotrexate (MTX). Various clinical and epidemiological features, the transplant proceedings, graft composition, conditioning regimens, as well as the duration and coherence of aGvHD prophylaxis were analyzed as potential risk factors for aGvHD.

**Results.** The incidence of II–IV aGvHD in the study cohort was 58.6%. The median time of the diagnosis of aGvHD was 18 days post-HSCT. In the multivariate analysis, risk factors significantly associated with grades II–IV of aGvHD were: myeloablative conditioning regimen containing total body irradiation (TBI-MAC) (RR (relative risk): 1.69; $p = 0.03$), premature termination of CsA administration due to its toxicity (RR: 1.99; $p = 0.0003$) and HSCT performed before the year 2009 (RR: 1.97; $p = 0.0001$). Donor and recipient age, donor–recipient sex mismatch, stem cell source, risk of disease, and amount of infused CD34+ cells seem to be insignificant as risk factors for aGvHD. The overall survival (OS) of patients with aGvHD was noticeably worse that in those who were aGvHD-free: 60.8% vs 74.1% ($p = 0.08$).

**Conclusions.** The conditioning regimen and the proper aGvHD prophylaxis, including continuous CsA administration, have a major impact on aGvHD occurrence. According to our results, the termination of CsA therapy should be carefully considered, and avoided if possible.

**Key words:** risk factors, acute graft-versus-host disease, hematopoietic stem cell transplantation, prophylaxis

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## Introduction

Acute graft-versus-host disease (aGvHD) remains one of the most common life-threatening complications after allogeneic hematopoietic stem cells transplantation (allo-HSCT), contributing significantly to morbidity and mortality.[1] Several studies have reported numerous risk factors associated with increased incidence of aGvHD in the adult population. Among them, human leukocyte antigen (HLA) mismatch, type of the conditioning regimen, female donor to male recipient, and higher recipient age have proven to be the most valid.[2–6] However, it needs to be highlighted that pediatric HSCTs differ remarkably from those in the adult population, particularly regarding the indications for transplant, existing comorbidities, previous treatment, and transplantation regimens.

In the pediatric population, the reported incidence of aGvHD varies from 30% to 80%, despite given prophylaxis.[2,7–10] First-line treatment for aGvHD is based mostly on high-dose steroids and turns out to be abortive in about 50% of cases. There are many possible second-line treatment agents and protocols, but, even when providing promising results, none of them have been proven unequivocally effective.[11,12] On that account, not only is an intensive quest for successful second-line treatment for aGvHD needed, but first and foremost, a precise evaluation of its risk factors, especially for a pediatric cohort of patients.

The aim of this single-center, retrospective cohort study was to assess which potential risk factors have a significant influence on the frequency of grades II–IV of aGvHD in children with a malignant disease after the 1st HSCT from an unrelated donor (UD). An analysis of the general outcome, overall survival (OS) and transplant-related mortality (TRM) in the studied cohort was the secondary aim of this research.

## Patients and methods

A retrospective analysis of the medical records of the patients who underwent HSCT in the Department of Pediatric Hematology, Oncology and Bone Marrow Transplantation of Wroclaw Medical University (Poland) in the years 2004–2015 was performed. According to the inclusion criteria, all patients under 21 years who were suffering from a malignant disease and underwent the 1st allo-HSCT procedure from an UD were considered in this study. Due to their small number, patients who received cord blood were excluded from the study. The final cohort included a total amount of 237 children and young adults (Table 1).

The grafted cells were obtained from human leukocyte antigen (HLA)-allele matched or mismatched UDs (age: 19–56 years; median age: 31 years). The HLA typing was performed at the high-resolution level (4 digits) in A*, B*, Cw*, DRB1*, and DQB1* alleles. A matched donor was defined as 10/10 or 9/10 HLA-compatible donor. More than 1 mismatched allele was defined as a mismatched unrelated donor (MMUD). According to the ALL-SCT BFM International 2008 criteria, all 10 alleles were high-resolution types.

Table 1. Study cohort characteristics and analyzed risk factors for aGvHD

| Patients' characteristics | n | % |
|---|---|---|
| Diagnosis | | |
| acute leukemia | 162 | 68.4 |
| chronic myeloblastic leukemia | 28 | 11.8 |
| myelodysplastic syndrome | 32 | 13.5 |
| lymphoma | 15 | 6.3 |
| Patient sex | | |
| male | 148 | 6.3 |
| female | 89 | 37.6 |
| Donor–recipient sex mismatch | | |
| matched | 142 | 59.9 |
| male to female | 44 | 18.6 |
| female to male | 51 | 21.5 |
| Patient age [years] | | |
| ≤5 | 53 | 22.4 |
| >5 and ≤15 | 129 | 54.4 |
| >15 | 55 | 23.2 |
| Donor age [years] (median: 31) | | |
| ≤median | 123 | 51.9 |
| >median | 114 | 48.1 |
| Amount of CD34+ cells (median: 8.29 × $10^6$/kg) | | |
| ≤median | 119 | 50.2 |
| >median | 118 | 49.8 |
| Amount of CD34+ cellls (quartile – cut-off value) | | |
| Q1 (5.01 × $10^6$/kg) | 60 | 25.3 |
| Q2 (8.29 × $10^6$/kg) | 59 | 24.9 |
| Q3 (12.09 × $10^6$/kg) | 60 | 25.3 |
| Q4 (51.85 × $10^6$/kg) | 58 | 24.5 |
| Stem cells source | | |
| peripheral blood | 201 | 84.8 |
| bone marrow | 36 | 15.2 |
| Conditioning regimen | | |
| TBI-MAC | 84 | 35.4 |
| Bu-MAC | 105 | 44.3 |
| other (RTC/RIC) | 48 | 20.3 |
| Premature cessation of CsA | | |
| yes | 47 | 19.8 |
| no | 190 | 80.2 |
| Risk of disease | | |
| high | 115 | 48.5 |
| standard | 122 | 51.5 |
| Year of transplantation (median: 2009) | | |
| ≤2009 | 126 | 53.2 |
| >2009 | 111 | 46.8 |
| Year of transplantation (quartile) | | |
| Q1 (2004–2006) | 72 | 30.4 |
| Q2 (2007–2009) | 54 | 22.8 |
| Q3 (2010–2012) | 58 | 24.5 |
| Q4 (2013–2015) | 53 | 22.3 |

TBI-MAC – myeloablative conditioning regimen containing total body irradiation; Bu-MAC – myeloablative conditioning regimen containing busulfan; RTC – reduced-toxicity conditioning; RIC – reduced-intensity conditioning; CsA – cyclosporine.

## Graft-versus-host disease prophylaxis

Within the standard prophylaxis for aGvHD, all patients, since the day before HSCT received intravenous cyclosporine (CsA) in a unified initial dose of 1.5 mg/kg twice per day in 2-hour infusions. Further dosage of CsA was adjusted to the CsA trough level (target level: 100–200 µg/L), measured every second day in the majority of patients. Therefore, the dosage was modified to maintain the target level. Cyclosporine was switched into oral formulation when patient was able to tolerate oral intake. According to standard protocols, in patients without signs of GvHD, CsA administration was discontinued on day 120 in the majority of cases, preceded by gradual dose reduction. The 2nd prophylactic agent, methotrexate (MTX), was administered threefold in a standard dose of 10 mg/m$^2$ on days 1, 3 and 6 after HSCT. All patients were given in vivo T-cell depletion by either rabbit anti-thymocyte globulin (ATG) (n = 229) (ATG-Fresenius/Grafalon® (Neovii Biotech GmbH, Gräfelfing, Germany) – median: 45 mg/kg; or Thymoglobuline® (Genzyme Europe B.V., Naarden, the Netherlands) – median 7.5 mg/kg) or Campath-1H (Genzyme Europe BV) (n = 8) (median: 1 mg/kg).

## Acute graft-versus-host disease diagnosis and staging

The diagnosis of aGvHD was based on the clinical findings and/or histopatological findings in the skin, gastrointestinal tract mucosa and liver biopsies.[13–15] Grading and staging of aGvHD was performed using pediatric-specific criteria published by Jacobsohn.[15] In the presented research, only grades II–IV were considered. Patients who presented symptoms of aGvHD after donor lymphocyte infusion were excluded from this study.

## Risk factors and definitions

Potential risk factors for developing aGvHD were carefully analyzed and are listed in Table 2. The patients' risk status was defined by our own study-specific modification of the classification proposed by Meisel et al.[16] Acute leukemia (AL) in 1st complete remission (1CR), chronic myeloblastic leukemia (CML) in chronic phase (CP), myelodysplastic syndrome-refractory cytopenia (MDS), and non-Hodgkin lymphoma (NHL) in complete remission (CR) was qualified as a standard risk. Acute leukemia in ≥2CR or non-remission, CML in equal to or greater than accelerating phase, MDS-refractory anemia with an excess of blasts, NHL in non-remission, and juvenile myelomonocytic leukemia (JMML) were defined as a high risk. Early termination of CsA administration was defined as termination before day 60 post-HSCT due to the toxicity of CsA. Cyclosporine-induced nephrotoxicity was diagnosed according to the following criteria: fall in baseline glomerular filtration rate – GFR >25% or doubling the serum creatinine level.[17] Neurotoxicity was defined as seizures, polyneuropathy, ataxy, impaired consciousness, or dizziness.[18] Children with other obvious causes of neuro- and nephrotoxicity were excluded. Only patients who presented first symptoms of aGvHD after the cessation of CsA administration were considered under the influence of this risk factor.

## Conditioning regimens

As many as 189 patients received myeloablative conditioning regimen (MAC) based either on myeloablative doses (12.8–19.6 mg/kg) of busulfan (Bu-MAC; n = 105), or on total body irradiation (TBI) at a median dose of 12 Gy (TBI-MAC; n = 84). Thirty-eight patients received reduced-toxicity conditioning (RTC), consisting mainly of fludarabine (160 mg/m$^2$) and treosulfan (36–42 mg/kg) with thiotepa (10 mg/kg), melphalan (140 mg/m$^2$) or cyclophosphamide (120 mg/kg). Ten patients received reduced-intensity conditioning (RIC) regimen, consisting of fludarabine with either melphalan or low, non-myeloablative doses of busulfan (2 mg/kg).[19,20] In the statistical analysis, patients receiving RIC and RTC were considered collectively as 1 group.

## Statistical analysis

The statistical analysis was performed using R statistical software (https://www.r-project.org/). Variables were analyzed in terms of their prognostic impact on aGvHD, OS and TRM. First, powered by the $\chi^2$ test, we compared the baseline characteristics of patients with II–IV aGvHD and those with either no aGvHD or grade I aGvHD. Thereafter, patient data was entered into a competing risk regression model. We prosecuted 5 variants of regression models, containing either all or only the significant factors. Using the Bayesian Information Criterion (BIC) test, we selected the most adequate model. Acute graft-versus-host disease was defined by the abovementioned criteria, analyzed as time to event with death, relapse or rejection without aGvHD as a competing event. The p-values <0.05 were considered significant. The survival analysis was performed using the Kaplan–Meier estimation and the survival graphs were compared using the log-rank test. Since aGvHD is a time-dependent variable, we applied the landmark analysis at day 100 to the poor-prognosis group in order to avoid bias connected with autoselection. All patients who died or did not undergo a follow-up before day 100 were deleted from the survival analysis.

# Results

A total of 237 patients underwent the 1st allo-HSCT from an UD. Two hundred thirty patients received stem cells from matched donor and 7 patients received stem cells

from MMUD (8/10). One hundred thirty-nine patients (58.6%) developed aGvHD stage II–IV within 100 days post-HSCT. The time of aGvHD diagnosis varied from 5 days post-HSCT up to 92 days post-HSCT (median: 18 days). Seventy-eight patients (32.9%) did not develop aGvHD or developed only stage I with no need for systemic treatment. Twenty (8.5%) of those who did not develop aGvHD encountered a competing event, such as graft rejection, relapse or death before day 100.

Forty-seven patients (19.8%) presented CsA-associated toxicity and required cessation of the therapy. The most common CsA toxicity observed in the study population was nephrotoxicity (n = 22) and neurotoxicity (n = 19). Six patients presented other, more unspecific symptoms, such as allergic reactions (n = 5) and microangiopathy (n = 1). The beginning of CsA treatment discontinuation varied from 1 day post-HSCT up to 52 days post-HSCT (median: 15 days). Instead of CsA, the majority of patients (n = 45; 96%) received Mycofenolate Mofetil (MMF) intravenously at a standard prophylactic dose (20 mg/kg/day). Four of those patients received additional steroids at a standard dose of 1 mg/kg/day. Two (4%) patients received steroids alone.

Initially, we performed the univariate statistical analysis. In this analysis, 3 factors significantly increased the probability of aGvHD ($p < 0.05$): TBI-MAC ($p = 0.0272$),

**Table 2.** Univariate analysis of risk factors for acute graft-versus-host disease (aGvHD)

| Risk factors | aGvHD | | | p-value |
|---|---|---|---|---|
| | yes n [%] | no (without a competing event) n [%] | no (with a competing event) n [%] | |
| Patient sex | | | | |
| male | 87 (62.6) | 53 (67.9) | 8 (40) | 0.0705 |
| female | 52 (37.4) | 25 (32.1) | 12 (60) | |
| Sex mismatch | | | | |
| matched | 85 (61.2) | 48 (61.6) | 9 (45) | 0.1221 |
| male to female | 27 (19.4) | 15 (19.2) | 2 (10) | |
| female to male | 27 (19.4) | 15 (19.2) | 9 (45) | |
| Patient age [years] | | | | |
| ≤5 | 33 (23.7) | 13 (16.7) | 7 (35) | 0.4084 |
| >5 and <15 | 76 (54.7) | 45 (57.7) | 8 (40) | |
| ≥15 | 30 (21.6) | 20 (25.6) | 5 (25) | |
| Donor age [years] (median: 31) | | | | |
| ≤median | 74 (53.2) | 41 (52.6) | 8 (40) | 0.5358 |
| >median | 65 (46.8) | 37 (47.4) | 12 (60) | |
| Amount of CD34+ cells (median: 8.29 × $10^6$/kg) | | | | |
| ≤median | 65 (46.8) | 42 (53.8) | 12 (60) | 0.3985 |
| >median | 74 (53.2) | 36 (46.2) | 8 (40) | |
| Amount of CD34+ cells (quartile – cut-off value) | | | | |
| Q1 (5.01 × $10^6$/kg) | 32 (23) | 22 (28.2) | 6 (30) | 0.7970 |
| Q2 (8.29 × $10^6$/kg) | 33 (23.7) | 20 (25.6) | 6 (30) | |
| Q3 (12.09 × $10^6$/kg) | 35 (25.2) | 21 (26.9) | 4 (20) | |
| Q4 (51.85 × $10^6$/kg) | 39 (28.1) | 15 (19.3) | 4 (20) | |
| Stem cells source | | | | |
| peripheral blood | 115 (82.7) | 68 (87.2) | 18 (90) | 0.5425 |
| bone marrow | 24 (17.3) | 10 (12.8) | 2 (10) | |
| Conditioning regimen | | | | |
| TBI-MAC | 59 (42.4) | 39 (50) | 7 (35) | 0.0272 |
| Bu-MAC | 59 (42.4) | 19 (24.4) | 6 (30) | |
| other (RTC/RIC) | 21 (15.2) | 20 (25.6) | 7 (35) | |
| Premature discontinuation of CsA | | | | |
| yes | 36 (25.9) | 7 (9) | 2 (10) | 0.0201 |
| no | 103 (74.1) | 71 (91) | 18 (90) | |
| Risk of disease | | | | |
| high | 71 (51.1) | 36 (46.2) | 8 (40) | 0.5711 |
| standard | 68 (48.9) | 42 (53.8) | 12 (60) | |
| Year of transplantation (median: 2009) | | | | |
| ≤2009 | 88 (63.3) | 33 (42.3) | 5 (25) | 0.0004 |
| >2009 | 51 (36.7) | 45 (57.7) | 15 (75) | |
| Year of transplantation (quartile) | | | | |
| Q1 (2004–2006) | 54 (38.8) | 17 (21.8) | 1 (5) | 0.0003 |
| Q2 (2007–2009) | 34 (24.5) | 16 (20.5) | 4 (20) | |
| Q3 (2010–2012) | 32 (23) | 17 (21.8) | 9 (45) | |
| Q4 (2013–2015) | 19 (13.7) | 28 (35.9) | 6 (30) | |

preemptive discontinuation of CsA (p = 0.0201) and transplantation performed before year 2009 (p = 0.0004). Donor and recipient age, patient sex, donor–recipient sex mismatch, amount of infused CD34+ cells, stem cell source, and underlying disease stage seemed to be insignificant as risk factors for aGvHD (Table 2).

The same factors as in the univariate analysis proved to be significant in the multivariate analysis. The myeloablative conditioning regimen containing total body irradiation puts patients in a greater risk of aGvHD (RR (relative risk): 1.69; 95% confidence interval (95% CI) = 1.047–2.74; p = 0.0320). Premature discontinuation of CsA significantly increased the risk of aGvHD (RR = 1.99; 95% CI = 1.369–2.89; p = 0.0003). Transplantation performed before 2009 enhanced the possibility of aGvHD (RR = 1.97; 95% CI = 1.400–2.78; p = 0.0001). The relative risk regression model containing all significant factors is presented in Table 3.

**Table 3.** Results of multivariate analysis, competing risk regression model

| Risk factors | RR (95% CI) | p-value |
|---|---|---|
| Conditioning regimen | | |
| other (RTC/RIC) | 1 | – |
| Bu-MAC | 1.11 (0.673–1.85) | 0.67 |
| TBI-MAC | 1.69 (1.047–2.74) | 0.0320 |
| Early cessation of CsA | | |
| no | 1 | – |
| yes | 1.99 (1.369–2.89) | 0.0003 |
| Year of transplantation (median: 2009) | | |
| >median | 1 | – |
| ≤median | 1.97 (1.400–2.78) | 0.0001 |

RR – relative risk; CI – confidence interval.

The median follow-up in the study cohort, which was 2.3 years after HSCT, revealed that 138 patients remained alive. The general outcome in the aGvHD cohort was noticeably worse (TRM = 22.5%; OS = 60.8%) compared to aGvHD-free patients (TRM = 12%; OS = 74.1%). The difference between OS (p = 0.08) and TRM in both groups was at the limit of statistical significance (p = 0.08). Transplantation performed before 2009 contributed significantly to a worse survival (p = 0.019) and increased TRM (p = 0.049) (Fig. 1,2). Other factors, including those which statistically impact the incidence of aGvHD, did not influence OS and TRM (data not shown). Complete OS and TRM statistics are presented in Table 4.

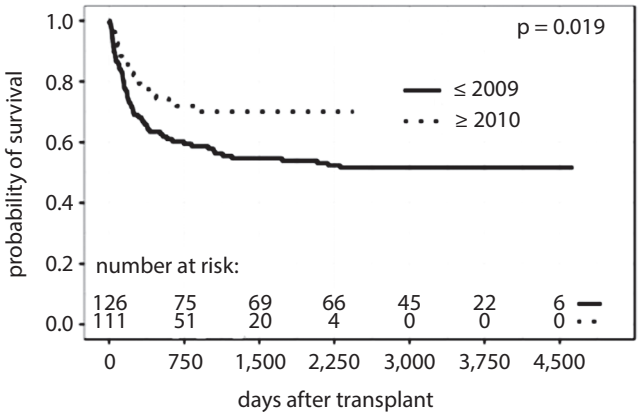


**Fig. 1.** Comparison of overall survival (OS) for patients receiving hematopoietic stem cell transplantation (HSCT) before and after 2009

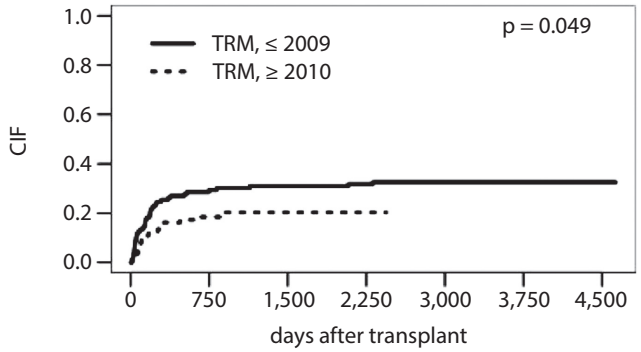


**Fig. 2.** Comparison of transplant-related mortality (TRM) for patients receiving hematopoietic stem cell transplantation (HSCT) before and after 2009

CIF – cumulative incidence function.

## Discussion

Acute graft-versus-host disease as a major factor affecting morbidity and mortality in the early period post-HSCT still remains a challenge for clinicians and researchers worldwide. There is a great number of published studies attributing both the incidence of aGvHD and the general outcome to the influence of potential risk factors. Repeatedly, those studies demonstrate different or even contradictory results. However, it should be emphasized that most of the studies were performed on adult patients and lack of great cohort research conducted on the pediatric population is unquestionable.

**Table 4.** Comparison of overall survival (OS) and transplant-related mortality (TRM) in patients with or without acute graft-versus-host disease (aGvHD)

| Time post HSCT | OS [%] | | p-value | TRM [%] | | p-value |
|---|---|---|---|---|---|---|
| | aGvHD | no aGvHD | | aGvHD | no aGvHD | |
| 1 year | 87.0 | 90.3 | | 13.8 | 8.7 | |
| 3 years | 72.2 | 85.3 | | 18.6 | 12.2 | |
| 5 years | 60.4 | 74.1 | 0.084 | 25.9 | 12.8 | 0.145 |

The incidence of aGvHD in our study (58.6%) is very similar to that presented in other studies, but there are also some reports referring to a much higher frequency of this complication, reaching the level of 80%.[1,2,9,10,23,24] Such discrepancies may be due to meaningful differences in the studied cohorts. In our study, patient selection was defined by strict criteria; therefore, the analyzed cohort was relatively homogenous.

A comparison of conditioning regimens Bu-MAC vs TBI-MAC vs other (RTC & RIC) shows TBI as a major risk factor for aGvHD in the pediatric population.[21,22] Tissue damage, particularly in the gastrointestinal tract, undoubtedly exacerbated by TBI, is thought to be the 1st stage of inflammatory reaction through the activation of host antigen-presenting cells (APCs) and cytokine production. This may initiate an inflammatory cascade, leading to the development of aGvHD.[28] Lack of significant differences between the Bu-MAC patients and the group receiving RTC and RIC may suggest that chemo-based myeloablative regimens would not influence aGvHD development.[29,30] This finding only highlights the contribution of TBI to aGvHD. There are also some studies suggesting that in younger recipients, a low dose of TBI may have a beneficial effect without increasing the risk for aGvHD. This indicates that reduced-toxicity regimens, containing low doses of TBI, could be further investigated also in pediatric HSCT recipients. However, in our study, the best survival rate was noted in the group of patients with Bu-MAC (data not shown). This finding may suggest that chemo-based MAC could be a golden standard for pediatric patients.[31]

Premature discontinuation of CsA increased the probability of aGvHD. There are a lot of reports in the literature about the essential role of calcineurin inhibitors in aGvHD prophylaxis. A large part of these reports focus on the superiority of one of them (CsA or tacrolimus) over the other. Another great part of the literature proves that a low plasma concentration level of CsA, especially in the early period post-HSCT (i.e., 2–3 weeks post-HSCT) is associated with a much higher risk of aGvHD.[26,32–35] To our knowledge, this is the only study providing results that refer to the influence of early cessation of CsA on the occurrence of aGvHD. There is only one reference regarding premature discontinuation of CsA – the research conducted by Cadenas et al.[32] Although this study was performed on a completely different population of patients, it contains a brief suggestion that there is no connection between discontinuation of CsA and aGvHD. The quoted study primarily focused on low CsA plasma concentration levels as a risk factor for aGvHD. To date, the optimal therapeutic approach for CsA therapy as aGvHD prophylaxis after HSCT remains unclear in terms of doses as well as the monitoring strategy.[36,37] However, our results indicate that calcineurin inhibitors as part of aGvHD prophylaxis may be irreplaceable.

Hematopoietic stem cells transplantation performed before 2009 proved to be a crucial risk factor for the development of aGvHD and contributed to a worse general outcome. This finding is a reflection of all the improvements in donor selection, transplantation regimens and supportive care that were made during the analyzed period of time. Furthermore, improved OS and reduced TRM after 2009 result from relevant advances in aGvHD therapy that were made during these years. Among all implemented GvHD treatment strategies, extracorporeal photopheresis seems to be promising both in our center (data not shown) and worldwide.

The CD34+ cell dose is one of the most ambiguous factors that have an impact on aGvHD and survival after HSCT. The results of our study showed no correlation between a greater amount of transplanted CD34+ cells and a higher incidence of aGvHD. In parallel, we found no interdependence between the CD34+ cells dose and either OS or TRM. Several studies, performed in both the adult and pediatric population of patients, confirm our findings. Previous research conducted in our center by Kałwak et al. also proves that there is no correlation between the CD34+ cell dose and aGvHD; notwithstanding, a greater amount of transplanted CD34+ cells contributed to better OS and general outcome.[9] Similar results were reported by Pulsipher at al. in a multi-center study performed on a great cohort of adult and pediatric patients.[38] Another study demonstrating no correlation between CD34+ and aGvHD was carried out by Tsirigotis et al.[39] It also reports that the number of infused CD34+ cells has no influence on the general outcome. On the contrary, there are some reports suggesting that a higher number of infused CD34 cells may be a risk factor for aGvHD.[5,40] The extremely discrepant findings concerning the impact of the CD34+ cell dose on aGvHD show that this variable should be analyzed individually, following the patient requirements. This knowledge can be beneficial, allowing clinicians to tailor the composition of the graft in accordance with the patient status and other peri-transplantation variables.

Other analyzed variables, such as donor and recipient age as well as donor–recipient sex mismatch, are well-known risk factors for aGvHD in the adult population. In children, however, the data is sparse. In our study, female donor to male recipient did not prove to be a risk factor for aGVHD, which was, however, noted as a risk factor in our population of male matched sibling donor recipients, when no ATG was given (data not shown).

## Conclusions

To conclude, conditioning regimen and adequate immunosuppressive prophylaxis, including continuous CsA use, remain major factors affecting the incidence of aGvHD in children with malignant disorders undergoing UD-HSCT. However, to date, it is not possible to provide strict guidelines for minimizing the risk of aGvHD. According to our results,

the decision to stop CsA administration should be carefully considered, and avoided if possible. Choosing an approach may be discussed if CsA needs to be discontinued for some reason. There is no clear answer. Both MMF and steroids could be useful, but in our belief, MMF seems to be the most appropriate option, with less toxicity.

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# Continuous electronic fetal heart monitoring versus intermittent auscultation during labor: Would the literature outcomes have the same results if they were interpreted following the NICHHD guidelines?

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## Abstract

**Background.** All guidelines regarding electronic fetal heart monitoring (EFM) before 2008 were designed to avoid more hypoxia than acidosis. In addition, the results of the Cochrane meta-analysis of 2013 do not show a significant improvement in neonatal outcomes using EFM or intermittent auscultation (IA).

**Objectives.** We retrospectively evaluated the results on delivery outcomes arising from a comparison between EFM and IA during labor of 2 specific and high-quality trials. We hypothesized that revisiting the delivery outcomes through the adoption of the recent National Institute of Child Health and Human Development (NICHHD) guidelines, the reported delivery outcomes would be different.

**Material and methods.** The study retrospectively evaluated the results on delivery outcomes arising from the comparison between EFM and IA during labor of the "Dublin trial" and "Vintzileos trial" published, respectively, in 1985 and 1993. A translational model was constructed to recalculate these results, applying a correction factor to estimate the number of pathological patterns using the NICHHD guidelines for EFM.

**Results.** After the reevaluation of the 2 trials using the proposed correction factor, the comparison of the re-calculated cesarean section and operative delivery rates for fetal distress between EFM and IA group were no longer statistically significant, both in the Dublin trial and Vintzileos trial. Even the comparison of the re-calculated incidence of the rate of non-reassuring fetal heart rate (FHR) patterns in the EFM and IA groups has not given any indication of significance for the Vintzileos trial.

**Conclusions.** Our results lead to reconsidering the results of the Dublin trial and Vintzileos trial in terms of operational rates of births, hypothesizing that these results would have been significantly lower if FHR traces were interpreted using the current NICHHD guidelines, which aim to identify potential acidotic fetuses rather than hypoxic ones.

**Key words:** fetal heart rate, intermittent auscultation, fetal hypoxia, fetal acidosis, electronic fetal heart monitoring

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## Introduction

Electronic fetal monitoring (EFM) was introduced into clinical practice in the seventies and since then it has become the most commonly used method for the surveillance of fetal well-being during labor. Since the first EFM classification of Boylan in 1987,[1] the major international scientific societies have developed specific guidelines that, based on objective parameters, classify the EFM into risk categories with the goal of addressing obstetricians in order to act appropriately to reduce neonatal morbidity, and to avoid inappropriate cesarean section and operative delivery.

Most of the recommendations and guidelines focused on avoiding fetal hypoxia, but in 2008 a consensus of the National Institute of Child Health and Human Development (NICHHD), the American College of Obstetricians and Gynecologists (ACOG) and the Society for Maternal Fetal Medicine clearly stated that the aim of EFM was to avoid the birth of acidotic fetuses and that the concept of fetal acidosis should replace the concept of fetal hypoxia.[2] The consensus reviewed and updated the definitions of fetal heart rate (FHR) tracings with the development of new guidelines that were subsequently revisited in 2009 by the ACOG with the definition of specific clinical management for each FHR category.[3,4] This interpretation of EFM tracings clearly highlighted that FHR deceleration represents a reversible sign of fetal hypoxia in utero in terms of a defense mechanism of the fetus towards the hypoxic stimulus, while the alteration of the FHR variability represents the highest risk factor for fetal acidosis.

From the introduction of EFM, several randomized trials have also compared its efficacy to intermittent auscultation (IA) of FHR during labor, suggesting that EFM reduces perinatal mortality, but it may increase the cesarean delivery rate without performing an assessment of fetal acid-base status.[5–8] The synthesis of all these reports was summed up in 2013 by a Cochrane review, which reported that EFM during labor is associated with a reduction in neonatal seizures, but with no significant differences in cerebral palsy, infant mortality or other standard measures of neonatal well-being; this review also confirmed that EFM was associated with an increase in cesarean and operative delivery.[9] Moreover, even technologies adjunctive to conventional intrapartum EFM, such as fetal ECG ST-segment analysis, did not improve perinatal outcomes or decrease operative-delivery rates.[10] However, this Cochrane review also showed that many of the epidemiological studies included had limitations and only a few studies were randomized and based on a larger population. In particular, only 2 of the 13 clinical trials included in the review were considered to be of high quality: the Dublin trial published in 1985 and the trial published in 1993 by Vintzileos et al.[11,12]

Based on these premises, the present study aims to retrospectively evaluate the 2 specific and high-quality trials that have contributed to the Cochrane meta-analysis – the Dublin and Vintzileos trials. Because it was not possible for us to re-evaluate all the single tracings, we hypothesized that, after revisiting the operative delivery and cesarean section rates, through the adoption of NICHHD guidelines throughout the construction of a translational model, these outcomes will be different in the light of recent guidelines.

**Table 1.** Characteristics of the Dublin trial and Vintzileos trial

| Characteristics | Dublin trial | Vintzileos trial |
|---|---|---|
| Study population, n | 12,964 | 1,428 |
| EFM | 6,530 | 746 |
| IA | 6,554 | 682 |
| Inclusion criteria | singleton<br>gestation age ≥28 weeks<br>active labor<br>No meconium-stained amniotic fluid | singleton<br>gestation age ≥26 weeks<br>spontaneous labor |
| Exclusion criteria | fetal malformation | congenital or fetal chromosomal defects |
| Setting | National Maternity Hospital, Dublin, Ireland | Alexandra Hospital, Athens, Greece<br>Marika Iliadi Hospital, Athens, Greece |
| Type of midwifery assistance | one to one | one to one |
| Type of EFM interpretation | Boylan Classification (1987) | ACOG Classification (1989) |
| Type of IA | every 15 min during the I stage of labor<br>every interval between contraction during the II stage of labor<br>1 min auscultation | every 15 min during the I stage of labor<br>every 5 min during the II stage of labor<br>1 min auscultation |
| Support techniques to EFM | fetal Ph from scalp blood sampling | none |
| Measures outcome | intrapartum death<br>neonatal death<br>neurological sequelae<br>other neurological problems | neonatal complications<br>intrapartum fetal death<br>neonatal and perinatal death |

EFM – electronic fetal monitoring; IA – intermittent auscultation.

## Material and methods

The study retrospectively evaluated the results on delivery outcomes arising from the comparison between EFM and IA during labor of the "Dublin trial" and "Vintzileos trial" published, respectively, in 1985 and 1993.[11,12] The characteristics of the trials are summarized in Table 1.

The Dublin trial used the specific criteria for EFM classification that was analogous to the Boylan classification for EFM further published in 1987.[1] Fetal heart rate patterns were considered "suspicious or ominous" in the case of at least one of the following criteria: marked tachycardia or bradycardia, moderate tachycardia or bradycardia with reduced variability, a pattern of late decelerations, moderate and severe variable decelerations, and other confusing patterns which could not be interpreted (Table 2). In the Dublin trial, if one of these patterns lasted for at least 10 min and conservative measures failed, the fetal pH was assessed with scalp blood sampling during the 1st stage of labor, or if delivery had been completed, during the 2nd stage of labor. Childbirth was performed as soon as possible in the case of pH < 7.20. If the pH was between 7.20 and 7.25 and the FHR pattern was not reassuring, delivery was completed as soon as possible; if the pH was greater than 7.25 and the pattern was not reassuring, the pH of the scalp was repeated after 0.5–1 h. Similarly, in the IA group, FHR auscultation was considered not reassuring when FHR >160 bpm or <100 bpm during 3 contractions without response to conservative measures.

The Vintzileos trial applied the ACOG guidelines of 1989[13] and EFM tracings were considered "non-reassuring" in the presence of at least of one the following criteria: late decelerations, persistent prolonged decelerations, severe variable decelerations, variable decelerations with loss of variability, persistent tachycardia with decreased variability, persistent decreased variability and sinusoidal pattern, early decelerations, late decelerations, and severe variable decelerations with loss of variability (Table 2). The IA was considered non-reassuring when the FHR was persistently lower than 100 bpm during and after uterine contraction or persistently higher than 160 bpm. Both groups were managed similarly in the case of non-reassuring FHR, with the application of conservative measures for at least 20 min and after that a cesarean section was performed.

In order to compare the delivery outcomes of the 2 studies, we structured a summary table for each trial in which the specific results were reported regarding fetal distress (Tables 3,4). In particular, the Dublin trial included, as an indication for operative or cesarean delivery for fetal distress, all the "suspicious FHR patterns" for fetal distress, while the Vintzileos trial referred to "suspected fetal distress" as an indication for operative or cesarean delivery in the presence of "not-reassuring FHR patterns". Both trials referred to intrapartum EFM anomalies as suspected fetal distress, since the resulting neonatal outcomes were analyzed as distinct parts and were not included in our revision.

In a second instance, we construed a translational model by recalculating these results, applying a correction factor to estimate the number of pathological patterns using the NICHHD guidelines for EFM. The NICHHD guidelines defined as "category III" the presence of sinusoidal patterns or a low FHR variability associated with at least one of the following criteria: recurrent late decelerations, recurrent variable decelerations and bradycardia (Table 2).[2]

The correction factor was calculated using as a reference a large randomized prospective study evaluating

**Table 2.** Pathological EFM patterns according to the different classifications

| Boylan Classification 1987 | ACOG classification 1989 | NICHHD classification 2008 |
|---|---|---|
| – marked tachycardia or bradycardia,<br>– moderate tachycardia or bradycardia with reduced variability,<br>– pattern of late decelerations,<br>– moderate and severe variable decelerations,<br>– other confusing patterns which could not be interpreted. | – late decelerations,<br>– persistent prolonged decelerations,<br>– severe variable decelerations – variable decelerations with loss of variability,<br>– persistent tachycardia with decreased variability,<br>– persistent decreased variability,<br>– sinusoidal pattern,<br>– early decelerations,<br>– late decelerations and severe variable decelerations with loss of variability. | – sinusoidal pattern,<br>– low FHR variability associated to at least 1 of the following criteria:<br>1. recurrent late decelerations,<br>2. recurrent variable decelerations,<br>3. bradycardia. |

EFM – electronic fetal monitoring; FHR – fetal heart rate.

**Table 3.** Outcomes of the Dublin trial for cesarean section and spontaneous operative delivery for fetal distress (modified from MacDonald et al.)[11]

| Type of delivery | Dublin trial | | p-value |
|---|---|---|---|
| | EFM (n = 6,474) | IA (n = 6,490) | |
| Cesarean section<br>Fetal distress | 158 (2.4)<br>25 (0.4) | 144 (2.2)<br>10 (0.2) | 0.0161* |
| Operative spontaneous delivery<br>Fetal distress | 528 (8.2)<br>190 (2.9) | 407 (6.3)<br>75 (1.2) | <0.0001* |

*p < 0.05; EFM – electronic fetal monitoring; IA – intermittent auscultation.

Table 4. Outcomes of the Vintzileos trial for obstetrical interventions for fetal distress and non-reassuring FHR patterns (modified from Vintzileos et al.)[12]

| | Vintzileos trial | | p-value |
|---|---|---|---|
| | EFM (n = 746) | IA (n = 682) | |
| Non-reassuring FHR patterns | 175 (23.4) | 73 (10.7) | 0.0001* |
| Total obstetrical intervention | 84 (11.2) | 33 (4.8) | 0.0001* |
| Operative delivery for fetal distress | 44 (5.8) | 17 (2.4) | 0.002* |
| Cesarean section for fetal distress | 40 (5.3) | 16 (2.3) | 0.005* |

*p < 0.05; EFM – electronic fetal monitoring; IA – intermittent auscultation.

the relationships among abnormal cardiotocography and umbilical cord blood pH, Apgar score, and meconium-stained amniotic fluid. Steer et al.[14] analyzed 698 FHR patterns, defining as "abnormal" 289 patterns that were comparable to category III of NICHHD. In particular, they excluded from abnormality traces with a baseline between 120 bpm and 160 bpm, a baseline variability of 15 bpm, the presence of accelerations, synchronous decelerations with uterine contraction and an amplitude of less than 40 bpm in the 1st stage of labor. Decelerations during the 2nd stage of labor were always considered normal, as well as a baseline between 100 bpm and 120 bpm with normal variability. Considering the reported rate of abnormal FHR patterns and relating that to the total patterns, from this proportion we calculated that 41.4% (289/698) of the traces were homologated to category III of NICHHD.

After this estimation, the number of pathological EFM tracings of the 2 trials that hesitate in a cesarean section or instrumental delivery for fetal distress were recalculated by subtracting 58.6% and approximated with excess.

All data was analyzed using SPSS software (IBM SPSS v. 23; IBM Corp, Armonk, USA). Delivery characteristics were compared in univariate statistical analyses using Pearson's $\chi^2$ test and Fisher's exact test. The results were reported as numbers and percentages, as appropriate. P-value <0.05 was considered statistically significant.

## Results

Evaluating the results of the Dublin trial, the number of cases of fetal distress that constituted the indication for cesarean section in the EFM group decreases from 25 (0.4%) to 10.3 (0.1%); similarly, the number of operative deliveries for fetal distress in the EFM group is reduced from 190 (2.9%) to 78.6 (1.2%) (Table 5).

The reevaluation of the Vintzileos trial showed that the rate of obstetrical intervention for fetal distress, both cesarean section and operative delivery, in the EFM group decreases from 84 (11.2%) to 34.8 (4.6%). In particular, in the EFM group, the rate of operative deliveries changes from 44 (5.8%) to 18.2 (2.4%) and the rate of cesarean sections decreases from 40 (5.4%) to 16.6 (2.2%) (Table 6). Similarly, the number of non-reassuring FHR tracings among patients who underwent EFM evaluation goes down from 175 (23.4%) to 72.4 (9.7%) (Table 6).

Table 5. Recalculated outcomes of the Dublin trial for cesarean section and spontaneous operative delivery for fetal distress (modified from MacDonald et al.)[11]

| Type of delivery | Dublin trial | | | Dublin trial revisited | | |
|---|---|---|---|---|---|---|
| | EFM (n = 6,474) | IA (n = 6,490) | p-value | EFM (n = 6,474) | IA (n = 6,490) | p-value |
| Cesarean section | 158 (2.4) | 144 (2.2) | 0.0161* | 10.3 (0.1) | 10 (0.2) | 0.8299 |
| Fetal distress | 25 (0.4) | 10 (0.2) | | | | |
| Operative spontaneous delivery | 528 (8.2) | 407 (6.3) | <0.0001* | 78.6 (1.2) | 75 (1.2) | 0.1342 |
| Fetal distress | 190 (2.9) | 75 (1.2) | | | | |

*p < 0.05; EFM – electronic fetal monitoring; IA – intermittent auscultation.

Table 6. Recalculated outcomes of the Vintzileos trial for obstetrical interventions for fetal distress and non-reassuring FHR patterns (modified from Vintzileos et al.)[12]

| | Vintzileos trial | | | Vintzileos trial revisited | | |
|---|---|---|---|---|---|---|
| | EFM (n = 746) | IA (n = 682) | p-value | EFM (n = 746) | IA (n = 682) | p-value |
| Non-reassuring FHR patterns | 175 (23.4) | 73 (10.7) | 0.0001* | 72.4 (9.7) | 73 (10.7) | 0.5163 |
| Total obstetrical intervention | 84 (11.2) | 33 (4.8) | 0.0001* | 34.8 (4.6) | 33 (4.8) | 0.8019 |
| Operative delivery for fetal distress | 44 (5.8) | 17 (2.4) | 0.002* | 18.2 (2.4) | 17 (2.49) | 0.9224 |
| Cesarean section for fetal distress | 40 (5.4) | 16 (2.3) | 0.005* | 16.6 (2.2) | 16 (2.3) | 0.7974 |

*p < 0.05; EFM – electronic fetal monitoring; IA – intermittent auscultation.

After the reevaluation of the 2 trials using the proposed correction factor, the comparison of the recalculated cesarean section and operative delivery rates for fetal distress between the EFM and IA groups was no longer statistically significant, both in the Dublin trial and Vintzileos trial (Tables 5,6). Even the comparison of the recalculated incidence of the rate of non-reassuring FHR patterns in the EFM and IA groups does not give any indication of significance for the Vintzileos trial (Table 6).

## Discussion

Our study aimed to reevaluate the results of the 2 most representative trials reported in scientific literature and by the Cochrane review on the subject of EFM vs IA during labor using the EFM tracings indications in the recent NICHHD guidelines. Our retrospective reevaluation noted that the previous differences in terms of obstetrical intervention for delivery in the case of fetal distress (cesarean section or operative spontaneous delivery) disappear after reading the EFM tracings using the NICHHD classification.

We suppose that the interpretative key of these results is represented by the increasing importance of the role of fetal acidosis as a "unique" indication for cesarean delivery or instrumental delivery. As stated in the NICHHD guidelines, the concept of fetal hypoxia should be replaced by the concept of fetal acidosis since, in the past, fetal hypoxia, often represented by fetal deceleration, was considered the indication for delivery; nowadays hypoxia is no longer an indication. Specifically, decelerations, if not associated with absent variability, should be considered an aspect of fetal reactivity, instead of fetal distress. Fetal deceleration represents a compensatory reaction that may subsequently worsen in a decompensatory phase represented by reduction of variability. So, if fetal hypoxia is embodied by FHR deceleration, fetal acidosis is mostly represented by the reduction of FHR variability that should therefore assume the most relevant value in the readings of EFM patterns. Category III of the NICHHD guidelines is, in fact, clearly defined by the presence of sinusoidal patterns or absent baseline FHR variability in association with at least one among bradycardia and recurrent late or variable decelerations. Category III embodies an increased risk of fetal acidosis at the time of observation and, if unresolved, these traces should be treated with a prompt delivery.[4]

Reviewing the 2 trials, it appears clear that suspicious or non-reassuring FHR characteristics have been revisited with the introduction of the NICHHD guidelines and some criteria that were previously used to define pathological patterns have since been reconsidered with the NICHHD classification. In both classifications used in the trials, the Boylan classification from 1987 and the ACOG classification from 1989, a definition of normal FHR baseline between 120 bpm and 160 bpm was used, while nowadays the lower limit for this parameter is fixed at 110 bpm. Furthermore, applying the Boylan classification, the Dublin trial considered "dangerous" and an indication for cesarean section at least 1 criterion among severe tachycardia with low variability, severe bradycardia, and late and variable decelerations irrespective of FHR variability, which is an essential criterion to allocate the tracings in category III of the NICHHD classification. Similarly, in the Vintzileos trial, the reduction of FHR variability is an isolated parameter sufficient to expedite delivery, without any association of other tracing characteristics, like in category III of the NICHHD.

Recently, Clark et al.[15] revisited the current NICHHD approach to the management of category II FHR patterns, indicating operative or cesarean delivery in cases of FHR patterns less severe than those of category III. Considering that algorithm for category II, the results of our reevaluation of the Dublin and Vintzileos trials could possibly be less impressive, but we chose not to include the Clark algorithm in our analysis since we preferred to focus only on consolidated national guidelines on intrapartum monitoring. Future research will probably elucidate this interesting approach to category II and its impact on clinical practice and previous trials.

From our comparison, it is evident that the indications for obstetrical intervention in the 2 randomized trials fail when EFM incorporates the concept of acidosis. This difference was less pronounced in the Dublin trial where the fetal pH was assessed with scalp blood sampling.

In the clinical practice, the biggest hazard for the obstetrician is represented by the misinterpretation of the EFM tracings, since EFM has an extreme intra-observer and inter-observer variability. Blackwell et al. recently demonstrated that while the intra-observer agreement is substantial for the reading of EFM tracings with the NICHHD guidelines, the inter-observer agreement is moderate for category I and II, but poor for category III.[16]

The misjudgment of physiological FHR variations as signs of hypoxia may inevitably expose inappropriate obstetrical interventions. The ACOG recently reported that the second most common indication for primary cesarean section is the presence of non-reassuring or indeterminate EFM tracings, accounting for about 23% cases of cesarean section.[17,18] Given that, a standardized approach to EFM tracing management could reduce the rate of inappropriate cesarean sections. Surgical intervention is frequently determined by the presence of abnormal EFM that does not represent a clear situation of hypoxia or acidosis, since FHR variations may occur before the onset of acidosis. Electronic fetal heart monitoring may identify fetuses that are not exposed to hypoxic stimuli (category I), fetuses that are able to overcome the hypoxic stimulus to which they are exposed by compensatory responses (category II) and fetuses that are incapable of overcoming the hypoxic stimulus and are confronted with decompensation (category III).

The strength of our study is represented by the critical revision of the well-established association between EFM and the increment of operative delivery in comparison with IA in low-risk labor, demonstrating that a proper reading of the FHR tracings in view of the recent guidelines does not increase the incidence of surgical intervention. Moreover, our study points out the concept of acidosis in reading the FHR traces that may alter the outcome of previous trials; similarly, the use of fetal ECG ST-segment analysis has been questioned as an adjunct to conventional intrapartum electronic FHR monitoring, since a recent multicenter trial demonstrated that it did not improve the perinatal outcomes or decrease operative-delivery rates.[10]

A limitation of our study is that our results were obtained by recalculating previous data with a correction factor rather than through a direct assessment of individual cases. Other limitations are represented by the intrinsic bias of the 2 trials examined. Concerning the Dublin trial, the major confounding factors are the use of support techniques such as pH measurement of the fetal scalp and the transition from one group to another (EFM and IA), which may have masked some benefits arising from one or the other of the monitoring techniques, and may have excluded high-risk patients for meconium-stained amniotic fluid or prolonged labor.

Different aspects may alter the results of the Vintzileos trial. As before, no additional methods of fetal surveillance were used (i.e., pH of the fetal scalp). Moreover, both low-risk and high-risk pregnancies were included in the study as well as preterm labors, and the study was conducted based on a previous statistical assessment carried out to identify the sample number needed to demonstrate the real difference in perinatal mortality rates between the 2 groups.

In conclusion, all guidelines regarding EFM before 2008 were designed more to avoid hypoxia than acidosis. In addition, the results of the Cochrane meta-analysis of 2013 do not show a significant improvement in neonatal outcomes using EFM or IA. These considerations lead us to reconsider the results of the Dublin trial and Vintzileos trial in terms of operational rates of births (cesarean sections and operative spontaneous delivery), hypothesizing that these results would have been significantly lower if FHR traces had been interpreted using the current NICHHD guidelines which aim to identify potential acidotic fetuses rather than hypoxic ones. Future clinical prospective trials will be needed to resolve any doubts and confirm our results.

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# Exploration of susceptible genes associated with Henoch–Schönlein purpura by whole exome sequencing

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## Abstract

**Background.** Henoch–Schönlein purpura (HSP) is a systemic small-vessel vasculitis caused by environmental and inherent factors. Although recent research has advanced our understanding of the role of genetic susceptibility in HSP, there are still significant gaps in our knowledge.

**Objectives.** In this study, we aimed to explore some susceptibility genes likely associated with HSP.

**Material and methods.** Three DNA samples from a family with HSP were used to perform whole exome sequencing with Illumina Hiseq 2500 high-throughput sequencing. The relevant single nucleotide variants (SNVs) were screened according to specific filter conditions and the screened SNVs were then verified with Sanger sequencing. The Sanger sequencing results were further screened according to available literature. Finally, candidate genes were validated in 92 samples from children with HSP, and also in 1 child with HSP from HSP family, using the polymerase chain reaction technique (PCR).

**Results.** Our analysis revealed that the *MIF* gene and the *MGAT5* gene related to immunity remained after screening. Among the 93 children with HSP, there were 3 patients with *MIF* mutations and 2 patients with *MGAT5* mutations.

**Conclusions.** Our findings are helpful for providing new methods and ideas for understanding the pathogenesis of HSP by detecting and analyzing gene mutations at the whole-exome level including multi-generation sequencing. *MIF* and *MGAT5* may be new susceptibility loci for HSP, but their roles in the pathogenesis of HSP are worthy of further study.

**Key words:** Henoch–Schönlein purpura, whole exome sequencing, *MIF*, *MGAT5*

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# Introduction

Henoch–Schönlein purpura (HSP) is a small-vessel vasculitis that often occurs in children and can cause various clinical issues, such as skin purpura, arthritis, and/or arthralgia, abdominal pain, and renal involvement.[1] Some of the renal damage can progress to renal insufficiency, which determines the long-term prognosis of HSP.[2] Although the first HSP report was made nearly 200 years ago, the exact etiology and pathogenesis of the disease are still unclear.

Some studies have shown that the incidence of HSP has a tendency for familial aggregation.[3–5] In other words, genetic factors may play an important role in the pathogenesis of HSP. The past few years have seen a tremendously large amount of research in quest for genetic variants which may influence HSP susceptibility. Candidate gene investigations have since revealed roles in HSP susceptibility of an increasing number of genes, including *TGF-β*, *MEFV (E148Q)*, *RAS*, *P-selectin*, *C1GALT1*, *HSP70-2*, *TNF-α*, *IL-17 (rs2275913)*, *CTLA-4*, *PAF-AH*, *eNOS*, *iNOS*, *TLR-4*, *ACE*, etc., all of which have been validated in independent Chinese populations.[6–17] The above studies, however, were basically designed to study the correlation between specific genes and HSP, but these selected genes are not necessarily related to the incidence of hereditary HSP.

Besides, the occurrence of HSP is related to immune disorders, such as a Th1/Th2 imbalance and overactive Th2 cells.[18] Therefore, we set out to perform whole exome sequencing (WES) in a family with HSP to find genes related to immune abnormalities which are likely associated with HSP, so as to provide clues for researching the pathogenesis of HSP.

# Material and methods

## Patients

The study focused on a Chinese family, consisting of a boy with HSP, his father (who has had HSP since he was 10 years old) and the boy's mother, who does not suffer from HSP. Other 92 samples from unrelated patients with HSP were recruited from the Department of Rheumatism at Shanghai Children's Medical Center. Henoch–Schönlein purpura was diagnosed according to criteria established by the European League Against Rheumatism (EULAR) in 2010.[19]

The 12-year-old boy (sample No. WES-741) presented with HSP on both of his lower limbs 2 days prior; this was accompanied by joint pain, but no renal involvement. His father (sample No. WES-742) had suffered from HSP since the age of 10, accompanied by abdominal pain and gastrointestinal bleeding, though his mother (sample No. WES-743) had no history of HSP.

The study was approved by the Medical Ethics Committee of Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, China, and informed consent was obtained from the parents of all participants.

# Whole exome sequencing

The genomic DNA of the 3 samples was isolated from 2 mL of peripheral blood samples collected from the veins using a QIAamp Blood DNA Mini Kit® (Qiagen GmbH, Hilden, Germany). A total of 3 µg of DNA from the sample was processed through shearing using a Covaris® M220 Ultrasonicator system (Covaris Inc., Woburn, USA) resulting in an amplicon of 150–200 bp. The genomic DNA library was prepared with an Agilent SureSelect Target Enrichment System (Agilent Technologies, Inc., Santa Clara, USA) according to the manufacturer's instructions. Both coding exons and the flanking intronic regions were enriched with SureSelect XT Human All Exon V6 (Agilent Technologies). Clusters were then generated with isothermal bridge amplification using an Illumina cBot station, and sequencing was performed on an Illumina HiSeq 2500 System (Illumina Inc., San Diego, USA).

Base calling and sequence read quality assessment were performed using Illumina Sequence Control Software with Real Time Analysis software (Illumina). The sequence reads to a reference human genome (Human 37.3, SNP135) were aligned using NextGENe (SoftGenetics, State College, USA). All single-nucleotide variants (SNVs) and indels were saved in VCF format and uploaded to Ingenuity Variant Analysis (Ingenuity Systems, Redwood City, USA) for biological analysis and interpretation.

## Single nucleotide variant screening

The screening parameters of the candidate variants used with the Ingenuity software consisted of 5 parts: 1) exclusion of low confidence variants; 2) allele frequency under 1.0% in the 1000 Genomes Project, 1.0% of the NHLBI ESP exomes, 1.0% of the Allele Frequency Community, or 1.0% of the ExAC database, with an area of analysis including each exon and about 20 bp of exon–intron boundaries; 3) exclusion of benign variants, including synonymous, harmless missense predicted by PolyPhen-2 and SIFT software, when those predicted had no impact on splicing using MaxEntScan software; 4) a filtering index of clinical symptoms of immunity for analysis of the resulting candidate variants; and 5) taking the intersecting set of variants in the cases of WES-741 and WES-742.

## Sanger sequencing verification

The primers for the amplicon of the screened SNVs gained by University of California, Santa Cruz (UCSC Genome Browser Home) were designed using Primer3 online

software (http://genome.ucsc.edu/cgi-bin/hgGateway) and synthesized with Map Biotechnology (Shanghai, China). The amplicon length was 300–500 bp and the annealing temperature was set at 60°C.

The exons and the exon–intron boundaries were amplified using polymerase chain reaction (PCR). The PCR products (5 µL) were examined on a 1% agarose gel and purified using an ExoSAP-IT Kit (GE Healthcare, Cleveland, USA). Sequencing reactions were prepared with the BigDye® Direct Cycle Sequencing Kit (Thermo Fisher, Waltham, USA). The final products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen) and the capillary electrophoresis sequencing was performed by using an ABI Prism 3730XL Genetic Analyzer (Thermo Fisher) with the forward and reverse primers. The sequence data were analyzed using Mutation Surveyor® software v. 4.0.4 (SoftGenetics).

## PCR of *MIF* and *MGAT5* and analysis of data

All exons of *MIF* and *MGAT5* were amplified using PCR from the genomic DNA of 93 children with HSP. The primers for the amplicons of *MIF* (GenBank accession No. NM_002415) and *MGAT5* (GenBank accession No. NM_002410) were designed using UCSC ExonPrimer online software (http://genome.ucsc.edu/index.html) and were synthesized using Map Biotechnology (Shanghai, China). The primers designed for *MIF* and *MGAT5* are listed in Table 1 (A and B). The exons and the exon–intron boundaries were amplified using PCR. The reaction mixture for each amplicon of *MIF* contained 1 × Premix Taq (Ex Taq v. 2.0; cat. No. RR 003; Takara/Biotechnology, Co. Ltd., Kusatsu, Japan), 100 ng of genomic DNA, and 1 µL of forward and reverse primer in a final volume of 25 µL. The reaction was performed under the following PCR conditions: initial denaturation at 95°C for 5 min; then 19 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 45 s; 14 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s; and a final elongation step at 72°C for 5 min using a C1000™ Thermal Cycler PCR instrument (Bio-Rad Laboratories Inc., Hercules, USA). The PCR products were examined on a 1% agarose gel (Sangon Biotechnology Co. Ltd., Shanghai, China) with electrophoresis and purified using a QIAquick Gel Extraction kit (Qiagen). The purified DNA was sequenced using an ABI3730XL sequencer (Thermo Fisher) with forward and reverse primers. The sequence data was analyzed using Mutation Surveyor DNA Variant Analysis v. 4.0.4 (SoftGenetics). The same reaction system and conditions were used for the amplification of *MGAT5*.

After PCR analysis, we used Bioedit (V.7.1.3) (Tom Hall, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to find pathogenic predictions of all variants of *MIF* and *MGAT5* and analyzed their conservation; the properties of amino acids were found using the Hphob./Kyte & Doolittle scale (http://web.expasy.org/protscale/).

Table 1A. The PCR primer sequence for the *MIF* gene

| Primer name | Primer sequence (5'-3') |
|---|---|
| MIF-E1F | CAGGCGGTGACTTCCCCA |
| MIF-E1R | CCCCGAACGTCCACTTCG |
| MIF-E2-3F | AGGGGACAGGAAGAGGGG |
| MIF-E2-3R | GACCTCGCTCCCCAATATCC |

Table 1B. The PCR primer sequence for the *MGAT5* gene

| Primer name | Primer sequence (5'-3') |
|---|---|
| MGAT5-E1F | AGTGAAAACATGGCTTCTGGT |
| MGAT5-E1R | GGCTCTCTGTGTGCAATTCA |
| MGAT5-E2F | CGGCCCTTGTCATCTTTCAG |
| MGAT5-E2R | ACTTATCTTTACAATGGCCATGC |
| MGAT5-E3F | TATATTGGAGCTGGAGGGGC |
| MGAT5-E3R | CATAGCCACAGACAAAGCCG |
| MGAT5-E4F | TGCACACCTGGCTATCTTGA |
| MGAT5-E4R | GGGAGAGTAATGAAGGACGGA |
| MGAT5-E5F | TCATCTGAGAGGGCACTTGT |
| MGAT5-E5R | TCCACAGAGACGAATACAGAAGT |
| MGAT5-E6F | CAGTTTGCAATCCTGGGACC |
| MGAT5-E6R | AGACTGAGCAGATTCACCCT |
| MGAT5-E7F | CCAAGGGCTGCAGTGATTTT |
| MGAT5-E7R | ATGAAGCCTCTGGAGCCTTC |
| MGAT5-E8F | TTTGAAAGGGCCATGCTGTC |
| MGAT5-E8R | GCAACACTCCATACAGCTGA |
| MGAT5-E9F | TCTTGTCTGGTCCTGCTTCT |
| MGAT5-E9R | ACAGCACACACTTCTACAGC |
| MGAT5-E10F | ACTGGGAATGTGTGGGAGAG |
| MGAT5-E10R | GCAGGTACATACAGCCTTGG |
| MGAT5-E11F | TCCACAAAGTCCAGCCAGAT |
| MGAT5-E11R | GCTGCCATTGGTCTAGCAAA |
| MGAT5-E12F | AGCCTTCCAAAGTGAGGTGT |
| MGAT5-E13R | AGCCTCTATACCCTCACCCT |
| MGAT5-E13F | TCAAGGGGAAGGACACAGTG |
| MGAT5-E13R | CCAAATCCTGCAGCTTGGTT |
| MGAT5-E14F | GCTTGAGATCACGACTTGCA |
| MGAT5-E14R | GGTGACCATGACAGTGCTAC |
| MGAT5-E15F | CTCCCAACTCTTCCCCGTC |
| MGAT5-E15R | CTTATCCCCACAGCCCAGTA |
| MGAT5-E16F | GATTGCAGACGAAGAGGGTG |
| MGAT5-E16R | GCAATGCCAATTCAGAACCTTC |

# Results

## DNA sequencing

The 3 samples from the HSP family were screened for selected variants using WES. The sequencing quality parameters are listed in Table 2. After being screened for selected variants, the filtered SNVs were verified using Sanger sequencing; the results are listed in Table 3. Functional prediction and biological relevance analysis led to 2 genes: *MIF* and *MGAT5*. One variant was in *MGAT5* (c.673C>G; p.L225V) and the other variant was in *MIF* (c.295T>C; p.Y99H). The 2 variants were both missense mutations which were heterozygous in both the patient and his father (Fig. 1). The pathogenicity analysis of all variants of *MGAT5* and *MIF* are listed in Table 4.

## PCR analysis of the *MGAT5* gene in 93 children with HSP

The sequencing results of *MGAT5* showed that there were 3 synonymous variants (c.141C>T, c.267A>G, and c.348A>G) and 2 missense variants (c.673C>G and c.1117A>G). The 3 synonymous variants do not cause changes in the corresponding amino acid and every synonymous variant exists in only 1 HSP patient. The other 2 missense variants are both heterozygous mutations and are located in the encoding region of *MGAT5*. Base mutation can cause amino acid change (c.1117A>G, p.Met373Val; c.C673G, p.Leu225Val). Also, each missense variant exists in only 1 HSP patient.

Conservation analysis of MGAT5 amino acid sequences in different species was performed with Bioedit software (Fig. 2). The results showed that the above amino acid sites

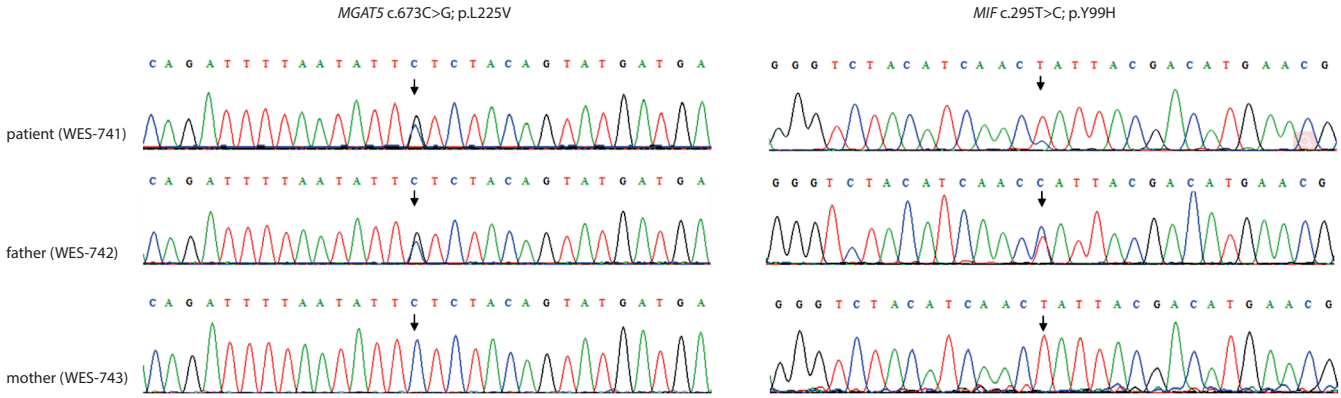


Fig. 1. Sanger sequencing of *MGAT5* and *MIF* genes. The *MGAT5* c. 673C>G; p.L225V and *MIF* c.295T>C; p.Y99H variants were detected in the patient and his father. The patient's mother had no variants. The corresponding variant bases are indicated by arrows


Fig. 2. Conservation analysis of mutations in MIF. One variant (c.295T>C, p.Y99H; in yellow) is well-conserved in vertebrates except *B. taurus* and *D. rerio*. Another variant (c.338C>T, p.T113I; in green) is well-conserved across species

Table 2. The sequencing quality parameters of the 3 cases included in this study

| Sample | Total reads | Average read depth (×) | Percent of ROI with 1× [%] | Percent of ROI with 20× [%] | Unaligned reads | Off-target percent of reads [%] |
|---|---|---|---|---|---|---|
| Patient (WES-741) | 59938725 | 101.95 | 96.897 | 99.873 | 2625970 | 28.88 |
| Father (WES-742) | 69050718 | 115.8 | 97.658 | 99.884 | 3382470 | 29.353 |
| Mother (WES-743) | 57841691 | 101.44 | 97.044 | 99.776 | 2191901 | 27.118 |

NA – not available; ROI – region of interest.

**Table 3.** The screened SNVs verified using Sanger sequencing

| Gene | NM_ ID | Transcript variant | Protein variant | PolyPhen-2 function prediction | Associated disease (OMIM) | Function |
|---|---|---|---|---|---|---|
| *KIF1B* | NM_015074.3 | c.1717C>T | p.R573C | probably damaging | Charcot-Marie-Tooth disease type 2A1; neuroblastoma; pheochromocytoma | transport of mitochondria |
| *ABCA4* | NM_000350.2 | c.3265A>T | p.T1089S | probably damaging | Stargardt disease; retinitis pigmentosa age-related macular dystrophy; fundus flavimaculatus | transport of an essential molecule (or ion) either into or out of photoreceptor cells |
| *MGAT5* | NM_002410.4 | c.673C>G | p.L225V | possibly damaging | NA | catalyzes the addition of β-1,6-N-acetylglucosamine to the α-linked mannose of biantennary N-linked oligosaccharides |
| *MUC4* | NM_018406.6 | c.11079G>T | p.Q3693H | NA | NA | protection of the epithelial cells and epithelial renewal and differentiation |
| *NMUR2* | NM_020167.4 | c.451C>T | p.P151S | probably damaging | NA | regulation of food intake and body weight |
| *HIST1H2AEHIST1H2BG* | NM_021052.2; NM_003518.3 | c.-554C>A; c.223G>T | p.A75S | | NA | basic nuclear proteins that involved in the nucleosome structure of the chromosomal fiber in eukaryotes |
| *ITPR3* | NM_002224.3 | c.6262A>T | p.M2088L | benign | NA | mediates the release of intracellular calcium |
| *SMARCA2* | NM_003070.4; | c.705_707 dupGCA | p.Q238dup | NA | Nicolaides–Baraitser syndrome | has helicase and ATPase activities and regulate transcription of certain genes |
| *PKP2* | NM_004572.3; | c.473G>A | p.R158K | benign | arrhythmogenic right ventricular cardiomyopathy | may regulate the signaling activity of beta-catenin |
| *MUC19* | NM_173600.2 | c.4910-3C>A | NA | NA | NA | may be involved in disruption of the ocular surface in Sjögren syndrome |
| *F10* | NM_000504.3 | c.706G>T | p.V236L | probably damaging | factor X deficiency | encodes the vitamin K-dependent coagulation factor X |
| *STRC* | NM_153700.2 | c.5243G>A | p.R1748Q | probably damaging | autosomal recessive deafness 16 | involved with mechanoreception of sound waves |
| *ZNF276* | NM_001113525.1 | c.493C>T; c.268C>T | p.R165W; p.R90W | possibly damaging | NA | zinc finger protein 276 |
| *FAM129C* | NM_173544.4 | c.2013G>C | p.Q671H | possibly damaging | NA | unknown |
| *LIPE* | NM_005357.3 | c.2200G>A | p.V734M | possibly damaging | familial partial lipodystrophy-6 | converts cholesteryl esters to free cholesterol in steroidogenic tissues or hydrolyzes stored triglycerides to free fatty acids in adipose tissues |
| *BPIFA1* | NM_130852.2; | c.726T>G | p.I242M | possibly damaging | NA | has antibacterial activity against Gram-negative bacteria |
| *KRTAP19-3* | NM_181609.3 | c.70_75dupGGCTAT | p.G24_ Y25dup | NA | NA | unknown |
| *PI4KA* | NM_058004.3 | c.2158A>G | p.I720V | NA | perisylvian polymicrogyria; cerebellar hypoplasia; arthrogryposis | catalyzes the first committed step in the biosynthesis of phosphatidylinositol 4,5-bisphosphate |
| *MIF* | NM_002415.1 | c.295T>C | p.Y99H | possibly damaging | systemic juvenile rheumatoid arthritis | regulation of macrophage function in host defense through the suppression of anti-inflammatory effects of glucocorticoids |

OMIM – Online Mendelian Inheritance in Man; NA – not available.

Table 4. Pathogenic predictions of all mutations of the *MGAT5* and *MIF* genes

| Gene | Transcript ID | DNA change | AA change | Translation Impact | Mutation Taster | PolyPhen2 | SIFT | PROVEN | HSF | MAF in various databases | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | ESP | gnomAD | 1000genomes |
| *MGAT5* | NM_002410.4 | c.673C>G | p.Leu225Val | missense | disease_causing | probably_damaging | deleterious | neutral | NA | NA | 5.54E-05 | NA |
| | | c.1117A>G | p.Mer373Val | missense | disease_causing | benign | tolerated | neutral | NA | NA | 0.0003724 | NA |
| | | c.141C>T | p.Arg47Arg | synonymous | disease_causing | NA | NA | neutral | potential alteration of splicing | NA | 2.86E-05 | NA |
| | | c.267A>G | p.Leu89Leu | synonymous | disease_causing | NA | NA | neutral | potential alteration of splicing | NA | NA | NA |
| | | c.348A>G | p.Ser116Ser | synonymous | disease_causing | NA | NA | neutral | potential alteration of splicing | NA | 4.47E-05 | NA |
| *MIF* | NM_002415.1 | c.295T>C | p.Tyr99His | missense | disease_causing | probably_damaging | deleterious | deleterious | NA | NA | NA | NA |
| | | c.282-6C>G | | Framshift | NA | NA | NA | NA | potential alteration of splicing | 0.1385 | 0.1924 | 0.174 |
| | | c.338C>T | p.Thr113Ile | missense | disease_causing | probably_damaging | deleterious | deleterious | NA | NA | 0.0001558 | 0 |

were highly conserved among different species. This suggests that the mutation of the 2 conserved sites may be important factors affecting the functioning of *MGAT5*, so the mutations are not caused by natural selection in evolution.

According to the Hphob./Kyte & Doolittle scale, the individual values for methionine, valine and leucine are 1.9, 4.2 and 3.8, respectively. These amino acids are hydrophobic and their properties do not change significantly. Because of the 2 highly conserved sites, the 2 gene mutations may still cause structural and functional changes of the MGAT5 protein.

## PCR analysis of the *MIF* gene in 93 children with HSP

The sequencing results of *MIF* showed that the 3 variants, c.282-6C>G, c.295T>C and c.338C>T, were all heterozygous mutations. The c.282-6C>G variant (rs2070766) is located in the splicing region of *MIF*. Among the 93 children, 35 patients (37.6%) carried this mutation. These data are a close match to those of the European population (32.2%) and the East Asian population (28.6%) according to the ExAC database, and the allele frequency is greater than 3%, so these sites are the single nucleotide polymorphism (SNP) site.

The c.295T>C and c.338C>T variants are both located in the encoding region of *MIF*. Base mutation can result in a change of the corresponding amino acid (c.338C>T; p.Thr113Ile and c.295T>C; p.Tyr99His). Among the 93 patients with HSP, there were 2 (2.15%) c.338C>T variants and 1 (1.08%) c.295T>C variant.

Conservation analysis of MIF amino acid sequences in different species were also performed with Bioedit software (Fig. 3). The results showed that the 113$^{th}$ amino acid was highly conserved among different species. This suggests that the variant may be one of the factors that affect the functioning of *MIF*, rather than natural selection in evolution. And the 99$^{th}$ amino acid is conserved in other vertebrates except cattle and zebrafish, in which it is semi-conserved.

Using the Hphob./Kyte & Doolittle scale, the individual values for tyrosine, histidine, threonine, and leucine are −1.3, −3.2, −0.7, and 4.5, respectively. The p.Tyr99His variant leads to a significant change in the structure of the amino acid side chain, while p.Thr113Ile can cause its hydrophobicity to change greatly. The amino acid side chain structure changes as well. Therefore, the 2 amino acids change more significantly.

# Discussion

Henoch–Schönlein purpura is one of the most common types of vasculitis in children, but its cause is still unknown and, so far, no specific genetic abnormality has been described in patients with HSP.[20–22] In this study, a WES analysis was performed in a family with HSP. Considering

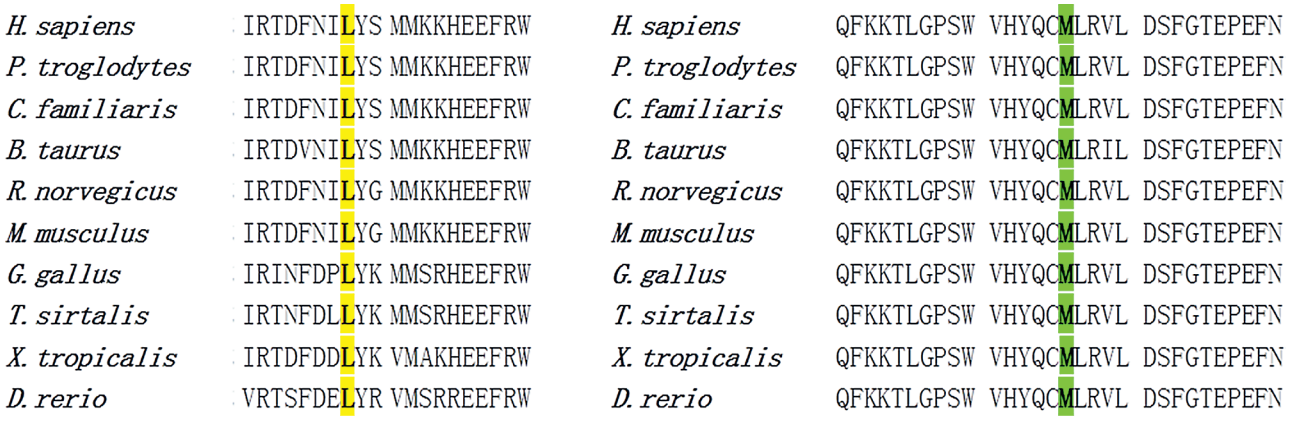


Fig. 3. Conserved analysis of mutations in *MGAT5*. The 2 variants (c.673C>G, p.L225V; left, in yellow, and c.1117A>G, p.M373V; right, in green) are both highly conserved across species

that HSP is associated with immune imbalance, we gained a total of 20 SNVs in the 20 different genes screened. Among the screened markers for immunity in our whole exome association study, *MGAT5* and *MIF* were of particular interest by functional prediction and biological relevance analysis.

The *MGAT5* gene, also known as *GNT-V*, encodes beta-1,6 N-acetyl-glucosaminyltransferase, an enzyme which catalyzes the addition of beta-1,6 GlcNAc- to N-glycans on glycoproteins. An *MGAT5* deficiency increases the number of T-cell receptors recruited to the antigen-presenting surface, thereby reducing the need for CD28 co-receptor engagement. *MGAT5* functions as a negative regulator of T-cell activation thresholds and susceptibility to immune diseases, such as multiple sclerosis or rheumatoid arthritis.[23–25] The expression of beta-1,6 GlcNAc-branched N-linked glycans selectively inhibits Th1 cell differentiation and enhances the polarization of Th2 cells.[26] In our study, PCR analysis of *MGAT5* in the 93 HSP patients showed that there was 1 patient (1.08%) with the c.1117A>G variant and another 1 child (1.08%) with the c.673C>G variant. Our result for the c.673C>G variant (1.08%) is much higher than that of the East Asian population (0.83%) and the European population (0%). The frequency of the c.1117A>G variant in HSP patients (1.08%) is also far higher than that of the East Asian population (0.43%) and the European population (0%). According to the analysis of *MATG5* gene sequence conservation and amino acid properties, these 2 amino acids did not change significantly, but because the 2 sites are highly conserved their mutation may still lead to structural and functional changes of the MGAT5 protein. In addition, the HSP patient with the c.673C>G variant, whose father also had HSP in childhood, had joint pain at his initial diagnosis. Once he had received enough prednisone therapy, his rash was under control and his joint pain was relieved. The patient with c.1117A>G had a rash which lasted 2 months, with a longer course and kidney involvement, and he recovered after 1 year of prednisone treatment. We speculate that the mutation of the *MGAT5* gene may be related to the occurrence of HSP, severity of HSP and the duration of glucocorticoid treatment through some unknown mechanism. Of course, more research is needed on the reliability of these findings and on the intrinsic mechanism of the disease.

Another interesting marker in our screening is macrophage migration inhibitory factor (MIF). It is a pro-inflammatory cytokine that is produced by immune T cells, macrophages and monocytes, and plays pivotal roles in innate and acquired immunity.[27] It has been reported to inhibit random migration and concentration of macrophages at sites of inflammation and to induce pro-inflammatory cytokine secretion, leading to an excessive inflammatory response.[28,29] The functional effects of *MIF* gene polymorphisms have been identified and associated with susceptibility or severity of several acute, chronic and autoimmune inflammatory conditions and vasculitides.[30] The promoter region of the MIF –173G/C polymorphism has been identified as a functionally relevant variant of this gene.[31] Our previous study and other studies have found increased levels of MIF in the urine and renal tissues of children with HSP.[32,33] However, Nalbantoglu et al. and Amoli et al. showed that the *MIF* gene -173G/C polymorphism was not associated with HSP.[34,35] The above results indicate that the -173G/C polymorphism in the *MIF* gene promoter does not appear to be a genetic risk factor for HSP, and that HSP may be related to abnormal regulation of inflammation by the MIF protein, abnormal levels of which are caused by other mutations in *MIF*. Our study demonstrated 2 new variants in the *MIF* gene. One is the c.295T>C variant; it was found in 1 patient (1.08%) with HSP and the site had never been reported before. Therefore, the gene frequency cannot be compared with the normal population of the ExAC database. The other variant is the c.338C>T variant. There were 2 children (2.15%) carrying this variant, and its frequency is much higher than that of the populations of East Asia (0.28%) and Europe (0%). According to the analysis of *MIF* sequence conservation and amino acid properties, the 99$^{th}$ amino acid is semi-conserved, the 113$^{rd}$ is highly conserved and the properties of the 2 amino acids change significantly. Therefore, the 2 variants

are likely to lead to changes in the structure and function of MIF, which is related with HSP.

Additionally, glucocorticoid (GC), which has a broad spectrum of anti-inflammatory and immune-regulatory effects on host immune responses, can inhibit the activation of immune cells and reduce the production of pro-inflammatory mediators, including cytokines, prostaglandins and reactive oxygen species.[36] Because of these properties, GC is effective in the treatment of some inflammatory diseases.[37] As a unique trans-regulator of GC, MIF can antagonize endogenous and exogenous glucocorticoid activity, so as to maintain a balance in immune regulation.[38] However, the research on the systemic lupus erythematosus (SLE) showed that *MIF* was associated not only with disease severity,[39] but also with steroid resistance.[40] In our study, there were 2 children with HSP and the c.338C>T variant. One was diagnosed with HSP nephritis, which was refractory and lasted for 2 years. The proteinuria was relieved after prednisone treatment combined with immunosuppressant. The other was a girl with severe rash (both lower limbs and both upper limbs) at her initial diagnosis who was treated with prednisone. The patient with the c.295T>C variant had joint pain at his initial diagnosis, and his rash was controlled after sufficient prednisone treatment. Thus, we speculate that *MIF* may be related to the severity of HSP. The specific mechanism between *MIF* and the occurrence of HSP, the relationship between *MIF* and the severity of the disease, and the drug resistance of the hormone still need to be studied further.

Because the sample size in this study is small, it needs to be verified in larger samples. At the same time, it also needs to be further studied in gene function and animal models. So far, few studies have reported a relationship between *MGAT5* or *MIF* and the pathogenesis of HSP, but our study provides a new direction for future research.

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# Does oral dryness influence quality of life? Current perspectives in elderly dental care

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## Abstract

**Background.** The oral condition can functionally, socially and psychologically influence the quality of life. Oral dryness often occurs in the elderly due to the presence of systemic diseases and medications, which favors the development of many lesions and complaints, including dental caries, candidiasis, retention of full removable denture, taste disturbance and it enforces a change in nutrition.

**Objectives.** The aim of this study was to assess the impact of oral dryness on oral health-related quality of life in older subjects.

**Material and methods.** Five hundred subjects of both genders, aged 65 and over (mean 74.4 ±7.4) were involved in the study. Oral dryness was evaluated clinically with use of the Challacombe scale (CODS, Clinical Oral Dryness Score). The oral health-related quality of life was assessed by the Oral Health Impact Profile scale (OHIP-14).

**Results.** Oral dryness occurred in 32.8% of subjects, most often on a mild level (29.6%). The average value of the OHIP-14 scale was 8.01 ±13.59. The regression analysis demonstrated a significant association between oral dryness and two domains of the OHIP-14 scale, i.e. functional limitation ($p < 0.01$) and psychological disability ($p < 0.05$).

**Conclusions.** Oral dryness is substantially related to the oral health-related quality of life, which indicates the necessity of monitoring oral dryness as part of routine dental care.

**Key words:** oral dryness, Oral Health Impact Profile, elderly, OHIP-14

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Extended human lifespan resulting from civilizational development along with decreased birth rate leads to an increase in the percentage of the elderly in comparison to younger people. This is the reason why, in recent years, the number of ageing members of society has been increasing, especially in highly developed countries.[1,2] The ageing process of the human body is an inevitable phenomenon and it concerns all the organs. Changes related to the ageing process occurring in the oral cavity involve teeth, the periodontium, oral mucosa, bones of the jaws, temporomandibular joints, and salivary secretion. In elderly people, reduced salivary secretion (hyposalivation) is frequently observed; this condition leads to oral dryness (xerostomia). It is estimated that 25–30% of people aged 65 and over suffer from this condition and experience its symptoms.[3,4] Data regarding stimulated salivary secretion among healthy elderly people does not indicate a significant and age-dependent reduction in secretion, but only an insignificant reduction in the secretion of seromucous glands (but not the serous gland, i.e. the parotid gland) in the conditions of minimal or prolonged stimulation. It has been observed that as people get older, the number of acini decreases, and the adipose and fibrous tissue increase. Small salivary glands also undergo these changes. However, reduction in the acinar part of the glands resulting from the ageing process does not significantly reduce the secretory function of the salivary glands. Therefore, it is currently assumed that the production of saliva and its composition does not change with age in healthy people. However, the observed dysfunction of salivary glands is mainly caused by consequent systemic diseases, pharmacotherapy and radiotherapy of the head and neck area.[3,5–7] Taking medications is considered to be the most common cause of oral dryness, as most elderly people take at least one medication which negatively impacts salivary secretion.[8] Medications that cause xerostomia include, among others, antihypertensive, anticholinergic, anti-reflux and cytotoxic medications, as well as diuretics, antidepressants and beta blockers.[4,6] Furthermore, some common diseases at an older age may lead to the hypofunction of salivary glands (e.g., diabetes type 2, and rheumatoid arthritis). Other causes include radiotherapy in the form of external radiation applied as part of head and neck cancer treatment, which destroys the tissues of the salivary glands and leads to long-lasting xerostomia.[6,7]

Reduced salivary secretion implies numerous clinical consequences, such as increased susceptibility to caries and periodontal diseases, which may then result in tooth loss, reduced denture tolerance, increased risk of fungal infections and taste disorders. People suffering from oral dryness have problems with eating (especially dry food, such as crackers or biscuits), speaking, swallowing and wearing removable partial dentures (their retention, pain experienced when wearing the dentures), as well as with the tongue tending to stick against the palate. Patients often report halitosis, burning mouth syndrome and intolerance to spicy food, excessive mucus or food accumulation.[8] Moreover, patients frequently complain about oral dryness experienced particularly at night, as this is the time of the lowest salivary secretion. Intensified difficulties in speaking and eating may deteriorate the quality of social interactions.[9]

Oral health considerably affects the overall quality of life. It leads to a reduction in everyday activity and to lack of physical and/or mental wellbeing. Therefore, the notion of Health-related Quality of Life – HRQoL has been introduced. It is based on the WHO (World Health Organization) definition of health defined as "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity".[10–13] The relation between oral health and the quality of life can be determined by assessing to what extent oral health influences the quality of life through performing functions, such as chewing, biting, swallowing, speaking, as well as through mental wellbeing (satisfaction with dental aesthetics, self-esteem), physical wellbeing (comfort while eating and lack of discomfort or pain), and social wellbeing (feeling comfortable while speaking to others).[14] One of the most commonly used assessment tools is the Oral Health Impact Profile (OHIP) scale that consists of 49 questions, or its shortened version, which consists of 14 questions (OHIP-14).[15,16]

## Objectives

The aim of the study was to determine the influence of oral dryness on the oral health-related quality of life among elderly people.

## Material and methods

### Recruitment and study duration

The study was conducted for 24 months (from January 2015 to December 2016) after obtaining consent of the Bioethics Committee of Wroclaw Medical University KB 420/2015.

The study involved 500 subjects of both genders (180 men and 320 women) at the age of ≥65 (mean age 74.4 ±7.4, age range 65–99) who were urban residents of Wrocław. Data on the total number of 65 and over subjects living in the city was derived from the Central Statistical Office (Demographic Yearbook of Poland 2015). A minimum sample size was calculated based on data concerning caries prevalence in this age group with the assumed significance level of 95% and with ±5% error tolerance. It turned out to be 383; therefore, the number of the examined subjects exceeded this figure.

### Survey inclusion/exclusion criteria

The inclusion criteria for the study were the following: age of 65 and over, place of residence (citizens of Wrocław), able to communicate and a written consent to participate in the survey.

The exclusion criteria were lack of a written consent or mental disorders which would have made filling out the questionnaire impossible.

The participation in the survey was voluntary. The patients were enrolled based on information provided in press, on the radio and on the Internet, as well as in Senior Citizens Associations, at the University of the Third Age, church organizations, primary care outpatient clinics, pharmacies, and residential homes.

## Survey execution

### Sociomedical examination

The demographic and personal information, and medical history were obtained from the subjects. The data included the date of birth and gender, marital status, level of education, and amount of monthly income before the appointment; the patients were asked to provide written information about current and past diseases, as well as medications currently taken by them. Medical data comprised the type of past and present diseases and medicine taken.

### Clinical oral examinations

Oral dryness was determined according to the Challacombe Scale which serves as the Clinical Oral Dryness Score – CODS. It includes 10 characteristics: 1) mirror sticks to oral mucosa; 2) mirror sticks to tongue; 3) saliva frothy; 4) no saliva pooling in floor of mouth; 5) tongue shows generalized shortened papillae (mild depapillation); 6) altered gingival architecture (i.e. smooth); 7) glassy appearance of oral mucosa, especially palate; 8) tongue lobulated/fissured; 9) cervical caries (more than two teeth); 10) debris on palate or sticking to teeth. One score is assigned to each sign. Scores 1–3 indicate a mild, 4–6 a moderate and 7–10 a severe oral dryness.[17,18]

### Psychometric examination

Oral health-related quality of life was assessed with the use of the Oral Health Impact Profile-14 scale (OHIP-14).[16,17] It consists of 14 questions related to problems which have occurred within the past month, and which are connected with teeth, oral cavity or wearing dentures. The problems include: 1) trouble with pronouncing words, 2) worsened taste, 3) pain, 4) discomfort while eating, 5) self-consciousness, 6) emotional tension, 7) unsatisfactory diet, 8) interrupted meals, 9) difficulty with relaxing, 10) embarrassments, 11) irritability, 12) inability to complete everyday tasks, 13) reduced satisfaction with life, (14) complete inability to function. The answers to the questions were categorized in accordance with the frequency of occurrence on a five-level scale ranging from never (score 0), hardly ever (score 1), occasionally (score 2), fairly often (score 3) to very often (score 4). Oral health-related quality of life is expressed as the sum of the score for the 14 questions (OHIP-14 sum, maximum 56 points). Furthermore, the 14 questions constitute 7 domains. The domains are related to functional limitations, pain (physical), psychological discomfort, physical disability, psychological disability, social disability and handicap (infirmity). The OHIP-14 scale has been validated for the Polish population.[17]

## Statistical analysis

Continuous variables were presented as mean values with standard deviation or, if not normally distributed, as median and range, while categorical variables were reported as percentages and numbers. As the empirical distributions of quantitative variables differed significantly from the theoretical normal distribution (they were skewed), they were transformed using the Box-Cox technique. Continuous variables were compared by *t*-test, while categorical variables were compared by $\chi^2$ test or Fisher's exact test. An analysis of variance (ANOVA) was performed to compare the mean values of transformed value between the OHIP-14 domains. The significance of the relationship between the oral dryness and the predictors analyzed was verified using the Pearson chi-square test or the exact Fisher test. For each predictor, odds ratio adjusted by age and sex was calculated. Its significance was verified using the Mantel-Haenszel test. A two-tailed value of $p < 0.05$ was used to reject the null hypothesis. All statistical analyses were performed using STATISTICA v. 13 (StatSoft, Inc., Tulsa, USA).

# Results

## Sociodemographic characteristics of the subjects

Most of the subjects lived with a family member (62.0%) and were independent (90.8%). Secondary education was completed by 51.0% of the subjects, primary education by 16.2%, and higher education by 32.8%. High monthly income per person was declared by 27.2%, medium income by 45.2%, and low income by 25.6%. The remaining 1.8% did not provide their answers.

## The subjects' medical history

Only 14% of the subjects did not report the occurrence of a chronic systemic disease. Most often, there were two coexisting systemic disorders (27.0%). Over 60% of the subjects reported hypertension, and over 20% diabetes (Table 1).

Table 1. General information of the patients

| Parameters | Quality variables | Quantitative variables |
|---|---|---|
| | % (n) | Me (min–max) |
| Age [years] | | 73 (65–99) |
| Female | 64.0 (320) | |
| Oral dryness | 32.8 (164) | |
| OHIP-14 (total score) | | 1 (0 – 56) |
| D1 – functional limitation | | 0 (0 – 8) |
| D2 – pain | | 0 (0 – 8) |
| D3 – psychological discomfort | | 0 (0 – 8) |
| D4 – physical disability | | 0 (0 – 8) |
| D5 – psychological disability | | 0 (0 – 8) |
| D6 – social disability | | 0 (0 – 8) |
| D7 – handicap | | 0 (0 – 8) |
| Number of medications | | 2 (0 – 6) |
| Number of diseases | | 2 (0 – 7) |
| Hypertension | 62.4 (312) | |
| Diabetes | 21.8 (109) | |
| Hypercholesterolemia | 18.4 (92) | |
| Rheumatoid arthritis | 14.6 (73) | |
| Hypothyroidism | 11.2 (56) | |
| Reflux | 9.8 (49) | |
| Heart failure | 7.4 (37) | |
| Kidney disease | 7.6 (38) | |
| Asthma | 6.6 (33) | |
| Infarction | 5.2 (26) | |
| Cancer | 5.8 (29) | |
| Hyperthyroidism | 4.4 (22) | |

## Oral dryness

Oral dryness was found in 32.8% of the subjects of both genders (Table 1), including mild dryness in 29.6, moderate – in 3.0% and severe – in 0.2%. Among women, the condition was significantly more common than among men (36.9% vs 25.6%, p = 0.025). The most frequent symptoms included the mouth mirror tending to stick against buccal mucosa – 26.6% (more often among women than men 30.6% vs 18.3%, p = 0.000) or against the tongue – 22.2% (slightly more often in women than in men 25.3% vs 17.8%, p = 0.068); the least common symptoms were cervical caries involving >2 teeth – 1.0% (0.6% in men and 1.3% in women, p = 0.779) and debris on palate or sticking to teeth 0.8% (1.1% in men, 0.6% in women, p = 0.622).

## OHIP-14 scale

The total OHIP-14 score (sum) for all the subjects was 8.01 ±13.59 and it did not reveal a gender-related difference (7.20 ±12.90 in men and 8.50 ±14.00 in women, p = 0.280).

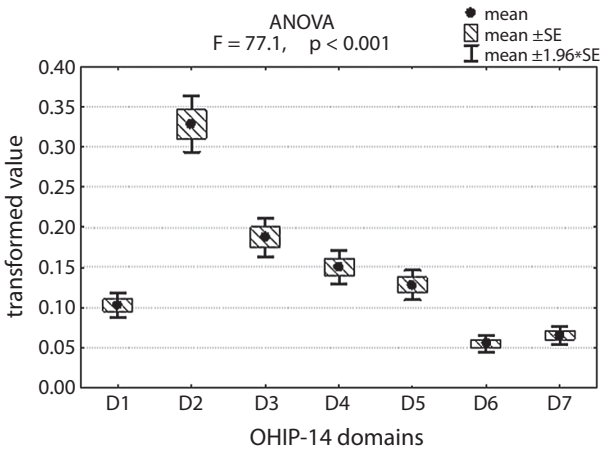


Fig. 1. Domains of OHIP-14

D1 – functional limitations; D2 – pain (physical); D3 – psychological discomfort; D4 – physical disability; D5 – psychological disability; D6 – social disability; D7 – handicap.

Regarding domains, it turned out that the subjects most frequently reported physical pain (D2) and physical discomfort (D3) (Fig. 1). Moreover, significant differences among all the domains were found, except for domains D6 and D7. When considering OHIP-14 scale transformed values adjusted by sex and age in groups that differed in oral dryness, we found higher mean total OHIP-14 score in a subgroup with oral dryness (p = 0.027). The subjects with oral dryness symptoms significantly more often reported trouble with pronouncing words (0.054 ±0.088 vs 0.029 ±0.071, p = 0.001), worsened taste (0.073 ±0.115 vs 0.045 ±0.098, p = 0.004), difficulty with relaxing (0.061 ±0.105 vs 0.039 ±0.089, p = 0.015), and complete inability to function (0.036 ±0.076 vs 0.021 ±0.061, p = 0.02) (Table 2).

## Regression analysis

Results of multiple regression between OHIP-14 domains (transformed) and the studied variables (Table 3) showed the correlation of domain 1 (functional limitation) and 5 (psychological disability) with oral dryness, domain 2 (pain) with number of medication, domain 3 (psychological discomfort) and 5 (psychological disability) and kidney diseases, and domain 4 (physical disability) with cardiac infarction (Table 3).

Gender, age over 70 years, the value of the OHIP-14 scale higher than 0, as well as some coexisting systemic diseases, such as hypertension, cardiac infarction, kidney disease and hypothyroidism, were independent predictors of oral dryness. It was noticed that women had almost as twice as high probability to suffer from oral dryness than men (OR = 1.70). A similar observation was made with regards to patients aged over 71 years who provided a positive answer to at least 1 question included in the OHIP-14 scale. Patients suffering from cardiac infarction were twice as prone to suffer from oral dryness as those who were not afflicted with the disease (OR = 2.04). Patients

**Table 2.** Mean transformed values of OHIP-14 scale adjusted by sex and age in relation to oral dryness

| No | Items | No oral dryness | Oral dryness | p-value * |
|---|---|---|---|---|
| | | $M^T$±SD | $M^T$±SD | |
| 1 | Trouble with pronouncing words | 0.029 ±0.071 | 0.054 ±0.088 | 0.001 |
| 2 | Worsened taste | 0.045 ±0.098 | 0.073 ± 0.115 | 0.004 |
| 3 | Pain | 0.131 ±0.203 | 0.150 ±0.208 | 0.321 |
| 4 | Discomfort while eating | 0.157 ±0.232 | 0.173 ±0.234 | 0.480 |
| 5 | Self-consciousness | 0.119 ±0.190 | 0.135 ±0.196 | 0.401 |
| 6 | Emotional tension | 0.089 ±0.153 | 0.098 ±0.157 | 0.542 |
| 7 | Unsatisfactory diet | 0.088 ±0.153 | 0.097 ±0.159 | 0.532 |
| 8 | Interrupted meals | 0.058 ±0.114 | 0.072 ±0.123 | 0.203 |
| 9 | Difficulty with relaxing | 0.039 ±0.089 | 0.061 ±0.105 | 0.015 |
| 10 | Embarrassment | 0.071 ±0.134 | 0.092 ±0.146 | 0.113 |
| 11 | Irritability | 0.041 ±0.105 | 0.047 ±0.106 | 0.437 |
| 12 | Inability to complete everyday tasks | 0.024 ±0.064 | 0.030 ±0.071 | 0.305 |
| 13 | Reduced satisfaction with life | 0.045 ±0.095 | 0.048 ±0.097 | 0.733 |
| 14 | Complete inability to function | 0.021 ±0.061 | 0.036 ±0.076 | 0.020 |
| Total OHIP-14 (sum) | | 0.717 ±0.821 | 0.891 ±0.812 | 0.027 |

* t-test values.

**Table 3.** Results of multiple regression – standardized coefficient regression values (Beta) between OHIP-14 domains (transformed) and the studied variables

| Variables | Functional limitation (D1) | Pain (D2) | Psychological discomfort (D3) | Physical disability (D4) | Psychological disability (D5) | Social disability (D6) | Handicap (D7) |
|---|---|---|---|---|---|---|---|
| $Age^T$ | –0.022 | 0.028 | –0.019 | –0.013 | 0.002 | –0.021 | –0.027 |
| Female | –0.037 | 0.030 | –0.015 | 0.017 | –0.005 | –0.022 | 0.015 |
| $NoM^T$ | –0.062 | 0.200* | 0.097 | –0.033 | 0.093 | –0.061 | 0.065 |
| Hypertension | –0.017 | –0.104 | –0.096 | –0.049 | –0.089 | 0.040 | –0.073 |
| Infarction | 0.061 | 0.044 | 0.047 | 0.108* | 0.012 | –0.046 | 0.070 |
| Kidney disease | –0.012 | –0.055 | –0.109* | 0.032 | –0.096* | –0.009 | 0.018 |
| Hypothyroidism | 0.064 | –0.020 | –0.001 | –0.009 | –0.051 | –0.041 | 0.059 |
| Oral dryness | 0.176** | 0.042 | 0.051 | 0.060 | 0.107* | 0.068 | 0.071 |

$Age^T$ – transformed age of patients; $NoM^T$ – transformed number of medication; significance level: *p < 0.05, **p < 0.01, ** p < 0.001.

suffering from kidney disease were 3 times more likely to develop oral dryness than patients who did not have the disease (OR = 2.75). In subjects with hypothyroidism, oral dryness was over 2 times more likely to occur than in patients who did not suffer from the disease (OR = 2.17) (Table 4).

## Discussion

Oral dryness resulting from reduced salivary secretion can be detected with the use of various methods: by asking about symptoms (e.g., the Fox's test), asking about some systemic diseases and medications taken, measuring the stimulated and unstimulated salivary flow (sialometry), physical examination, salivary glands imaging and biopsy, and analyzing biochemical components of the saliva and serum.[6,8] During the clinical examination, there is an attempt to identify symptoms of oral dryness, including glassy appearance of oral mucosa, mirror sticking to buccal mucosa, dry, painful and lobulated tongue, sticky and frothy saliva, no saliva pooling in mouth floor, turbid saliva flowing out of the salivary duct, as well as enlarged and painful salivary glands. Reduced salivary secretion results in cervical caries, accumulation of food particles due to oral clearance reduction, candidiasis, and angular cheilitis. Among subjects wearing removable dentures, reduced retention or denture intolerance resulting from lower lubrication of the tissue base and the denture plate are observed. This leads to reduced adhesion and disturbance in the distribution of occlusal forces.[19–23]

In our study, oral dryness was clinically assessed with the use of the Challacombe Scale that includes 10 characteristic symptoms related to saliva shortage in the oral

Table 4. Results of chi-square test

| Oral dryness predictors | Oral dryness | | Chi-square test | | OR | 95% CI |
|---|---|---|---|---|---|---|
| | Yes n = 164 | No n = 336 | $\chi^2$ | p-value | | |
| Female | 72.0 (118) | 60.1 (202) | 6.70 | 0.010 | 1.70 | 1.14–2.55 |
| Age >71 years | 64.6 (106) | 51.8 (174) | 7.38 | 0.007 | 1.70 | 1.16–2.50 |
| OHIP-14 >0 | 46.1 (155) | 59.2 (97) | 4.34 | 0.037 | 1.62[a] | 1.10–2.40 |
| Hypertension | 71.3 (117) | 58.0 (195) | 4.23 | 0.040 | 1.67[a] | 1.11–2.52 |
| Hypercholesterolemia | 19.5 (32) | 17.9 (60) | 0.06 | 0.608 | 1.00[a] | 0.60–1.68 |
| Diabetes | 26.8 (44) | 19.4 (65) | 1.34 | 0.248 | 1.46[a] | 0.91–2.32 |
| Infarction | 8.5 (14) | 3.6 (12) | 4.55 | 0.033 | 2.04[a] | 1.04–4.28 |
| Heart failure | 6.1 (10) | 8.0 (27) | 0.35 | 0.552 | 0.74[a] | 0.35–1.57 |
| Asthma | 7.3 (12) | 6.2 (21) | 0.07 | 0.795 | 1.18[a] | 0.57–2.47 |
| Reflux | 8.5 (14) | 10.4 (35) | 0.44 | 0.507 | 0.80[a] | 0.42–1.54 |
| Kidney disease | 12.8 (21) | 5.1 (17) | 5.14 | 0.023 | 2.75[a] | 1.19–6.36 |
| Hyperthyroidism | 6.7 (11) | 3.3 (11) | 2.33 | 0.127 | 2.12[a] | 0.90–5.01 |
| Hypothyroidism | 17.1 (28) | 8.3 (28) | 7.61 | 0.006 | 2.17[a] | 1.14–4.16 |
| Cancer | 3.7 (6) | 6.8 (23) | 1.51 | 0.220 | 0.52[a] | 0.21–1.30 |
| Rheumatoid arthritis | 19.5 (32) | 12.2 (41) | 1.38 | 0.241[a] | 1.57[a] | 0.93–2.64 |

[a] – adjusted by gender and age; [b] – Fisher's exact test; [c] – adjusted by age.

cavity.[17] The Clinical Oral Dryness Score (CODS), which consists of a 10-point scale with each point representing a feature of dryness in the mouth, may, in accordance with the current state of knowledge, be used individually during routine clinical assessment to detect hyposalivation.[18]

Overall, oral dryness was determined in 32.8% of the subjects and that included 29.6% of mild dryness, 3.0% of moderate dryness and 0.2% of severe dryness. Among women, dry mouth occurred significantly more frequently than in men (36.9% vs 25.6%, p = 0.025), which was consistent with the results of other studies.[24–28] Niklander et al. found the occurrence of xerostomia in among 13% of women and half that many in men (6.1%) at the age of 18–83 years with a mean age of 46 years.[29] They observed the highest prevalence in subjects aged 68–77 years (33.3%), followed by patients aged 78–83 years (22.27%).

A study conducted in India revealed that 58.6% of the 55–75-year-old and older subjects perceived dryness of mouth.[30] Among Korean subjects aged 55 years and over, the occurrence of at least 1 symptom of xerostomia was observed in 70.1% of the subjects, and in 25.8%, the observed symptoms were severe.[31] At the age of 60 and over (average age of 67.5 years), reduced secretion of stimulated saliva was observed in 64% subjects from Saudi Arabia.[32] Age-dependent occurrence of xerostomia was identified and it ranged from 5% in the group aged 18–25 years to 16% in the group aged 65–67 years, and to as much as 26% in the group aged 75 and over.[28]

Salivary glands function can be injured by many systemic diseases based on different mechanisms with the resulting complication of oral dryness.[33] Our data showed a significantly higher number of co-morbid diseases in the subjects with clinical symptoms of dry mouth. Elderly people suffering from chronic diseases are on long-term therapy using polypharmacy. Hundreds of medicines induce oral dryness; some of them can exert a synergistic effect on the salivary secretion. Hence, drug-induced dry mouth is one of the most common oral problems in elderly individuals.[34] Our data showed a significantly higher mean number of administered medicines among the subjects presenting oral dryness symptoms compared to these with no symptoms. Similar observations were made by Bogucki et al., who identified a correlation between xerostomia, medication taken by patients, age, gender and past diseases.[35]

The total OHIP-14 score in the examined subjects aged 65 and over was 8.01 ±13.59 and it did reveal a significant difference related to the gender. Comparing the figure in the same age group of different populations, we noticed a slightly higher value (8.88) in Spain[36], 2.8-fold higher (22.4) in Iran[37] and 2.7-fold lower (2.9) in the United Kingdom.[38] In our study, a significantly higher total OHIP-14 score was found in the subjects with clinical symptoms of oral dryness, indicating a poorer quality of life related to oral health (OHRQoL). Similar conclusions were made by Locker, who conducted research among a group of 225 subjects in a long-term care setting (average age: 83 years) and who observed a major influence of xerostomia on the well-being and quality of life of the population.[39]

The finding was consistent with the study performed in subjects aged 18–75 years and over in whom the total OHIP-14 score was ca. 50% higher (6.3 vs 3.5) in the patients with xerostomia determined by a response to one

item: "How often does your mouth feel dry?"[28] Another study involving subjects aged 18–87 years also presented a significantly higher total OHIP-14 score in patients with xerostomia than in patients without xerostomia (20.1 vs 12.7).[29] A Japanese study performed on the elderly (mean age 66.1) showed that higher values of the OHIP-14 score were related to the perception of dry mouth on eating and hyposalivation.[40] Our results indicated a relationship between oral dryness and 2 OHIP-14 domains: functional limitation and psychological disability. The Chilean study, which revealed more reduced OHRQoL in a group of patients with xerostomia, concluded that the highest impact was detected for the domains of psychological discomfort, psychological incapacity and physical pain.[29]

Own data indicated that gender, age over 70 years, the value of the OHIP-14 scale higher than 0, as well as some coexisting systemic diseases were independent predictors of oral dryness.

Several limitations have to be considered in a discussion about the results of this study. Firstly, this study was conducted using a self-reported questionnaire to report data such as OHIP-14, which could lead to identification bias. However, some studies showed the use of this scale to be a valid and reliable method of screening for oral health-related quality of life.

Secondly, the use of survey data did not allow us to explain temporal relationships or to show inferences on causality. Thirdly, while analyzing the obtained results, it is essential to bear in mind a possible self-selection error of the study participants, i.e., the participants who joined the study were worried that they were having dental problems, or they were aware of their dental problems and were looking for help; also, there may have been those who refused to participate in the study due to dental fear. Moreover, another interfering factor resulted from the application of the exclusion criteria. Finally, excluding patients with a mental disorder might have constituted another interfering factor.

# Conclusions

Individuals with oral dryness symptoms had poorer oral health-related quality of life having a higher mean of the total OHIP-14 score than their counterparts without any symptoms. The findings support the view that oral dryness negatively affects everyday life; therefore, there is a necessity to monitor oral dryness during the routine dental care as well as to ensure the cooperation of the dentist with general practitioner to diminish oral dryness severity caused by medication.

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# Biological and psychological components of depression in patients receiving IFN-alpha therapy for hepatitis C

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## Abstract

**Background.** Depressive symptoms are frequent side effects of interferon α therapy (IFN-α). Both biological and psychological processes may occur concomitantly during hepatitis C treatment.

**Objectives.** This study was carried out to determine the impact of biological (immune response) and psychological factors on formation of depressive symptoms and major depressive disorder (MDD) during hepatitis C treatment.

**Material and methods.** A total of 99 patients receiving pegylated IFN-α and ribavirin for chronic C type hepatitis participated in the prospective cohort study. Symptoms of depression were assessed with the Montgomery-Åsberg Depression Rating Scale (MADRS) during treatment and 24 weeks after treatment. Neuroticism was measured with the Eysenck Personality Questionnaire – Revised (EPQ-R/N). Diagnosis of MDD was made using the Present State Examination (PSE-10) and DSM-IV-TR criteria. Factor analysis was used to detect factors adding up to total severity of depressive symptoms. Predictors of MDD were investigated using logistic regression analysis.

**Results.** Factor analysis returned 3 factors: 1st – MADRS scores at weeks 0–12, 2nd – MADRS and N scores before treatment, 3rd – MADRS at the 24th week of treatment and 24 weeks after treatment. The total severity of depressive symptoms consisted of 3 components: personality-related before treatment, IFN-α-related during treatment and dependent on the effect of treatment. Regression analysis showed that a history of psychiatric disorders (OR = 4.8) and MADRS scores before treatment (OR = 1.25) were predictors of MDD, as opposed to level of neuroticism.

**Conclusions.** The severity of depressive symptoms and MDD during the hepatitis C treatment was related to general depressive vulnerability, not to psychological factors related to neuroticism.

**Key words:** depression, hepatitis C, interferon α, neuroticism trait

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# Introduction

Interferon α (IFN-α) in combination with ribavirin used to be the main pharmacological treatment for the chronic hepatitis C virus (HCV) infection. Interferon α is known to induce several neuropsychiatric side effects including depression, anxiety, psychosis, hypomanic mood, and cognitive impairment.[1] Depression is a particularly common side effect and in some rare cases it may be associated with suicidal ideation or suicide attempts.[2] Previous psychiatric history was a strong risk factor for depression, anxiety and other psychiatric disorders during treatment, which might suggest that the overall susceptibility to depression and anxiety, including susceptibility of a psychological origin, underlay neuropsychiatric side effects induced by IFN-α.

Interferon-α-induced neuropsychiatric symptoms have been attributed to the release of pro-inflammatory cytokines modulating several neurophysiological and neuroendocrine systems involved in mood regulation.[3,4] This is a multidirectional action involving neurotransmitter systems in the central nervous system (CNS), biochemical changes in the CNS and in the hypothalamic–pituitary–adrenal (HPA) axis. Interferon α is a potent inducer of pro-inflammatory cytokines including interleukins 1 and 6 (IL-1, IL-6) and tumor necrosis factor α (TNF-α). These cytokines play an important role in the development of sickness behavior – a set of psychological and behavioral changes that may lead to depressive symptoms in approx. half of the patients treated with IFN-α. The presumably depressogenic effect is not directly caused by cytokines but is a consequence of sickness behavior. This cytokine-induced behavioral syndrome is associated with alterations in the metabolism of neurotransmitters such as serotonin, norepinephrine and dopamine in brain regions essential to the regulation of emotions, including the limbic system, as well as the regulation of psychomotor and reward functions, including the basal ganglia.[3–6] Cytokines activity is associated with: 1) significant alterations in diurnal HPA axis activity including the flattening of the adrenocorticotropin (ACTH) and cortisol diurnal fluctuations, and an increase in evening ACTH and cortisol concentrations[4]; 2) increased activity of the metabolic enzyme indoleamine 2,3-dioxygenase responsible for degrading tryptophan to kynurenine which is then metabolized to quinolinic acid interfering with CNS activity[7]; 3) decreased brain-derived neurotrophic factor (BDNF) levels[8], and 4) altered function of the glucocorticoid receptors.[9]

The effect of IFN-α seems to be purely biological rather than psychological, but patients during an antiviral treatment are going through a difficult phase in their life caused by neuropsychiatric symptoms such as emotional lability, cognitive decline and insomnia, as well as somatic symptoms including fever, nausea, lack of appetite, and weakness. In vulnerable individuals, this stressful situation may cause depression. Significantly, both biological and psychological processes may occur concomitantly during hepatitis C treatment; in this light, the present paper offers an analysis of their mutual interdependence.

# Material and methods

## Participants and treatment

A total of 99 patients (50 men and 49 women) were included in the study. All of them had a chronic HCV infection with a detectable serum HCV-RNA concentration and a compensated liver disease. The patients were scheduled for treatment with pegylated IFN-α and ribavirin in the Ward of Infectious Diseases at the Department of Infectious Diseases, Liver Diseases and Acquired Immune Deficiences at Wroclaw Medical University, Poland. All participants were over 18 years of age and were recruited from native Polish populations in the Lower Silesian region. The inclusion scheme was based on the order in which the patients had showed up for hepatitis C treatment, with sex stratification used to compensate for the number of men and women in the study group. The exclusion criteria entailed comorbidity with severe somatic disease, autoimmune diseases, neurological disorders (including dementia or brain injury), substance dependence (except for nicotine), active psychotic disorders, depression, and pregnancy. The study protocol was approved by the Wroclaw Medical University Ethics Committee. All patients provided their written informed consent.

Initially, the subjects were receiving weekly doses of 180 µg Pegintron® (PEG-IFN-a2a, Hoffmann-LaRoche, Basel, Switzerland) combined with either 1,000 mg/day or 1,200 mg/day of Rebetol (ribavirin, Schering-Plough Corporation, Kenilworth, USA), depending on their body weight (those weighing 75 kg and more were administered the higher dose). Except for patients infected with genotype 3 virus, who were treated for 24 weeks; in the case of all other subjects the treatment lasted 48 weeks.

## Psychiatric assessments

The study followed a prospective longitudinal cohort design. The subjects were evaluated before the treatment (week 0), at weeks 2, 4, 8, 12, and 24, and 24 weeks after the conclusion of the treatment. Because patients with HCV genotype 3 were treated for 24 weeks and the remainder for 48 weeks, assessment point at the end of treatment was omitted in analysis. At each assessment point blood samples were collected and 2 psychometric scales were administered: Montgomery-Åsberg Depression Rating Scale (MADRS) and the Present State Examination (PSE-10). Neuroticism score was assessed once, at the beginning of the study, with the Eysenck Personality Questionnaire – Revised (EPQ-R/N).

The MADRS scale consists of 10 items assessing symptoms associated with depression, each item scored from 0 to 6 according to severity.[10] We used the MADRS scale as adapted by Mazurek et al., which proved its good psychometric properties.[11] The PSE-10 scale is a part of the Schedules for Clinical Assessment in Neuropsychiatry (SCAN 2.1) – a set of instruments and manuals aimed at assessing, measuring and classifying psychopathology and behavior associated with major psychiatric disorders of adult life.[12] We utilized only sections 6 and 7 of the PSE-10 (i.e., depressed mood and ideation, dysthymia, recurrent brief depressive disorder and thinking, concentration energy, interests), adopting time criteria for depression. The Polish adaptation of SCAN was a collateral result of the international EDEN (European Day Hospital Evaluation) project and as such was successfully tested in several clinical applications.[13–15] The PSE-10 served as a diagnostic tool for depression according to the DSM-IV criteria at all time points. Psychiatric disorders prior to treatment were assessed by a psychiatrist following a clinical interview. The scale of neuroticism was derived from the EPQ-R questionnaire. It consists of 24 items referring to a minor emotional distress. Neuroticism is an indicator of vulnerability to depression understood as a reaction to stressful life events.[16–18] We used the Polish adaptation of the EPQ-R authored by Brzozowski and Drwal.[19]

Depressive symptoms resulting from stressful life events are the effect of the interaction between the burden of stressful life events (SLE) and neuroticism level (N).[17] Depressive symptoms due to biological processes are not dependent on personality structure, and thus are not related to neuroticism level. We assumed that the severity of depressive symptoms is the sum of 2 components: the biological and psychological. Therefore, part of the variation of depressive symptoms related to neuroticism can be considered to be of psychogenic origin, while the remainder of the variance of depressive symptoms – independent of neuroticism – of a biological one.

The subjects had a diagnosed baseline mental condition, and their psychiatric history was evaluated by a psychiatrist. None of the participants were receiving any antidepressant or anxiety medications within 6 months prior to inclusion in the study. Antidepressant prescription during the study, if introduced, was dictated by the clinical judgment of the responsible physician and was not further controlled with study protocol. Nevertheless, concomitant medications including antidepressants were recorded by study personnel at each assessment point.

## Statistics

To analyze the relationships between the severity of depressive symptoms at different time points, the principal components factor analysis method with varimax rotation was used. This method detects the heterogeneity of the depressive process and enables its further investigation in the biological (IFN) and psychological (neuroticism, response to treatment effects) context.

The logistic regression/stepwise model method was used to determine predictors for major depressive disorder (MDD). Demographic, biological and psychiatric variables were included in the marginal results table using logistic regression forward stepwise procedure with sigma-restricted parameterization for categorical predictors. The dependent MDD variable was coded as MDD diagnosis for a good code and no MDD for a bad code. Variables which reached a statistically significant impact on the accuracy of a discriminative function were included in the analysis. All the statistical computations were performed with Dell STATISTICA v. 13.1 for Windows (Microsoft, Armonk, USA).

# Results

Out of 99 patients enrolled, 85 (85.9%) completed the treatment. The authors decided to perform analysis for MDD predictors in all patients except for 1 who had dropped out after 2 weeks of the treatment. The remainder stayed in treatment for at least 8 weeks which was considered sufficient for depressive symptoms to emerge. Table 1 shows pre-treatment demographic and clinical characteristics of the sample. None of the patients was diagnosed with MDD before the treatment but 14 patients (14.1%) reached MADRS score of 13 and over, and 4 patients reached MADRS score of 18 and above, which meant that some of them were actually depressed although they did not meet the formal DSM-IV-TR criteria for a major depressive episode.[20] The initial MADRS mean score in men (5.7) was slightly lower than in women (7.0), but the difference was not significant ($t = -1.38$, $p = 0.17$). Of the 98 patients 42 (42.9%) met criteria for MDD at any time within the 24 weeks of treatment, women more often (49.0%) than men (36.8%), but this difference was not statistically significant ($\chi^2 = 1.5$, $p = 0.22$); neither was the difference in mean maximal MADRS score between men (16.4) and women (18.5) significant ($t = -1.44$, $p = 0.15$).

The mean severity of depressive symptoms fluctuated in the course of treatment: it doubled during the first 4 weeks, then remained stable until week 24, and dropped slightly below the initial level 24 weeks after the treatment (Fig. 1).

In order to assess the structure of depressive symptoms and their association with the level of neuroticism (i.e., psychological factors), factor analysis was performed in 85 (85.9%) patients that completed the treatment. Eigenvalues of the first 3 factors were 5.62, 0.88 and 0.62, respectively. The remaining eigenvalues were below 0.35, so analysis for a model with 3 factors was carried out. Factor analysis (Table 2) separates MADRS scores during treatment (weeks 2, 4, 8, 12, and 24 – factor 1) from those after treatment (week 24 of the treatment and the 24$^{th}$ week after

Table 1. Comparison of pre-treatment demographic and clinical characteristics between patients with and without major depressive disorder during antiviral treatment

| Baseline | All (n = 98) | No depression (n = 56) | Major depressive episode (n = 42) |
|---|---|---|---|
| Male, n (%) | 49 (50) | 31 (63.2) | 18 (36.8) |
| Age [years], mean (SD)<br>range | 46.5 (11.1)<br>22–68 | 45.6 (11.0)<br>24–68 | 47.7 (11.2)<br>22–68 |
| Genotype, n (%)<br>1<br>2<br>3<br>4 | <br>67 (68.4)<br>0 (0)<br>24 (24.5)<br>7 (7.1) | <br>39 (69.7)<br>0 (0)<br>12 (21.4)<br>5 (8.9) | <br>28 (66.7)<br>0 (0)<br>12 (28.6)<br>2 (4.7) |
| MADRS, mean (SD)<br>range | 6.5 (4.9)<br>0–20 | 4.1 (3.1)<br>0–13 | 9.4 (4.9)<br>2–20 |
| Neuroticism, mean (SD)<br>range | 10.0 (5.7)<br>0–24 | 7.9 (4.8)<br>0–21 | 12.7 (5.7)<br>3–24 |
| Body weight, mean (SD)<br>range | 78.9 (16.1)<br>47–126 | 80.1 (16.2)<br>47–126 | 77.3 (17.3)<br>53–117 |
| Alcohol abuse, n (%) | 10 (10.2) | 8 (15.8) | 2 (4.8) |
| Substance abuse, n (%) | 13 (13.1) | 8 (14.2) | 5 (11.9) |
| Smoking, n (%) | 56 (57.1) | 34 (60.7) | 22 (52.4) |
| Psychiatric history, n (%) | 22 (22.5) | 5 (8.9) | 17 (40.5) |

SD – standard deviation.

Table 2. Results of factor analysis with the application of the 3-factor model

| Variables | Factor loadings | | |
|---|---|---|---|
| | factor 1 | factor 2 | factor 3 |
| MADRS 0 | 0.55 | 0.72 | 0.05 |
| MADRS 2 | 0.85 | 0.33 | 0.23 |
| MADRS 4 | 0.88 | 0.34 | 0.21 |
| MADRS 8 | 0.86 | 0.14 | 0.40 |
| MADRS 12 | 0.77 | 0.22 | 0.48 |
| MADRS 24 | 0.64 | 0.28 | 0.60 |
| MADRS 24a | 0.28 | 0.16 | 0.90 |
| Neuroticism 0 | 0.16 | 0.91 | 0.24 |
| Explained variance | 3.66 | 1.75 | 1.72 |
| % of total | 0.46 | 0.22 | 0.21 |

the treatment – factor 3) and before treatment (neuroticism and MADRS before treatment – factor 2). The association between the severity of depressive symptoms and neuroticism scores observed in this model points towards a psychogenic origin of these symptoms. This association is only significant before the treatment, so the rise of the severity of depressive symptoms during treatment is not related to initial neuroticism level, which is attributable to personality factors. The 1st and most significant factor is a direct consequence of the pro-inflammatory action of IFN-α. The 3rd factor included MADRS scores at the 24th week of treatment and 24th week after the treatment. It shows the difference in the depressive response

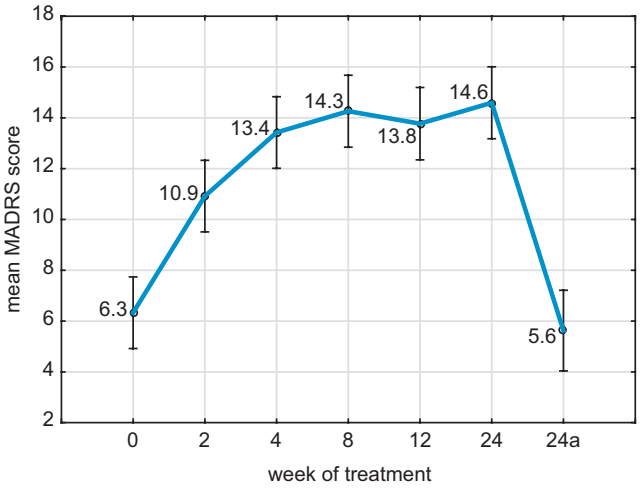


Fig. 1. The average MADRS scores during treatment of hepatitis C with IFN-α and ribavirin

when the treatment came to an end. The severity of depressive symptoms decreased to varying degrees in patients and hence the last 2 measurements were less correlated with other.

To identify MDD predictors, the procedure performed was stepwise logistic regression method with bidirectional elimination testing at each step for variables to be included or excluded. The model included the following variables: age, sex, weight, years of formal education, MADRS score before the treatment, the Eysenck's neuroticism scale score before the treatment, HCV genotype 1,4/2,3, grading, staging before treatment, tobacco smoking, alcohol abuse, psychoactive drugs abuse, family history of psychiatric disorders, and individual history of psychiatric disorders.

Stepwise logistic regression method returned 3 variables that were found to significantly affect the diagnosis of MDD, i.e., the initial MADRS score, the initial neuroticism score and individual history of psychiatric disorders. After these variables had been entered into the logistic regression model, only 2 proved to be statistically significant, i.e., the initial MADRS score and individual history of psychiatric disorders. The results of logistic regression calculations are presented in Table 3.

## Discussion

Shifts in the severity of depressive symptoms during antiviral treatment may appear as a continuous process. Mild symptoms which occur prior to treatment increase during treatment and decrease after treatment. In our cohort study, we managed to capture the diversity of this process. Factor analysis revealed 3 different components of depression changing with time. The main component (factor 1 in the 3-factor model) relates to IFN action. Depressive symptoms due to IFN administration persisted all through the treatment. The biological impact of IFN

**Table 3.** Results of logistic regression analysis

| Effect | Wald statistics | OR | OR lower CL (95%) | OR upper CL (95%) | p-value |
|---|---|---|---|---|---|
| Intercept | 8.5 | – | – | – | 0.0035 |
| MADRS 0 | 8.16 | 1.25 | 1.07 | 1.46 | 0.0043 |
| Neuroticism | 0.46 | 1.04 | 0.93 | 1.17 | 0.49 |
| History of psychiatric disorders | 6.18 | 4.79 | 1.40 | 16.47 | 0.013 |

OR – odds ratio; CL – confidence limit.

on mood did not depend on psychological factors related to neuroticism. On the other hand, the 2 remaining components, which appear to be much weaker, seemed to be related to psychological factors. The 3rd factor includes depressive symptoms at 2 timepoints, i.e., at the 24th week of the treatment and 24 weeks after the treatment. Therefore, it might be assumed that this component of depression is an effect of stress which in fact corresponds to worries over the outcome of the antiviral treatment – those patients who had recovered were less depressive. Towards the end of treatment, the patients might have already begun to suspect what the results of their treatment would be, and at the endpoint of this study (24 weeks after the treatment) some of them gained certainty – for example, after receiving information about the presence of HCV RNA in blood plasma at the end of treatment. Given that the second factor includes MADRS and neuroticism ratings before treatment, it reflects the psychological reaction to a stressful life situation that the liver disease certainly is. Patients suffering from different somatic diseases are known to have depressive symptoms correlating with the level of their neuroticism: the more severe illness, the stronger the correlation between neuroticism and depression.[21] Somatic illness as a stressful life situation may cause depressive symptoms in vulnerable individuals, and the neuroticism score is a measure of this vulnerability.[22] The results of this study did not confirm this relationship – the severity of depressive symptoms before treatment turned out to be a stronger predictor of MDD than the level of neuroticism. This relationship is quite common in other studies on depression in which the initial severity of depressive symptoms was a strong predictor of depressive symptomatology.[23–26] Depressive symptoms in general seem to be the main predictor of depressive disorders and broad psychiatric and somatic symptomatology. A similar situation has been replicated in the present study – depressive symptoms, despite their origin, foreshadowed MDD in patients treated for hepatitis C. Moreover, the diagnosis of depression during treatment depended solely on the initial severity of depressive symptoms.

In the 3-factor model, the pre-treatment depressive symptoms are almost evenly dispersed between factors 1 (biological) and 2 (psychological). The initial severity of symptoms seems to be a superposition of 2 depressive processes, i.e., the biological one which is related to HCV infection and evolves considerably during treatment, and the psychological one which is bound to neuroticism ratings and gradually loses its significance. Factor 1 related to the IFN-induced depressive symptoms (46% of variance) proved much stronger than factor 2 related to neuroticism (22% of variance); the point of reference for such a distinction is the overall severity of symptoms among which IFN-induced depression predominates. Seemingly, there exists a direct link between pre-treatment depression and IFN-induced depression. The inflammation processes, endogenic cytokines activity and direct effects of administered INF-α constitute the base for this interconnection.

Hepatitis C infection causes a chronic mild inflammation affecting both the liver and the brain, especially its white matter.[27,28] Hepatitis type C virus replicates in the mononuclear cells of the immune system and within the brain cells.[29,30] The inflammation is usually very mild and does not cause noticeable signs of encephalitis, but may still trigger a mild cognitive dysfunction in the form of attention and memory decline observable in the results of neuropsychological cognitive tests. Moreover, the inflammation may also be detected with the use of sophisticated neuroimaging methods such as the computed tomography diffusion tensor.[27,31] It is very likely that IFN-induced cytokines activity during the treatment is the continuation of inflammation-induced cytokines activity from before the treatment. Both may lead to MDD through illness behavior.

The results of the study showed that patients who previously suffered from mental disorders, as well as patients with a high level of depressive symptoms before treatment, are more susceptible to the development of a depressive disorder during treatment with IFN-α. Giving more attention to this group of patients will facilitate a rapid implementation of antidepressant treatment, which may prevent cases of treatment discontinuation.

The results of the study suggest that active assessment of the severity of depressive symptoms during control examinations (not only responding to patients' complaints) is necessary to capture the depressive disorder at the subclinical stage, in order to start a more effective treatment.

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# Pediatric unmanipulated haploidentical hematopoietic stem cell transplantation with post-transplant cyclophosphamide and reduced intensity, TBI-free conditioning regimens in salvage transplantations

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## Abstract

**Background.** Unmanipulated haploidentical stem cell transplantation (haploSCT) with post-transplant cyclophosphamide is an option for patients with advanced hematologic malignancies. It offers a platform both for non-major histocompatibility complex-restricted alloimmunity due to killer-like immunoglobulin receptor (KIR)-mediated mechanisms of natural killer lymphocyte regulation and for classical T-cell mediated antileukemic effects.

**Objectives.** The devastating long-term sequelae after total body irradiation (TBI) in children are encouraging omission of irradiation techniques in pediatric stem cell transplantations (SCT).

**Material and methods.** Five children, 4 with acute leukemia and 1 with hemophagocytic lymphohistiocytosis, aged from 1 to 10 years, underwent haploSCT with post-transplantation cyclophosphamide. In all children, the conditioning regimen consisted of chemotherapy without TBI. The graft material was bone marrow (BM) in 4 cases and peripheral blood stem cells in 1 case. Three out of 5 leukemic patients showed better KIR haplotype associated with augmented alloreactivity.

**Results.** Engraftment with complete donor chimerism was achieved in 4 patients, and 1 recipient died before leukocyte recovery. Three patients developed skin acute graft-versus-host-disease (aGvHD), 1 gut aGvHD and 1 liver aGvHD. In 2 recipients, chronic graft-versus-host-disease (cGvHD) was observed (1 limited and 1 extensive). The 4 engrafted patients were alive and in complete remission 3, 9, 32, and 36 months after transplantation. A T-cell count of 200 cells/uL was reached 90 days after haploSCT in all patients.

**Conclusions.** HaploSCT with TBI-free protocols can be a viable option for heavily pretreated patients with advanced malignancies.

**Key words:** hematopoietic stem cell transplantation, pediatric, haploidentical, post-transplant cyclophosphamide, TBI-free

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## Introduction

The limited availability of matched stem cell donors was, in the past, a vital problem for patients requiring allogeneic stem cell transplantation (SCT) because partially matched donors could not have been accepted due to the high risk of a fatal graft-versus-host-disease (GvHD) and high risk of rejection. The first successful attempt in SCT crossing the human leukocyte antigen barrier was achieved in 1983 with the development of T-lymphocyte depletion by differential agglutination with soybean agglutinin (SBA) and subsequent E-rosette depletion.[1] Haploidentical stem cell transplantation (haploSCT) had become a part of transplant methods in children since the introduction of immunomagnetic selection in 1996.[2] Techniques enabling physical removal of donor T-lymphocytes from the graft material were highly effective, but high cost and the requirements for an established graft processing lab were limiting interest in haploSCT despite further refinements. Another milestone in haploSCT was marked by successful application of post-transplantation GvHD prophylaxis with cyclophosphamide (PTCY),[3] which in turn started an avalanche of clinical trials with unmanipulated (T-cell replete) haploSCT. Cyclophosphamide administered from 48 h to 72 h after haploSCT eliminates T lymphocytes that have been activated by recognition of host minor histocompatibility antigens and prevents GvHD.[3] Still, despite successful engraftment after PTCY, the patients can develop acute GvHD (aGvHD) and chronic GvHD (cGvHD), although with incidence rates comparable with matched donor transplantations. The most popular conditioning regimen in PTCY is the total body irradiation (TBI)-containing Baltimore protocol.[4] Administration of TBI in children is restricted below the age of 3 and older children can require a TBI procedure under sedation. Due to the many limitations and unacceptable TBI-related late effects, radiotherapy-free conditioning regimens are being investigated in children.[5,6]

HaploSCT offers a platform both for non-major histocompatibility complex-restricted natural killer (NK)-lymphocyte alloimmunity and for classical T-cell mediated antileukemic effects. Killer-like immunoglobulin receptors (KIR) are involved in the activation or inhibition of NK-cells, and KIR genes constitute an inheritable haplotype on chromosome 19. Two basic KIR haplotypes can be found in humans: group A, containing predominantly inhibitory receptors (with the exception KIR2DS4), and group B haplotypes, with combinations of 1 or more B-specific genes: KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR2DL2, and KIR2DL5.[7,8]

## Material and methods

In the years 2014–2018, in the local Department of Pediatric Hematology and Oncology at Wroclaw Medical University, Poland, 5 children underwent haploSCT with PTCY GvHD prophylaxis. The medical procedures and this retrospective study were conducted according to local

**Table 1.** Patient characteristics and clinical data

| UPN | Sex/age [years] | Indication for haploSCT | Summary of previous therapy | Previous transplantations (conditioning) | Months from previous SCT | Source (donor) | HLA match | KIR B-content | Dose × $10^6$ $CD34^+$/kg |
|---|---|---|---|---|---|---|---|---|---|
| 1 | F/2 | infant B-cell precursor ALL, 4.CR | Interfant 99, IntreALL HR, 2 months of blinatumomab | I MUD (Bu+Flu+TT+ATG)<br>II MUD (Treo+Cy+VP16+Thymoglobulin) | 4.8 | PBPC (mother) | 04/06 | better | 6.95 |
| 2 | F/7 | B-cell precursor ALL, 3.CR | ALLIC 2009, IntreALL 2010 HR, 1 month of blinatumomab | MSD (TBI+VP16) | 25.3 | BM (father) | 03/06 | better | 3.37 |
| 3 | M/10 | B-cell precursor ALL/t-MDS-AML | ALLIC 2002, FLA cytoreduction in MDS | Alpha-Beta T-cell depleted HAPLO (Bu+Cy+Mel+ATG) | 0.9 | BM (father) | 06/10 | better | 2.79 |
| 4 | M/1 | familial HLH | HLH 2004 protocol | Alpha-Beta T-cell depleted HAPLO (Bu+Flu+TT+ATG) | 1.2 | BM (mother) | 05/10 | neutral | 7.18 |
| 5 | M/9 | B-cell precursor ALL, 3.CR | ALLIC 2009, IntreALL 2010 SR, IntreAll 2010 HR, 2 months of blinatumomab | MSD (TBI+VP16) | 16.5 | BM (father) | 06/10 | neutral | 5.6 |

haploSCT – haploidentical stem cell transplantation; AML – acute myeloid leukemia; ALL – acute lymphoblastic leukemia; BM – bone marrow; CR – complete remission; FLA – fludarabine + cytarabine; HLH – hemophagocytic lymphohistiocytosis; MSD – matched sibling donor; MUD – matched unrelated donor; PBPC – peripheral blood progenitor cells; t-MDS – treatment-induced myelodysplastic syndrome; UPN – unique patient number. Abbreviations for conditioning regimens: ATG/Thymo – antithymocyte globulin; Bu – busulfan; Cy – cyclophosphamide; Flu – fludarabine; Mel – melphalan; TBI – total body irradiation; Treo – treosulfan; TT – thiotepa; VP16 – etoposide.

Table 2. Patient post-transplant data

| UPN | Engraftment | Opportunistic infections in post-transplant period | Non-hematologic toxicities > grade 2 | GvHD acute or chronic | GvHD therapy | GvHD outcome | Follow-up |
|---|---|---|---|---|---|---|---|
| 1 | donor 100% | neutropenic fever | mucositis | aGvHD skin III, gut III Chronic GvHD extensive. | aGvHD: methylpredniosolone 2 mg/kg, tapered and discontinued at +3 months, etanercept discontinued at +2 months. cGvHD: treated with steroids and mycophenolate mofetil | CR | alive in 4th CR |
| 2 | donor 100% | neutropenic fever | mucositis | aGvHD skin III/ chronic limited | methylpredniosolone 1 mg/kg, tapered and discontinued at + 2 months | CR | alive in 3rd CR |
| 3 | not achieved | *Achromobacter xylosoxidans* sepsis | mucositis | not applicable | – | not applicable | died – non-relapse mortality |
| 4 | donor 100% | neutropenic fever, CMV replication, possible splenic candidiasis | mucositis | aGvHD skin II, liver II | methylpredniosolone 2 mg/kg, tapered | partial response, on therapy | alive in CR |
| 5 | donor 100% | neutropenic fever, CMV replication | mucositis | no | – | not applicable | alive in 3rd CR |

UPN – unique patient number; CR – complete remission; GvHD – graft-versus-host-disease; aGvHD – acute graft-versus-host-disease; cGvHD – chronic graft-versus-host-disease; CMV – cytomegalovirus.

regulations. Demographic and clinical data is presented in Table 1. The choice of haploidentical donor was justified by the unavailability of a matched donor at the time of planned transplantation. All recipients had experienced failures of 1 or 2 allogeneic transplantations, with at least 1 based on full intensity, myeloablative conditioning, and the time from previous transplantation in the range of 0.9–25.3 months. Two out of 3 patients with acute lymphoblastic leukemia relapsing after SCT (UPN 1 and 2) were conditioned with a modified FCM (fludarabine, cyclophosphamide and melphalan) protocol, and 1 (UPN 5) with treosulfan, fludarabine and thiotepa. Finally, 2 patients (UPN 3 and 4) underwent transplant after rejection of alpha-beta T-cell depleted haploidentical grafts. Patient UPN 3, who was aplastic at the time of salvage transplantation, was treated with treosulfan and fludarabine. Patient UPN 4 showed previous graft rejection with autologous recovery and underwent a second transplantation with treosulfan, fludarabine and cyclophosphamide. Melphalan in patients 1 and 2 and treosulfan in patients 3–5 were added due to contraindications for TBI therapy. The conditioning regimen details are shown in Table 2. The graft source was bone marrow (BM) in 4 cases and peripheral blood apheresis (PBPC) in 1 case. Post-transplant immunosuppression consisted of 2 cyclophosphamide doses on days +3 and +4 after transplantation. Cyclosporine and mycophenolate mofetil were used in all recipients from the 5th day after haploSCT.

## Infection prophylaxis

Herpes simplex virus and varicella-zoster virus prophylaxis included intravenous acyclovir (5–10 mg/kg or 250–500 mg/m$^2$ every 8 h) or, if tolerated, oral (10 mg/kg every 6 h). Prophylaxis with trimethoprim-sulfamethoxazole against *Pneumocystis jirovecii* pneumonia was started after neutrophil engraftment and continued for as long as immunosuppressive therapy was given. Granulocyte-colony stimulating factor at a dose of 5 µg/kg per day was started at day 5 after haploSCT until a neutrophil count of 500/µL was reached. The weekly monitoring of plasma for the Epstein–Barr virus, cytomegalovirus (CMV), adenovirus, BK virus, and urine for adenovirus and BK virus was performed.

## KIR B-content analysis

The DNA isolated from donor blood samples was used for KIR genotype testing with a KIR-typing kit (Miltenyi, Bergisch Gladbach, Germany). The genotyping results were used for calculation of B-content score groups classified as neutral/better/best according to relapse protection in T-cell replete unrelated donor transplantations for acute myeloid leukemia according to Cooley.[7]

Statistical analysis and data presentation were performed with the computer software GraphPad Prism (GraphPad Software, La Jolla, USA).

# Results

The neutrophil recovery was achieved between days 10 and 18 in 4 patients (Fig. 1A). One patient died before leukocyte recovery on day +10 due to gut associated *Achromobacter xylosoxidans* sepsis. Three engrafted patients

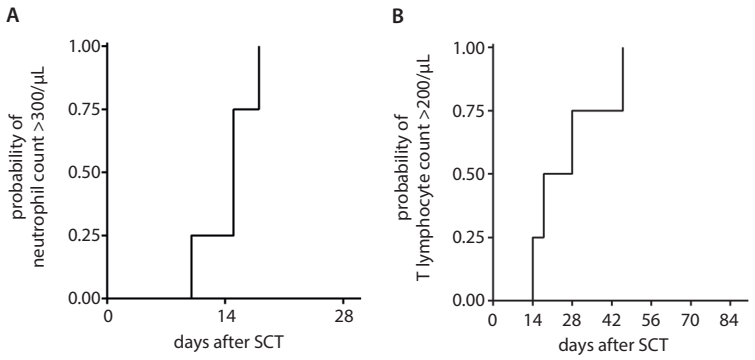


Fig. 1. Probability of absolute neutrophil recovery >500/µL (A) and T-lymphocyte recovery >200/µL (B)

developed skin aGvHD (maximal stage III), 2 of them acute gut GvHD (both stage III) and 1 liver aGvHD (stage II). In 2 recipients, cGvHD was observed (1 limited, 1 extensive). All patients showed full donor chimerism from the time of engraftment. Immune recovery in all surviving patients occurred early, and in all patients, a complete T-lymphocyte count of 200 cells/µL was reached before day +60 after transplantation (Fig. 1B). During the transplant period, CMV replication was diagnosed and treated in 1 patient, and asymptomatic BK virus viruria without signs of hemorrhagic cystitis was observed in 2 patients. None of the patients developed EBV- or adenovirus-related diseases. Immunosuppression was discontinued in UPN 2 6 months after haploSCT. At the time of this analysis, all 4 engrafted patients were alive, without significant comorbidities and in complete remission with normal blood results 3, 9, 32, and 36 months after transplantation.

## Discussion

Although at the moment of preparing this manuscript, the number of peer-reviewed papers on haploSCT with PTCY is over 400, the number of pediatric reports roughly constitutes 1/10 of the whole number and a TBI-free strategy is published only in single papers. The advantage of haploSCT with PTCY lies in low cost, relatively low technical difficulty, easy access to haploidentical donors within the patient's family, and with transplant outcomes similar to matched unrelated donor transplantations.[9,10] The next advance in haploSCT with PTCY is expected to be in the development of TBI-free conditioning, which can overcome the limited access to expensive and time-consuming techniques, especially in developing countries.

The strategy of PTCY GvHD prophylaxis can be combined with standard conditioning in different diseases, like in sickle cell disease, which was reported by Pawlowska.[11] Llosa et al. included melphalan in the chemotherapy backbone, although without elimination of low-dose TBI.[12] Similarly, Shima reported the combination of TBI with melphalan.[13] The usage of TBI as a part of haploSCT with a PTCY regimen is continued even in patients with Fanconi anemia[14,15] or in combination with busulfan.[16] Some reports in adults have investigated the possibility of a TBI-free conditioning regimen by replacing it with thiotepa and melphalan, but the trial was complicated by limited access to thiotepa and the group reversed to TBI.[17] The largest up-to-date pediatric study summarizing an Italian experience with 33 children transplanted with PTCY reported a subgroup of 10 patients treated with a myeloablative, busulfan-fludarabine-thiotepa regimen, and 2 with remote ischemic conditioning protocols, although the paper mentions some not-haploidentical transplantations, too.[18] The TBI-free regimens can be based on busulfan containing regimens, although at risk of hepatic venoocclusive disease and might not be well-tolerated in heavily pretreated patients. A number of successful haploSCT

Table 3. Conditioning regimen details

| Day before/after haploSCT | | −6 | −5 | −4 | −3 | −2 | −1 | 0 | +3 | +4 |
|---|---|---|---|---|---|---|---|---|---|---|
| UPN1, UPN2 | melphalan 4 mg/kg | | | | | | • | haploSCT | | |
| UPN3 | treosulfan 10 g/m$^2$ | | | • | • | • | | | | |
| UPN4 | treosulfan 10 g/m$^2$ | • | • | | | | | | | |
| UPN5 | treosulfan 14 g/m$^2$ | • | • | • | | | | | | |
| | thiotepa 2 × 5 mg/kg | | | | | • | | | | |
| Common backbone | cyclophosphamide 14.5 mg/kg (except for UPN5) | • | • | | | | | | | |
| | fludarabine 30 mg/m$^2$ | • | • | • | • | • | | | | |
| | cyclophosphamide 50 mg/kg | | | | | | | | • | • |

HaploSCT – haploidentical stem cell transplantation; UPN – unique patient number.

procedures with busulfan-fludarabin regimen, PTCY and antithymocyte globulin (ATG) have been reported in upfront transplantations in primary immunodeficiencies,[19,20] and by Bahr in malignant infantile osteopetrosis.[21]

Our paper shows experience with TBI-free preparative regimens in heavily pretreated patients, who were spared from toxic and myeloablative protocols, which can help in establishing new transplant standards. The conditioning regimens were given as a part of salvage therapies for highly resistant disease in heavily pretreated patients or after rejection of graft from the same donor. The FCM protocol presented in our series of patients was well-tolerated and proved its potential for full donor engraftment.

The choice of drugs in reduced intensity conditioning has not been established in the pediatric population. Previously-reported experiences with treosulfan-based transplantations in children were an encouraging alternative conditioning regimen in patients who rejected the first transplantation.[6] The immunosuppressive properties of treosulfan against antigen-presenting cells and T-lymphocytes supported its administration in children that had recently experienced graft rejection from the same donor.[22] Low-intensity conditioning with the treosulfan-fludarabine combination was successful in a patient with chronic granulomatous disease and autologous recovery.[23] Combination with treosulfan in a patient who had rejected the graft but not recovered autologously (UPN 4) resulted in significant mucositis with *Achromobacter xylosoxidans* sepsis and death, which discourages treosulfan-based conditioning in children with mucositis and aplastic BM.

Another crucial point of the procedure is related to the donor's choice. An analysis shown by Berger et al. identified a superior outcome in a case of using the mother as a donor due to significantly lower relapse incidence.[18] The explanation of this fact may be sensitization during the pregnancy by the father's haplotype, and in consequence building an antitumor reaction. The expected alloreactivity can be used to improve the donor selection algorithm. In our series, we tested the KIR haplotype and KIR-ligand mismatch, which could have helped in choosing a better donor. Retrospective analysis in 3 out of the 5 leukemic patients showed better KIR haplotype, which may be partially responsible for the lack of acute lymphoblastic leukemia relapses, as was shown in haploSCT with T-cell depletion.[24] The alloreactivity and anti-leukemic effects after haploSCT with PTCY are exerted through NK-cell activity affected by better KIR haplotype and KIR receptor-ligand mismatch, which should be considered during the donor selection process.[25,26] The effect of infused NK cells is diminished by the elimination of NK cells with cyclophosphamide infusion.[27]

Another issue observed in our patients was a high incidence of GvHD, although only 1 patient transplanted with PBPC developed cGvHD. Most haploSCTs with PTCY are carried up with BM as a source of stem cells. Transplantation with PBPC and PTCY is reported to show a high incidence of aGvHD and cGvHD (45% and 53%, respectively).[28] This report is particularly worth mentioning due to the fact that all children, even at the age of 1 year, underwent TBI. In the literature, the preference of PBPC over BM as a graft source is associated with an increased risk of aGvHD incidence (stage II–IV: HR-2.1; $p < 0.001$ and stage III–IV: HR-3.8; $p < 0.001$) after haploSCT with PTCY, as shown by Ruggeri et al.[29] In 1 pediatric series, after PBPC, an excessive 80% incidence of aGvHD was reported.[30] Although the BM is proven to reduce the risk of aGvHD without affecting non-relapse mortality, at the same time the risk of malignancy relapse is increased (HR-1.49; $p = 0.009$).[31] In our series, BM was preferred (4 of 5 cases), and in the remaining case, PBPC was selected due to the advanced disease and need for maximal immunotherapeutic effect. Still, the risk of GvHD is not always warranted and both graft rejection and GvHD after haploSCT with PTCY can be reduced by the addition of serotherapy, as reported by Wiebking et al.[32] The group reported 3 haploSCT in sickle-cell disease, with administration of a treosulfan, fludarabine and thiotepa protocol with pre- and post-transplant cyclophosphamide and low doses of MabCampath, which resulted in full donor engraftment without GvHD incidence. Another strategy has been tried with the addition of ATG, that can decrease the GvHD incidence to less than 20%.[33] Thus, serotherapy in haploSCT with PTCY can be recommended in patients with non-malignant disorders, who do not benefit from a T-cell mediated graft-versus-leukemia effect. Reduction of graft rejection risk can be another advantage of serotherapy included in the conditioning backbone.

## Conclusions

Haploidentical stem cell transplantation can be a viable option for patients with advanced malignancies, who relapse after transplantation and are heavily pretreated, but due to limited experience, further trials are required. Alternative, reduced-intensity TBI-free protocols can be used as conditioning in haploSCT with PTCY, especially in pediatric patients.

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# Effectiveness of subcutaneously administered methotrexate in patients with rheumatoid arthritis

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## Abstract

**Background.** Subcutaneous methotrexate (sMTX) administration is considered more effective than the oral route due to better bioavailability and a lower rate of adverse drug reactions (ADRs); however, clinical data supporting this hypothesis is scarce.

**Objectives.** The aim of the study was to evaluate the efficacy and tolerability of sMTX in patients with active rheumatoid arthritis (RA), including a subset classified as an early stage of RA.

**Material and methods.** A post-marketing, multicenter, open-label, non-randomized, non-interventional study enrolled 771 adult patients with active RA treated with sMTX (Metex®) for 2–6 weeks. The evaluation of therapy effectiveness (DAS28-ESR or DAS28-CRP) and monitoring of ADRs was an element of routine patient management. Therapy effectiveness was scored as the achievement of remission or response (according to European League Against Rheumatism (EULAR)).

**Results.** Among 761 (98.7%) patients that continued sMTX (after 25–31 weeks), clinical response was achieved by 69.5%, remission by 19.2% and low disease activity by 34.2%. Patients aged >60 years were less likely to achieve both remission (odds ratio (OR) = 0.61 (95% confidence interval (95% CI) = 0.39–0.93)) and clinical response (OR = 0.82 (95% CI = 0.71–0.95)), while overweight/obese patients (OR = 1.11 (95% CI = 1.00–1.24)) and those with early RA had greater chance to reach a clinical response (OR = 1.18 (95% CI = 1.03–1.34)). There were 16 ADRs (no serious or severe). In addition, at least 2-fold increase in alanine transaminase (ALT) activity was noted in 10 patients (1.3%).

**Conclusions.** After 6-month therapy with sMTX, about 70% of patients with RA achieve a clinical response, and remission was observed in 20%. Younger age, overweight/obesity and an early stage of the disease are factors increasing therapy effectiveness; sMTX is well tolerated.

**Key words:** methotrexate, rheumatoid arthritis, tolerance, subcutaneous, response rate

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## Introduction

Rheumatoid arthritis (RA) is the most common form of inflammatory arthropathy and affects 1–2% of the population worldwide.[1] The disease may have a clinical course that is difficult to predict, and the vast majority of patients eventually develop the disease progression with bone erosions and structural damage to the joints followed by functional impairment and increased mortality. The management of RA is aimed to control disease activity and prevent irreversible joint damage. To achieve these goals, early and aggressive therapy is required. New therapies including anti-cytokine drugs, interleukin antagonists or B-cell-depleting agents have revolutionized the treatment of RA. However, therapy using conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) is still recognized as the first-line treatment that provides a satisfactory clinical response, especially when initiated early. The treatment of RA should be initiated with csDMARDs, a group of various chemical compounds with a not-fully-known mechanism of action, that possess the ability to target inflammation and reduce structural joint damage.

Among several csDMARDs, methotrexate (MTX; a folic acid antagonist) is still recognized as an anchor drug,[2] recommended by the European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) as the first-choice drug for the management of RA.[3,4] The recommendations point out that very early use of MTX results in higher clinical response ratio.[3,4] Moreover, the treatment outcome is related to the activity of the disease that patients experience throughout the period of therapy. That is why the substantial reduction of disease activity and maintenance of clinical remission are now targets of all strategies used in patients with RA. The philosophy of optimal therapy is to achieve remission or at least minimal disease activity, commonly known as "treat to target" (T2T) strategy, nowadays considered as a standard of care in daily clinical practice.[5]

Effective treatment should be initiated at the very early stages of the disease, otherwise known as the "window of opportunity", when therapeutic intervention may lead to halting disease progression or even long-lasting remission.[6]

Recognizing MTX as the first-line treatment for all patients with RA is based on the results of numerous clinical trials. However, it is still unknown whether the route of MTX administration (orally or parenterally) may affect the outcome, which is of most significance in early stages of RA when the window of opportunity is wide open. Taking into account the better bioavailability of MTX when given parenterally, and less adverse drug reactions (ADRs), the parenteral route seems to be a better therapeutic option. Unfortunately, such therapy is more expensive and may result in patient discomfort related to injections. That is why it is important to assess whether patients treated with subcutaneous MTX (sMTX) may experience some additional benefits overshadowing the higher cost of therapy and self-injection difficulties.

The aim of the study was to evaluate the efficacy and tolerability of sMTX (Metex®; Medac Gesellschaft für klinische Spezialpräparate GmbH, Wedel, Germany) in patients with active RA, including a subset of those with an early stage of the disease.

## Material and methods

A post-marketing, multicenter, open-label, non-randomized, non-interventional study was conducted by 98 rheumatologists with the participation of 771 adult patients diagnosed with active RA on the basis of EULAR criteria.[7]

The only inclusion criterion was therapy with sMTX (Metex®) for 2–6 weeks before enrollment. In line with the Summary Product Characteristics, patients with hypersensitivity to MTX or any of the excipients contained in the Metex® formulation, liver failure, alcohol abuse, severe renal impairment (creatinine clearance below 20 mL/min), recognized blood dyscrasias (bone marrow hypoplasia, leukopenia, thrombocytopenia, or clinically significant anemia), interstitial lung disease, severe acute or chronic infections (tuberculosis, HIV or other immunodeficiency), ulceration of the oral mucosa, and the diagnosis of active gastric ulcer or duodenal ulcer, simultaneous vaccination with live vaccines, as well as pregnant and breastfeeding women, were excluded from the study.

The study was designed as a post-authorization efficacy study (PAES) (Register: EUPAS18973), in line with Article 1(15) of Directive 2014/357/EC, as a study related to an authorized medicinal product and conducted within an authorized therapeutic indication aim of complementing available efficacy data in the light of well-reasoned scientific uncertainties on aspects of the evidence of benefits that is to be, or can only be, addressed post-authorization. According to Polish law, PAES studies are not medical experiments and do not require either Bioethical Committee approval or the need to obtain informed consent from the patients for inclusion. As such, neither ethical approval nor informed consent from patients for inclusion into this study was sought.

The evaluation of therapy effectiveness and monitoring of ADRs was an element of routine patient management by the rheumatologists. The study methodology included a collection of effectiveness and safety data during 3 consecutive visits performed in about 12-week intervals, that is, the routine clinical check-ups during therapy. The data was entered into a study questionnaire completed within the 3 subsequent control visits between May 2015 and November 2016.

### Monitoring of subcutaneous methotrexate therapy and its effectiveness

Collection of data included: laboratory results (total blood count, erythrocyte sedimentation rate (ESR),

C-reactive protein (CRP), aspartate transaminase (AST), alanine transaminase (ALT), and creatinine – if performed); an assessment of therapy effectiveness with Metex® including Disease Activity Score with 28-joint counts (DAS28), based on ESR (DAS28-ESR) or CRP (DAS28-CRP), on center discretion, performed during all 3 visits; compliance with a Medication Adherence Questionnaire (MAQ); and reported ADRs.

## Data analysis

Using body mass and height, body mass index (BMI) was calculated. Normal weight, overweight and obesity were defined according to the World Health Organization (WHO) criteria (BMI values of 18.5–24.9 kg/m$^2$, 25–29.9 kg/m$^2$ and ≥30 kg/m$^2$, respectively). Rheumatoid arthritis activity was scored based on DAS28-ESR or DAS28-CRP criteria (on center discretion). DAS28 of less than 2.6 indicates remission, 2.6 and higher but less than 3.2 indicates low disease activity, 3.2 and higher but less than 5.1 indicates moderate disease activity, and higher than 5.1 indicates high disease activity.[8] The primary endpoint was the number of patients achieving remission. The secondary endpoint was response rates defined as a decrease in DAS28 > 1.2 with the shift to a lower class activity. Adherence to sMTX was scored on the basis of the MAQ as follows: adherent (≤2 points) or non-adherent (>2 points).

## Statistical analysis

Analyses were performed using STATISTICA v. 10.0 PL (StatSoft Inc., Tulsa, USA). All data is expressed as percentages or means with standard deviation (SD). The $\chi^2$ and $\chi^2$ for trend tests were used to compare qualitative data. Changes in quantitative data accross the visits were analyzed with a single-factor repeated-measures analysis of variance (ANOVA) for normally distributed or Friedman test for not normally distributed variables. Logistic regression analysis was used for calculation of odds ratios (OR) and 95% confidence intervals (CI). A p-value of less than 0.05 was considered statistically significant.

# Results

## Study group characteristics

The characteristics of the study group of 771 patients (mean age 56.7 ±11.3 years) diagnosed with RA is shown in Table 1. There was a high predominance of women (3.7:1). Patients with early RA (up to 6 months after the onset of symptoms) accounted for 9.5% of all studied patients.

Laboratory tests carried out before the 1st visit (treated with sMTX from 2 to 6 weeks in 70.4%) showed a more than 2-fold elevated activity of ALT and AST (>80 U/L) in 0.6% and 0.3% of patients, respectively. Leukopenia and thrombocytopenia occurred in 1.4% and 1.0% of patients, respectively. Anemia occurred in 4.8% and chronic kidney disease (estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m$^2$) was observed in 8.3% of patients.

At the 1st visit, 3.5% of patients already obtained remission, 10.4% presented low activity, 39.3% moderate, and 46.9% very active disease. There was no association between the disease activity and nutritional status (data not shown).

Table 1. Study group characteristics (n = 771)

| Variables | Value |
|---|---|
| Sex [n; %] | |
| men | 163; 21.1 |
| women | 608; 78.9 |
| Age [years] | 56.7 ±11.3 |
| BMI [kg/m$^2$] | |
| <18.5 kg/m$^2$ [n; %] | 3; 0.4 |
| 18.5–24.9 kg/m$^2$ [n; %] | 260; 33.7 |
| 25.0–29.9 kg/m$^2$ [n; %] | 356; 46.2 |
| ≥30.0 kg/m$^2$ [n; %] | 152; 19.7 |
| Red blood cells [10$^6$/μL] | 4.0 ±0.8 |
| Hematocrit [%] | 39.5 ±6.3 |
| Hemoglobin [g/dL] | 13.0 ±1.3 |
| <11.0 g/dL [n; %] | 37; 4.8 |
| White blood count [10$^3$/μL] | 7.4 ±2.3 |
| <4 × 10$^3$/ μL [n; %] | 11; 1.4 |
| Platelets [10$^3$/μL] | 282 ±80 |
| <150 × 10$^3$/μL [n; %] | 8; 1.0 |
| ALT [U/L] | 26.0 ±20.8 |
| >80 U/L [n; %] | 5; 0.6 |
| AST [U/L] | 24.0 ±15.9 |
| >80 U/L [n; %] | 2; 0.3 |
| Bilirubin [mg/dL] | 0.73 ±0.06 |
| Creatinine [mg/dL] | 0.84 ±0.01 |
| eGFR-MDRD [mL/min/1.73 m$^2$] | 83.9 ±21.7 |
| eGFR <60 mL/min/1.73 m$^2$ [n; %] | 64; 8.3 |
| Duration of RA [n; %] | |
| >6 months | 692; 89.7 |
| <6 months | 73; 9.5 |
| missing data | 6; 0.8 |

BMI – body mass index; ALT – alanine transaminase; AST – aspartate transaminase; eGFR-MDRD – estimated glomerular filtration rate – modification of diet in renal disease; RA – rheumatoid arthritis.

## Course of treatment with subcutaneous methotrexate

The mean weakly prescribed dose of sMTX at the 1st visit (18.0 ±4.9 mg) was increased during the subsequent 2 visits by 2.2 mg to 20.2 ±4.2 mg. There were only 2 dropouts, 1 patient did not accept the subcutaneous route of drug administration, and 6 patients discontinued therapy due to ADR occurrence. In 1 patient, therapy was discontinued due to detection of anti-HCV antibodies. According to the MAQ, the compliance to therapy was over 99% (Table 2).

**Table 2.** Effectiveness and compliance with subcutaneous methotrexate (MTX). Data shown as means ± standard deviation (SD) or medians with interquartile range (^)

| Variable | Visit I [n = 771] | Visit II [n = 770] | Visit III [n = 761] | ANOVA $\chi^2$ |
|---|---|---|---|---|
| Number of patients discontinuing therapy, n | – | 1 | 8 | – |
| Number of patients loss to follow-up, n | – | 0 | 2 | – |
| Duration of follow-up [days] | – | 77 ±20 | 160 ±30 | – |
| MTX dose [mg/week] | 18.4 ±4.5 | 19.6 ±4.2 | 20.2 ±4.3 | <0.001 |
| Disease activity measures:<br>tender joints count, n<br>swollen joints count, n<br>patient global assessment of disease activity<br>ESR [mm/h]^<br>CRP [mg/L]^ | <br>9.0 ±5.4<br>5.7 ±4.2<br>56 ±19<br>30 (20–42)<br>11.0 (6.1–18.5) | <br>5.9 ±3.9<br>3.8 ±3.1<br>39 ±17<br>20 (14–30)<br>6.1 (4.0–10.8) | <br>4.1 ±3.2<br>2.0 ±2.3<br>28 ±16<br>16 (11–21)<br>5.0 (3.0–7.0) | <br><0.001<br><0.001<br><0.001<br><0.001<br><0.001 |
| DAS28 ≤2.6 (remission, n (%)) | 27 (3.5) | 55 (7.3) | 148 (19.2) | <0.001* |
| DAS28 2.6–3.2 (low activity, n (%)) | 80 (10.4) | 144 (18.6) | 264 (34.20 | <0.001* |
| DAS28 3.2–5.1 (moderate activity, n (%)) | 303 (39.3) | 460 (59.7) | 311 (40.3) | <0.001 |
| DAS28 >5.1 (high activity, n (%)) | 361 (46.9) | 111 (14.4) | 48 (6.3) | <0.001* |
| Compliance (MAQ ≤ 2 points, n (%)) | 766 (99.3) | 766 (99.3) | 769 (99.7) | 0.97 |

*$\chi^2$ – trend; ANOVA – analysis of variance; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; DAS28 – Disease Activity Score 28; MAQ – Medical Adherence Questionnaire.

## Therapy effectiveness

Among the 761 patients continuing therapy with sMTX for 23 ±4 weeks (3rd visit), remission was obtained by 19.2% and 34.2% achieved a low activity of the disease (Table 2). Response (decrease in DAS28 > 1.2 with a shift to a lower-class activity) was achieved by 529 patients (69.5% of included). There were 22 patients with RA exacerbation (2.9% of included) during the therapy.

Older patients (aged >60 years) were less likely to achieve both remission or response to the therapy with sMTX (OR = 0.61 (95% CI = 0.39–0.93) and 0.82 (95% CI = 0.71–0.95), respectively), while overweight/obese patients and those with early RA had a greater chance to get a response (OR = 1.11 (95% CI = 1.00–1.24) and 1.18 (1.03–1.34), respectively) (Table 3).

**Table 3.** Factors affecting the effectiveness of therapy with subcutaneous methotrexate (sMTX) in patients with RA

| Factor | Variables | Patients that obtained remission during the therapy [%] | Patients that obtained response to the treatment [%] | The probability of obtaining remission during the therapy | | The probability of obtaining response to the treatment | |
|---|---|---|---|---|---|---|---|
| | | | | OR (95% CI) | p-value | OR (95% CI) | p-value |
| Sex | women | 19.0 | 69.6 | Ref | | Ref | |
| | men | 19.9 | 69.3 | 1.05 (0.73–1.49) | 0.80 | 0.99 (0.88–1.12) | 0.93 |
| Age | ≤40 years | 27.3 | 76.2 | Ref | | Ref | |
| | 41–60 years | 19.8 | 72.3 | 0.73 (0.49–1.09) | 0.12 | 0.95 (0.83–1.09) | 0.45 |
| | >60 years | 16.5 | 62.6 | 0.61 (0.39–0.93) | <0.05 | 0.82 (0.71–0.95) | <0.01 |
| Duration of disease | non-early RA | 18.1 | 68.2 | Ref | | Ref | |
| | early RA | 25.7 | 80.0 | 1.42 (0.93–2.19) | 0.11 | 1.18 (1.03–1.34) | <0.01 |
| Nutritional status | normal weight | 20.7 | 65.0 | Ref | | Ref | |
| | overweight/obesity | 18.6 | 72.6 | 0.90 (0.66–1.22) | 0.48 | 1.11 (1.00–1.24) | <0.05 |
| eGFR | ≥60 mL/min/1.73 m$^2$ | 18.2 | 69.8 | Ref. | | Ref. | |
| | <60 mL/min/1.73 m$^2$ | 12.0 | 68.0 | 0.72 (0.25–2.08) | 0.54 | 0.97 (0.74–1.27) | 0.83 |

OR – odds ratio; eGFR – estimated glomerular filtration rate.

## Adverse drug reactions

In the period from initiation of Metex® use up to the 1st visit and the subsequent 160 days of follow-up, ADRs were reported in 16 patients (2.1%): nausea (n = 8; 1.0%), headache (n = 3; 0.4%), alopecia (n = 3; 0.4%), loose stools (n = 1; 0.1%), and injection site reaction (n = 1; 0.1%). There were no serious or severe ADRs, or reactions not included in the summary of product characteristics (SPC). As a consequence of ADRs, therapy with Metex® was discontinued in 6 patients, and doses applied were reduced in 2 patients. An at least 2-fold increase in ALT activity was observed in 10 patients (1.3%).

# Discussion

The results of the study confirmed the role of MTX as an anchor drug for patients with RA and are in agreement to numerous previous studies showing the efficacy and good tolerability of this drug. Unfortunately, the majority of these studies utilized oral MTX in monotherapy or in combination with other synthetic DMARDs and biologic agents, so the final conclusion on the therapeutic potential of MTX may not be free of bias. However, when we extracted data on sMTX administration from completed trials, they were in high concordance with those obtained in our study. During the 25–31 weeks of active treatment (including a 2–8 week period of MTX treatment before formal inclusion into the study) complete remission was obtained in 19.2% patients. This is in agreement with the results of Bijlsma et al.,[9] where the remission ratio in the sMTX arm was as high as 20% in week 20 followed by 40% and 44% in week 40 and 104, respectively. Quite recently, Müller et. al.[10] published an analysis on the effectiveness of sMTX in patients with early RA from the St. Gallen RA cohort. This real-world analysis showed higher rates of remission (75.7%) and low disease activity (81.1%) than our study. However, some differences between these studies need to be addressed. The St. Gallen cohort comprised only patients with early RA (n = 70), in whom the remission rate is usually higher than in patients with long-lasting disease.[10] Moreover, as many as 37 of the patients from the analyzed cohort had been switched to biologic therapy due to the lack of MTX effectiveness.[10] After recalculation, the rate of clinical response to the achievement of low disease activity is about 40%, which is in agreement with our data.

The Treatment of Early Aggressive Rheumatoid Arthritis (TEAR) study was designed to assess the need for combination therapy in patients initially treated with MTX, and enrolled 775 patients with early RA.[11] In that study, 28% of patients treated with MTX had remission (DAS28 ≤ 2.6) at week 24, which is a slightly better result in comparison to our study, with remission attained in 19.2% of patients. The inclusion of selected patients with early RA may explain the higher rate of clinical response than in our study. However, when we analyzed the data from a subgroup of patients with early RA, the results from both studies showed a similar effectiveness of MTX (25.7% in early RA in our study). The better clinical response in early RA (defined in our study as clinically overt disease duration of less than 6 months) than in patients with long-term disease shown in our study (OR = 1.18 (95% CI = 1.03–1.34)) is similar to that reported in a Swedish cohort where remission was observed in 30% patients on MTX monotherapy.[12] This is an important finding, as a relatively high clinical response rate in early RA in MTX monotherapy contributes significantly to the substantial reduction of disease-associated joint damage and overall better prognosis in line with the philosophy of the "opportunity window". The efficacy of MTX in early RA can be seen in clinical trials with numerous biological agents where MTX served as the comparator. In one of them, the ACR 70 response index (roughly equal to remission in EULAR) in MTX alone reached the level of 20–25%,[13] which is again in perfect line with results from our study. The weak point of this comparison, however, is a lack of the specification of what route of administration was used in patients in these trials.

The high remission and low disease activity ratio observed in the current study (53.4% of patients) and the good clinical response observed in 69.5% of them may be explained by rapid tittering of MTX dose on the basis of clinical assessment during control visits, resulting in the increase in mean MTX dose by 2.2 mg per week. This indicates the need for detailed routine assessment of patients and for adjustment of the drug dose to obtain better clinical outcomes. In the present study, patients received relatively high MTX doses (20.2 ±4.2 mg). It is known that the bioavailability of greater MTX doses (over 15 mg) differs depending on the route of administration. The bioavailability of oral MTX administration reaches a plateau for doses over 15 mg, whereas the bioavailability of sMTX shows further linear absorption.[14] This easily translates to better clinical response and a lower DAS index in patients on sMTX treatment, as was shown recently in early RA.[15] The superiority of sMTX over oral MTX was shown by Braun et al.[16] in a phase IV randomized double-blinded trial. This corresponds to the satisfactory response in our study that has been attained in a relatively high percentage of patients. There is also growing evidence that subcutaneous administration is superior to oral MTX in regard to its effectiveness and toxicity.[15]

It should be stressed that 1/3 of patients on sMTX in our study did not experience any clinical response. Such patients may benefit from switching to biologic agents; however, before the initiation of such therapy, combined treatment, including glucocorticosteroids, and MTX dose adjustment, especially in the patients without risk factors for the progressive disease, should be considered.[3,7]

Our study demonstrates a high adherence to sMTX therapy, with only 1% non-compliant patients. Similar data comes from 2 retrospectives studies, where a higher rate

of therapy persistence in patients on sMTX than those receiving the drug orally has been observed.[15,17] There are several factors that may explain such good results. Firstly, sMTX is characterized by better tolerance and fewer ADRs. Secondly, the good clinical response and reduction of disease-associated symptoms observed in the majority of our patients contribute significantly to the very high continuation rate.

It is estimated that as many as 66% of patients on oral MTX experience adverse events, usually of low or moderate intensity, and in approx. ½ of the cases, the adverse events are related to the drug (ADRs). The most common ADRs reported in patients on MTX are gastrointestinal disturbances (nausea, vomiting, loss of appetite, and diarrhea).[18] Their occurrence precludes obtaining clinical benefits of MTX therapy. However, several reports emphasize that the frequency of ADRs may be reduced by choosing the subcutaneous route of administration.[19,20]

In addition, we showed a better response to MTX in overweight and obese patients. This is not surprising, as nutritional status somehow reflects the activity of the disease.[21] The rapid reduction of body weight in patients with RA is largely due to the release of pro-inflammatory cytokines (mainly but not exclusively to tumor necrosis factor α (TNF-α)). Such an explanation of the relation between response to MTX and nutritional status does not seem to be fully justified, as we failed to find an association between RA activity and nutritional status assessed at the 1st visit.

The study showed the high efficacy and tolerance of sMTX in a real-world rheumatological practice and confirmed observations from heretofore completed controlled trials. The good efficacy of sMTX and low frequency of ADRs, which were only or mostly moderate and acceptable by the patients and did not cause therapy cessation in the study, seems to increase the adherence to the therapy by satisfied patients. During follow-up, including an earlier 2–8 week initial treatment period and subsequent 23 weeks of Metex® use, only 16 ADRs were reported. The most common ADR were: nausea (occurring in 1% of patients), alopecia (0.4%) and headache (0.4%). There were no ADRs not included in the SPC, nor were there serious or severe ADRs. As a consequence of ADRs, Metex® therapy was stopped in 6 patients, and in 2 patients the dose was reduced. Reported laboratory results show a more than 2-fold increase in ALT in 1.3% of patients only. However, the limited scope of the collected data does not allow a full interpretation of the results of the laboratory tests, and an explanation of the reasons for the increase in ALT and not reporting it as an ADR. In some cases, it may have been coexisting liver disease, concomitant use of statin or alcohol intake.

This study has some limitations related to the lack of data concerning therapy previously used, including other DMARDs and corticosteroids. The activity of RA was monitored with 2 DAS28 scoring systems with good clinical correlation, but not equivalence.[8]

# Conclusions

After 6-month therapy with sMTX, about 70% of patients with RA achieved a clinical response and remission was observed in 20%. Younger age, overweight/obesity and an early form of the disease are factors increasing therapy effectiveness. Subcutaneous MTX is well tolerated.

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# *NFE2L2* is associated with *NQO1* expression and low stage of hepatic fibrosis in patients with chronic hepatitis C

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## Abstract

**Background.** Oxidative stress is extremely important in the pathogenesis of chronic hepatitis C virus (HCV). In response to oxidative stress, adaptive antioxidant defenses are upregulated in the liver. The balance between antioxidant response and oxidative stress plays a key role in hepatic injury in HCV infection.

**Objectives.** The objective of this study was to assess the hepatic expression of the antioxidant genes *GFER* (growth factor erv1-like) and *NQO1* (NAD(P)H:quinone oxidoreductase-1) and the regulatory gene *NFE2L2* (nuclear factor erythroid 2-related factor-2) in liver biopsy specimens obtained from chronic HCV patients with regard to selected clinical parameters and histology, and to determine whether *GFER* and *NQO1* expression is dependent on *NFE2L2*.

**Material and methods.** The study group consisted of 42 patients with chronic HCV. Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the expression of antioxidant and regulatory genes in liver biopsy samples.

**Results.** Positive correlation was observed between the hepatic expression of *NFE2L2* and *NQO1* in the chronic HCV patients ($p < 0.0001$). The hepatic expression of *NFE2L2* was significantly lower in patients with advanced liver fibrosis ($p = 0.05$). However, there was no significant difference in the hepatic expression of *GFER* and *NQO1* in relation to the progression of liver steatosis, inflammation and fibrosis.

**Conclusions.** The hepatic expression of *NFE2L2* is associated with *NQO1* and low stage of hepatic fibrosis in patients infected with HCV.

**Key words:** hepatitis C, liver fibrosis, antioxidant genes, regulatory gene

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# Introduction

Despite increasing access to effective antiviral therapies, HCV infection remains an important epidemiological issue worldwide, with about 3 to 4 million cases reported each year.[1] Approximately 75–85% of HCV-infected patients develop chronic HCV infection, 20% of whom progress to cirrhosis.[2]

It is well known that oxidative stress plays a significant role in HCV pathogenesis.[3,4] Hepatitis C virus core proteins induce the mitochondrial production of reactive oxygen species (ROS), leading to the peroxidation of membrane lipids. In response to this oxidative stress, adaptive antioxidant defenses such as antioxidant enzymes are upregulated in the liver. The main regulator of this self-protective antioxidant mechanism is nuclear factor erythroid 2-related factor-2 (Nrf2), encoded by the *NFE2L2* gene, which activates the expression of antioxidant proteins such as glutathione perioxidase, NAD(P)H-quinone oxidoreductase-1, superoxide dismutase, catalase, glutathione S-transferases, glutamate-cysteine ligase, heme oxygenase-1, thioredoxin reductase-1, and augmenter of liver regeneration.[5] NQO1(NAD(P)H:quinone oxidoreductase-1) is a cytosolic flavoprotein encoded by the gene *NQO1* that inhibits oxidative stress by the detoxification of superoxide anions and the regeneration of reduced forms of protective endogenous antioxidant compounds.[6]

Augmenter of liver regeneration (ALR) is encoded by the growth factor erv1-like (*GFER*) gene and is responsible for liver regeneration through the upregulation of the mitogen-activated protein kinase pathway, as well as interleukin-6 (IL-6) and tumor necrosis factor-α.[7]

The aim of the present study is to assess the hepatic expression of the antioxidant genes *GFER* and *NQO1* and the regulatory gene *NFE2L2* in liver biopsy specimens obtained from patients with chronic HCV in relation to selected clinical parameters and histological factors, and to determine whether *GFER* and *NQO1* expression is dependent on *NFE2L2*.

# Material and methods

The study included 42 adult patients with chronic HCV. Inclusion criteria were as follows: 1. The presence of positive serum anti-HCV antibodies and detectable HCV viremia lasting more than 6 months; 2. HCV genotype 1. Exclusion criteria:

1. other systemic or inflammatory diseases;
2. other causes of liver disease;
3. cirrhosis (revealed by liver biopsy);
4. previous anti-HCV treatment;
5. treatment with other medications including antioxidant vitamin and diet supplements;
6. HIV/HBV confection;
7. pregnancy.

The study was approved by the ethical committee of the Faculty of Medicine, Medical University of Lodz, Poland, and informed written consent was obtained from every patient. Liver biopsies were performed as part of the routine standard of care for the subjects studied. The grade of inflammation and the state of fibrosis were determined according to the Batts and Ludwig scale. Steatosis was assessed with Bruntet and Kleiner's histological scoring. Hepatitis C virus RNA was measured using reverse transcription PCR (RT-PCR) with COBAS AMPLICOR HCV v. 2.0 (Roche Diagnostic Inc., Basel, Switzerland). Hepatitic C virus genotype was assessed using a hybridization method (InnoLipa HCV; Innogenetics, Ghent, Belgium).

## Statistical analysis and polymerase chain reaction

### Total RNA isolation

Total RNA was isolated using the mirVana™ miRNA isolation kit (Ambion® Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol.

### mRNA expression

Homo sapiens-specific TaqManGene Expression Assay (Applied Biosystems Inc., Foster City, USA) for *GFER*, *NQO1* and *NFE2L2* mRNA was used for gene expression assays. cDNA generation was performed using 250 ng of total RNA with High Capacity cDNA Reverse Transcription Kits according to the manufacturer's protocols (Applied Biosystems). Expression levels of mRNA were determined using beta-actin (ACTB) as an endogenous control.

### Real-time polymerase chain reaction analysis

TaqMan PCR assays were determined using Sequence Detection System v. 2.0 software (Thermo Fisher Scientific). Fold induction values were measured according to $2\Delta\Delta Ct$, where $\Delta Ct$ defines the differences in cycle threshold numbers between the target gene and endogenous control, and $\Delta\Delta Ct$ defines the relative change in these differences between study and control groups.

### Statistical analysis

The Mann–Whitney test was used to determine differences between the examined groups. Correlations between the hepatic expression of antioxidant genes *GFER* and *NQO1* and the regulatory gene *NFL2L2* were assessed with Spearman's rank correlation coefficient. Values of $p \leq 0.05$ were considered to be statistically significant.

# Results

## Study group

The study group consisted of 42 patients with chronic HCV infection: 11 women and 31 men. The median age of patients was 32 years (LQ 21 years–UQ 49 years). The median plasma HCV viral load was 4,145,000 IU/mL (LQ 388,000 IU/mL–UQ 6,890,000 IU/mL). The characteristics of the patients are presented in Table 1.

**Table 1.** Characteristics of the study group

| | HCV infection (n = 42) | | |
|---|---|---|---|
| | median | LQ–UQ | min–max |
| Age [years] | 32 | 21–49 | 18–67 |
| ALT U/L | 54 | 39–83 | 23–336 |
| HCV-RNA IU/mL | 4,145,000 | 388,000–6,890,000 | 118,000–118,000,000 |
| *GFER* | 1.46 | 0.98–2.05 | 0.06–3.98 |
| *NQO1* | 1.46 | 0.98–1.69 | 0.01–17.47 |
| *NFE2L2* | 2.30 | 1.50–3.83 | 0.21–12.33 |

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotranferase.

## Hepatic expression of *GFER*, *NQO1*, *NFE2L2*, and HCV viral load

The study group was divided into 2 subgroups: one with HCV-RNA > 600,000 IU/mL and the other with HCV-RNA < 600,000 IU/mL. No significant difference in the hepatic expression of *GFER*, *NQO1* and *NFE2L2* was observed between the 2 groups (Table 2).

## Hepatic expression of *GFER*, *NQO1* and *NFE2L2* in the context of inflammation, steatosis and fibrosis

Advanced fibrosis (S ≥2) was detected in 34 patients. No significant difference in the hepatic expression of *GFER* or *NQO1* was observed in relation to the progression of liver fibrosis. However, the hepatic expression of *NFE2L2* was significantly lower in patients with advanced liver fibrosis (p = 0.05) (Table 3). No relationships were found between the hepatic expression of *GFER*, *NQO1* or *NFE2L2* and the grade of inflammatory activity (Table 4). Again, the hepatic expression of *GFER*, *NQO1* or *NFE2L2* was not associated with the presence of liver steatosis (Table 5). However, older age was reported in patients with the higher grade of liver inflammation (p < 0.0001) and HCV-RNA > 600,000 IU/mL (p = 0.03) (Tables 2,4).

## Correlations between hepatic expression of *GFER*, *NQO1* and *NFE2L2*

A significant positive correlation was found between the hepatic expression of *NFE2L2* and *NQO1* in chronic HCV patients (p < 0.0001). However, the hepatic expression of *NFE2L2* was not associated with *GEFR* (Table 6).

**Table 2.** Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to HCV viral load

| | HCV-RNA < 600,000 IU/mL (n = 15) | | | HCV-RNA > 600,000 IU/mL (n = 27) | | | p-value |
|---|---|---|---|---|---|---|---|
| | median | min–max | LQ–UQ | median | min–max | LQ–UQ | |
| Age [years] | 26 | 18–59 | 21–48 | 37 | 20–67 | 29–56 | 0.03 |
| ALT U/L | 55 | 24–336 | 37–91 | 52 | 23–201 | 39–77 | >0.01 |
| *NFE2L2* | 2.42 | 9.21–12.32 | 1.72–4.40 | 2.01 | 0.22–6.03 | 1.00–3.50 | >0.01 |
| *GFER* | 1.45 | 0.09–17.47 | 0.97–2.57 | 1.46 | 0.68–2.01 | 0.05–2.55 | >0.01 |
| *NQO1* | 0.89 | 0.02–17.47 | 0.47–2.03 | 0.58 | 0.38–1.62 | 0.01–2.36 | >0.01 |

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotranferase.

**Table 3.** Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to liver fibrosis

| | Staging <2 (n = 8) | | | Staging ≥2 (n = 34) | | | p-value |
|---|---|---|---|---|---|---|---|
| | median | min–max | LQ–UQ | median | min–max | LQ–UQ | |
| Age [years] | 26 | 18–61 | 20–33 | 35 | 18–67 | 26–50 | >0.01 |
| ALT U/L | 54.5 | 24–336 | 42–83 | 41.5 | 23–95 | 25–79.5 | >0.01 |
| HCV viral load | 580,000 | 392,000–11,800,000 | 2671,000–7,535,000 | 3,040,000 | 118,000–9,780,000 | 381,000–6,390,000 | >0.01 |
| *NFE2L2* | 3.96 | 0.21–12.32 | 2.44–6.16 | 2.05 | 0.22–7.76 | 1.42–3.13 | =0.05 |
| *GFER* | 1.14 | 0.09–3.76 | 0.35–1.82 | 1.48 | 0.05–3.98 | 1.04–2.14 | >0.01 |
| *NQO1* | 0.79 | 0.24–9.32 | 0.02–17.47 | 0.77 | 0.01–5.48 | 0.44–1.68 | >0.01 |

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotranferase.

Table 4. Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to liver inflammation

| | Grading <2 (n = 30) | | | Grading ≥2 (n = 12) | | | p-value |
|---|---|---|---|---|---|---|---|
| | median | min–max | LQ–UQ | median | min–max | LQ–UQ | |
| Age [years] | 26 | 18–51 | 20–33 | 53 | 27–201 | 48.5–2.68 | <0.0001 |
| ALT U/L | 51 | 23–336 | 35–73 | 76 | 24–67 | 53–108 | >0.01 |
| HCV viral load | 4,760,000 | 118,000–11,800,000 | 452,000–7,200,000 | 809,000 | 221,000–9,780,000 | 4,522,000–4,895,000 | >0.01 |
| *GFER* | 1.50 | 0.06–3.76 | 0.97–2.14 | 1.26 | 0.07–3.98 | 0.89–1.96 | >0.01 |
| *NQO1* | 0.79 | 0.01–17.24 | 0.39–1.80 | 0.76 | 0.05–3.66 | 0.52–1.34 | >0.01 |
| *NFE2L2* | 2.51 | 0.21–12.32 | 1.72–4.40 | 1.84 | 0.22–6.03 | 1.13–2.68 | >0.01 |

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotranferase.

Table 5. Hepatic expression of *GFER*, *NQO1* and *NFE2L2* according to the presence of liver steatosis

| | Without steatosis (n = 30) | | | With steatosis (n = 12) | | | p-value |
|---|---|---|---|---|---|---|---|
| | median | min–max | LQ–UQ | median | min–max | LQ–UQ | |
| Age [years] | 27 | 18–67 | 21–48 | 38.5 | 18–59 | 30–52.5 | >0.01 |
| ALT U/L | 52 | 23–201 | 32–73 | 76 | 39–336 | 52–96 | 0.03 |
| HCV viral load | 4,370,000 | 118,000–11,800,000 | 392,000–6,390,000 | 2,930,000 | 221,000–8,750,000 | 383,499.5–7,085,000 | >0.01 |
| *GFER* | 1.45 | 0.06–3.98 | 0.90–1.96 | 1.46 | 0.68–3.32 | 1.15–2.30 | >0.01 |
| *NQO1* | 0.76 | 0.03–17.47 | 0.41–1.68 | 0.87 | 0.01–3.17 | 0.52–1.85 | >0.01 |
| *NFE2L2* | 2.26 | 0.21–12.32 | 1.75–4.16 | 2.34 | 0.80–6.03 | 1.46–3.21 | >0.01 |

LQ – lower quartile; UP – upper quartile; ALT – alanine aminotranferase.

Table 6. Correlations between hepatic expression of *GFER*, *NQO1* and the *NFE2L2* in patients with chronic HCV

| | *GFER* | | *NQO1* | |
|---|---|---|---|---|
| | Spearman's rank correlation ρ | p-value | Spearman's rank correlation ρ | p-value |
| *NFE2L2* | 0.22 | NS | 0.59 | <0.0001 |

NS – not significant.

## Discussion

Polymerase chain reaction proteins induce the production of more ROS in the liver than other hepatitis viruses.[8–10] As excessive production of ROS can result in hepatocyte damage, an adequate antioxidant response is extremely important. The transcription factor Nrf2 plays a central role in stimulating the expression of various antioxidant-associated genes. Numerous reports describe that the antioxidant response is disturbed in subjects with HCV infection.[11,12]

It is possible that HCV can directly affect regulatory genes responsible for the antioxidant response, such as *NFE2L2*. Carvajal-Yepes et al. indicate that HCV prevents the entry of Nrf2 in the nucleus by the delocalization of sMaf proteins, thereby inhibiting the induction of Nrf2/ARE-regulated genes.[13] Reduced levels of glutathione and other antioxidants have been reported in liver biopsies in chronic HCV.[14,15] Interestingly, these results correspond to those of an in vitro study conducted by Wen at al., which indicates the decreased induction of HMOX-1 in hepatocyte cell lines expressing the HCV core protein in response to oxidative stress.[12] Conversely, Inanov et al. demonstrated that HCV proteins activate the Nrf2/ARE pathway in HCVcc infected Huh7.5 cells and induce the transcription of antioxidant genes.[9] Our results confirm the involvement of Nrf2 in the regulation of the antioxidant defense system, in that a positive correlation was observed between *NFE2L2* and *NQO1* expression.

Interestingly, the literature presents several contradicting opinions on the role of antioxidant enzymes in HCV replication. Zhu et al. demonstrated that heme oxygenase-1 overexpression is associated with decreased HCV replication in vitro.[16] In contrast, Ghaziani et al. found the heme oxygenase-1 gene to be upregulated in Huh-7 cells expressing HCV proteins.[17] It is still not clear whether antioxidant enzymes directly affect HCV replication in vivo; however, our present findings indicate that the hepatic expression of *GFER, NQO1* and *NFE2L2* was not related to HCV viremia.

A number of studies have examined the role of the antioxidant response in hepatocyte injury.[18,19] In a study

conducted by Kumar et al., the decreased hepatic expression of ALR was found in alcohol-induced fibrosis in mice model.[19] Conversely, Cheng et al. reported the expression of *NQO1* to be increased in alcoholic cirrhosis.[20]

Our present findings did not indicate any association between hepatic expression of *GFER* and *NQO1* with the progression of liver inflammation and fibrosis in chronic HCV. However, higher hepatic expression of *NFE2L2* was observed in patients with a lower stage of fibrosis.

Interestingly, Nrf2 activation directly inhibits the functioning of TGF-β1 (transforming growth factor-β1) and subsequently suppresses the collagen-producing hepatic stellate cells in a liver fibrosis mouse model.[21] These findings are in accordance with Jiang et al., who reported stronger Nrf2 staining in HCV infected Huh 7.5.1 hepatocytes and in liver biopsy specimens obtained from chronic HCV patients with a low grade of inflammation and fibrosis.[22]

Moreover, experimental studies show that the decreased expression of ALR and Nfr2 can result in liver steatosis by lowering the activity of several enzymes important for fatty acid β-oxidation and the promoting factors that regulate adipocyte differentiation and lipogenesis.[19,23,24]

However, our findings do not confirm the influence of *NFE2L2* and *GFER* on the presence of liver steatosis in HCV infection in vivo. The pathogenesis of liver steatosis in HCV infection is complex. Not only the host but also viral mechanisms likely contribute to the development of hepatic steatosis in HCV infection.[25,26]

The limitation of this study is the lack of the assessment of oxidative stress markers in the liver. Therefore, further studies are needed to precisely clarify the simultaneous role of the oxidative stress and antioxidant response in pathogenesis of chronic HCV. In conclusion, the hepatic expression of *NFE2L2* is associated with *NQO1* and low stage of fibrosis in patients infected with HCV. However, the study subgroup with mild fibrosis was limited to 8 patients.

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# The influence of statin monotherapy and statin-ezetimibe combined therapy on FoxP3 and IL 10 mRNA expression in patients with coronary artery disease

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## Abstract

**Background.** FoxP3 is a marker of human T regulatory cells (Tregs), which are supposed to play an important role in the pathophysiology of atherosclerosis. Interleukin 10 (IL-10) is a cytokine with pleiotropic, immunoregulatory properties, produced mostly by Tregs and B regulatory cells. Due to their anti-inflammatory action, both Tregs and IL-10 are believed to inhibit plaque development and decrease atherosclerosis progression. The effect of hypolipidemic drugs – statins or ezetimibe – on FoxP3-positive Tregs and anti-inflammatory cytokines, such as IL-10, is still unclear.

**Objectives.** The objective of the study was to investigate the effects of 3 different therapies of equivalent hypolipidemic activity: atorvastatin, rosuvastatin, and combination therapy of atorvastatin and ezetimibe on FoxP3–Tregs transcription factor and IL-10 mRNA expression in peripheral blood mononuclear cells (PBMCs) from patients with stable coronary artery disease (CAD).

**Material and methods.** Sixty-five patients with diagnosed CAD participated in the study. They were randomly assigned to 3 therapeutic groups: atorvastatin at a dose of 40 mg/day (A40 group); rosuvastatin 20 mg/day (R20 group); and atorvastatin 10 mg/day combined with ezetimibe 10 mg/day (A10+E10 group). After 1 month and 6 months of therapy, the mRNA expression for FoxP3 and IL-10 in PBMCs was evaluated using real-time polymerase chain reaction (RT-PCR) and lipid parameters.

**Results.** An improvement in lipid parameters was observed in each of the groups studied; however, hypolipidemic treatment did not induce any change in FoxP3 and IL-10 mRNA expression. After 6 months, an increase in FoxP3 mRNA expression was noted in A40 group as compared to R20 group.

**Conclusions.** None of the therapies of equal hypolipidemic efficacy affected FoxP3 and IL-10 mRNA expression in patients with stable CAD.

**Key words:** statins, ezetimibe, FOXP3, IL-10, regulatory T cells

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## Introduction

Statins are well-established medications for both the primary and secondary prevention of cardiovascular disease. If a patient does not achieve low-density lipoprotein cholesterol (LDL-C) goal of <70 mg/dL during conventional statin therapy, 3 other therapeutic options are available: firstly, an increase in statin daily dose (however, the expected improvement is estimated at only 6–8%[1]); secondly, implementation of statin with more potent hypolipidemic features, such as rosuvastatin in a dose of 40 mg/day or atorvastatin in a dose of 80 mg/day; and finally, a combination of statin with ezetimibe-inhibitor of cholesterol absorption in the brush border of small intestine.[2] One should emphasize that in spite of many different statins available there is no consensus regarding which of the above therapeutic options is optimal. Not only equal hypolipidemic effect of these options, but also divergent pleiotropic and immunomodulatory properties should be considered as the reason of differences in their clinical efficacy. Cases concerning anti-inflammatory features of statins and their effect on the regulation of Th1/Th2 homeostasis are well-documented.

FoxP3 is a marker of human T regulatory cells (Tregs), which are supposed to play an important role in the pathophysiology of atherosclerosis not only by the inhibition of pro-atherogenic Th1 and Th17 lymphocyte-mediated immune mechanisms, but also by the activation and migration of dendritic cells towards the plaque, suppression of inflammatory macrophages and their conversion into foam cells, and finally, reduction in the activation of endothelial cells.[3]

Interleukin 10 (IL-10) is a cytokine with pleiotropic, immunoregulatory properties, produced mostly by Tregs and B regulatory cells.[4,5] Due to their anti-inflammatory properties, both Tregs and IL-10 are believed to inhibit the plaque development and decrease atherosclerosis progression.[6] The effect of 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase inhibitors on FoxP3-positive Tregs and anti-inflammatory cytokines, such as IL-10, is still unclear.[7–10]

Therefore, the aim of our study was to investigate the effects of 3 different therapies of equivalent hypolipidemic activity: atorvastatin, rosuvastatin, and combination therapy of atorvastatin and ezetimibe on both FoxP3, the Tregs transcription factor, and IL-10 mRNA expression in peripheral blood mononuclear cells (PBMCs) from patients undergoing the secondary prevention therapy. The second aim of the study was to assess the association of hypolipidemic activity of atorvastatin, rosuvastatin, and a combination of atorvastatin and ezetimibe with the mRNA expression of FoxP3 and IL-10 in PBMCs from these patients.

This project was approved by the Bioethics Committee of the Medical University of Lodz, Poland (approval No. RNN/846/12/KB).

## Material and methods

Sixty-one patients aged 44–76 years participated in the study. They had undergone a myocardial infarction and/or percutaneous coronary intervention (PCI) and/or coronary artery bypass grafting (CABG) in the period over 6 months, and despite ongoing hypolipidemic treatment (simvastatin 10–40 mg/day, lovastatin 10–40 mg/day, atorvastatin 10–30 mg/day, rosuvastatin 5–15 mg/day) did not achieve the level of LDL-C < 70 mg/dL.

Exclusion criteria were the following: type 1 and type 2 diabetes mellitus, chronic kidney disease at stage IV or V (estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 $m^2$), New York Heart Association (NYHA) class III–IV heart failure with ejection fraction (EF) <40%, severe hypertension, acute infection within the past 2 weeks, autoimmune and allergic diseases, thyroid diseases, liver diseases and impairment of hepatic functions at active stage or with persistent elevated serum aminotransferase activity (more than 3 times the upper normal limit of non-defined cause), myopathy, myalgia, the state after rhabdomyolysis, documented cases of cancer, administration of glucocorticoids and other immunomodulatory drugs, HIV infection, pregnancy and lactation, women of childbearing potential not using effective contraception, alcohol abuse, smoking during the last 6 months, hypersensitivity to statin and ezetimibe components, statin or ezetimibe intolerance, therapy with rosuvastatin at a dose ≥20 mg/day, atorvastatin ≥40 mg/day, or combination therapy with statin and ezetimibe prior to randomization.

Qualified patients were randomly assigned to 3 therapeutic groups. Twenty patients received atorvastatin 40 mg/day (A40 group); 21 patients – rosuvastatin 20 mg/day (R20 group); and 20 patients – combination therapy: atorvastatin 10 mg/day with ezetimibe 10 mg/day (A10+E10 group). The study material was collected from patients at 3 different timepoints of the treatment: before the treatment, and after 1 month and 6 months of the treatment.

Therapeutic groups were homogeneous. They did not differ in terms of lipid parameters, body mass index (BMI), liver enzymes, and glycemia. However, they differed in high-sensitivity C-reactive protein (hsCRP) – its highest significant values before the treatment were observed in A40 group (mean values did not exceed the upper limit) and lowest in R20 group (Table 1). No significant changes in the activity of liver enzymes or glycemia were observed in the therapeutic groups. There were no muscular complaints and no significant increase in creatine kinase (CK).

### Peripheral blood mononuclear cell isolation

Blood collected by venipuncture in sodium heparin vacuum tubes was diluted in phosphate-buffered saline (PBS) (1:3) (Biomed, Lublin, Poland) and centrifuged on Histopaque 1077 density gradient (Sigma-Aldrich, St. Louis, USA) at 800 g.

## mRNA extraction, complementary DNA (cDNA) preparation, RT-PCR

mRNA was isolated using a RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Potential genomic DNA contamination was removed with on-column DNase I digestion, and 10 µg of mRNA was reverse-transcribed with a High-Capacity cDNA Kit (Applied Biosystems, Foster City, USA). Polymerase chain reaction (PCR) was then carried out using the Applied Biosystems 9700HT Fast Real-Time PCR System (Applied Biosystems). The PCR mixture consisted of cDNA solution, SYBR-Green PCR Mastermix (Applied Biosystems) and both sense and antisense primers. The reaction was conducted as follows: 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Elongation factor-1α (EF1-α) was amplified as a housekeeping gene. FoxP3 and IL-10 mRNA expression was normalized to EF1-α using ΔΔCt calculation. The following primers were used:

FoxP3 forward: 5'-CGGACCATCTTCTGGATGAG-3', reverse: 5'-TTGTCGGATGATGCCACAG-3';
IL-10: forward: 5'-GTGATGCCCCAAGCTGAGA-3', reverse: 5'-CACGGCCTTGCTCTTGTTTT-3';
EF1-α: forward: 5'-CTGAACCATCCAGGCCAAAT-3', reverse: 5'-GCCGTGTGGCAATCCAAT-3'.

## Laboratory tests

Complete blood count was determined using a 5diff hematology analyzer (UniCel DxH 800 Coulter Cellular Analysis System, USA). Glucose level and lipid profile (LDL-C was determined with direct method) were evaluated with the enzymatic method. The creatinine level was determined using the compensated Jaffe colorimetric method. Estimated glomerular filtration rate (eGFR) was calculated from the Modification of Diet in Renal Disease (MDRD) equation. Alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using the kinetic method with nicotinamide adenine dinucleotide hydrogen (NADH) and Tris buffer (according to the International Federation od Clinical Chemistry and Laboratory Medicine (IFCC). Creatine kinase was determined with the N-Acetyl-Cysteine (NAC) method.

Table 1. Characteristics of the selected parameters of 3 therapeutic groups (A40, R20 and A10+E10) prior to the treatment

| Parameter | A40 | R20 | A10+E10 |
|---|---|---|---|
| Age [years] | 61.80 (±7.10) | 61.95 (±6.71) | 63.65 (±7.39) |
| Men/women, n | 18/2 | 12/9 | 16/4 |
| BMI [kg/m$^2$] | 26.66 (±2.60) | 26.41 (±2.25) | 25.76 (±2.18) |
| TC [mg/dL] | 185.45 (±28.21) | 181.62 (±38.20) | 183.40 (±36.62) |
| LDL-C [mg/dL] | 111.85 (±20.22) | 114.52 (±27.83) | 110.70 (±30.49) |
| HDL-C [mg/dL] | 52.70 (±12.42) | 54.77 (±16.69) | 53.49 (±9.32) |
| Non-HDL-C [mg/dL] | 132.75 (±28.1) | 126.85 (±32.96) | 129.92 (±38.26) |
| TG [mg/dL] | 130.95 (±55.40) | 134.43 (±57.19) | 131.80 (±57.77) |
| SBP [mm Hg] | 129.75 (±15.77) | 127.14 (±10.79) | 125.65 (±14.60) |
| DBP [mm Hg] | 78.25 (±10.92) | 80.71 (±6.76) | 74.35 (±7.17) |
| HR [beats/min] | 67.10 (±9.44) | 68.38 (±9.54) | 72.90 (±8.74) |
| AST [U/L] | 24.10 (±8.77) | 25.52 (±6.74) | 23.05 (±6.25) |
| ALT [U/L] | 26.95 (±15.63) | 25.60 (±10.56) | 28.63 (±10.52) |
| CK [IU/L] | 123.45 (±38.85) | 131.35 (±44.20) | 155.00 (±96.88) |
| hsCRP [mg/L] | 2.27 (±1.45)[a] | 1.21 (±0.91)[a] | 1.58 (±1.44) |
| Glucose [mg/dL] | 93.83 (±6.66) | 94.47 (±10.12) | 93.88 (±8.06) |
| WBC [× 10$^3$/µL] | 6.86 (±1.84) | 6.34 (±1.45) | 5.89 (±1.57) |
| RBC [× 10$^6$/µL] | 4.84 (±0.38) | 4.60 (±0.32) | 4.67 (±0.37) |
| Hb [g/dL] | 15.10 (±1.07) | 14.64 (±1.00) | 14.77 (±1.15) |
| Ht [%] | 45.15 (±3.02) | 43.86 (±2.76) | 44.54 (±3.32) |
| PLT [× 10$^3$/µL] | 181.58 (±36.86) | 199.10 (±43.10) | 202.61 (±38.75) |
| Creat [mg/dL] | 0.96 (±0.12) | 0.79 (±0.16) | 0.92 (±0.16) |
| eGFR [mL/min/1.73 m$^2$] | 83.40 (±10.31) | 96.53 (±17.39) | 87.36 (±15.29) |

Data is presented as mean ± standard deviation (SD); p-value <0.05 is considered statistically significant; [a] $p < 0.05$; BMI – body mass index; TC – total cholesterol; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; Non-HDL-C – non-high-density lipoprotein cholesterol; TG – triglycerides; SBP – systolic blood pressure; DBP – diastolic blood pressure; HR – heart rate; ALT – alanine aminotransferase; AST – aspartate transaminase; CK – creatine kinase; hsCRP – high sensitivity C-reactive protein; WBC – white blood cells; RBC – red blood cells; Hb – hemoglobine; Ht – hematocrit; PLT – platelets; Creat – creatinine; eGFR – estimated glomerular filtration rate.

## Statistical analysis

The results obtained were subject to statistical analysis using STATISTICA PL v. 7.1. (StatSoft Polska, Kraków, Poland) and PQSTAT v. 1.6.2. (PQStat Software, Poznań, Poland). For comparison of quantitative variables measured at subsequent time intervals, the Student's t-test was used for repeated measurements if the condition of normality of distributions (comparing the results of 2 measurements) was fulfilled. The Wilcoxon signed-rank test (for 2 measurements) and the nonparametric Friedman's analysis with the Dunn–Bonferroni post-hoc test (for 3 measurements) were used to compare repeated results in the absence of normal distribution. For comparison of patient groups, when the assumption of normality of distributions was fulfilled, the analysis of variance was used (equality of variances was determined with Levene's test) and, in the case of rejecting the hypothesis of equality of variances, the modified Brown–Forsythe's test was applied. In the absence of normal distribution, the non-parametric Mann–Whitney U test (for 2 groups) and the Kruskal–Wallis test with the Dunn–Bonferroni POST-HOC test (for 3 groups) were used.

Correlations were tested with Spearman's tests. In all tests, a p-value lower than 0.05 was considered statistically significant.

# Results

## The effect of atorvastatin, rosuvastatin and combination therapy: atorvastatin with ezetimibe on the FoxP3 and IL-10 mRNA expression in PBMCs

The value 1 was regarded as the level of the FoxP3 and IL-10 mRNA expression before the treatment. Relative changes related to the value before the treatment, as well as after 1 month and 6 months of therapy, were defined. No statistically significant differences in FoxP3 and IL-10 mRNA (Fig. 1) expression induced by hypolipidemic therapy were detected in any study group and in any patient after 1 month and 6 months of the treatment. While comparing all the groups with each other at the same timepoints, the statistically significant differences were noted between groups A40, R20 and A10+E10 for FoxP3 mRNA expression after 6 months of therapy (p = 0.015). The comparison of the groups in pairs revealed statistically significant differences only between A40 group and R20 group. An increase in FoxP3 mRNA expression in A40 group after 6 months of therapy was significantly greater as compared to R20 group (p = 0.012); however, it did not significantly differ from FoxP3 mRNA expression in A10+E10 group (p = 0.466) (Fig. 1).

## The effect of atorvastatin, rosuvastatin and combination therapy

In each therapeutic group, a significant improvement in lipid parameters was observed. In A40 group, after 6 months of therapy, the level of LDL-C decreased by 21% (p = 0.006) and non-high-density lipoprotein cholesterol (non-HDL-C) by 22% (p = 0.003); in R20 group, a reduction by 24% (p < 0.001) and 21% (p = 0.001), respectively, was found; and in A10+E10 group, the LDL-C level decreased by 28% (p < 0.001) and non-HDL-C by 29% (p < 0.001) (Fig. 2). There were no statistically significant differences between the levels of lipids (TC, LDL-C, HDL-C, non-HDL-C, TG) in particular groups of patients at defined timepoints (prior to the treatment, after 1 month and 6 months of the treatment).

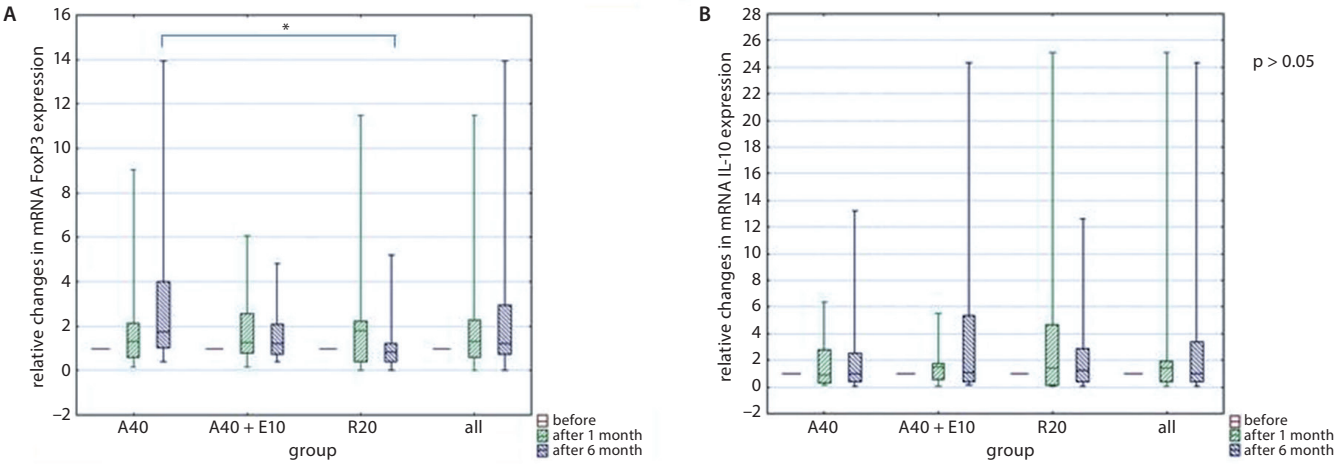


Fig. 1. A – relative changes in FoxP3 mRNA expression for particular groups (A40, A10+E10 and R20) and all patients after 1 and 6 months of therapy (p < 0.05); B – relative changes in IL-10 mRNA expression for particular groups (A40, A10+E10 and R20) and all patients after 1 and 6 months of therapy

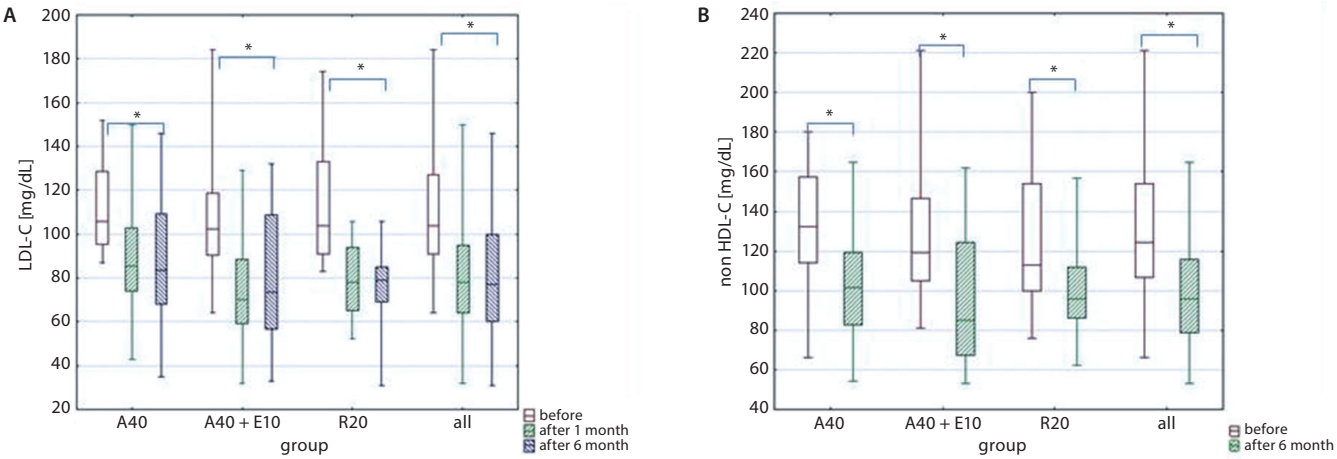


Fig. 2. A – comparison of the LDL-C level for particular study groups (A40, A10+E10 and R20) and in all patients after 1 and 6 months of therapy (p < 0.05). B – comparison of the non-HDL-C level for particular study groups (A40, A10+E10 and R20) and in all patients after 1 and 6 months of therapy (p < 0.05)

## FoxP3 and IL-10 mRNA expression in patients achieving and not achieving the expected LDL-C level goal of <70 mg/dL after 1 and 6 months of therapy

In A40 group after 1 month of therapy, the level of LDL-C <70 mg/dL was achieved by 4 of 20 patients, and after 6 months by 6 of 20 patients. In R20 group, 7 of 21 and 6 of 21 patients achieved the goal, respectively, and in A10+E10 group – 10 of 20 and 9 of 20 patients, respectively. Due to the small number of patients with LDL-C <70 mg/dL, the analysis was carried out in all therapeutic groups. Table 2 shows the changes in FoxP3 and IL-10 mRNA expression, taking into consideration the division of patients in relation to LDL-C level. There were no statistically significant changes in FoxP3 and IL-10 mRNA expression in patients with achieved and non-achieved LDL-C goal <70 mg/dL, both after 1 month and 6 months of hypolipidemic treatment ($p > 0.05$).

## Correlations between the decrease in the LDL-C level and changes in FoxP3 and IL-10 mRNA expression for particular groups and all patients after 1 month and 6 months of therapy

No correlations were observed between the reduction in the LDL-C level and changes in FoxP3 and IL-10 mRNA expression related to hypolipidemic therapy in particular groups as well as in all patients studied.

Positive correlations were recorded between changes in FoxP3 and IL-10 mRNA expression for R20 group ($r = 0.47$, $p = 0.031$) after 1 month of therapy and for A40 group ($r = 0.582$, $p = 0.007$) and R20 group ($r = 0.48$, $p = 0.026$) after 6 months. There were no such relationships observed for A10+E10 group. A significant positive correlation for all studied population occurred between those parameters after 1 month ($r = 0.395$, $p = 0.002$) and 6 months ($r = 0.269$, $p = 0.036$) (Table 3).

**Table 3.** The association between relative changes of FoxP3 mRNA and IL-10 mRNA expression for A40, A10+E10 and R20 groups and all studied population after 1 month and 6 months of therapy

| Period | Group | R Spearman's | p-value |
|---|---|---|---|
| After 1 month | A40 | 0.438 | 0.054 |
| | A10+E10 | 0.130 | 0.586 |
| | R20 | 0.471 | **0.031** |
| | all | 0.394 | **0.002** |
| After 6 months | A40 | 0.582 | **0.007** |
| | A10+E10 | –0.125 | 0.600 |
| | R20 | 0.485 | **0.026** |
| | all | 0.269 | **0.036** |

Spearman's R – Spearman's correlation; IL-10 – interleukin 10; statistically significant p-values are marked in bold.

# Discussion

Apart from hypolipidemic features, statins were shown to have anti-inflammatory properties, which may vary depending on the particular medicine. However, their effect on immunomodulatory mechanisms regarding Tregs and Treg-driven mechanisms is still not clear. Interestingly, decreased activity of Tregs and a lower production of IL-10 is believed to be engaged in the atherosclerosis development, leading to the domination of pro-inflammatory Th1-related immune mechanisms. Therefore, it is hypothesized that statins might exert an anti-atherosclerotic effect not only by lowering cholesterol levels, but also through the induction of Tregs-dependent immunosuppressive responses.

In our study, neither rosuvastatin nor atorvastatin influenced FoxP3 and IL-10 mRNA expression in PBMCs from patients with stable coronary artery disease (CAD) upon 1 month and 6 months of therapy. Similarly, ezetimibe added to atorvastatin did not trigger any additional effect. Our results are in line with the data indicating that systemic administration of atorvastatin and pravastatin to mice did not affect FoxP3 mRNA expression and population of Tregs.[8] In contrast, in Rodriguez-Perea et al. 1-month study treatment with atorvastatin (20 mg/day) or lovastatin (40 mg/day) increased both FoxP3-positive cell number and FoxP3 mRNA expression.[9] Moreover,

**Table 2.** Characteristics of relative changes in the FoxP3 and IL-10 mRNA expression of patients with LDL-C ≥ 70 mg/dL and LDL-C < 70 mg/dL after 1 and 6 months of therapy

| Time period | FoxP3 mRNA expression | | | IL-10 mRNA expression | | |
|---|---|---|---|---|---|---|
| | LDL-C < 70 | LDL-C ≥ 70 | p-value | LDL < 70 | LDL ≥ 70 | p-value |
| After 1 month | 1.49 (±1.32) | 2.05 (±2.35) | NS | 2.97 (±6.39) | 2.56 (±3.66) | NS |
| After 6 months | 1.42 (±1.32) | 2.24 (±2.49) | NS | 4.04 (±5.72) | 2.61 (±4.34) | NS |

Data is presented as mean (±SD); p-value <0.05 is considered statistically significant; LDL-C – low-density lipoprotein cholesterol; IL-10 – interleukin 10; NS – not significant.

Mausner-Fainberg et al. evidenced that after a 8-week course of simvastatin (20 mg/day) or pravastatin (10–40 mg/day) of hypercholesterolemic patients, Tregs number and FoxP3 mRNA expression were increased as compared to the timepoint before implementation of therapy.[8] One should consider that those patients were not receiving any statins prior to the inclusion into the study. Similarly, if compared to placebo, atorvastatin was also shown to increase the number of Tregs, FoxP3 mRNA expression and IL-10 serum levels in patients with a history of myocardial infarction.[10–12]

Why do our results differ from the clinical data? Firstly, in our study, only patients who were already receiving statins before being included to the study were analyzed, but still did not achieve proper LDL-C level (<70 mg/dL) and, therefore, they needed intensification of treatment. One may assume that previous therapy with statins might have already affected FoxP3 and IL-10 mRNA expression. Secondly, we could not establish placebo arm, as statin-based hypolipidemic therapy is obligatory for the secondary prevention of cardiovascular events according to all current guidelines.[2] This may be the cause why we did not observe any statistically significant effect of atorvastatin or rosuvastatin on immunological parameters in our study. Our data revealed higher FoxP3 mRNA expression in the group treated with atorvastatin for 6 months, as compared to the group receiving rosuvastatin. All this data taken together suggests that the effect of statins on FoxP3 and IL-10 mRNA expression and Tregs population may depend on the type and dose of statin as well as the period of the treatment. One should underline that there was a strong positive correlation between FoxP3 and IL-10 mRNA expression, which indicates that IL-10 is produced mainly by FoxP3-positive Tregs in PBMCs.

The effect of hypolipidemic therapy on FoxP3 and IL-10 mRNA expression was analyzed with regard to its influence on lowering of LDL-C in our study. After 6 months, LDL-C level significantly decreased by 21% in atorvastatin group, in rosuvastatin group by 24% and in patients receiving atorvastatin with ezetimibe by 28%. In spite of significant mean hypolipidemic effect in each group, there were patients who did not achieve cholesterol level below 70 mg/dL. However, there was no effect of cholesterol lowering therapy on FoxP3 and IL-10 mRNA expression, independently on the drugs received, in the group with LDL-C concentrations below 70 mg/dL achieved, and in the group with LDL-C above 70 mg/dL. Furthermore, we did not observe any correlation between relative changes in LDL-C concentrations and FoxP3 or IL-10 mRNA expression. Our data is consistent with studies of other authors, showing no association between the lipid profile with changes in FoxP3 and IL-10 mRNA expression in patients receiving statins.[11] This indicates that cholesterol levels are not associated with the mRNA expression of FoxP3 and IL-10.

In summary, none of the therapies of equal hypolipidemic efficacy, such as atorvastatin at a dose of 40 mg/day, rosuvastatin 20 mg/day, and atorvastatin 10 mg/day combined with ezetimibe 10 mg/day, affected FoxP3 and IL-10 mRNA expression in patients with history of CAD. However, therapy with atorvastatin seems to determine higher expression of these immunoregulatory parameters as compared to rosuvastatin. As most patients had already been on cholesterol-lowering therapy before inclusion to the study and they received average doses of statins during our study, one cannot exclude that maximal doses of statins used in lipid-lowering therapy (i.e., atorvastatin 80 mg, rosuvastatin 40 mg) might affect both FoxP3 and IL-10 mRNA expression in PBMCs of CAD patients. Therefore, further investigations are required.

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# Single-nucleotide polymorphisms of *APE1* associated with risk and prognosis of vitiligo in a Han Chinese population

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## Abstract

**Background.** The single-nucleotide polymorphisms (SNPs) of apurinic/apyrimidinicendonuclease 1 (APE1), which has been implicated in cancers and the DNA base excision repair (BER) process, have not been thoroughly investigated in association with the risks of oxidative stress-related vitiligo.

**Objectives.** The aim of this study is to investigate associations between APE1 single-nucleotide polymorphisms 141T >G and 1349T >G and risk and prognosis of vitiligo.

**Material and methods.** From June 2013 to June 2015, a total of 460 vitiligo patients were randomly recruited as a case group; 200 of these patients received narrow bound ultraviolet B (NB-UVB) treatment. Meanwhile, 460 healthy controls were included as a control group. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed to explore the distribution frequencies of genotypes.

**Results.** Significant differences were detected between the case group and the control group in the frequencies of the 141T >G and 1349T >G genotypes. At 141T >G, compared with patients carrying the TG + GG genotype, male patients carrying the TT genotype aged more than 20 years with active non-segmental vitiligo, without a family history of vitiligo or other autoimmune diseases, exhibited an increased risk of vitiligo. Binary logistic regression analysis demonstrated that the TT genotype at 141T >G and the non-TT genotype at 1349T >G were independent risk factors for vitiligo development. At 1349T >G, compared with patients carrying the TT genotype, male patients carrying the TG + GG genotype aged more than 20 years with active non-segmental vitiligo, without a family history of vitiligo or other autoimmune diseases, exhibited an increased risk of vitiligo. Moreover, patients carrying 141TG + GG or 1349 TT genotypes had better photochromic effects, lower cumulative radiation doses, shorter treatment times, and earlier first photochromic times.

**Key words:** vitiligo, APE1, 141T >G, Asp148Glu, risk of vitiligo

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# Background

Vitiligo is a depigmentation skin disorder characterized by the destruction of melanocytes, resulting in the presence of noticeable pale milk-white areas of depigmented skin.[1,2] The incidence of vitiligo varies from region to region, and it is estimated that the global prevalence is less than 1% in most populations.[3] Vitiligo may occur at any time, with the most prevalent onset of the disease estimated at around 24 years.[4] Among all the therapies for vitiligo, including topical steroids, calcineurin inhibitors and phototherapy, narrow band ultraviolet B (NB-UVB) phototherapy is found to be the most effective treatment.[5–7]

Patients with vitiligo have a very high risk of recurrences of segmental vitiligo.[8,9] Though not entirely explained, the pathogenesis of vitiligo is commonly believed to be related to autoimmune and genetic factors, and to oxidative stress.[10] Melanin synthesis is induced by oxidation reactions and superoxide anion and hydrogen peroxide ($H_2O_2$) generation.[11] However, over-accumulation of $H_2O_2$ could lead to oxidative damage and therefore bring about white spots and finally vitiligo.[12] In this context, oxidative stress regulation may be a useful lever in melanin synthesis. As the name suggests, DNA base excision repair (BER) is a process repairing base damage, including single-strand breaks, non-bulky adducts, oxidative DNA damage, alkylation adducts, and damage induced by ionizing radiation.[13] Evidence has shown that the induction of oxidative damage and expression of several BER genes are significantly increased in melanocytes.[14] The apurinic/apyrimidinic endonuclease (*APE1*) gene is the most important gene reported in close association with oxidative-stress-related disorders.[15,16] In addition, *APE1* is also known as redox effector factor 1 (Ref-1), and participates in the regulation of reoxidation and transcription.[17] Taking all this into account, the authors propose the hypothesis that *APE1* may be involved in the development of vitiligo.

The aim of the current study, which included 460 patients with vitiligo and 460 healthy controls, was to investigate any association between *APE1* SNPs 141T >G and 1349T >G and the risk of vitiligo. In addition, 200 patients who received NB-UVB treatments were followed up in order to observe the influence of *APE1* polymorphisms on the prognosis in vitiligo.

# Material and methods

## Ethical statement

The study design was reviewed and approved by the ethics committee of the Affiliated Yantai Yuhuangding Hospital (Yantai, China). All the subjects signed written informed consent to undergo diagnostic and therapeutic procedures at the time of hospitalization. All the procedures in this study were in compliance with the Declaration of Helsinki.[18]

## Subjects

From June 2013 to June 2015, a total of 460 patients diagnosed with vitiligo at the Affiliated Yantai Yuhuangding Hospital were randomly recruited as the case group in the study; among them, 200 received NB-UVB treatment. The diagnosis of vitiligo was confirmed based on three-dimensional computed tomography (CT) of the skin with commercially available reflectance confocal microscopy (Vivascope 1500; Lucid Inc., Rochester, USA) and the diagnostic criteria of vitiligo.[19] In patients with vitiligo, as presented in the CT image, the pigment rings at the dermo-epidermal junction totally disappeared in the lesion areas and appeared half-ring-shaped or fan-shaped in the surrounding non-lesion areas. The case group consisted of 231 males and 229 females with a mean age of 28.6 ±13.9 years. Only Han Chinese subjects were included in the current study to avoid genotype frequency variations among ethnic groups. The study's exclusion criteria included melanoma, dermatoma or other disease that may result in hypopigmentation or depigmentation of the skin and mucosa, including amelanotic nevus, pityriasis simplex and acquired pigment loss. The subjects' vitiligo was classified into stable stage and active stage, based on whether the leukoplakia had spread within the previous 6 months, whether the pigment loss was worsening and whether new leukoplakia was appearing. Based on the spreading of the leukoplakia, the vitiligo cases were grouped into non-segmental vitiligo and segmental vitiligo. Meanwhile, the healthy controls (n = 460, including 224 females and 236 males, with a mean age of 30.6 ±15.0) were enrolled from among patients undergoing physical examinations in the outpatient department. The exclusion criterion for the control group was a family history of vitiligo.

## Data collection

For each of the 200 vitiligo patients receiving NB-UVB treatment, the researchers recorded the clinical classifications, pigmented skin lesions, the course of the disease, cumulative exposure dosage, total treatment time, the time of initial repigmentation, and overall repigmentation. Pigmented skin lesions include lesions on the patients' faces, bodies, arms, legs, hands and/or feet. The course of the disease refers to the period from the initial depigmentation to the patient's referral to the hospital. Cumulative exposure dosages were calculated as exposure dose × exposure time. The total treatment time was recorded by month. The time of initial repigmentation is the time when repigmentation was first occurred in the skin lesions. Evaluation of the overall repigmentation was based

on the repigmentation area or the absence of leukoplakia, i.e., improvement (a decrease in or disappearance of leukoplakia, repigmentation/skin lesions ≥50%) or no improvement (reappearance of or increase in leukoplakia, repigmentation/skin lesions <50%).[20]

## *APE1* SNP detection

Fasting venous blood (5 mL) drawn from the forearm of each subject was placed in EDTA collecting tubes. The blood samples were centrifuged at 2,700 rpm for 10 min at room temperature to collect the cellular layer, then stored at −80°C. DNA extraction (DNA Extraction Kit; Beijing Biotech Ltd. Inc., Beijing, China) and content measurement using an ultraviolet spectrophotometer (Beckman Coulter Inc., Brea, USA) were performed. Polymerase chain reaction (PCR) amplification was conducted using synthesized primer sequences (Sangon Biotech Co. Ltd., Shanghai, China) (Table 1). The PCR reaction system includes 0.1 μg of genomic DNA, 10 mmol/L of Tris-HCl (pH = 8.0), 50 mmol/L of KCl, 2.5 mmol/L of $MgCl_2$, 200 μmol/L of dNTPs, 1U of TaqDNA polymerase, and 0.4 μmol/L of oligonucleotide primer. The amplification was conducted under the conditions of initial denaturation at 94°C for 3 min, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 55.7°C for 40 s and extension at 72°C for 40 s. The amplification was finished with final extension at 72°C for 10 min. Subsequently, 8.5 μL of PCR product was added with 5U of BgLII, 5U of BfaI and 1 μL of 10 × buffer solution (total 10 μL). Then, 10 μL of the reaction system was subjected to water bath digestion for 3 h, followed by electrophoresis with agarose gel containing 2% of ethidium bromide. The observation was performed under ultraviolet (UV) light and images were photographed for analysis.

Table 1. The primer sequences used for polymerase chain reaction amplification

| Position | Sequence |
|---|---|
| 141T > G | Forward: 5′-CTAACTGCCAGGGACGCGA-3′ |
| | Reverse: 5′-ACCACTGACTTAAGATTCTAACTA-3′ |
| 1349T > G | Forward: 5′- ACGGCATAGGTGAGACCCTA-3′ |
| | Reverse: 5′-GCTGTTACCAGCACAAACGA-3′ |

## Statistical analysis

Continuous data was presented as mean ± standard deviation (SD), while categorical data was expressed as percentages. Genotype frequencies between the case group and the control group were compared using the $\chi^2$ test. The $\chi^2$ goodness-of-fit test was employed to identify whether the genotype distributions fulfilled the Hardy–Weinberg equilibrium. The relative risk was presented as odds ratios (OR) with 95% confidence intervals (95% CI). Logistic regression analysis was conducted to screen out the risk factors for vitiligo development. P-values <0.05 were considered statistically significant. The data analysis was conducting using SPSS v. 21.0 software (IBM Corp., Armonk, USA).

# Results

## Clinico-pathological data

The demographic and clinical data between the case group and the control group are presented in Table 2. The $\chi^2$ test found no significant differences in terms of age, sex or smoking history between the case and control groups (all p > 0.05). Among the 460 vitiligo patients, 374 (81.3%) were diagnosed as being in the active stage and the remaining 86 patients (18.7%) were in the stable stage; 36 patients (7.8%) had segmental vitiligo, while 424 (92.2%) had vitiligo vulgaris; 72 patients (15.7%) had a family history of vitiligo and 388 patients (84.3%) did not; 10 patients (2.2%) also had an autoimmune disease, while 450 patients (97.8%) had no autoimmune disease.

Table 2. Comparisons of the clinical baseline information of the case group and control group

| Baseline characteristics | Case group (n = 460) | | Control group (n = 460) | | p-value |
|---|---|---|---|---|---|
| | n | % | n | % | |
| Age [years] | | | | | 0.37* |
| ≤20 | 150 | 32.6 | 164 | 35.7 | |
| >20 | 310 | 67.4 | 296 | 64.3 | |
| Sex | | | | | 0.32* |
| female | 229 | 49.8 | 224 | 48.7 | |
| male | 231 | 50.2 | 236 | 51.3 | |
| Smoking history | | | | | 0.84* |
| yes | 256 | 55.7 | 260 | 56.5 | |
| no | 204 | 44.3 | 200 | 43.5 | |
| Disease stage | | | | | |
| active stage | 374 | 81.3 | | | |
| stable stage | 86 | 18.7 | | | |
| Clinical phenotype | | | | | |
| segmental | 36 | 7.8 | | | |
| non-segmental | 424 | 92.2 | | | |
| Family history of vitiligo | | | | | |
| yes | 72 | 15.7 | | | |
| no | 388 | 84.3 | | | |
| Autoimmune diseases | | | | | |
| yes | 10 | 2.2 | | | |
| no | 450 | 97.8 | | | |

* two-sided $\chi^2$ test.

## *APE1* genotype analysis

*APE1* was located in chromosome14q11.2. *APE1* 1349T >G (rs1130409) was a mutation of T >G in the 5th exon, containing the 1349T >G TT genotype (238bp, 403bp), 1349T >G TG genotype (165bp, 238bp, 403bp) and 1349T >G GG genotype (165bp, 403bp), while 141T >G (rs1760944) was

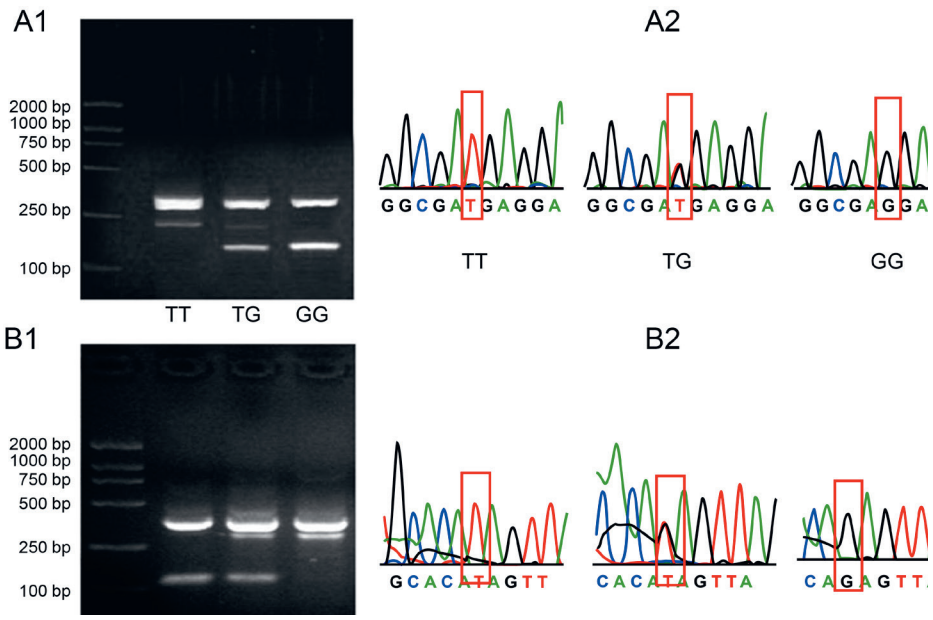


**Fig. 1.** The genotypes for apurinic/apyrimidinic endonuclease 1 (*APE1*)1349T >G and 141T >G (A – the agarose electrophoresis results for 1349T > G; B – the agarose electrophoresis results for 141T > G)

a mutation of T >G in the promoter region, including the TT genotype (116bp 443bp), GG genotype (327bp, 443bp) and TG genotype (116bp, 327bp, 443bp) (Fig. 1).

## Genotype frequencies of *APE1* 141T >G and 1349T >G

The distributions of *APE1* 141T >G and 1349T > G genotype frequency in both the vitiligo patients and the healthy controls fulfilled the Hardy–Weinberg equilibrium (all $p > 0.05$). Taking the TT genotype of 141T >G as a reference, the subjects with the GG genotype and TG + GG genotype had a decreased risk of vitiligo (both $p < 0.05$). When the TT genotype of 1349T >G was taken as a reference, the subjects with the GG genotype and the combined TG + GG genotype had an elevated risk of vitiligo (both $p < 0.05$) (Table 3).

## Associations between *APE1* SNPs and vitiligo phenotypes

Using the TT genotype as reference, the 141T >G, TG + GG genotype was associated with a decreased risk of vitiligo in the subgroups male ($p = 0.035$), more than 20 years

**Table 3.** Distribution of genotypes at 141 T > G and 1349T >G in the case group and control group

| Genotypes | Case group (n = 460) | | Control group (n = 460) | | p-value | Adjusted OR (95% CI) |
|---|---|---|---|---|---|---|
| | n | % | n | % | | |
| *APE1* 141 T >G | | | | | | |
| TT | 176 | 38.3 | 144 | 31.3 | | 1.00 (reference) |
| TG | 214 | 46.5 | 218 | 47.4 | 0.140 | 0.80 (0.60–1.07) |
| GG | 70 | 15.2 | 98 | 21.3 | 0.006 | 0.58 (0.40–0.85) |
| TG + GG | 284 | 61.7 | 316 | 68.7 | 0.032 | 0.74 (0.56–0.97) |
| Allele | | | | | | |
| T | 566 | 61.5 | 506 | 55 | | 1.00 (reference) |
| G | 354 | 38.5 | 414 | 45 | 0.005 | 0.76 (0.34–0.92) |
| *APE1* 1349 T >G | | | | | | |
| TT | 127 | 27.6 | 158 | 34.3 | | 1.00 (reference) |
| TG | 228 | 49.6 | 232 | 50.4 | 0.200 | 1.22 (0.91–1.65) |
| GG | 105 | 22.8 | 70 | 15.3 | 0.002 | 1.87 (1.27–2.74) |
| TG + GG | 333 | 72.4 | 302 | 65.7 | 0.032 | 1.37 (1.04–1.82) |
| Allele | | | | | | |
| T | 482 | 52.4 | 548 | 59.6 | | 1.00 (reference) |
| G | 438 | 47.6 | 372 | 40.4 | 0.002 | 1.34 (1.12–1.61) |

OR – odds ratios; 95% CI – 95% confidence intervals.

old (p = 0.019), non-segmental vitiligo (p = 0.008), active vitiligo (p < 0.001), no family history of vitiligo (p = 0.017), and no other autoimmune diseases (p = 0.049). When the 1349T >GTT genotype was used as the reference, an elevated risk of vitiligo associated with the combined 1349T >GTG + GG genotype was more obvious in the subgroups more than 20 years old (p = 0.045), male (p = 0.022), non-segmental vitiligo (p = 0.031), active stage (p < 0.001), and no family history of vitiligo (p = 0.031) (Table 4).

## Risk factors for vitiligo detected >G in the logistic regression analysis

With presence of vitiligo as the dependent variable and age, sex, smoking history, the TT genotype at 141T >G, a non-TT genotype at 141T >G, the TT genotype at 1349T >G, and a non-TT genotype at 1349T >G as independent variables, a binary logistic regression analysis was performed. As shown in Table 5, the TT genotype at 141T >G and a non-TT genotype at 1349T >G were independent risk factors for vitiligo development (both p < 0.05), while age, sex and smoking history had no significant association with vitiligo development (all p > 0.05).

## Associations between *APE1* SNPs and the prognosis of vitiligo patients receiving NB-UVB treatment

A total of 200 vitiligo patients received NB-UVB treatment, among whom 127 patients (63.5%) had depigmentation on their faces, 110 (55.0%) had depigmentation on their bodies, 82 patients (41.0%) had depigmentation on their arms and legs (apart from the hands and feet), and 77 patients (38.5%) had depigmentation on their hands and feet. Compared with subjects with the TT genotype of *APE1* 141T >G, patients with the TG + GG genotype had better repigmentation ($\chi^2$ = 6.453, p = 0.012), lower cumulative exposure dosages ($\chi^2$ = 7.227, p = 0.011), lower total treatment times ($\chi^2$ = 6.315, p = 0.015), and earlier initial repigmentation ($\chi^2$ = 10.250, p = 0.002). Compared

**Table 4.** *APE1* SNPs associated with clinical phenotypes of vitiligo

| Clinical phenotype | 141 T >G | Adjusted OR (95% CI) | 1349 T >G | Adjusted OR (95% CI) |
|---|---|---|---|---|
| | TT/TG + GG | | TT/TG + GG | |
| Age [years]<br>≤20<br>>20 | 176/284<br>69/81<br>107/203 | 0.62 (0.42–0.92) | 127/333<br>32/118<br>95/215 | 1.63 (1.03–2.58) |
| Sex<br>female<br>male | <br>99/130<br>77/154 | 0.66 (0.45–0.96) | <br>52/177<br>75/156 | 1.64 (1.08–2.48) |
| Smoking history<br>yes<br>no | <br>83/173<br>93/111 | | <br>63/193<br>64/140 | |
| Disease stage<br>active stage<br>stable stage | <br>160/214<br>16/70 | 0.37 (0.25–0.54) | <br>65/309<br>62/24 | 12.28 (7.14–21.11) |
| Clinical phenotype<br>segmental<br>non-segmental | <br>22/14<br>154/270 | 0.39 (0.19–0.78) | <br>4/32<br>123/301 | 3.27 (1.13–9.44) |
| Family history of vitiligo<br>yes<br>no | <br>37/35<br>139/249 | 0.53 (0.32–0.88) | <br>12/60<br>115/273 | 2.11 (1.09–4.06) |
| Autoimmune diseases<br>yes<br>no | <br>7/3<br>169/281 | 0.26 (0.07–1.01) | <br>1/9<br>126/324 | 3.50 (0.44–27.91) |

SNP – single-nucleotide polymorphism; OR – odds ratio; 95% CI: 95% confidence interval.

**Table 5.** Risk factors for vitiligo development screened out using logistic regression analysis

| Variables | B | S.E. | Wald | Sig. | Exp (B) | 95% CI for EXP(B) |
|---|---|---|---|---|---|---|
| 141T >G | –0.35 | 0.14 | 6.16 | 0.013 | 1.42 | 1.08–1.87 |
| 1349T >G | 0.36 | 0.15 | 6.17 | 0.013 | 0.70 | 0.53–0.93 |
| Age | –0.22 | 0.15 | 2.09 | 0.148 | 0.80 | 0.59–1.08 |
| Sex | 0.06 | 0.14 | 0.20 | 0.654 | 1.07 | 0.81–1.41 |
| Smoking history | 0.08 | 0.14 | 0.36 | 0.549 | 1.09 | 0.83–1.43 |

B – regression coefficient; SE – standard error of regression coefficient; Wald – test statistics; Sig. – significance; Exp (B) – index of the regression coefficients; CI – confidence interval.

Table 6. Association between *APE1* SNPs and the prognosis of vitiligo patients receiving NB-UVB treatment

| Variables | 141 T >G TT | 141 T >G TG + GG | $\chi^2$ | p-value | 1349T >G TT | 1349T >G TG + GG | $\chi^2$ | p-value |
|---|---|---|---|---|---|---|---|---|
| Re-pigmentation | | | 6.453 | 0.012 | | | 9.924 | 0.002 |
| re-pigmentation area ≤50% | 76 | 55 | | | 51 | 80 | | |
| re-pigmentation area >50% | 27 | 42 | | | 43 | 26 | | |
| Cumulative exposure dosage [J/cm$^2$] | | | 7.227 | 0.011 | | | 5.138 | 0.033 |
| ≤36 | 42 | 58 | | | 55 | 45 | | |
| >36 | 61 | 39 | | | 39 | 61 | | |
| Total treatment time [months] | | | 6.315 | 0.015 | | | 8.348 | 0.004 |
| ≤6 | 36 | 51 | | | 51 | 36 | | |
| >6 | 67 | 46 | | | 43 | 70 | | |
| Initial time of re-pigmentation [months] | | | 10.250 | 0.002 | | | 5.335 | 0.027 |
| ≤1 | 64 | 80 | | | 75 | 69 | | |
| >1 | 39 | 17 | | | 19 | 37 | | |

SNP – single-nucleotide polymorphism; NB-UVB – narrow band ultraviolet B.

with subjects with the combined TG + GG genotype of *APE1* 1349T >G, subjects with the 1349T >G TT genotype had better repigmentation ($\chi^2$ = 9.924, p = 0.002), lower cumulative exposure dosages ($\chi^2$ = 5.138, p = 0.033), reduced treatment times ($\chi^2$ = 8.348, p = 0.004) and earlier initial repigmentation ($\chi^2$ = 5.335, p = 0.027) (Table 6).

## Discussion

This study investigated associations of the SNPs 141T >G and 1349T >G in the *APE1* gene with the risk of vitiligo and its prognosis. The results showed that a mutation of the SNPs at141T >G was associated with a decreased risk of vitiligo, while a mutation of 1349T >G confers a highly increased risk of vitiligo. Moreover, the results also showed that the combined 141TG + GG and TT genotypes at 1349T >G were associated with a better prognosis in vitiligo patients.

Vitiligo is a depigmentation disease usually treated by NB-UVB and characterized by irregular white spots on the face, body and/or extremities.[21] It has been suggested that the loss of melanocytes from lesional skin may be a cause of the white spots, and research has shown an increase in DNA damage in the leucocytes of patients with vitiligo compared with healthy controls.[22] The epidermis of vitiligo patients presents multiple signs of oxidative stress, including allantoin, a well-known indicator for reactive oxygen species (ROS)-induced stress.[23] The *APE1* gene, along with 8-oxoguanine glycosylase-1 (*OGG1*) and X-ray repair cross-complementing-1 (*XRCC1*), which are significantly implicated in the BER pathway, mediates the removal of oxidatively damaged DNA and is involved in eukaryotic transcriptional regulation of gene expression.[23,24] *APE1* is a master regulator of ROS production and contributes greatly to the maintenance of genome stability.[26] Therefore, it was reasonable to speculate that SNPs of *APE1* are implicated in the occurrence and development of vitiligo by removing ROS-induced DNA damage. Several sequence variants have been identified in the *APE1* gene, including an amino acid change from aspartic acid to glutamic acid (1349T >G), which may be associated with hypersensitivity to ionizing radiation.[27] A number of case-control studies have documented an association between the *APE1*1349T >G SNP and susceptibilities to disease.[15,28] 1349T >G GG may have altered endonuclease and DNA-binding activity, and a reduced ability to communicate with other BER proteins.[29]A recent study reported that *APE1* expression in the epidermal lesion and non-lesion patches of vitiligo patients is higher than in healthy controls, and showed that *APE1*1349T >G variant genotypes confer an increased risk of vitiligo.[23] A functional study revealed that individuals with the *APE1*1349T >GGG and 1349T >GTG genotypes showed higher levels of damage,[30] supporting the present study's findings that compared to 1349T >G TT genotype, the combined 1349T >G TG + GG genotype was associated with an increased risk of vitiligo. In addition, the present study also found that the combined 141T >G TG + GG genotype was associated with a reduced risk of vitiligo compared with the TT genotype. Similarly, a previous study showed that a variant allele G of *APE1* 141T >G was associated with an increased risk of abnormal melanocyte proliferation and then cutaneous melanoma.[31] Moreover, the association was more pronounced in male vitiligo patients and those with active vitiligo, nonsegmental vitiligo, no family history of vitiligo, and an age of more than 20 years. To sum up, TT at 141 T >G and non-TT at 1349 T >G may be potential risk factors for vitiligo development, which was further confirmed by a logistic regression model in the present study.

This study also investigated associations between *APE1* 141T >G and 1349T >G SNPs and the prognosis for vitiligo, and the results showed that subjects with the combined 141TG + GG or 1349T >G TT genotype showed a better response to NB-UVB treatment, with better repigmentation, lower cumulative exposure dosages, reduced treatment times, and earlier initial repigmentation.

It was of great importance to identify protective factors in these vitiligo prognoses, since the therapeutic options and prognoses are quite different in different individuals.[6] It has been shown that a younger age, segmental vitiligo, short disease duration, and lesions located on the fleshy regions of the body may have a better chance of recovery.[32]

In conclusion, the present study provided evidence that *APE1* SNPs are associated with the risk of vitiligo. The G allele at *APE1*1349T >G combined with the 1349T >G TG + GG genotype may increase the risk of vitiligo, while the combined TG + GG genotype may reduce the risk of vitiligo. In addition, the study also found that 141T >G and 1349T >G SNPs may be significantly associated with the prognosis of this disease. However, as an important member of the BER pathway, it is possible that *APE1* functions with other genes in this pathway that are involved in the occurrence and development of vitiligo. The authors are undertaking a further study to focus on the interaction of *APE1* SNPs with related molecules in vitiligo.

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# Beyond the lungs: Alpha-1 antitrypsin's potential role in human gestation

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## Abstract

Alpha-1 antitrypsin (AAT) is an acute-phase protein with strong inhibitory activity towards proteolytic enzymes, mainly elastase but also trypsin, chymotrypsin and thrombin. The biological role of the protein and the effects of its deficiency have been subjects of scientific research for years, yet in many areas our knowledge remains incomplete. Alpha-1 antitrypsin deficiency (AATD), a defect in AAT synthesis and functionality, is one of the most frequently inherited genetic disorders among Caucasian populations. Its severe form is characterized by very low serum levels of AAT, and it most often affects the lungs (causing early-onset emphysema or chronic obstructive lung disease (COPD)) and/or liver (leading to jaundice and liver cirrhosis in children and adults). However, little is known about other possible clinical consequences of AAT deficiency. We discuss AAT's potential role in mechanisms regulating human fertility and gestation, with a particular emphasis on the clinical context and on indications for AATD diagnostic testing.

**Key words:** diagnosis, pregnancy, alpha-1 antitrypsin, oocyte maturation, alpha-1 antitrypsin deficiency

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Alpha-1 antitrypsin (AAT) is an acute-phase glycoprotein that functions primarily as a protease inhibitor (PI), acting on elastase, trypsin, chymotrypsin, and thrombin. It is synthesized mainly by hepatocytes and delivered to other tissues via the plasma, with significant action in the lungs, where it protects the alveolar space from proteolytic damage by neutrophil elastase.[1] Alpha-1 antitrypsin deficiency (AATD) is a common inherited genetic disorder among Caucasians. It is estimated that approx. 1 in 3000–4500 individuals suffers from this autosomal codominant condition, which most often manifests itself as very low concentrations of circulating AAT, a consequence of pathogenic mutations in the *SERPINA1* gene.[2] As might be expected, the disorder mainly affects the lungs (resulting in airflow obstruction, and/or early onset emphysema and/or bronchiectasis), the liver (causing jaundice and/or liver disease of unexplained etiology in newborns, children and adults), and rarely, the skin (manifested as panniculitis). The most common symptoms associated with AATD are widely known and well-documented (Table 1). Despite the fact that classic AATD-related symptoms are quite well-defined, the disease is under-recognized, with less than 10% of the expected number of cases reported, and an average of 5–8 years of delay between the initial symptoms and diagnosis.[5,6] In addition, current knowledge of other symptoms and clinical consequences of AAT anomalies remains incomplete. Given the large number of studies focused on this protein, such a vague understanding of its functions and the wider implications of its deficiency is cause for concern.

This paper discusses the potential role of AAT in mechanisms regulating human fertility and gestation, with a particular emphasis on the clinical context and indications for diagnostic testing for AATD.

Table 1. The main indications for diagnosing alpha-1 antitrypsin deficiency according to current recommendations[3,4]

| |
|---|
| Emphysema, especially with early onset (before 45 years of age) |
| Symptomatic form of chronic obstructive pulmonary disease, regardless of exposure to tobacco smoke |
| Bronchial asthma with persistent airway obstruction |
| Persistent airway obstruction confirmed by function tests and exposure to occupational factors or tobacco smoke, regardless of whether symptoms occur |
| Bronchiectasis of unclear etiology |
| Vasculitis with the presence of c-ANCAs |
| Liver disease of unclear etiology |
| Necrotizing panniculitis |
| Family members with confirmed alpha-1 antitrypsin deficiency |
| A family history of one of the aforementioned disorders |

## The alpha-1 antitrypsin protein and human reproduction

Encoded by the *SERPINA1* gene (MIM #107400), AAT is part of a family of structurally unique serine protease inhibitors, referred to as serpins, implicated in the pathogenesis of the so-called serpinopathies. These include neurodegenerative diseases, angioedema and coagulation abnormalities.[6,7] Abnormalities in AAT and other serpins can have a number of consequences.

Serum levels of AAT increase during pregnancy and during estrogen therapy.[8–10] However, it should be emphasized that pregnancy-related AAT increases in women with severe AATD does not reach levels considered normal. This is of potential clinical importance, as AAT promotes angiogenesis and vascularization of the endometrium, and inhibits the activity of cathepsins, tissue plasminogen activator and kallikrein, implicating it in trophoblast invasion and implantation.[11–13] Alpha-1 antitrypsin has also been shown to serve in the placenta as a substrate for the human serine protease high temperature requirement A1 (HTRA1), which in turn is supposed to support trophoblast apoptosis.[14] Furthermore, AAT may play a role in fertility regulation, pregnancy loss and other obstetric pathologies.[15–22] Finally, there is data from maternal serum protein profiling showing that alpha-1-antitrypsin is among the markers for non-invasive prenatal diagnoses of trisomy 21, 18 and 13.[23]

## Oocyte maturation

Alpha-1 antitrypsin in the follicular fluid (FF) originates from the circulation as well as from follicular secretions. Follicular fluid provides an optimal environment for the development and maturation of the oocyte, and is thought to have an important role in follicular maturation and, as an indicator of oocyte maturity, in controlling the release of mature oocytes.[24,25] Wu et al. provided preliminary data documenting differences in protein expression profiles (i.e., AAT) in FF between controlled ovarian hyperstimulation (COH) and natural ovulatory cycles.[15] That study included a group of 12 infertile women (6 receiving COH and another 6 with natural cycles) undergoing in vitro fertilization. Alpha-1 antitrypsin concentrations were significantly higher in FF from follicles with immature oocytes than in follicles with mature oocytes. The authors suggested that the higher AAT level in FF from women during the COH cycle may inhibit oocyte maturation, leading to the reduced fertilization rate seen in these patients. It can further be concluded that abnormalities in protein profiles as a result of increased immune and inflammatory responses might be a factor in adverse effects of controlled ovarian hyperstimulation on oocyte vitality, contributing to poor in vitro fertilization and embryo transfer outcomes.

## Spontaneous abortions

Changes in AAT concentration are a relevant factor in spontaneous as opposed to elective abortions.[16]

In a prospective case-control study, serum levels of AAT and cytokines were assessed in 14 patients with recurrent spontaneous abortions, 15 with sporadic spontaneous abortions and 11 controls who had undergone elective abortions of normal pregnancies. Women with recurrent and sporadic pregnancy loss had significantly lower AAT concentrations than those with normal pregnancies. However, antiproteolytic activity was significantly lower in the elective abortion and sporadic abortion groups. Interestingly, in both spontaneous abortion groups, these findings were accompanied by elevated levels of circulating proinflammatory cytokines.

## Preeclampsia

Preeclampsia (PE) affects 2–8% of pregnancies and is characterized by hypertension and proteinuria after 20 weeks of gestation.[26] It is classified as early-onset preeclampsia (EOPE) if it results in a preterm delivery before 34 weeks of gestation. The mechanisms governing PE are still not fully understood. Some recently identified factors include alpha-1-antitrypsin, postulated as a protective factor acting by activating Smad2 and the inhibitor of DNA binding 4 and/or by suppressing oxidative stress via downregulation of the p38MAPK signaling pathway.[27,28]

Preeclampsia and in particular EOPE are characterized by high mortality rates. Therefore, the identification of novel, sensitive prognostic biomarkers is of particular interest. Kolialexi et al. demonstrated a 3-fold increase in plasma concentrations of AAT in prospectively analyzed samples from 10 women with subsequent EOPE, compared with those of 40 controls with normal pregnancies, matched for gestational age and duration of sample storage.[22] This is in line with other reports of significantly increased AAT levels in patients with clinical symptoms of severe PE vs those with uncomplicated pregnancies.[19,29,30] Obviously, this might simply result from inflammatory components of pregnancies complicated by EOPE, but AAT should also be considered a candidate biomarker or part of a biomarker panel in early screening for EOPE.

Interestingly, for PE per se, the published data on plasma levels of AAT are contradictory. Twina et al. prospectively analyzed the link between a relative decline in AAT levels and enzymatic activity in maternal blood from PE and normal-pregnancy patients.[18] They analyzed samples from 41 individuals (singleton pregnant females within 24–42 weeks of gestation), including 23 patients with severe preeclampsia and 18 patients without preeclampsia who were admitted in labor. Severe PE was defined as hypertension associated with any of the following: proteinuria after 20 weeks of pregnancy and systolic blood pressure > 160 mm Hg and/or diastolic blood pressure >110 mm Hg, severe proteinuria (≥5 g per day), or multi-organ involvement. The authors documented significantly lower AAT concentrations and activity among patients with severe PE compared to parturient women without PE.

Feng et al., who looked at differences in protein expression profiles between normal full-term pregnancy, early-onset severe preeclampsia (ES-PE) and late-onset severe preeclampsia (LS-PE), obtained very similar results.[19] A cohort of 30 patients (10 per group) was included in the study, and a total of 20 differentially expressed proteins were identified; AAT expression differed among the 3 groups. Its level was the highest in the normal full-term pregnancy group (1.85 ±0.15 g/L), moderate in the ES-PE group (0.77 ±0.14 g/L) and lowest in the LS-PE group (0.42 ±0.07 g/L; $p < 0.05$).

## Preterm labor

Alpha-1 antitrypsin expression in the amnion, which is the inner layer of the fetal membrane lining the amniotic cavity, is regulated by cytokines (such as tumor necrotic factor alpha, interleukin-6 and oncostatin M).[31] Izumi-Yoneda et al. noted that in amnion from pregnancies with premature rupture of the membrane (PRM), AAT activity was significantly lower, probably as a result of its oxidation.[31] Thus, an imbalance between oxidation and AAT expression/activity may contribute to PRM.

Deficiency in AAT, as a protective factor against tissue damage from enzymes released by inflammatory cells, has also been proposed by Baron et al. to be involved in preterm labor, in PRM and preterm PRM (PPRM).[21] Their study defined PRM as rupture of membranes at least 1 h prior to the onset of labor or after 37 weeks of gestation, and PPRM as PRM prior to 37 weeks of gestation. The authors measured AAT concentration and activity in blood samples from 71 patients in a prospective case-control study. No significant differences in circulating AAT levels or activity were detected between patients with preterm and term labor, or between those with PRM and those with PPRM. AAT deficiency was observed in only 2 women, who, notably, belonged to the 15-patient PPRM group. Taking into account that inherited AAT deficiency is largely underdiagnosed, the authors suggested further investigation into whether AATD is undetected in pregnant women and affects the risk of obstetric complications.

However, to the best of our knowledge, pregnancy-related issues in women diagnosed with AATD have not been described to date. Of course, such issues may not occur, but a possible reason is that when AATD is eventually recognized, it is usually beyond the 5$^{th}$ decade of life, due to a progressive decline in lung function or to emphysema. During the first 4 decades of life, the major health problem of affected individuals may be liver dysfunction (in the form of chronic elevation of liver enzymes or cirrhosis). In other words, symptoms of AATD would not commonly be expected during the usual range of childbearing years.

## Alpha-1 antitrypsin deficiency awareness in obstetrics

To expand our current knowledge of possible and rare clinical consequences associated with decreased AAT, and following the current recommendations (Table 1), we would like to encourage gynecologists and obstetricians to check serum concentrations of AAT in female patients with persistent airflow obstruction (as demonstrated by spirometry), a family history of emphysema (particularly in individuals ≤45 years) and/or unexplained chronic liver disease, or a positive family history of AATD.

It is important to bear in mind that the optimal counselling time for determining genetic risk and carrier status, introducing prophylactic factors and discussing the availability of prenatal testing is before pregnancy. An AATD diagnosis should also be considered in neonates and infants with prolonged jaundice after birth and/or abnormal liver enzymes.

It should be noted that AATD diagnosis is available in Poland and relatively simple. All subjects with serum concentrations of AAT protein below 100 mg/dL should be referred for more detailed testing. This consists of pheno- and genotyping or sequencing and is easily performed from blood spot samples (whole blood samples dried on dedicated blotting paper). These samples can be delivered by regular post. Since 2009, the National Institute of Tuberculosis and Lung Diseases in Warsaw, Poland has been providing a nationwide testing program, free of charge for all patients with respiratory disorders.[32]

## Conclusions

There is evidence that AAT plays a role in early-onset preeclampsia and fetal loss. Consequently, the potential of AAT as a biomarker for identifying patients at increased risk of EOPE needs further investigation. On the other hand, severe AATD in mothers or fetuses does not seem to affect conception or pregnancy outcomes, respectively. Nevertheless, it is advisable to consider testing for AATD (serum concentrations of AAT and/or *SERPINA1* gene analyses) in female patients with a personal or family history of chronic respiratory disorders and/or chronic liver pathology.

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# Branched chain amino acids: Passive biomarkers or the key to the pathogenesis of cardiometabolic diseases?

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## Abstract

The metabolomic approach to research on lifestyle diseases has led to the discovery of new potential biomarkers of pathological conditions as well as key metabolic pathways that may become targets of therapeutic intervention. Current evidence supports plasma branched chain amino acids (BCAAs) as potential diagnostic and prognostic biomarkers of cardiometabolic diseases. However, the biological mechanisms of the associations that have been identified are still not completely understood and should be clarified before implementing BCAA-based biomarkers in the clinical setting. The most crucial issue that needs to be solved first is determining whether BCAA plasma profile disturbances are only passive biomarkers or whether they facilitate dysmetabolic processes. In this context, further research is also warranted to investigate the role of dietary BCAAs. Gaining this knowledge would be significant progress in molecular nutrition research, providing perspective for target therapeutic and prophylactic interventions. This paper provides a comprehensive review of the main hypotheses and mechanistic models that consider circulating BCAAs both as passive biomarkers and as contributors to cardiometabolic diseases.

**Key words:** amino acids, biomarker, metabolomics, BCAA, cardiometabolic diseases

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The metabolomic approach to research on lifestyle diseases has led to the discovery of new potential biomarkers of pathological conditions as well as key metabolic pathways that may become targets of therapeutic interventions.[1] Evidence from recent studies indicates that there is a close association between the plasma concentrations of branched chain amino acids (BCAAs) and cardiometabolic diseases (CMDs) such as diabetes (DM2), insulin resistance (IR), cardiovascular disease (CVD), obesity, and metabolic syndrome (MS).[2] Moreover, it has been shown that BCAAs have diagnostic and prognostic value, correlate with positive outcomes of therapeutic interventions, and can be used to differentiate metabolically healthy from metabolically unhealthy obese patients. Branched chain amino acids have therefore been proposed as valuable diagnostic and prognostic biomarkers with considerable clinical potential.[2,3]

Branched chain amino acids comprise 3 essential amino acids (AA): leucine (Leu), isoleucine (Ile) and valine (Val), which account for 35–40% of the indispensable dietary amino acids in body protein and 14% of the total amino acids in skeletal muscle. They share common membrane transport systems and enzymes for their transamination and irreversible oxidation.[4] Branched chain amino acids are not only sources of energy and passive substrates for synthesis, but are also very important as signaling molecules and cell regulators. They influence glucose, protein and lipid metabolism. Changes in amino acid availability have profound effects on cell signaling, gene expression, brain, and neuroendocrine functions.[5]

The mechanisms underlying the relationship of plasma BCAAs to diseases processes are still not completely understood and need to be clarified prior to the use of BCAAs as biomarkers in clinical practice.[3] The most crucial issue that needs to be solved first is determining whether BCAA plasma profile disturbances are only passive biomarkers or whether they facilitate dysmetabolic processes; this has not yet been determined.[6] Paradoxically, there is a great deal of evidence showing the benefits of BCAA supplementation and diets high in BCAA-rich proteins.[7] The reasons for these seemingly contradictory observations, however, remain elusive.

Branched chain amino acid catabolism impairment in 1 or more tissues, changes in cellular uptake from the blood, increases in proteolysis, IR or excessive intake have been identified as potential causes of elevated plasma BCAA concentration. It has been suggested that BCAAs may have a causative role in CMD development, mainly through nutrient-sensitive factor overstimulation and IR promotion,[8] but also by direct and indirect influence on brain function.[9,10] The association of BCAAs with coronary artery disease seems to be independent of IR and diabetes.[11] There are suggestions that some disturbances of BCAA metabolism could be part of an early, yet to be elucidated, metabolic change that precedes CMD development, and links DM2 and CVD pathogenesis.[12] Some of the essential issues regarding BCAA metabolism in the context of CMD pathogenesis are highlighted in this review.

# BCAAs as passive CMD biomarkers

## Excessive intake

Considering that BCAAs are essential AAs, which means that they cannot be synthesized in the organism de novo, plasma BCAA concentration depends on the intensity of their utilization, protein turnover and the amount of dietary supply.[6]

The robust regulation and capacity of the BCAA catabolic pathway makes increased intake a highly doubtful cause of elevated plasma BCAA levels. In the postprandial state the BCAA metabolism rate increases with supply and the plasma concentration remains within a narrow range. The capacity of BCAA catabolism reaches a plateau and the plasma BCAA concentration increases only when intake greatly exceeds the normal daily supply.[13] Studies showing an association between CMDs and plasma BCAA concentration have mostly been performed in prandial situations. Previous studies have showed no association between prandial plasma BCAA concentration and dietary intake. Within 5 h of a meal the plasma BCAA concentration should bounce back to the baseline.[14] However, excessive intake should be considered a meaningful trigger in the situation of BCAA catabolic pathway disruption.

## Impairment of BCAA catabolism

The metabolism of BCAAs has 3 stages (transamination, oxidative decarboxylation and oxidation), is very effective and is strictly regulated to avoid toxic excess.[16] The first, reversible step takes place in peripheral tissues and is catabolized by the mitochondrial isoform of branched-chain aminotransferase (BCATm, encoded by BCAT2). The second, highly regulated, irreversible step, which is critical for BCAA catabolism, is catalyzed by the multienzyme mitochondrial branched-chain-ketoacid dehydrogenase complex (BCKDC). The BCKDC irreversibly oxidizes BCAAs to their respective ketoacids. The ketoacids formed are further metabolized by multiple enzymatic steps within the mitochondrial-matrix, eventually forming lipogenic, ketogenic or glucogenic substrates (acetoacetyl-CoA, acetyl-CoA and propionyl-CoA).[17]

In humans BCAA metabolism takes place mainly in muscles and adipose tissue.[18] Studies on rodents indicate that BCATm and BCKDC in adipose tissue have direct and significant influence on plasma BCAA concentration.[19]

The expression and activity of BCKDC can be altered by numerous metabolic factors. The overall enzymatic activity is controlled by the phosphorylation (inactivation)/dephosphorylation (activation) cycle. Intense physical activity, high abundance of branched-chain ketoacids (from

a high-protein diet, BCAA supplementation, starvation, etc.), glikokortykosteroids or clofibrate cause reductions in kinase activity. Insulin, free fatty acids (FFAs), a low-protein diet, hyperthyroidism, and 17β-estradiol enhance kinase activity.[20]

Visceral adipose tissue in metabolically unhealthy obese people and rodents is characterized by reduced expression of BCKDC enzymes. It has been suggested that insulin action or a healthy metabolic/inflammatory phenotype is responsible for the modulation of BCAA catabolic enzyme expression.[21] Morbidly obese women with MS have been found to have reduced BCAA catabolic enzyme expression compared to equally obese but metabolically healthy controls.[21] Treatment with PPAR agonists has been shown to lead to increases in BCAA catabolic pathway transcripts.[22] Bariatric surgery in morbidly obese patients leads to reductions in plasma BCAA concentration of approx. 35% and concomitant increases in mBCAT and BCKDC in omental and subcutaneous tissue. Moreover, decreases in plasma BCAA concentration predict metabolic improvement irrespective of body weight reduction.[23]

Manipulation of GLUT4 transporter expression in adipose tissue leads to a selective decrease in BCAA catabolic enzyme activity with a concomitant increase in plasma BCAA concentration. Both insulin and FFAs (both of which are increased in IR) are negative regulators of the BCKDC.[24] It is suspected that diminished muscle uptake of glucose with increased glucose availability and corresponding hyperinsulinemia lead to selective adipose tissue BCAA catabolism impairment and increase BCAA plasma concentration. This suggests that IR and obesity may cause impairment of BCAA degradation in adipose tissue.[18]

Genetic variations in the expression of the genes encoding key BCAA catabolic enzymes or the proteins that control protein synthesis and turnover may be considered further contributors to BCAA dysmetabolism. The *BCKDC* encoding gene has been identified as one of the major genes associated with DM2 and obesity-related metabolic dysfunction.[25] Studies on monozygotic twins indicate that BCAA metabolic pathway gene expression undergoes functional negative regulation in obese individuals, which leads to elevated plasma BCAA concentration.[18] Positive functional regulation of BCAA catabolic pathway gene expression with concomitant lower plasma BCAA concentration seems to be associated with higher levels of physical activity.[26]

It has been demonstrated that brain insulin plays a significant role in BCAA metabolism through positive regulation of hepatic BCKDC. It is suspected that hypothalamic IR is responsible for disruption of hepatic BCAA catabolism, and persistently increased BCAA concentrations can exacerbate hypothalamic IR by mTOR hyperstymulation, starting a vicious cycle.[27]

Taking all of the above together: BCAA catabolic pathway is dampened in obesity-related metabolic disorders. Regulation mainly involves the BCKDC in adipose tissue and takes place on several levels: direct enzyme regulation (activators, inhibitors, allosteric factors), post-translational modifications and gene expression. Insulin resistance, adipocyte dysfunction and physical activity have been identified as the most important factors influencing the BCAA catabolic pathway. Impairment of BCAA catabolism is functional and can be improved by interventions increasing insulin sensitivity.

## Increased protein turnover

Most studies indicating a relationship between BCAAs and CMDs have been performed in the fasting state, when protein anabolism is minimized; differences in protein synthesis rates are therefore unlikely to be a meaningful factor driving plasma BCAA differences. Similarly, increased protein and fat-free mass catabolism in IR and reasonably controlled DM2 should not be considered important in plasma BCAA differences, because they do not explain the selectivity of hyperaminoacydemia.[17]

## Gut microbiota

Gut microbiota are involved in the metabolism of several amino acids and have been shown to be important factors in the supply of BCAAs to mammalian hosts[28]; at the same time, BCAAs participate in bacterial metabolism and are considered important regulators of intestinal microbial species and diversity.[5] Disruption of gut microbial composition and function (dysbiosis) is implicated in the pathogenesis of CMDs such as obesity, IR, MS, and DM2.[29,30] It is conceivable that the altered composition of gut microbiota that is observed in DM2 and obese patients modulates the plasma BCAA profile.[28,31] This may also go some way to explaining why dietary protein content does not necessarily reflect changes in the plasma AA profile. The association between dysbiosis, plasma AA profile changes and CMDs needs further evaluation.

## BCAAs as contributors to CMDs

### mTOR overstimulation

Branched chain amino acids are very important signaling molecules and cell regulators that influence many key cell signaling pathways.[32] Some AA transporters also play the role of receptors and conduct information about the nutritional state, the quantity and quality of extra and intracellular AAs to nutrient-sensitive factors such as the general control nonderepressible 2 (GCN2) kinase and the mammalian target of rapamycin complex (mTORC).[33] However, sensing pathways are also affected

in negative ways. Under certain conditions BCAAs are considered IR-promoting factors through overstimulation of the adenosine monophosphate activated protein kinase (AMPK), mTOR and GCN2.[34]

mTORC1 is a nutrient-sensitive protein complex that promotes the synthesis of proteins and lipids, the growth, proliferation and differentiation of cells, and the biogenesis of mitochondria, while it inhibits autophagy.[35] The activity of the complex is regulated by pathways related to insulin and growth factors, and is independently stimulated by cell energy status, glucose and AA availability. Thus, mTORC1 integrates extra and intracellular signals to maintain balance in the organism's energy.[35]

Insulin and insulin growth factor (IGF) stimulate mTOR via the IRS/PI3K/Akt pathway and activation of the mTORC1 inducer GTPase Rheb. Rheb is regulated by the cell nutrient sensor AMPK. In states of high energy abundance, the ATP/cAMP ratio is high, repressive function of AMPK on Rheb is reduced and mTORC1 is activated.[36] It has been shown that AAs, particularly Leu, play a critical role in mTORC1 activation by enabling translocation of inactive mTORC1 complex to cell compartments with a high abundance of Rheb protein.[37] Moreover, BCAAs influence mTOR activity both directly and by inactivating other mTOR repressors, like GCN2. GCN2 inhibition also reduces repression of sterol regulatory element-binding protein 1 (SREBP-1), promoting lipogenesis.[8]

A negative feedback signal emanating from the mTOR pathway end-product – active p70 S6K – results in serine phosphorylation of insulin receptor substrate (IRS) and thus is involved in negative regulation of insulin signaling. Further, mTOC1 overstimulation by a high abundance of nutrients, proinflammatory cytokines and extra cell stimulation by insulin and IGF will result in IR on the IRS level, with concomitant lipid synthesis promotion via SREBP-1.[37] This is the foundation for the hypothesis that an energy-dense diet rich in AAs coming from dairy (high-insulinogenic, with the potential to evoke IGF action) will lead to IR via mTOR overstimulation.[37] Furthermore, taking into account the previous considerations, it should be noted that the effect could be exacerbated by impairment of BCAA catabolism.

## Brain function and the neurobiological origin of obesity and metabolic diseases

The central nervous system (CNS), particularly the hypothalamus, plays an important role in the regulation of food intake, energy balance and glucose homeostasis.[38] It has been suggested that disruptions in the brain signaling systems play an important role in the pathogenesis of DM2 and metabolic syndrome.[39,40]

Branched chain amino acids have both direct and indirect influence on brain neurochemistry. They compete for blood-brain barrier carriers with the precursors for neurotransmitters synthesis: aromatic amino acids (AAAs). Thus, the plasma AA pattern determines AA supply to the brain and consequently also determines neurotransmission.[41]

It has been posited that addiction and obesity have the same neurobiological origin and are based on serotonergic and dopaminergic transmission that regulates the neuron systems of reward, control, motivation, and conditioning.[42] Modulation of the proportions of BCAAs and AAAs leads to changes in serotonergic and dopaminergic transmission.[42]

The dopaminergic pathway, which relies on tyrosine (Tyr) and phenylalanine (Phe) supply, is involved in food-based rewards and is closely linked to hunger.[43] Serotonergic neurotransmission, which is affected by tryptophan (Trp) availability, is essential for the regulation of reward-related behaviors and is involved in the regulation of food intake, body weight, mood, and autonomic functions.[39,40] Receptor- and brain area-specific disruptions of serotonin signaling lead to hyperphagia, disturbed energy expenditure, obesity and reduced insulin sensitivity.[39] "Carbohydrate craving" behavior, which is considered a driver of obesity, restores serotonergic neurotransmission by increasing tryptophan's flux across the blood-brain barrier.[44] The appetite suppressant effects of fenfluramine and dexfenfluramine, which were formerly effectively used for obesity treatment, were based on increments in serotonin transmission.[45] Central administration of serotonin-depleting agents in rats has been shown to result in hyperphagia and increased body weight.[46] Some studies have suggested an association between reduced central serotonergic responsivity and MS.[40]

Insulin lowers plasma concentrations of BCAAs, Tyr and Phe. Tryptophan circulates in the blood mostly bound to albumin, so plasma Trp concentrations are not as strongly affected by insulin action.[41] Ingestion of carbohydrates increases the plasma ratio of tryptophan to other large neutral amino acids, leading to significantly increased serotonin synthesis and release. In contrast, ingestion of a protein-containing meal is usually reported to lower serum Trp concentrations in relation to competitors, to reduce brain Trp uptake and levels, and to diminish serotonin synthesis and release.[47] Based on this, we can assume that in IR, Trp uptake in the brain would be diminished, disrupting serotonin transmission in the complex neurobehavioral circuit that controls appetite. To benefit from higher serotonin release, exhibit positive mood changes and reach satiety, one must provide food with a much higher glycemic load, producing higher insulin release.

Leu acts on the CNS both directly, by activating the mTOR pathway, and indirectly, through its metabolites, and exerts both positive and negative effects on whole body metabolism. It has been linked to nutrient-sensitive hypothalamic neurons that affect the behavioral and physiological determinants of energy balance and are associated with the pathophysiology of obesity and metabolic diseases.[9]

Central Leu infusion decreases food intake and body

weight.[9,48] However, in most studies no central anorexogenic effect of clinical importance was observed when Leu was provided orally. The rationale for this discrepancy could be explained by the fact that increases in plasma Leu do not necessary reflect significant increments in the CNS. Direct Leu infusion into the CNS does not reproduce physiological conditions; however, it is conceivable that Leu has a central anorexogenic role in appetite regulation and obesity.[48]

Hypothalamic BCAA metabolism is involved in central glucoregulation through the brain-liver circuit and the evoked effects appear to oppose those that are exerted on the periphery.[49] The Leu metabolite malonyl-CoA, which is further utilized to oleoyl-CoA, is directly responsible for the glucoregulatory effects. Activation of hypothalamic $K_{ATP}$ channels generates a neurogenic signal that is relayed to the liver via the hepatic branch of the vagus nerve to reduce hepatic glucose production through a reduction in gluconeogenesis and glycogenolysis.[50] This model of central glucose regulation has been confirmed by studies using both central and systemic BCAA administration.[49,50] It has been shown that factors that disrupt central Leu-sensing can contribute to glycemic dysregulation and hyperglycemia.[49] Central glucoregulation can be also attenuated by diet. Animal studies have shown, for example, that diets high in saturated fat blunt the mediobasal hypothalamus response to Leu.[49,51]

Leu central signaling can also exert negative metabolic effects by inducing peripheral IR in chronic BCAA elevation, leading to persistent hypothalamic mTOR overstimulation.[27] The ambiguous effects of Leu can be explained by the dualistic mTOR/insulin sensitivity model, which assumes that the association of mTOR activity and insulin sensitivity seems to follow a U-shaped curve, where mTOR activity that is either too low or too high has a negative metabolic effect.[36]

## The detrimental effects of excessive BCAA catabolic flux

Some studies suggest that BCAA catabolism rather than BCAA concentration is associated with metabolic dysfunction.[52,53] Newgard et al. observed that IR is associated not only with BCAAs but also with BCAA catabolites, indicating enhanced BCAA catabolism. They hypothesized that enhanced BCAA catabolism may reflect overfeeding, particularly an overload of the BCAA catabolic pathway in obese subjects.[54] Experimental studies with rats showed that enhanced BCAA flux synergizes with hyperlipidemia, making a contribution to IR. The disruption was accompanied by chronic mTOR activation, phosphorylation of IRS1 (ser307) in skeletal muscles and accumulation of incompletely oxidized substrates, leading to mitochondrial stress.[37]

Another explanation for how excess catabolic flux of BCAAs can lead to IR involves a catabolic intermediate of valine – 3-hydroxyisobutyrate (3-HIB) – the paracrine metabolite that regulates the trans-endothelial flux of FFAs. It is suspected that excessive secretion of 3-HIB from muscles leads to excess trans-endothelial FFA import into muscles, accumulation of lipotoxic, incompletely esterified intermediates, and blunted insulin signaling.[53] Moreover, increased plasma levels of 3-HIB were found to be a marker of future DM2 development.[55]

## Does dietary BCAA matter?

There are multiple reports about the influence of BCAA-rich diets on metabolic health, but the conclusions are inconsistent.[24] Some of them indicate detrimental effects, such as the promotion of IR, metabolic dysfunctions and DM2.[56,57] Others suggest beneficial outcomes, including a decreased risk of DM2,[7,58] which, in the light of the above considerations, may seem paradoxical. However, it should be mentioned that both Leu and Ile have the capacity to increase glucose clearance by different modes of action: by the promotion of insulin secretion (Leu) as well as in insulin- and mTOR-independent mechanisms: by increasing cellular uptake (Leu and Ile), by promoting glycogenogenesis (Leu) and by downregulating gluconeogenesis (Ile).[59] It seems that the influence of dietary BCAA on glucose metabolism and insulin signaling are sophisticated, and that the final outcome depends on the context in which BCAAs are administered, such as actual insulin sensitivity, the overall energy balance, the dosage, the site of action, the duration, background lipid profiles, other macronutrients in the diet, and muscle mass.[60] Diet intervention studies have many limitations and inter-individual differences in the metabolomic effects of dietary interventions have been reported.[61] Certain individuals display greater stability in their metabolic phenotype.[61] The individual microbiome[28] and the potential of high-protein diets to increase satiety through the incretin-CNS axis should also be considered possible confounding factors.[58]

## Conclusions

Metabolomics research has identified plasma BCAA as a potentially valuable biomarker of CMD. However, despite numerous hypotheses, the mechanisms underlying the relationship of plasma BCAA to the disease process have still not been clarified. Determination of the place and role of plasma and BCAA diet in the pathogenesis of CMDs should be made prior to implementing BCAA-based biomarkers in clinical settings, and could provide a perspective for target therapeutic and prophylactic interventions.

The complexity of BCAA interactions with metabolic pathways, along with the multitude of diet-delivered and internal factors that influence them, constitutes a great challenge for molecular nutrition research, personalized nutrition and medicine.

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# Echocardiographic evaluation of left ventricular strain in severe aortic stenosis with therapeutic implications and risk stratification

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## Abstract

Degenerative aortic stenosis (AS) is an increasingly common acquired valvular heart disease in adults due to the extension of life expectancy in the population of developing countries. The occurrence of calcifications and associated severe aortic stenosis (SAS) increases with age and affects approx. 3–5% of people over 75 years of age. The basis for the decision on the date and type of therapy is echocardiographic evaluation of the severity of the AS and left ventricular (LV) function as well as clinical signs. It appears that the use of newer, more precise methods in echocardiography, especially in patients with preserved ejection fraction (pEF), may change our management in qualifying for valve replacement, especially in asymptomatic patients with SAS. The aim of this review study is echocardiographic strain analysis and evaluation of strain of LV myocardial fibers in patients with SAS, using the speckle tracking echocardiography (STE). This evaluation allows for risk stratification of a valve disease and the choice of the appropriate therapy method.

**Key words:** aortic stenosis, speckle tracking echocardiography, strain

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## Introduction

Degenerative aortic stenosis (AS) is an increasingly common acquired valvular heart disease in adults due to the extension of life expectancy in the population of developing countries. The occurrence of calcifications and associated severe aortic stenosis (SAS) increases with age and affects approx. 3–5% of people over 75 years of age.[1] The process of forming aortic valve lesions results from the deposition of lipids, infiltration of macrophages and T lymphocytes in the endothelium of aortic cusps and stimulation of their calcification processes (Fig. 1).

The consequence of an increase in AS is pressure overload and the resulting concentric left ventricular hypertrophy (LVH). At this stage, the cardiac output and filling pressure are normal. The left ventricular (LV) remodeling, in addition to stenosis, is also influenced by other factors such as the age and sex of the patient, e.g., in women the process of faster calcification of the valve and quicker appearance of the disease symptoms are observed.[2] The other important factors are the following: genetic diversity in the renin-angiotensin system, comorbid coronary artery disease, arterial hypertension, and significant aortic regurgitation. In patients with associated arterial hypertension, the LV afterload increases, which at a certain stage of the disease causes a decrease in the stroke volume and progressive deterioration of myocardial function. In patients with SAS and associated hypertension, a reduced survival rate was observed.[3]

A careful diagnosis of AS is important, not only regarding the size of the stenosis and transvalvular gradient of stenosis, but also the function of the LV and its impact on pulmonary pressure, the right ventricle and the left atrium. The current criteria for echocardiographic diagnosis of SAS are presented in the ESC/EACTS recommendations of 2017.[4]

Surgical treatment is level B class I recommendation in symptomatic patients with severe AS confirmed with echocardiography. Level C class I recommendation includes asymptomatic patients with SAS and with reduced left ventricular ejection fraction (LVEF) <50%, as well as asymptomatic patients with SAS and abnormal exercise test results in which symptoms were manifested during exercise clearly resulting from AS. Surgical aortic valve replacement (AVR) is recommended for patients from the low surgical risk group (STS or EuroSCORE II <4% or logistic EuroSCORE II <10% without other risk factors not included in the abovementioned scales such as frailty syndrome, porcelain aorta or history of chest irradiation) – this is level B class I recommendation. Transcatheter aortic valve implantation (TAVI) is recommended for patients who, in the opinion of the Heart Team, are not suitable candidates for AVR – level B class I recommendation.[5]

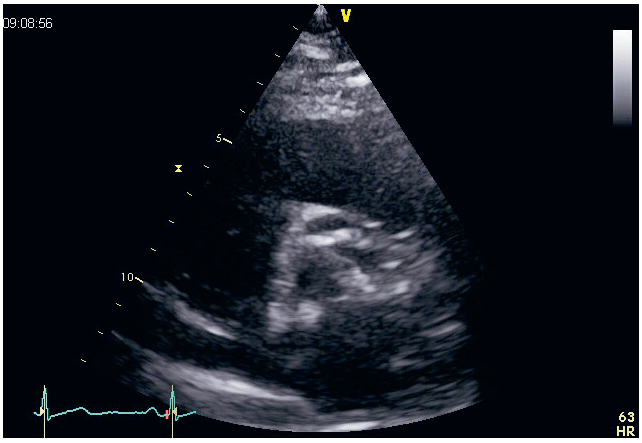


Fig. 1. Image of SAS in short axis parasternal transthoracic view

Management of patients in the asymptomatic period with preserved ejection fraction (pEF) remains debatable. The onset of symptoms and the deterioration of the LV function are mostly associated with poor prognosis. Left ventricular ejection fraction is still the basic echocardiographic parameter for assessing myocardial function. It appears that the use of newer, more precise methods in echocardiography, especially in patients with pEF, may change our management policy in the field of qualifications for AVR, especially in asymptomatic patients with SAS.

The aim of this review is an echocardiographic analysis and evaluation of a strain of LV myocardial fibers in patients with SAS using the speckle tracking echocardiography (STE).

## Risk factors for aortic stenosis

The process of AS formation is the result of many factors including clinical, genetic and anatomical factors. Clinical factors include old age, male sex, elevated low-density lipoprotein (LDL) and lipoprotein(a) (Lp(a)), arterial hypertension, smoking, diabetes, and metabolic syndrome. An increased risk of AS is also present in patients after mediastinal irradiation.[6]

The presence of bicuspid aortic valve as a congenital defect is more conducive to degenerative valve lesions. The congenital polymorphism of Lp(a) and an elevated level of this lipoprotein in serum are associated with the possibility of inheriting the tendency for calcification to form on the valves. The increase in AS from the period of sclerosis in which cusp calcifications and thickening develop to hemodynamically significant stenosis takes on average from 2 to 5 years in 10–15% of patients.[7]

In the period when the AS is mild and with echocardiographic evaluation of the transvalvular velocity results of about 2 m/s are noted, progression occurs in almost all patients and most of them require AVR. The average increase in AS in adults from a mild to moderate defect is manifested by a change in the following parameters: an increase in the transvalvular velocity by 0.3 m/s/year, an increase in the mean transvalvular gradient by 7 mm Hg/year and a decrease in the effective orifice area (EOA) by 0.1 $cm^2$/year.[8]

When significant valve stenosis is found, it is very important to differentiate between symptomatic and asymptomatic patients. A patient with the typical symptoms of SAS (angina pectoris, syncope, arrhythmias, and heart failure) requires intervention in the form of AVR within 30 days of symptoms onset, as this is the only way to reduce the symptoms and decrease mortality in this group of patients. The mortality of these patients over a 2-year period without surgical intervention is 50%.[9]

The management of asymptomatic patients with significant valve stenosis is still under debate. Some authors suggest earlier AVR based on 2 factors: 1. The risk of sudden cardiac death (about 1% per year in these patients), which approaches the risk of a surgical intervention averaging to about 2.5% and even less in experienced cardiac surgery centers. 2. The risk of complications, which may be even higher in patients with severe aortic valve calcification and a rapid increase of peak transvalvular velocity, e.g., >5.5 m/s, abnormal reaction or symptoms induced by the exercise test or a high level of serum natriuretic peptides.[10]

In observational studies covering the issues of natural history of asymptomatic patients with SAS, it was found that about 1/3 of them become symptomatic within 2 years and 2/3 of them underwent AVR or sudden cardiac death within 5 years.[11]

Survival in asymptomatic, non-operated patients is reported to be 99%, 98% and 93%, respectively, at the end of 1, 2 and 5 years of follow-up. It is worth noting that the survival rate of these patients was similar to the healthy population at a similar age and of the same sex.[12] The long-term prognosis worsens with the onset of symptoms.

In the management of an asymptomatic patient, the following principles should be respected: identification of high-risk patients, education for the symptoms of AS, periodic follow-up examinations, treatment of additional diseases, and optimization of the AVR time.

## Left ventricular pathophysiology in aortic stenosis

In order to optimize the time for AVR in SAS, it is necessary to evaluate in detail the hemodynamic consequences of the confirmed disease. The LV pressure overload in SAS results in a change in its geometry and function (hypertrophy, dilation and a decrease in EF). In order to maintain the heart function (pEF), the high stress of the ventricular wall leads to the hypertrophy of the middle layer of the muscle. Further increase in stress in this layer gradually intensifies myocyte hypertrophy, with time leading to fibrosis of extracellular space. At this stage, a progressive deterioration in heart function is noted, although the stroke volume and ejection fraction (EF) may remain normal. Development of valve stenosis causes progressing changes in myocardium, along with the death of cardiomyocytes and focal replacement fibrosis. Apart from the degree of the valve stenosis, muscle hypertrophy is also affected by the following factors: sex, age, genetically conditioned angiotensin-aldosterone system disorders, concomitant coronary artery disease, arterial hypertension, or significant aortic regurgitation.

In patients with concomitant arterial hypertension, there is an increase in peripheral vascular resistance which, accompanied by AS, increases the stress of the ventricular wall, at a later stage causing an increase in the afterload. The consequence of this may be a decrease in cardiac output, damage to the function of the myocardium and reduced survival rate. Long-term significant valve stenosis causes the exhaustion of cardiac compensatory mechanisms and contributes to an increase in LV diastolic stiffness and impairment of its filling function. The LV becomes incapable of maintaining an adequate stroke volume and this condition is called "afterload mismatch". During this period, there is no significant damage to the LV systolic function and EF is preserved (pEF), but only the correction of the heart defect results in a complete, beneficial improvement in LV function. However, diastolic dysfunction and unfavorable response to physical exercise in patients with pEF may still continue for many years after AVR.[13] A compensatory change of the LV geometry is associated with an increase in the relative wall thickness (RWT) with the unchanged function of the radial and circumferential fibers in the middle layer of the LV wall, which in turn allows the normal EF (pEF) to be maintained.

A common phenomenon in AS is impaired coronary flow reserve with decreased flow in the subendocardial layer, even in the absence of lesions in the coronary vessels. This is due to the severity of the valve stenosis and the associated LV hemodynamic load and the shortened diastolic perfusion time. These phenomena may be the basis for fiber dysfunction in the longitudinal dimension.

Early identification of myocardial contractility damage symptoms is important, especially in asymptomatic patients with AS, and helps in their earlier qualification for AVR surgery. Based on data from cardiovascular magnetic resonance, it has been found that LV remodeling in SAS in the first period takes the form of extracellular matrix diffuse fibrosis and is reversible, similarly to myocardial cellular hypertrophy. However, cardiomyocytes necrosis with replacement fibrosis (scar) is an irreversible process.

## Echocardiography

Echocardiography plays a central role in the assessment and management of patients with SAS. This examination method helps to stratify the risk in a heterogeneous group of asymptomatic patients with SAS. The main echocardiographic technique used to assess the severity of AS is Doppler echocardiography. The use of Doppler echocardiography, due to its high availability and versatility,

received Class I recommendations. Cardiac catheterization is not routinely performed. The use of this method is reserved for patients with ambiguous results of non-invasive examinations. The criterion for the diagnosis of SAS according to current recommendations (ESC and EACTS) of 2017 is as follows:

1. Peak transvalvular velocity >4 m/s;
2. Mean gradient (MG) >40 mm Hg;
3. Effective orifice area (EOA) <1.0 $cm^2$;
4. EOAI <0.6 $cm^2/m^2$.

Unfortunately, results that do not comply with the guidelines may appear in clinical practice. This occurs in approx. 25–30% of patients with SAS and preserved left ventricular ejection fraction (pLVEF). In this situation, there is a problem with the assessment of the severity of stenosis and the conditions where, e.g., EOA< 1.0 $cm^2$ and MG < 40 mm Hg are inconsistent with the guidelines for diagnosing SAS, making the decision to qualify the patient for AVR surgery difficult. It has been repeatedly stated that the EOA parameter obtained from the calculation of the continuity equation can be miscalculated (a more accurate measurement is obtained in 3D or computed tomography (CT)) and, therefore, it was assumed that the evaluation of the severity of AS is based mainly on the MG. The quite common underestimation of the severity of AS is associated with late qualification of patients for AVR and with poor long-term prognosis.[14]

Besides the interpretation of echocardiographic parameters, it is important to assess the blood pressure and the functional status of a patient. Patients with high blood pressure should be reevaluated after its normalization.[15]

Taking into account the assessed parameters, 4 categories of AS can be defined:

1. High-gradient AS, EOA < 1.0 $cm^2$, MG > 40 mm Hg – the stenosis can be determined as severe regardless of whether LVEF and flow are normal or decreased.

2. Low-flow, low-gradient AS with decreased EF – EOA < 1.0 $cm^2$, MG < 40 mm Hg, EF < 50%, indexed stroke volume (SVI) ≤35mL/$m^2$.

In this case, a dobutamine test with a low dose of dobutamine is recommended in order to differentiate a true-severe from pseudo-severe AS, which was defined as increasing the EOA > 1.0 $cm^2$ along with normalization of flow. The presence of flow reserve (contractility reserve, increasing the stroke volume >20%) is of prognostic importance, because it is associated with a better long-term prognosis.[16]

3. Low-flow, low-gradient AS with pEF called paradoxical low-flow – EOA < 1 $cm^2$, MG < 40 mm Hg, EF ≥ 50%, SVI ≤ 35 mL/$m^2$.

This occurs typically in elderly patients, it is associated with small size of the LV and marked LVH and often with a history of arterial hypertension.[17]

The diagnosis of SAS in this case requires ruling out measurement errors and other reasons for such an echocardiographic image. The degree of calcification assessed by means of multi-slice computed tomography (MSCT) is associated with the severity of stenosis and with prognosis.[18] Therefore, the evaluation of this parameter is of increasing importance in such cases.

4. Normal-flow, low-gradient AS with pEF, EOA < 1 $cm^2$, MG < 40 mm Hg, EF ≥ 50%, and SVI > 35 mL/$m^2$. Such patients generally present with moderate grade AS.

The second step in the echocardiographic assessment of AS is the assessment of its impact on the geometry and function of the LV and other heart structures. Linear LV dimensions measurements should be performed in all patients based on recommendations for further estimation of LV mass and RWT in order to classify the type of LV remodeling (Fig. 2).

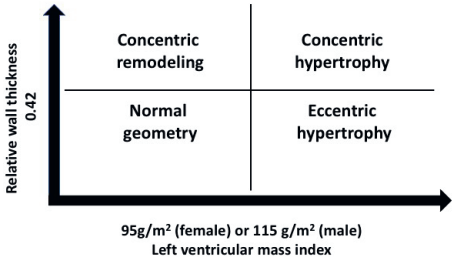


Fig. 2. Left ventricular measurements of left ventricular mass index and RTW

The estimation of the LV systolic function can be obtained with measuring the endocardial movement during the LV systole and diastole. Parameters – shortening fraction (SF) and EF – are calculated from the obtained parameters. Unfortunately, in the presence of concentric muscle hypertrophy (LVH) which we find in AS, there is often an overestimation of the systolic function. Another important parameter is mitral annular plane systolic excursion (MAPSE), which reflects the global LV longitudinal systolic function and is a sensitive index of systolic dysfunction comparable to LVEF. The MAPSE value decreases with the increase in stenosis severity, regardless of LVH, indicating a direct association with the increase in hemodynamic load in AS. A decrease in MAPSE is associated with an increase in subendocardial fibrosis. The cut-off point for the MAPSE value is 9 mm and is of high value in the differentiation between SAS and moderate AS.[19] The exact calculation of LV stroke volume (using LV outflow tract velocity time integral and diameter) must be included in the echocardiographic evaluation of AS patients, especially in patients with SAS (AV calculation <1.0 $cm^2$), pEF (>50%) and a low mean transvalvular gradient (MG < 40 mm Hg). A cut-off points value <35 mL/$m^2$ is an essential criterion for the diagnosis of paradoxical low-flow AS.[20]

Left ventricular ejection fraction below 50% is the only parameter of systolic LV function which obliges us to intervene in patients with SAS. But it is not a good parameter of myocardial contractility, because it is mainly determined by the radial function which can be normal for a long time, even in the presence of subendocardial fibrosis. At this point, we should remember about the structure

of the LV muscle. It has a double helical structure. Subepicardial fibers are going circumferentially, in the mid-wall part in radial and subendocardial in longitudinal direction. The longitudinal and circumferential fibers shorten, and the radial fibers thicken during systole.

Nowadays, it is recognized that LVEF is not a sensitive marker of myocardial dysfunction, and an impairment in EF is often a late manifestation that may not be reversible.[21] In symptomatic SAS, AVR is beneficial for improving their cardiac function. However, some patients do not experience cardiac function improvement after a successful AVR, and some of them even experience deterioration. Therefore, there is a great need to introduce a method that specifically evaluates the complex function of the heart muscle, which will allow patients to be qualified earlier for AVR surgery and will improve long-term prognosis. This role is attributed to tissue Doppler imaging (TDI) and two-dimensional STE (2D-STE). The introduction of 3D echo imaging increases the diagnostic possibilities to an even greater extent.

## Tissue Doppler imaging

Tissue Doppler imaging (TDI) is used to assess global and segmental LV systolic function. The determination of tissue movement in TDI is based on the same principles as the calculation of the flowing blood velocity in pulse wave Doppler or color Doppler. The use of TDI supported by numerous clinical trials allowed detection of changes in LV function in patients with SAS and pLVEF. Peak annular systolic velocities (S') were significantly reduced in patients with SAS and pLVEF.[22] Reductions of early diastolic mitral annular velocity (e') and higher values of E/E' ratio were found in asymptomatic patients with moderate AS, indicating an early impairment of diastolic function.[23] The prognostic value of TDI (S', E' and A' velocities) was evaluated in 183 asymptomatic patients with SAS and pLVEF. Patients who developed the symptoms had lower S' values, suggesting subclinical LV dysfunction compared to asymptomatic patients (6.6 cm/s vs 7.2 cm/s, p = 0.03).[24]

Novel myocardial deformation imaging (strain and strain rate and torsion) using STE has been proposed as a sensitive approach of assessing intrinsic myocardial contractility.

## 2D speckle tracking echocardiography

The 2D-STE technique is a relatively new method for assessing myocardial function. It allows the assessment of the global and segmental function of the heart in an objective, quantitative manner, independent from the angle of analysis. Interactions between the ultrasound beam and muscle fibers smaller than the ultrasonic wavelength are the reason for the heterogeneity of the grayscale echocardiographic image. The resulting point grains (speckles) are specific acoustic markers. The STE is based on the analysis of the change in their position in two-dimensional high-resolution echocardiographic images in the cardiac cycle. By tracking the displacement of acoustic markers using modern algorithms, one can obtain data on the velocity and the value of displacement, the strain rate and the strain of myocardium in 3 special directions: longitudinal, radial and circumferential, as well as on the LV rotation.[25]

The parameters assessed using STE include:

1. Strain – a measurement determining the degree of deformation of the analyzed myocardium area in relation to its initial position. It is expressed as a percentage. It is assumed to take negative values when the strain is shortening or thinning and positive when the strain consists in lengthening or thickening.

Types of strains:

A. Longitudinal strain (LS) – myocardial deformation directed from the base to the apex of the heart, determined in apical 3, 4.2-chamber views. In a healthy heart, in systole the longitudinal dimension of the LV is shortened, hence the negative values of longitudinal strain. Longitudinal strain allows data to be obtained on segmental and global LV function (global longitudinal strain – GLPS). Topographic presentation of peak longitudinal strain values is possible in the form of a planar map for 17 segments of the LV (bull eye) (Fig. 3).

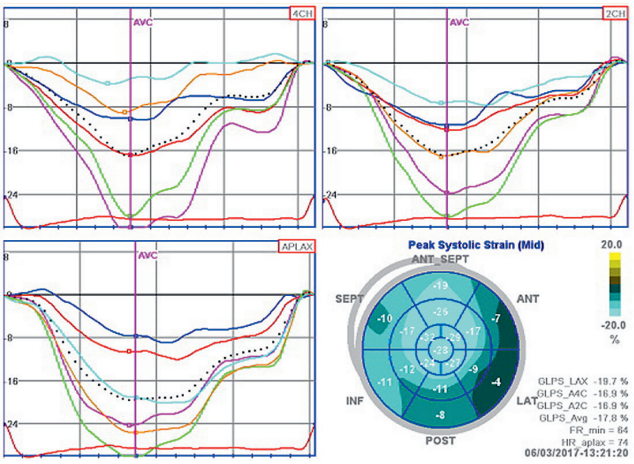


Fig. 3. Left ventricular longitudinal strain measured using STE in SAS

B. Radial strain (RS) – myocardial deformation directed to the center of the LV cavity evaluated in parasternal short axis views. In systole, the myocardium thickens; hence, the radial strain takes positive values in this phase of the cycle.

C. Circumferential strain (CS) – myocardial deformation consisting in the shortening of muscle fibers in a circular dimension leading to a reduction of the LV circumference. It is estimated in parasternal short axis views. It takes negative values.

2. Strain rate 1/s (SR) – a parameter showing the rate of the myocardium strain. It is calculated from the quotient of the velocity difference of 2 points in the area under examination and the distance between these points.

Using the algorithms based on acoustic marker tracking, it is also possible to analyze the LV rotatory mechanics. The LV rotatory strain is shown by:

1. Rotation (R) is a parameter describing the LV rotatory movement in the short-axis cross-section around a point located in the center of the mass of the LV. It assumes the value of the angle by which the myocardium deviates in the cross-section of interest and in a specific phase of the cardiac cycle in relation to its initial value. The rotational movement of the apex and base of the heart takes place in opposite directions. The apex rotates counterclockwise and the angle of rotation takes positive values. The base of the heart rotates in the clockwise direction and takes negative values.

2. Left ventricular systolic twist (Twist – in degrees) – the absolute difference between the maximal value of rotation at the level of the apex (Ar) and the maximal value of rotation at the level of the heart base (Br) (Fig. 4).

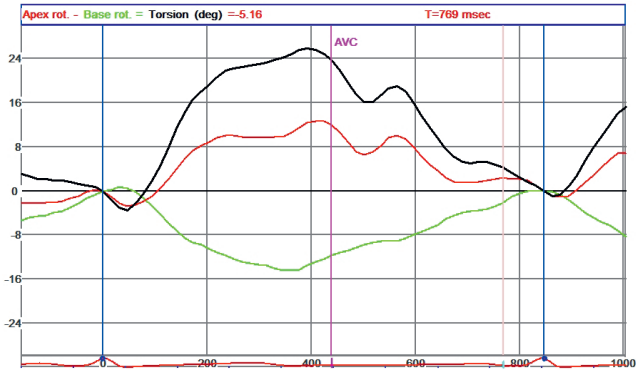


Fig. 4. Rotation and twist measured using STE in SAS

3. Systolic twist standardized by the LV longitudinal dimension (Torsion – in °/cm) – the quotient of LV systolic twisting and the mean value of LV longitudinal dimension of the LV in diastole – the distance between the apex and the base of the heart in 4 and 2 chamber views.

4. Diastolic LV torsional deformation (Untwist – in degrees) – the absolute difference between the values of LV torsion at the apex and at the base of the heart in diastole. In the early phase of diastole, the potential energy accumulated in the heart interstitium at the active stage of contraction is released and the myocardium passively deforms at individual levels in directions opposite to those observed in the phase of the systole.

5. Post-systolic shortening (PSS) is the difference between the maximum value of shortening during the entire cardiac cycle and the shortening values at the aortic valve close (AVC) point. It is presented in the form of the post-systolic index (PSI), which is the quotient of the PSS and the maximum value of shortening during the cardiac cycle, expressed as a percentage.

The results from clinical studies using 2D-STE in patients with SAS and pLVEF confirmed a significant decrease in LV longitudinal strain mainly in basal segments and showed its impact on exercise capacity and pure prognosis in asymptomatic patients. In these patients, more cardiac events and abnormal exercise responses were observed with a sensitivity of 68% and a specificity of 77%.[26] The measurements of longitudinal strain are not only related to the severity of AS but also to the type of LV remodeling, with lower values in patients with higher LV mass and RWT.[27] Ng et al. have showed that along the progression of AS severity there is a progressive impairment of strain in all kinds of muscle fibers ($p < 0.001$).[28] Decreased global longitudinal strain in AS groups was also reported by Miyazaki et al.[29] and Delgado et al.[30] Galli et al. were studying the influence of increasing valvular-arterial impedance (ZVa) on the longitudinal, circumferential and radial components of LV mechanics in patients with AS. The ZVa is a recently introduced parameter which permits the evaluation of global LV afterload in AS. It was calculated as the sum of the systolic arterial pressure and the mean transvalvular pressure gradient divided by SVI. They showed that global longitudinal strain (GLS) and global circumferential strain (GCS) are often reduced in the presence of increased ZVa. Global radial strain (GRS) was significantly altered only in patients with the highest ZVa. This means that mid-wall radial fibers are far less sensitive to LV afterload with respect to subendocardial longitudinal fibers. The radial fibers compensate for the reduction in longitudinal and CS, which contributes to maintaining the global LV function. When the global LV dysfunction is set, GRS significantly decreases and becomes strongly influenced by LV afterload.[31] Donal et al.[32] made the same observations in an animal model. The chronic exposure to a high global LV load developed the impairment of myocardial contractile function. Marechaux et al.[33] have shown a negative correlation between ZVa and GLS ($r = -0.41$, $p < 0.0001$).

The value of LV twist depends to a large extent on preload (it increases with preload increase) and afterload (it decreases with afterload increase as in AS).[34] Disturbed values of the torsion rate have been shown in clinical trials in patients with LV diastolic dysfunction secondary to AS.[35] The PPS parameter has also been proven to be a sensitive but nonspecific index of LV muscle dysfunction.[36] Stress testing (the low dobutamine or exercise stress tests) is recommended for asymptomatic patients with SAS, especially for the differentiation of true-severe from pseudo-severe AS. Global 2D LS has the big possibility to provide more accurate information about LV function during stress testing.[37] Donal et al.[38] and Lafitte et al.[26] have both showed that lower LS was detected in AS patients with the positive exercise test than in AS patients with the negative test. The assessment of LV contractile reserve

using the low dobutamine stress test has clear prognostic implication in patients with low-flow low-gradient AS and reduced LVEF. A decrease or limited increase in LVEF during exercise is associated with a markedly reduced midterm cardiac event-free survival.[39] Global longitudinal strain during exercise allows more precise detection of latent LV systolic dysfunction than LVEF.

The elevated LV afterload can induce alterations in torsion. Patients with AS manifested increased apical rotation (13° ±5.8° vs 7.7° ±2.6°, p < 0.001) and twist (19.7° ±5.7° vs 12.9° ±3.2°, p < 0.001), compared to a healthy group, while basal rotation remained normal.[40] Popescu et al. and van Dalen et al.[41] have had similar observations.

Carasso et al.[42] have demonstrated the differential mechanics between compensation and decompensation of AS using strain imaging. Compensatory LV showed an increase in the apical rotation angle in patients with >50% LVEF and high CS in patients with pLVEF, whereas decompensation showed a decrease in CS and the rotation angle.

## Prognostic value of echocardiographic parameters in aortic stenosis

The management of patients with asymptomatic SAS was also observed by Lech et al. They have found an increase in GLS during the exercise test in patients with SAS, but smaller than in the control group. The observation group was small (50 points) and further studies are needed. The authors have indicated a significant role of the relationship between the LV mass and GLS. A degree of myocardial hypertrophy could be a stronger argument for qualifying asymptomatic patients for surgical treatment. This requires further research.[43] In their latest research, Ng et al.[44] have presented the largest study to date, which included 688 AS patients, of which 294 had SAS. They observed patients with a wide range of LVEF and AS severity. The conclusions are the following: the LV GLS is an independent and superior prognosticator compared with LVEF in patients with AS. Severe AS patients with normal LVEF may present evidence of myocardial dysfunction and have an increased mortality risk, similar to that of SAS patients with impaired LVEF (log-rank p = 0.34). Therefore, LV GLS can further stratify risk in SAS patients and may influence the optimal timing of AVR. The prognostic value of LV GLS was confirmed by Kusunose et al. in a big group of AS patients (n = 395), mean age 70 ±14 years, 57% men, EOA < 1.3 $cm^2$ and pLVEF. The cut-off value of LV GLS > –12.1% was independently connected with increased mortality.[45] The patients with inconsistent grades of AS are very often asymptomatic. Lancellotti et al. have proposed a risk stratification model founded on the characteristic of flow and gradient. The patients with low-flow, low-gradient SAS have the lowest cardiac event-free survival compared with patients with low-flow, high-gradient SAS. The patients with normal-flow, high-gradient and normal-flow, low-gradient SAS demonstrate a higher frequency of survival.[46]

Myocardial deformation improved progressively after a successful AVR.[47] In a study by Rosa et al. it was indicated that there was no significant improvement in the strain observed 1 week after AVR along with unchanged LVEF; a longer follow-up still showed increased deformation 6 months after AVR.[48] Carasso et al. have showed that longitudinal systolic strain increased from –12.8% ±1.7% to –15.9% ±2.2%, whereas mid-LV circumferential strain decreased from –27.0% ±5.1% to –22.3% ±4.9% at an early follow-up post AVR (7 ±3 days) in AS patients with pLVEF. In another study, Carasso et al. have found in research on AS patients with EF < 50% that the circumferential strain that was decreased before AVR had a significant improvement after AVR.[49] A twist and apical rotation increased in compensated patients and normalized after AVR. Normalization of twist in decompensated patients after AVR was uncertain.

In echocardiographic imaging, we can also use the 3D STE test, which allows simultaneous imaging of strain in 3 perpendicular directions, thus giving us the unique possibility of performing a simultaneous quantitative analysis of strain in the longitudinal and circumferential directions and obtaining the so-called area strain. The advantage of 3D STE over 2D STE is the ability to obtain LV strain values based on data from 1 cardiac cycle. The main limitation of 3D STE, however, is the insufficient quality of echocardiographic images, from which 3D reconstructions are created. Others include a worse volume rate than in the case of 2D STE, and a lower level of validation and of clinical usefulness assessment.[50]

## Conclusions and perspectives

The consequences of increased afterload on LV should always be taken into account for the comprehensive assessment of patients with AS. Besides the conventional assessment of LV mass and EF, the measurements of LV strain (GLS) and myocardial fibrosis (when needed estimated by CMR) will probably be increasingly used in the decision-making process in patients with AS.

Echocardiographic parameters of longitudinal function are strongly linked to the extent of myocardial fibrosis, which has clear prognostic implications. Mid-wall myocardial fibrosis was associated with an 8-fold increase in all-causes mortality in patients with significant AS, while focal fibrosis was an independent predictor of increased preoperative risk and mortality in patients with AS undergoing surgical AVR.[51,52]

Therefore, the echocardiographic assessment of LV LS allows for the estimation of LV subendocardial fibrotic changes and becomes a tool for risk stratification in patients

with SAS. Nowadays the clinical utility of GLS is hindered by the lack of standardization on different echo cardiographic machines and the lack of specific cut-off values.

Exercise echocardiography with the application of GLS may provide the incremental prognostic value by assessing both exercise-induced symptoms and changes in valve hemodynamics, LV function and pulmonary pressure. This approach can aid in timing the intervention in asymptomatic patients with SAS and stratify risk in patients undergoing AVR.

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