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Study of antinociceptive effect of ketamine in acute and neuropathic pain models in rats

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Background. Glutamate N-methyl-D-aspartate (NMDA) receptors are known for their importance in the perseverance of chronic neuropathic pain. Ketamine, an intravenous anesthetic agent, is a non-competitive blocker of NMDA receptors. Applied in anesthetic doses, ketamine has anti-nociceptive effects in various animal pain models.

Objectives. The objective of this study was to investigate the anti-nociceptive effect of ketamine in acute and neuropathic pain models in rats.

Material and methods. To study the anti-nociceptive effect of ketamine on acute pain, 40 Wistar rats were divided into 5 groups (n = 8): control, positive control group and 3 experimental groups treated intraperitoneally (ip.) with 30 mg/kg bw, 40 mg/kg bw and 50 mg/kg bw ketamine, respectively. The anti-nociceptive effect was evaluated in hot plate, analgesy-meter and formalin tests. The model of neuropathic pain was induced by left sciatic nerve ligation. Twenty-four Wistar rats were divided into 3 groups (n = 8): sham-control group, model group and ketamine-treated group subsequently tested in hot plate and analgesy-meter tests.

Results. In the hot plate test, the rats treated with ketamine presented increased reaction latency at the 120th min and 180th min compared to the controls. In the analgesy-meter test, ketamine produced an anti-nociceptive effect at the 60th min compared to the controls. In the formalin test, the paw licking time across the early phase of testing was reduced in the rats treated with the 2 higher doses of ketamine. In a neuropathic pain model, ketamine increased the reaction latency at the 120th min and 180th min compared with the model group in the hot plate test. In the analgesy-meter test, in the ketamine-treated animals the paw withdrawal threshold increased at the 60th min compared with the model group.

Conclusions. Our results suggest that ketamine produces peripheral anti-nociceptive effect in an acute pain model. Also, it relieves thermal and mechanical allodynia after 14 days of treatment in a neuropathic pain model.

Key words: ketamine, rats, acute pain, neuropathic pain

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Pain is a subjectively unpleasant sensory and emotional experience caused by actual or potential tissue damage. It is classified by different criteria: duration, pathophysiology and anatomical localization. Depending on its duration, pain can be classified as acute or chronic. According to the pathophysiological mechanisms that cause it, pain could be nociceptive or neuropathic.[1]

Pain management is of great importance in clinical practice. Conditions involving acute or chronic pain account for a large proportion of presentations to the family general practitioner and in the emergency ward. There is evidence of the critical role of the excitatory neurotransmitter glutamate in the development and perseverance of chronic neuropathic pain.[2] Glutamate acts pre- and postsynaptically through the activation of diverse receptors: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA), and metabotropic receptors.

Ketamine, an intravenous anesthetic agent, is a non-competitive blocker of NMDA receptors.[3] Ketamine produces anti-inflammatory, antioxidant and immunosuppressive effects. The mechanism of action of ketamine has been associated with binding to the phencyclidine site in the glutamate receptor.[4] It affects opioid, dopaminergic and cholinergic transmission.[5] The effect on the glutamate mediation determines its analgesic, dissociative and neuroprotective effects, and by acting on opioid receptors, ketamine produces dysphoric effects. The sympathomimetic action is mediated by the response of the central and peripheral monoaminergic systems. Blocking the central and peripheral cholinergic mediation contributes to the anesthetic and psychomimetic action.[6] Ketamine has been shown to antagonize the effects of dopamine on dopamine type 2 (D2) receptors.[7] Ketamine supplied in anesthetic doses has neuroprotective effects in various animal models of brain injury. This effect is caused by a blockage of NMDA receptors and reduced influx of Ca^{2+} ions through the receptor channels.[8]

Chronic pain is caused by tissue damage that continues after the lesion has healed. For this reason, it is not caused by the activation of nociceptors, has no protective effect and lowers the quality of life of patients.

Neuropathic pain has been described as "the most terrible of all tortures which a nerve wound may inflict". The damaged nerve fibers in neuropathic pain send incorrect signals to the pain centers. Neuropathic pain is characterized by sensory abnormalities such as an unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia) and pain in response to a stimulus that does not normally provoke pain (allodynia).

The present study aims to investigate the anti-nociceptive effect of ketamine in acute and neuropathic pain models in rats.

Material and methods

Animals

Male Wistar rats with an average weight between 200 g and 220 g were used. The animals were housed under standard laboratory conditions: a 12:12 h light/dark cycle, room temperature of 26 ±1°C, and free access to food and water. After surgery, the animals were housed separately. The experiments were performed after the protocol was approved by the Animal Ethics Committee of the Medical University of Plovdiv (Bulgaria) and Bulgarian Food Safety Agency, and in compliance with the European Directive 2010/63/EU.

Experimental design

The anti-nociceptive effect of repeated doses of ketamine was studied in the 1st series of experiments. The animals were randomized into 5 groups (n = 8). The control group received only saline, the positive control group was treated with metamizole 150 mg/kg bw, and the 3 experimental groups were treated with ketamine in doses of 30 mg/kg bw, 40 mg/kg bw and 50 mg/kg bw, respectively. All agents were dissolved in saline as a vehicle and injected intraperitoneally (ip.) for 14 days. Three nociceptive tests were used: hot plate test, analgesy-meter and formalin test.

The effect of repeated doses of ketamine on the neuropathic pain model was studied in the 2nd series of experiments. A chronic constriction injury of the sciatic nerve model was used. After the operation, the animals were randomly allocated to receive ip. saline (model group) or ketamine 50 mg/kg bw. To study the analgesic effect of ketamine in neuropathic pain, hot plate and analgesy-meter tests were used.

Hot plate test

The original hot plate set up (Ugo Basile S.R.L., Germonio, Italy) was used. The temperature of the hot plate surface was set to 55 ±0.5°C. The reaction latency as well as the time between when the animals were placed and the onset of paw-licking or jumping behaviors were measured in seconds. To minimize tissue damage, a cut-off time of 30 s was adopted.

Analgesy-meter test

Mechanical nociceptive testing was conducted using an analgesy-meter (Ugo Basile). The rats were tested at the 60th min, 120th min and 180th min after ip. administration of the test substance. The force of the pressure was calibrated at 10 g/cm at a max force of 250 g. The force of the pressure at which the animal withdrew the test paw was expressed in conditional units (cm). The maximum possible pressure was 25 cm.

Formalin test

In this method, formalin (0.1 mL, 2%) was injected into the plantar surface of the left hind paw. Each animal was placed in an observation chamber and the nociceptive response was recorded for a period of 30 min. The sum of time (in seconds) spent licking and biting the injected paw in the first 10 min is known as the early or acute phase. The period between 20–30 min is considered the late or chronic phase.

Chronic pain model

The model of neuropathic pain was designed according to the model of chronic constriction injury (CCI) of the sciatic nerve described by Bennett and Xie.[9] The animals were anesthetized with pentobarbital sodium (50 mg/kg bw, ip.), which provided anesthesia for 20–40 min. The left sciatic nerve was found deep within the biceps femoral muscle. The nerve was gently removed from the surrounding tissues and 2 ligatures 2 mm apart were made. The sham control was subjected to identical left foot dissection without ligation of the sciatic nerve. After the operation, the animals were allowed to recover for 1 week.

Statistical analysis

The data obtained from the nociceptive tests was statistically processed with SPSS v. 17.0 (IBM Corp., Armonk, USA). Results were represented as the mean ± standard error of the mean (SEM). Comparison of the results for each score to the respective control group was performed using an independent sample t-test. A value of $p < 0.05$ was considered statistically significant. The Shapiro-Wilk test was applied as a normality test. In distributions different from normal, the Mann-Whitney U test was used for inter-group evaluation.

Results

Acute pain model

Effects on thermal nociception

The average latency of the controls in the hot plate test was between 7 s and 10 s. The metamizole-treated rats significantly ($p < 0.05$) increased the reaction latency at the 120th min and the 180th min compared with the control group. The animals treated with ketamine in all doses showed an increase in latency at the 120th min and 180th min ($p < 0.05$) compared with the respective day controls (Fig. 1).

Effects on mechanical nociception

In the analgesy-meter test, the control animals' average pressure reaction was between 7 cm and 9 cm. The animals treated with metamizole showed a significant increase ($p < 0.05$) in latency at the 60th min, 120th min and 180th min compared with the controls. The lower doses of ketamine of 30 mg/kg bw and 40 mg/kg bw did not exert significant changes on the reaction latency. The rats treated with ketamine in a dose of 50 mg/kg bw increased the paw withdrawal threshold at the 60th min ($p < 0.05$) compared with the control group (Fig. 2).

Effects on chemical nociception

In the controls, the duration of paw licking across the early phase was 35.37 ±4.02 s, and 47.75 ±6.3 s across the late phase. The paw licking time did not decrease in the metamizole-treated group during the 2 phases of the test compared to the control group. In the group treated with ketamine in a dose of 30 mg/kg bw, the time of paw licking did not significantly change in either phase of the formalin test compared with the controls. The paw licking time in the animals treated with the 2 higher doses of ketamine (40 mg/kg bw and 50 mg/kg bw) decreased only across the

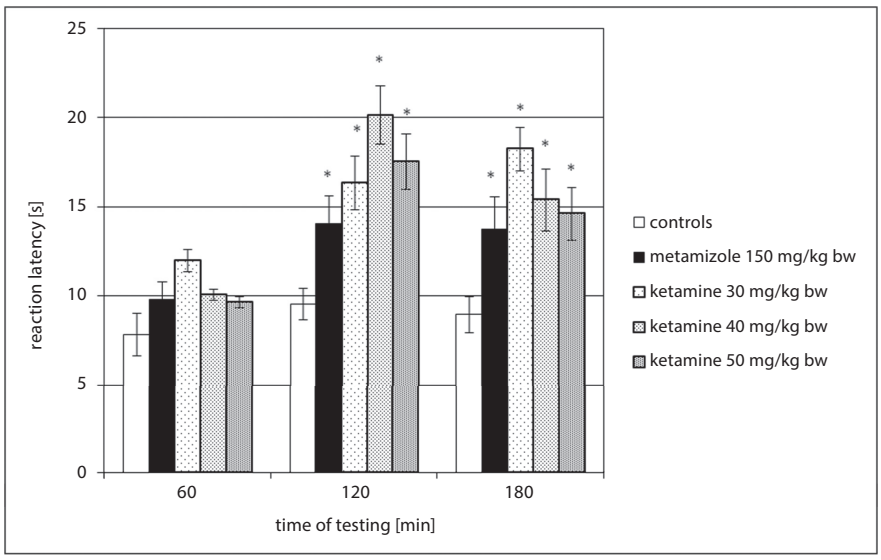

Fig. 1. Anti-nociceptive effect of ketamine in hot-plate test and acute pain model

* $p < 0.05$ compared with the control group.

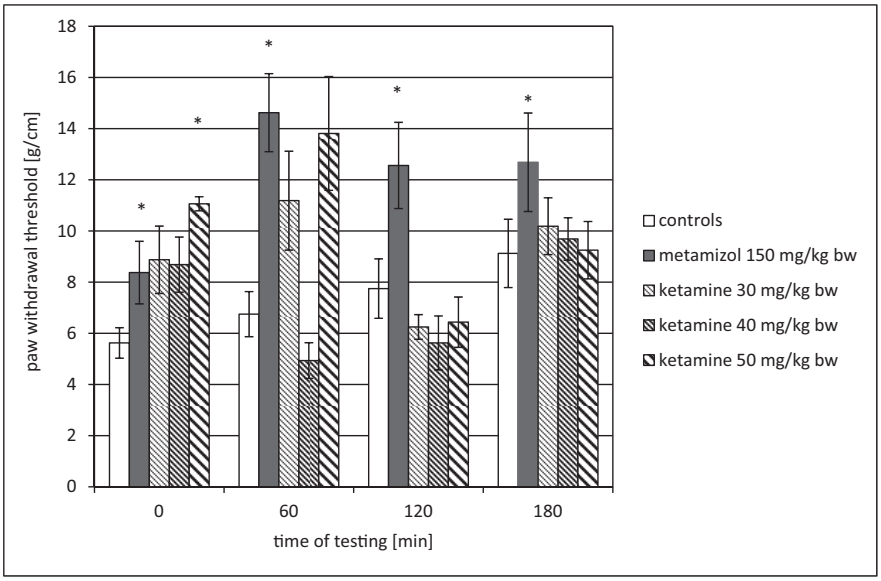

Fig. 2. Anti-nociceptive effect of ketamine in analgesy-meter test and acute pain model

* $p < 0.05$ compared with the control group.

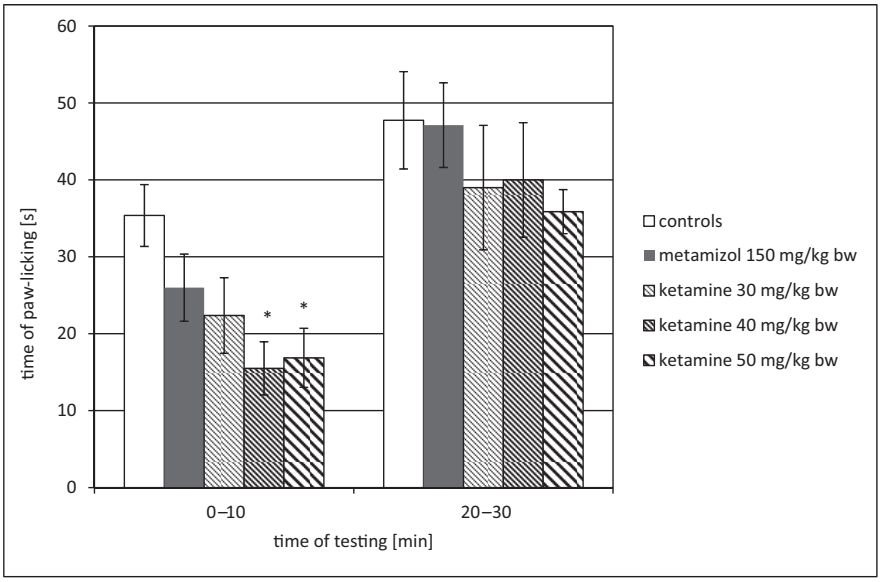

Fig. 3. Anti-nociceptive effect of ketamine in formalin test

* $p < 0.01$ compared with the control group.

early phase of testing ($p < 0.01$) compared with the control group (Fig. 3).

Neuropathic pain model

Effects on thermal nociception

In the hot plate test, the average latency of the sham control was between 14 s and 18 s. The model group significantly increased the latency time at the 60th min ($p < 0.01$) compared to the sham control. The animals treated with ketamine at a dose of 50 mg/kg bw significantly increased latency at the 120th min and 180th min ($p < 0.05$) compared to the model group (Fig. 4).

Effects on mechanical nociception

In the mechanical paw pressure test, the average latency of the sham control was between 9 cm and 12 cm. The model group did not show a significant increase in the pressure reaction as compared to the sham control. The group treated with ketamine 50 mg/kg bw showed increased ($p < 0.05$) latency of pressure reaction at the 60th min as compared to the model group (Fig. 5).

Discussion

The present study showed that ip. administration of ketamine produced an anti-nociceptive effect in models of acute and neuropathic pain. Ketamine exhibited more potent activity in regard to inhibiting thermal nociception (allodynia) than mechanical nociception. Thus, we assume that systemic ketamine does have an analgesic effect in normal and neuropathic pain conditions.

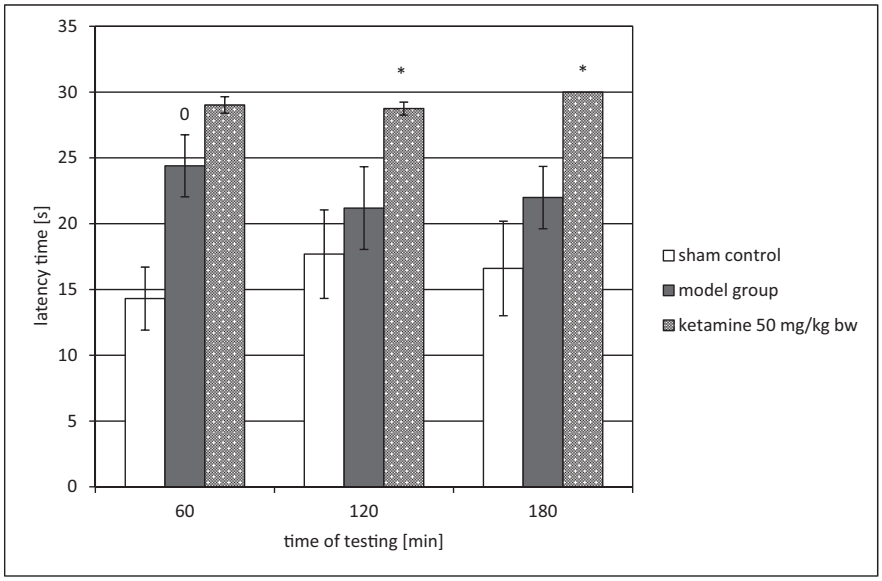

Fig. 4. Anti-nociceptive effect of ketamine in hot-plate test and neuropathic pain model

[0] $p < 0.01$ compared with sham control; * $p < 0.05$ compared with the model group.

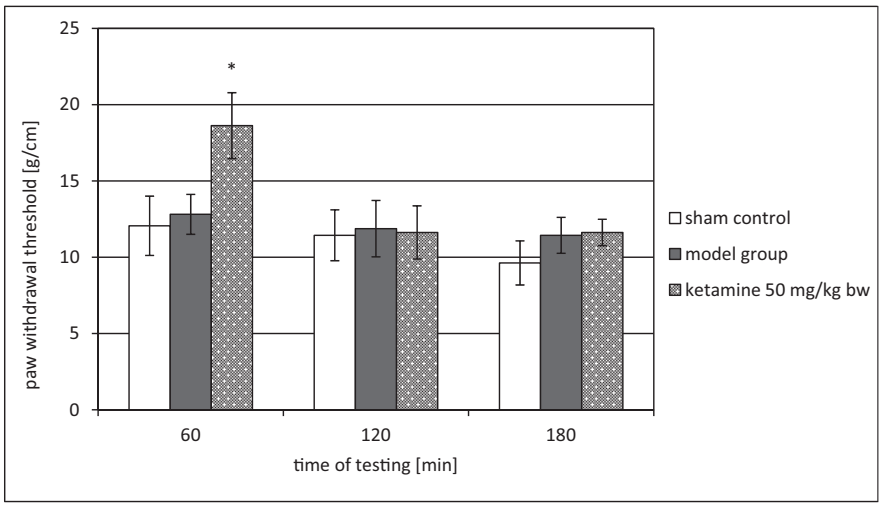

Fig. 5. Anti-nociceptive effect of ketamine in analgesy-meter test and neuropathic pain model

* $p < 0.05$ compared with the model group.

Anti-nociceptive effect of ketamine on acute pain

In the present study, all doses of ketamine showed a significant anti-nociceptive effect in the hot plate test. Koizuka et al. found that in normal rats ketamine produces anti-nociceptive effects through the activation of monoaminergic descending inhibitory pathways for acute thermal pain.[10] Our findings are in agreement with those of other authors, who reported a well-defined anti-nociceptive effect of ketamine in other nociceptive test with thermal stimulus – infrared rays.[11] We also observed that the highest dose of systemic ketamine produced early anti-nociception in the acute pain model with mechanical stimulus. The results of the present study are consistent with previously reported results.[12] This effect has been reported at doses lower than ours – 7.5 mg/kg bw and 10 mg/kg bw single dose, as well as in a model of hyperalgesia caused by intraplantarly injected prostaglandin E2. The analgesic effect of ketamine was found in postoperative pain in a number of clinical and experimental studies.[13]

The nociception response in the formalin test is biphasic. In the initial acute period of about 10 min (phase 1), the registered behavioral changes were due to axially activated primary afferent nerve terminals. These changes are mediated by activation of the transient receptor potential ankyrin 1 (TRPA1) channels.[14] The 2nd phase of the formalin test reflects the central sensitization of neurons in the dorsal horn and peripheral sensitization of nociceptors by formalin-induced local inflammatory response.[15] Our results indicate that ip. treatment with higher doses of ketamine significantly reduced nociceptive scores in the early phase of the formalin test. In contrast, Bulutcu et al. found a significant anti-nociceptive effect in the single systemic intrathecal administration of ketamine only during the late phase of the formalin test.[12] Perhaps this difference is due to the different route of administration of ketamine. Our results allow the conclusion that ketamine can be a reliable

analgesic in pain caused by chemical agents immediately after the onset of the damage rather than at a later stage in the development of the pain response.

Anti-nociceptive effect of ketamine on neuropathic pain

The CCI of the sciatic nerve is associated with intraneural edema, focal ischemia and Wallerian degeneration. Behavioral changes, like mechanical and thermal hyperalgesia, chemical hyper-reactivity and cold allodynia, are likely to develop within a week.[17]

Traumatic injury of the sciatic nerve in rats is a commonly used model for the study of peripheral nerve damage. There is data in the literature regarding the involvement of NMDA receptors in the pathogenesis of pain in peripheral tissues or nerves.[18,19] These receptors are found in the peripheral myelinated and unmyelinated somatic nerve fibers, spinal cord and cerebellum.[20] They play a role in the process of central sensitization. Accordingly, NMDA receptor antagonists such as ketamine effectively reduce pain responses in animal models and influence pain in clinical trials.[19,21]

In the model of neuropathic pain, ketamine at the studied dose showed an anti-nociceptive effect in the "hot plate" test. Burton et al. found that ketamine administration resulted in a sustained delay in the development of allodynia associated with spinal nerve damage in rats.[22] Other authors have found that the introduction of the short-acting NMDA antagonist reduces the development of opiate tolerance, a condition associated with the development of NMDA receptor hyperalgesia.[23] In experimental models, intrathecal or systemic administration of ketamine reduces hyperalgesia and inflammatory disease caused by peripheral nerve damage.[24,25] Other authors have reported that ketamine does not exhibit an anti-nociceptive effect in acute thermal pain in rats when administered intrathecally.[26] The anti-nociceptive effect of ketamine was also found in acute pain observed in thermal pain stimulus applied to the tail.[27] This effect results from a blockage of NMDA receptors and reduced influx of Ca^{2+} ions through the receptor channel.

In the nociceptive test with mechanical paw pressure, ketamine showed an early and short-lasting analgesic effect. Koizuka et al. found that ketamine reduced the hypersensitivity of neurons at an early stage in postoperative pain model in rats.[10] The same authors found that pretreatment with the α2-receptor or serotonin receptor antagonists reduced the effect of ketamine in this model. These results suggest that activation of monoaminergic downstream inhibitory pathways occupies an important place in the mechanisms of analgesic efficacy of ketamine in this pain model.

Mei et al. reported that in a model of neuropathic pain induced by spinal cord ligation, the intrathecal introduction of ketamine reduced mechanical allodynia.[28] These authors observed that the intrathecal injection reduced astrocytic activity in the dorsal neurons in the lumbar spinal cord, as measured by the immunohistochemical method. These results allowed them to conclude that the inhibition of astrocyte activation by blocking NMDA receptors on the astrocyte membrane is probably a new mechanism in the analgesic efficiency of ketamine at the spinal level.

Our results suggest that ketamine showed an anti-nociceptive effect in both the acute pain tests and chronic neuropathic pain model. Various experimental models of peripheral nerve damage have detected the involvement of nociceptive C-fibers in mechanical and thermal hyperalgesia in neuropathic pain. Vogelaar et al. observed reduced mechanical and thermal sensitivity in the damaged area after damage to the sciatic nerve.[17]

Conclusions

In conclusion, the present study revealed that systemic administration of ketamine produced anti-nociception in models of acute pain. Ketamine relieved mechanical and thermal allodynia in a chronic neuropathic pain model. These observations make ketamine an attractive alternative in the treatment of acute and chronic pain.

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Influence of temperature rise by 2.5°C on the increase of apoptosis of HL-60 cells treated with busulfan

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Abstract

Background. Hyperthermia is one of the new and still poorly known methods used in cancer treatment. It consists of raising the patient's body temperature for therapeutic purposes. The article presents the results of in vitro studies describing the effect of an elevated temperature of 39.5°C, the busulfan cytostatic and their combination on the level of apoptosis of human leukemia HL-60 cells.

Objectives. During the experiments, the influence of a 2.5°C temperature increase on the behavior of the population of 2 groups of HL-60 cells, with busulfan cytostatic and without the cytostatic, was investigated. The control group consisted of 2 groups of HL-60 cells incubated at 37.0°C with the cytostatic and without the cytostatic. Two questions were asked: 1. Is low-temperature hyperthermia likely to have an effect on the effectiveness of busulfan cytostatic? 2. Does the increase in temperature by 2.5°C have an effect on the level of apoptosis in the unsaturated HL-60 cell line?

Material and methods. Human promyelocytic leukemia cell line HL-60 was used in the experiments to examine the influence of temperature on apoptosis HL-60 in 2 separated incubators set to 37.0°C and 39.5°C for 3 h. Apoptosis was assessed with flow cytometry using Annexin V.

Results. An increase in mortality of HL-60 cells was found in the case of simultaneous exposure to elevated temperature and busulfan in comparison to the group of cells treated with the cytostatic alone. There was no observed effect of an elevated temperature of 39.5°C alone on the level of HL-60 cell apoptosis.

Conclusions. Analysis of the study results indicates that low-temperature hyperthermia may be used to increase the effectiveness of busulfan treatment. No effect of an elevated temperature of 39.5°C on the level of apoptosis in HL-60 cells that were not treated with busulfan was observed. There is a need to test the efficacy of other cytostatic agents at elevated temperatures.

Key words: apoptosis, in vitro, hyperthermia, busulfan, HL-60 cell line

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Introduction

Due to the steady increase of cancer incidence, new ways of combating this group of diseases are being sought. The ways of fighting cancer can be divided into conventional and modern methods. Conventional methods of treatment include chemotherapy, radiotherapy and surgery. Modern methods of cancer treatment are the following: immunotherapy, gene therapy, hyperthermia, photodynamic therapy, and hormone therapy.[1]

Hyperthermia is one of the new and still poorly known methods used in this field. It consists of raising the body temperature of a person for therapeutic purposes. Hyperthermia can be divided into low- and high-temperature hyperthermia, and due to its scope into regional tumor heating alone and global hyperthermia, which consists of raising the patient's body temperature. Hyperthermia can be obtained using various methods, including radio waves, electromagnetic fields (the capacitance method), infrared radiation, microwaves, and thermal bath.

The electromagnetic wave can cause the body temperature to rise and at the same time can cause other physical processes, e.g., flow of the electrical charges in cancer tissue affected by this physical factor. Therefore, temperature rise is not the only factor influencing the processes taking place in the structures of neoplastic cells.

The question therefore is to what extent the temperature itself influences the apoptosis of cancer cells and what is the role of accompanying physical factors in this process.

To answer the first part of the question, we carried out experiments with a short term (3 h) temperature rise to 39.5°C (caused only by thermal conductivity based on the principle of heat exchange between bodies of different temperatures remaining in direct contact with each other, i.e., on the transfer of kinetic energy of random movement of particles as a result of their collisions, which leads to temperature equalization between bodies) on cultured HL-60 cells. In parallel, the influence of a 2.5°C temperature rise on apoptosis induced by busulfan treatment in relation to the effectiveness of this cytostatic activity at 37.0°C was also assessed.

Material and methods

Cell cultures

Human promyelocytic leukemia cell line HL-60 was used for the experiments. The cells were cultured in a fully humidified CO_2 incubator (NuAire, Plymouth, USA) at 37°C, 5% CO_2, in RPMI1640 cell medium (IITD PAN, Wrocław, Poland) supplemented with 10% fetal calf serum (FCS; Invitrogen/Thermo Fisher Scientific, Warszawa, Poland) and 100 µg/mL of gentamicin (KRKA-Poland, Warszawa, Poland) culture medium. Twenty-four hours before starting the experiment, the cells were fed with an exchange of culture medium and the cell concentration was established at $3–4 \times 10^5$/mL.

To examine the influence of temperature on apoptosis, the HL-60 cells were suspended in fresh medium at a concentration of 2×10^5/mL with and without supplementation of 250 µg/mL of busulfan (Busilvex; Pierre Fabre Médicament, Paris, France), and incubated in 1 mL volume in 75 × 12 mm polystyrene culture tubes (Sarstedt, Warszawa, Poland) in 2 separated incubators set to 37.0°C (Hera Cell 150; Heraeus, Hanau, Germany) and 39.5°C (NuAire DH Autoflow CO_2 Incubator) for 3 h.

Apoptosis measurement

Apoptosis was assessed with flow cytometry using an Annexin V binding test (Annexin V-FITC Apoptosis Detection Kit I; Becton Dickinson-Pharmingen, San Diego, USA), according to the protocol provided by the manufacturer. In brief, HL-60 cells after 3 h of incubation were washed once by centrifugation, suspended in Annexin V binding buffer and incubated for 15 min at room temperature with Annexin V-FITC and propidium iodide (PI) solutions. Apoptosis was measured in a PAS (Partec, Görlitz, Germany) flow cytometer. Fifteen thousand cells were assessed in each measurement. Apoptosis was the sum of cells stained with Annexin V-FITC only (Annexin V+) – early apoptosis – and cells stained both with Annexin V-FITC and PI (AnnexinV+/PI+) – advanced apoptosis.

Statistical analysis

The t-test and confidence intervals (CIs) were calculated using Microsoft Excel 10 (Microsoft Corp., Redmond, USA) to assess the statistical significance of the results obtained. The CIs were determined for the arithmetic mean of the test samples; they were calculated for a confidence level of 0.95. P-values less than 0.05 were considered statistically significant. The study assumes that the general population is normal. Normal decomposition testing was performed using the Shapiro-Wilk test.

Results

In the experiments carried out, the influence of a 2.5°C temperature increase on the behavior of the population of 2 groups of HL-60 cells, with busulfan cytostatic and without the cytostatic, was investigated. The control group consisted of 2 groups of HL-60 cells incubated at 37°C with the cytostatic and without the cytostatic. Two questions were asked:

1. Does the 2.5°C increase in temperature have an effect on the level of apoptosis of cultured HL60 cells?

2. Is low-temperature hyperthermia likely to have an effect on the effectiveness of busulfan cytostatic treatment?

The study was conducted in 2 incubators. In the 1st incubator, the temperature was 37.0°C and in the 2nd one, the cells were subjected to a temperature of 39.5°C. The test time for each of the populations studied was 3 h.

The experiment was repeated 9 times. As the number of repetitions (sample size) was less than 30, normality verification of distribution was performed with the non-parametric Shapiro-Wilk test, which verifies the null hypothesis that the sample originates from normal distribution. At the significance level of 0.05, the null hypothesis was not rejected because the calculated p-value = 0.6079 for the test statistics W = 0.9551 was higher than 0.05. Therefore, there were no grounds for rejecting the hypothesis of normal distribution.

Spontaneous apoptosis of HL-60 cells at 37°C and 39.5°C was less than 3% and did not differ considerably between these 2 groups. Early apoptotic cells (Annexin V+/PI–) consisted of 63% of both apoptotic populations. There was a statistically significant increase of apoptosis (p = 0.0198) in the busulfan-treated population at 39.5°C compared to the busulfan-treated population at 37.0°C. The test results are shown in Table 1 and Fig. 1. The majority of apoptotic cells were in the early phase of apoptosis: 82% at 37.0°C and 88% at 39.5°C, as could be expected from the relatively short exposure time to the drug (3 h).

In the experiments conducted, photographs of particular groups of cells examined were also taken, illustrating their state at the end of the experiment (Fig. 2). No morphological signs of apoptosis were seen in the control populations (Fig. 2A,B), whereas prominent apoptotic bodies were observed in both populations of HL-60 cells exposed to busulfan treatment (Fig. 2C,D).

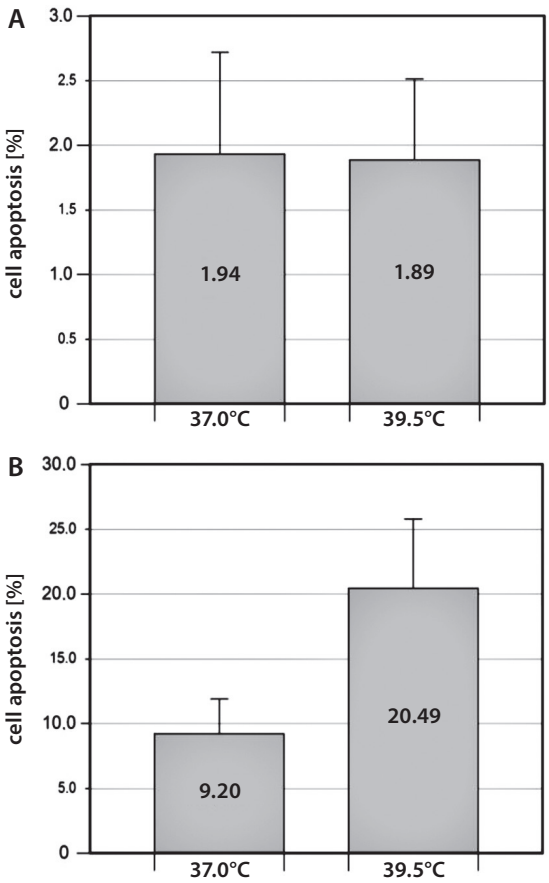

Fig. 1. Graphical illustration of apoptosis level of HL-60 cells subjected to 2.5°C temperature rise in the absence (A) or in the presence (B) of 250 µg/mL busulfan during 3-hour culture. The data on the graph is presented as mean values and confidence intervals

Table 1. Levels of apoptosis of HL-60 cell population as measured using flow cytometry. Values for control groups incubated at 37.0°C and 39.5°C, respectively, and the test groups (exposed to busulfan) at the same temperatures

Parameter	37.0°C – control	39.5°C – control	37.0°C with busulfan	39.5°C with busulfan
n	9			
m [%]	1.94	1.89	9.20	20.49
SD [%]	1.14	0.98	4.57	8.04
CIs [%]	0.62	0.51	2.49	4.38

n – number of experiments in the particular group (sample size); m – average value; SD – standard deviation; CI – confidence interval.

Discussion

The potential of low-temperature hyperthermia in cancer chemotherapy is a new, promising area, bringing hope for new treatment options for this type of disease. The literature analysis suggests that the action of low-temperature hyperthermia may have an impact on the effectiveness of cytostatics.[2] The most invasive form of hyperthermia is the local effect on the patient's body with a very high temperature (>60°C), which can destroy (in a way – to "boil") the cancer, which is called ablation.[3] It has also been shown that the natural reaction of the human body manifested by the rise in temperature is a positive response of the patient's body to the infection. It shortens the duration of the illness and increases the likelihood of survival.[4] Some researchers point to the relationship between fever caused by infection and a simultaneous retreat of cancer.[5]

There are reports that hyperthermia induced by Whole Body Hyperthermia (WBH) fever affects the activation of T lymphocytes.[6,7] It has also been shown that this type of hyperthermia affects the production and secretion of cytokines.[8]

Other researchers have shown that the combination of curcumin and hyperthermia has a beneficial anti-cancer effect and indicated that combination therapy significantly inhibited cell proliferation of MS-1 and LL/2 in vitro. It has also been shown that the combination therapy conducted in this way inhibited tumor growth and lengthened life expectancy.[9] There are also reports from clinical studies that the rise of the temperature in the tumor tissue has positive therapeutic effects (e.g., recurrent breast and melanoma cancer) and increases the chances of survival in cases

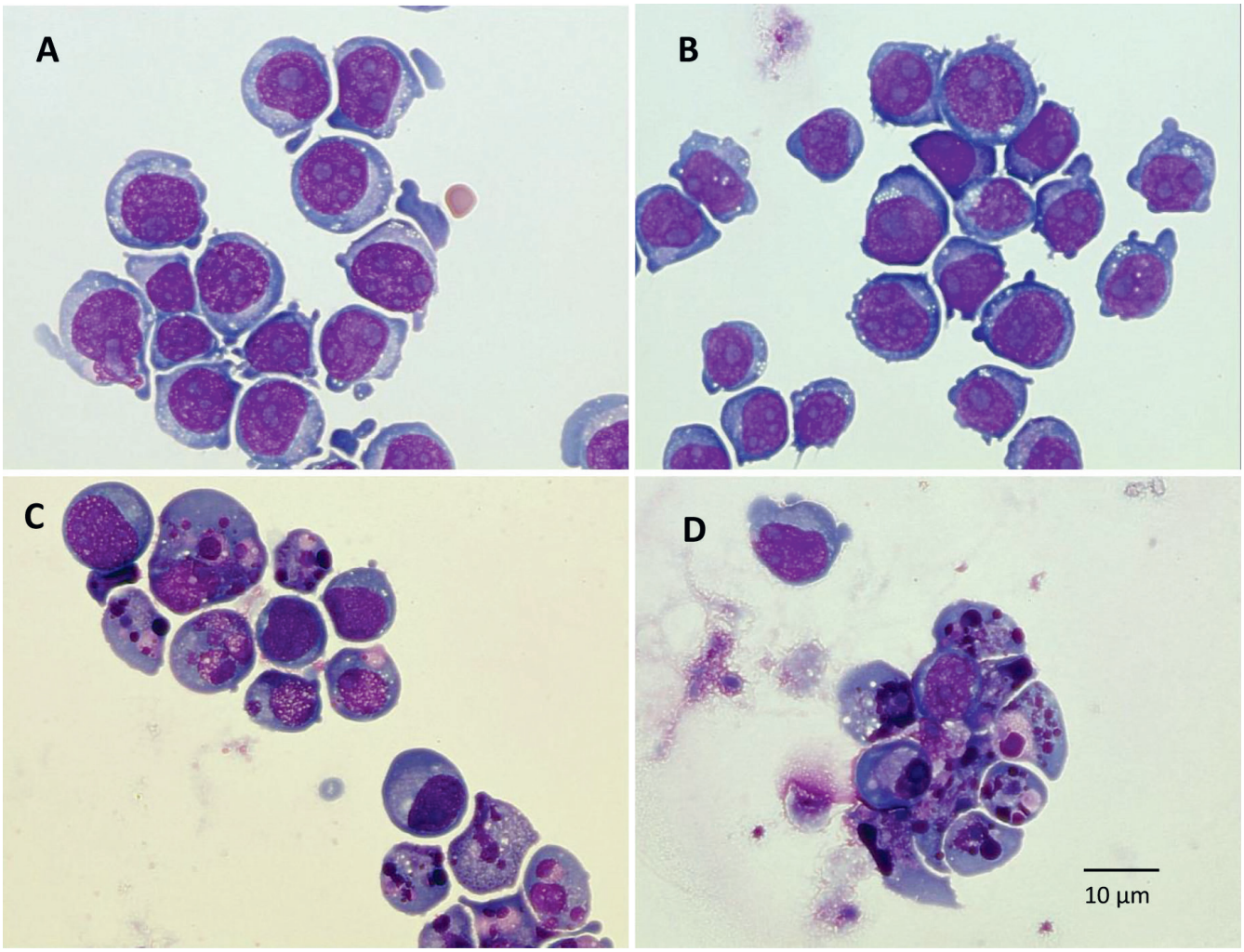

Fig. 2. May-Grünwald-Giemsa-stained HL-60 cells after 3-hour culture without the cytostatic drug at 37.0°C (A) and 39.5°C (B) and cells cultured in the presence of busulfan at the same temperature values (C and D, respectively)

such as metastases to the lymph nodes and neck, glioma and cervical cancer.[2]

It has also been shown that raising the temperature to 39.5°C increases the expression of the Toll-like 4 receptor (TLR4) on human macrophages and causes increased cytokine production.[10] Cancer cells exposed to elevated temperatures between 39.0°C and 45.0°C can be damaged or killed with minimal damage to normal tissues.[11]

The examples presented above show that there is still a need to carry out a number of studies aimed at identifying the most efficient and optimal procedures of dealing with these issues using hyperthermia. This is due to the scarce knowledge of biological mechanisms that cause the potential anticancer effects of hyperthermia. For these reasons, we have carried out studies to observe the effect of 2.5°C elevated temperature on the apoptosis of HL-60 cells treated with busulfan.

Our results showed statistically significant differences in apoptosis level between the control group and test group only in the case of cells subjected to busulfan. HL-60 cells cultured at 39.5°C without the cytostatic showed even a slight decrease of apoptosis in comparison to the control group (but statistically not significant).

The increase in the mean apoptosis level of cells exposed to a temperature of 39.5°C and busulfan was more than 11 percentage points, which means an increase in cell mortality by 223% as compared to the cells incubated at 37.0°C. The results obtained and the analysis of publications in this field suggest that the use of low-temperature hyperthermia in the cytostatic area may increase its effectiveness. For this reason, there is a real need to continue such experiments and to test the effectiveness of other cytostatic agents at elevated temperatures as well as the effects of busulfan at higher temperatures. This research could, in the future, allow the introduction of new, more effective cancer treatment procedures utilizing chemotherapy. A particularly interesting option is the local heating of a tumor, aimed at increasing the local cytotoxicity of applied chemical compounds. This local action may increase the level of apoptosis of tumor cells caused by cytostatic treatment with a relatively minor negative effect on the rest of the human body.

Continuation of research in this direction may show the efficacy of cytostatic drugs as a function of temperature rise, which may influence the determination of new, effective treatment procedures for cancer diseases with these chemical compounds, and the selection of cytostatic concentration applied depending on the current temperature of the patient's body.

Conclusions

The use of low-temperature hyperthermia combined with chemotherapy in cancer treatment is a new area that requires further thorough research. The study confirmed that a 2.5°C temperature increase may be used to improve the effectiveness of busulfan treatment. The same hyperthermia does not influence the apoptosis of cells not exposed to the drug. There is a need to test the efficacy of other cytostatic agents at elevated temperatures, which may influence the determination of new, effective treatment procedures for cancer diseases with these chemical compounds, and the selection of cytostatic concentration applied depending on the current temperature of the patient's body.

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Occurrence of dietary risk factors in inflammatory bowel disease: Influence on the nutritional status of patients in clinical remission

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Abstract

Background. Among the complex factors that may favor the occurrence of inflammatory bowel disease (IBD), genetic, immunological and environmental initiators, including nutritional factors, are listed. So far, there have been no previous studies on the type and frequency of dietary risk factors for IBD in Poland and their effect on the nutritional status of patients.

Objectives. The aim of the study was to assess the influence of the frequency and type of dietary risk factors for IBD on the nutritional status of patients with ulcerative colitis (UC) and Crohn's disease (CD).

Material and methods. In the study, the dietary habits and nutritional status of patients were assessed using the cross-check dietary history method and the Mini Nutritional Assessment (MNA) questionnaire. The study group consisted of 162 IBD patients: 61 individuals with CD and 101 with UC. The data was compared to the results of a control group (129 healthy volunteers).

Results. The results obtained showed that IBD patients during a period of remission disclosed such dietary risk factors as inadequate consumption of fiber and excessive consumption of red meat and meat products, animal fats, and sugars in comparison to the control group. Only low fiber intake was associated with a worse nutritional status of patients with UC. No consistent influence of the number of IBD dietary risk factors on the nutritional status of patients was found.

Conclusions. The nutritional status of IBD patients in remission was related to the type of dietary risk factors, but did not depend on the number of them.

Key words: nutritional status, dietary risk factors, inflammatory bowel disease

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Introduction

Inflammatory bowel disease (IBD) comprises chronic diseases of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC), also called IBD, affect up to 500 per 100,000 people in the Western world. The countries reporting the highest estimates of IBD are the USA, the UK and Sweden, as opposed to the countries of Southern Europe, South Africa and Australia.[1–3] Only a few scattered reports on IBD frequency come from the countries of Eastern and Central Europe.[2,3] An accurate count of the incidence of IBD in Poland has not yet been compiled.[3] Recent studies on the etiology of IBD suggest that these diseases are caused by a combination of genetic, environmental and immunological factors.[4,5] One of the most important environmental factors is nutrition. Many of the recent studies show dietary risk factors which may cause or intensify IBD symptoms. Dietary risk factors include high intake of sugar and sweets, hydrogenated fats, products with a high sulphur content, and a diet poor in fiber, vitamin C and n-3 fatty acids.[6–9] Hypothetically, dietary components could increase the risk of colonic inflammation by several mechanisms, which include a direct contact with the colonic mucosa, affecting the chemical composition of mucosal cell membranes or altering the balance of intestinal flora.[10] Moreover, it has also been suggested that the visceral fat tissue and adipocytokines may play a role in IBD development.[11] Nutritional treatment plays an important role in the clinical care of patients with IBD. Supportive nutritional therapy aims to correct the nutritional status and macronutrient deficiencies and to reverse their metabolic/pathological consequences, in addition to providing advice to patients on recommended dietary regimes. This should be considered in all patients with IBD. It should also be remembered that there is no single, common diet suitable for all IBD patients; each of them is unique and dietary recommendations must be individually developed for each patient, depending on the course of the disease. Studies of exclusive enteral nutrition, exclusion diets, a low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) diet, as well as semi-vegetarian diets suggest that minimizing the exposure of the intestinal lumen to selected foods may prolong the remission period of patients with IBD.[12–14] Poor nutritional status is common in patients with IBD, especially in active CD, and it is a result of reduced dietary intake, maldigestion and malabsorption. Several studies have shown weight loss in 70–80% of hospitalized IBD patients and in 20–40% of outpatients with CD.[9] The prevalence of malnutrition is lower in patients with UC, but nutritional deficiencies can quickly develop in these patients during periods of active disease.[3]

The aim of this study was to assess the influence of the frequency and type of dietary risk factors on the nutritional status of patients with IBD in remission.

Material and methods

Study population

The study group consisted of 162 IBD patients aged 18–81 years (72 females and 90 males): 61 individuals with CD and 101 with UC. Inflammatory bowel disease diagnosis was based on clinical manifestation, endoscopic and/or radiological findings, and pathological studies. Patients with unidentified colitis were excluded. All patients in the study were interviewed, after giving an informed consent, within 1 year of the onset of symptoms, most of them 4–6 months after the onset of symptoms; all of them were in remission. Remission was defined as having no need for oral steroids (either prednisolone or budesonide) for at least 3 months. All of the patients were in remission according to the Crohn's Disease Activity Index and the Mayo Activity Index for CD and UC, respectively. Control subjects were recruited from the healthy population in the study region. The control group was matched for gender and age to the IBD group (within the same 10-year age group, as follows: 18–29, 30–39, 40–49, 50–59, 60–69, or 70–81 years).

Data collection

In all subjects (patients and controls), the typical dietary intake over the month before the interview was assessed using the validated cross-check dietary history method.[15] The patients and controls were interviewed about their dietary intake in the same way by experienced dieticians. The questionnaire comprised of 2 parts. The 1st one consisted of sociodemographic characteristics, namely, age, gender, weight, height, place of residence, education level, and current smoking habits. In the 2nd part, concerning dietary habits, the subjects were asked about the average intake frequency of foods and dishes, including potential dietary risk factors for IBD.

Nutritional status

The nutritional status of all subjects (patients and controls) was evaluated by the Mini Nutritional Assessment (MNA) and by anthropometric measurements of the subjects: body mass index (BMI) and the percentage of body fat, determined by a bioelectrical impedance analysis, using a body-fat analyzer (Omron BF306; Omron Healthcare Co., Ltd., Matsusaka, Japan).[16] Malnutrition and risk for malnutrition were defined by the MNA. The questionnaire is composed of 18 items and involves anthropometric, general, dietary, and subjective assessment. The questionnaire consists of 2 main parts: screening and assessment. Screening includes questions related to changes in weight loss, oral intake, mobility, stress, etc. Assessment additionally includes medical history, some questions related to eating habits, and measurements of arm and calf circumferences.

A total score >23.5 indicates adequate nutritional status; a score of 17.0–23.5 denotes a risk for malnutrition, while <17.0 indicates malnutrition.[16]

Statistical methods

Using the χ^2 test, we compared the dietary habits of UC or CD patients to those of control subjects and those of UC or CD patients with different nutritional status. The significance of the test was assumed if $p \leq 0.05$. Statistical analyses were performed with STATISTICA v. 8.0 for Windows (StatSoft Polska Sp. z o.o., Kraków, Poland). The evaluation of nutrient intake derived from the dietary recall was performed using FoxPro software v. 2.6/Win and Excel 2000 by (Microsoft Polska Sp. z o.o., Warszawa, Poland). The dietary recall data from the studied men and women were combined into 1 group for statistical analysis.

This study was approved by Wroclaw Medical University Research Ethical Board (KB-216/2016).

Results

The background characteristics of UC and CD patients and control subjects are presented in Table 1. The mean body weight, BMI and body fat were lower among CD patients and slightly lower in UC patients than among control subjects. The highest percentage of patients with malnutrition or a risk for malnutrition was among CD patients; almost 100% of control subjects had adequate nutritional status.

The exposure to dietary risk factors for UC and CD patients with different nutritional status and control subjects is shown in Tables 2 and 3.

Low fiber intake was significantly more prevalent among UC patients with malnutrition and a risk of malnutrition than in UC patients with adequate nutritional status. Low fiber intake and high intake of red meat and meat products were significantly higher in UC patients than in control subjects. No significant differences in the exposure to dietary risk factors were observed between CD patients with different nutritional status. Low fiber intake and high intake of sugar and sweets, animal fats, and red meat and meat products were significantly higher in CD patients than in control subjects. Crohn's disease patients had a higher intake of animal fats, and sugar and sweets than UC patients. There was a trend toward a higher intake of red meat and meat products among UC patients with malnutrition and risk for malnutrition vs CD patients with the same nutritional status.

Most patients were exposed to 3–5 dietary risk factors, though the number of dietary risk factors was not associated with the nutritional status of UC and CD subjects (Table 4).

Discussion

Diet, as a source of luminal antigens, is thought to be an important factor in the immunopathogenesis of IBD, but whether antibodies against dietary antigens play a primary role in IBD etiology or are secondary to intestinal inflammation is yet to be determined. While many studies associate diet with IBD, there is still no conclusive evidence that any specific food or dietary factor directly contributes to the pathogenesis of either CD or UC. Low-fiber, high-sugar, high-animal-fat intake, and westernized diets have been proposed as risk factors for the development of IBD.[9,17,18] In the literature, there are many reports on possible associations between diet and IBD, with a range of foods being implicated, such as fast foods, refined sugar, margarine, and highly processed foods.[6,15,19,20]

This is the first population-based case-control study to investigate whether there is an association between the nutritional status of patients with IBD and the frequency and type of dietary risk factors during remission. The nutritional status of patients with IBD was evaluated by the MNA questionnaire. Since the MNA questionnaire is not routinely used in the nutritional assessment of IBD patients, we compared the mean values of biochemical factors

Table 1. Characteristics of study subjects

Characteristics	UC patients (n = 101)	CD patients (n = 61)	Control group (n = 129)
Age [years]	49.3 (19–79)	40.2 (18–81)	45.6 (18–82)
Gender (male/female)	60/41	32/29	71/58
Body weight [kg]	68.0 (40–108)	61.5 (42–105)	74.5 (49–115)
Body fat [%] (male/female)	21.9 (4.0–38.6)/ 31.5 (10.7–45.1)	18.3 (8.8–40.0)/ 27.3 (10.0–38.3)	21.6 (10.5–38.0)/ 33.4 (13.5–49.2)
BMI [kg/m^2]	23.5 (16.0–36.9)	21.7 (15.2–33.1)	24.8 (18.1– 43.0)
Obese/overweight/healthy weight/underweight by BMI (n, %)	6/30/57/8 (n), 6/30/57/7 (%)	3/7/39/12 (n), 5/11/64/20 (%)	8/38/81/2 (n), 6/29/63/2 (%)
Adequate nutritional status/risk for malnutrition/malnutrition by MNA (n, %)	54/42/5 (n), 54/42/4 (%)	22/31/8 (n), 36/51/13 (%)	127/2/0 (n), 98/2/0 (%)

BMI – body mass index; CD – Crohn's disease; MNA – Mini Nutritional Assessment; UC – ulcerative colitis; values expressed as means (min–max) or (n, %).

Table 2. Exposure to dietary risk factors of UC patients with different nutritional status vs control subjects

Dietary risk factor	UC patients with adequate nutritional state [%]	UC patients with a risk for malnutrition and malnutrition [%]	p-value*	Control subjects [%]	p-value**
	n = 54	n = 47		n = 129	
Fiber intake <20 g/day	71.7	88.9	0.0283	51.5	0.0004
Cheese intake >3 slices/day	25.6	17.4	NS	14.5	NS
Animal fat >3 servings/day	81.2	72.7	NS	67.3	NS
Sugar intake ≥15 g and/or sweets 3 times/day	39.1	39.8	NS	29.1	NS
Red meat and processed meat intake >4 servings/day	8.7	11.5	NS	0.9	0.0006
Oily sea fish intake <1 serving/week	65.9	70.2	NS	64.7	NS
Dark chocolate intake >4 servings/week	13.4	7.5	NS	14.3	NS
Milk chocolate intake >4 servings/week	3.6	9.7	NS	13.7	NS
Highly processed food intake >1 serving/week	3.7	4.1	NS	4.7	NS

NS – not significant; UC – ulcerative colitis; * comparison between UC patients with different nutritional status; ** comparison between UC patients and control subjects.

Table 3. Exposure to dietary risk factors of CD patients with different nutritional status vs control subjects

Dietary risk factor	CD patients with adequate nutritional state [%]	CD patients with a risk for malnutrition and malnutrition [%]	p-value*	Control subjects [%]	p-value**
	n = 22	n = 39		n = 129	
Fiber intake <20 g/day	62.3	74.9	NS	51.7	0.011
Cheese intake >3 slices/day	12.2	11.7	NS	14.4	NS
Animal fat >3 servings/day	87.2	88.7	NS	67.4	0.001
Sugar intake ≥15 g and/or sweets 3 times/day	37.9	55.3	NS	29.7	0.005
Red meat and processed meat intake >4 servings/day	8.5	0.0	NS	0.8	0.046
Oily sea fish intake <1 serving/week	66.9	49.9	NS	64.8	NS
Dark chocolate intake >4 servings/week	8.6	6.4	NS	14.3	NS
Milk chocolate intake >4 servings/week	12.7	16.5	NS	13.7	NS
Highly processed food intake >1 serving/week	0.0	2.6	NS	4.7	NS

NS – not significant; CD – Crohn's disease; * comparison between CD patients with different nutritional status; ** comparison between CD patients and control subjects.

of the patient groups with different nutritional statuses evaluated by the MNA in our previous work.[21] It was found that IBD patients' nutritional status assessment according to the MNA largely overlapped with changes in iron and cholesterol serum levels, as well as blood hemoglobin concentration. In this case-control study, low intake of fiber was associated only with the nutritional status assessed by the MNA for UC patients, though both UC and CD patients were exposed to a higher degree of these dietary risk factors than control subjects. Low fiber intake,

Table 4. Number of dietary risk factors in IBD patients with different nutritional status

Number of dietary risk factors	IBD patients with adequate nutritional status [%] (n = 76)	IBD patients with a risk for malnutrition and malnutrition [%] (n = 86)	p-value
0–2	18.1	16.0	NS
3–5	74.7	78.7	NS
6–8	7.2	6.4	NS

IBD – inflammatory bowel disease; NS – not significant.

especially fiber from fruit and vegetables, may elevate the risk of IBD.[7,20] It is not clear, however, whether the fiber content of fruits and vegetables is the seemingly protective factor or whether one or more of the micronutrient components of these foods, for example water or vitamin C, are involved.[7] Furthermore, the finding that the fiber intake from cereals, fruits and/or vegetables is lower in IBD patients may represent a response to the disease rather than an etiological factor. Patients may choose a low-fiber diet, consequently avoiding fruits and vegetables, in order to minimize symptoms such as diarrhea. This is a possible reason why UC patients with malnutrition and a risk for malnutrition had a higher incidence of low fiber intake than UC patients with adequate nutritional status in our study. A striking finding among individual food items was high intake of red and processed meat among both patient groups. High meat intake, as a source of sulfur, is more associated with an increased UC risk than with a CD risk.[22,23] Consumption of large amounts of sulfur and sulfate-rich foods is also associated with an increased risk of UC relapse.[23] High sulfur intake, either from sulfur amino acids or sulfate additives, results in the generation of hydrogen sulfide and mucosal damage in the colon. The acute toxicity of hydrogen sulfide appears to result from the inhibition of cytochrome oxidase, leading to mucosal damage, loss of barrier function and histological changes resembling UC.[22] In our study, the patients with CD were found to consume more animal fat, sucrose and refined carbohydrates from sweets compared to control subjects. Since the 1980s, various studies have indicated high consumption levels of these products in patients with IBD, to the extent that they are now considered a risk factor for CD and UC.[6,7,24,25] High consumption of animal fat is a risk factor for many diseases, but its mechanism of action in IBD is currently still unknown. The findings of a large epidemiological study suggest that high consumption of refined carbohydrates or added sugars may be true risk factors for IBD, while they may also be just an expression of a "modern lifestyle", which also involves other risk factors for the development of IBD.[7,26]

No statistically significant associations were detected between IBD patients and control subjects in regard to their exposure to other dietary risk factors studied. A similar result was reported in some other European studies, in which there was no correlation or a low correlation between a risk for IBD and a limited number of potential dietary risk factors.[10,20,23]

Canadian authors reported slightly different results in patients with active and inactive IBD. In their study, food avoidance among active and inactive IBD patients, in comparison to the control group, was prevalent for alcohol, popcorn, legumes, nuts, seeds, deep-fried foods, and processed deli meat, with a higher prevalence among those with active IBD. Patients with active IBD also consumed significantly more portions of sports drinks and sweetened beverages compared to those with inactive disease.[27]

In this study, we found no association between the number and type of dietary risk factors and the nutritional status of UC and CD patients. However, lower fiber intake and higher red meat intake in both patient groups, in comparison to the control group, as with higher animal fat and sugar intake in UC patients, indicate that those factors are important in the etiology of IBD. These results suggest that dietary factors are less statistically important than prior disease activity in the current nutritional status of UC and CD patients. The most important causes of malnutrition, excluding reduced food intake, are probably reduced absorptive surface due to inflammation, resection, diarrhea, and the enteric loss of nutrients during periods of disease activity, as well as during remission.[28–31] Previous studies have shown that UC and CD patients in remission are at risk of developing nutritional deficiencies in micro- and macronutrients, and that they are also exposed to dietary risk factors.[21,32,33] Future nutritional concepts for patients with IBD in remission should therefore include strategies for disease modification, e.g., involving immunomodifying substances or limiting the number of dietary risk factors, rather than focusing on the prevention and treatment of malnutrition alone.

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Histological examinations of the in vivo biocompatibility of oxycellulose implanted into rat skeletal muscle

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

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Abstract

Background. Recently it was shown that oxycellulose suppressed bone regeneration led to an accumulation of connective tissue as well as foam cells in bone defects.

Objectives. Since oxycellulose can be used as a hemostatic agent in general and dental surgery, the aim of the study was to examine muscle tissue response to implanted oxidized cellulose.

Material and methods. RESO-Cell® (Resorba Wundversorgung GmbH, Nuremberg, Germany) standard was implanted in the latissimus dorsi of 20 rats; subsequently, 12 samples were processed for histological evaluation after 4 and 8 weeks. The remaining 8 samples were processed for mRNA expression analyses of gene-encoding growth factors and collagens using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Results. Muscle tissue exposed to oxycellulose showed elevated mRNA levels of *COL1A1* compared to untreated muscle tissue. The histological analysis revealed that no undegraded oxycellulose was detectable after as little as 4 weeks. Furthermore, a strong accumulation of CD68-positive foam cells was found in the treated area.

Conclusions. In conclusion, the study has shown that oxidized cellulose can cause an inflammatory response after this material is implanted in skeletal muscle. Therefore, it is not recommended to leave this material in the body over a long period. However, it could be used as auxiliary material in the treatment of periodontal defects.

Key words: skeletal muscle, implantation, qPCR, oxidized cellulose, histiocytic reaction

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Introduction

One of the most common tissues for transplantation worldwide is bone. In the fields of orthopedics, neurosurgery and dentistry, bone grafts are used in order to reconstruct bone defects and stimulate bone healing after trauma and bone loss.[1,2]

There are several methods of bone transplantation; they include the use of autologous, allogeneic and xenogeneic bone tissues, as well as synthetic bone substitutes. Autologous bone is harvested from the patient's own body, while allografts are obtained from a bone bank. Xenogeneic bone, on the other hand, originates from other species. Synthetic variants can be created from ceramics, coralline hydroxyapatite, bioactive glasses, glass ionomers, and biological/synthetic composite grafts.[1–3] The use of synthetic bioactive bone substitute materials is of keen interest in modern dentistry as an alternative to autogenous bone grafts.

To date, diverse types of biomaterials for bone regeneration have been used, e.g., non-mineral and mineral-based materials, with and without natural or artificial polymer.[4,5] Heretofore, hyaluronic acid (HA)-based biomaterials, such as Geistlich Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland) or NanoBone® (Artoss Inc., Foley, USA), were the most abundant materials used in modern bone substitution. Calcium phosphate cements, such as Cerasorb® (Curasan AG, Frankfurt am Main, Germany) or Straumann Bone Ceramic® (Straumann Holding AG, Basel, Switzerland), currently play a secondary role in dentistry.[2]

It is well known that plants and animals naturally produce a variety of polymers which can serve as a solid source for biomaterials, such as collagen, silk or cellulose.[2,6] The use of vegetable fibers in polymeric composites, in particular bast fibers from flax, hemp or jute, has been reported in many studies.[7–10] Composites enhanced with natural fibers have been used in medicine as artificial scaffolds for tissues, drug-release systems, cardiovascular patches, or nerve cuffs.[11,12] Recently it was shown that wound treatment with flax dressings effectively reduced wound exudates and fibrin levels, and increased new granulation.[13] Furthermore, it has been demonstrated that cellulose fibers isolated from flax have good in vitro and in vivo biocompatibility and support bone regeneration.[7–9] However, flax composites are of limited use because, although cellulose is hydrophilic, it is insoluble in water as well as most organic solvents, and is not biodegradable.[10]

Oxidized cellulose is a water-insoluble derivative of cellulose and it is one of the most important biocompatible and bioresorbable polymers. It can be produced after oxidation of highly pure cellulose in many different forms, such as powder, textile, paper, or fibers.[14] Depending on the amount applied, oxycellulose can be absorbable.[15,16] It has recently been shown that bone defects treated with oxidized cellulose were completely filled with connective tissue with embedded foam cells and that natural bone regeneration was suppressed.[17] Contrary to these findings, oxidized cellulose has been long used as a hemostatic agent in general, oncoplastic breast and dental surgery.[14–16,18–23]

Tissue response to biomaterials depends on a variety of factors, including the physico-chemical properties of the implant, its biodegradability/bioresorbability, as well as the site of implantation.[24–26] Due to the fact that oxidized cellulose could also be applicable for short-term treatment of bleeding or periodontal defects in dentistry, the aim of this study was to evaluate the absorbability and biocompatibility of the oxycellulose material RESO-Cell® standard (Resorba Wundversorgung GmbH, Nuremberg, Germany) by implanting it in rat muscle tissue. In addition, the specific tissue response to the implanted oxycellulose has been characterized.

Material and methods

Test material

Reso-Cell® standard is a resorbable hemostat made from oxidized cellulose. It is used in the form of gauze strips produced from natural cellulose derived from cotton.

Experimental design and surgical procedure

Twenty adult Lewis 1A rats of both sexes (3 months old, body weight 250–350 g) were used. All the surgical and experimental procedures were approved by the Animal Welfare Committee of the State Government of Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-1.1-033/11). Each rat was anesthetized with an intraperitoneal injection of ketamine (10%; CEVA Tiergesundheit, Düsseldorf, Germany) and rompun (2%; Bayer HealthCare, Leverkusen, Germany) and a skin incision was made on the back of the lattisimus dorsi muscle as described previously.[7,27] After exposition and transection of the fascia, the defect was covered with oxycellulose (1 × 1 cm) and then subsequently closed with wound clips (Fig. 1). Five rats undergoing surgical procedures without any material insertion served as negative controls.

The rats were sacrificed after 4 (n = 14) or 8 weeks (n = 6) and the inserted material with the surrounding tissue of the latissimus dorsi muscle was harvested. The samples were processed for gene expression analysis (n = 9) and histological examination (n = 11).

For the molecular-biological examinations, the samples were shock-frozen in liquid nitrogen and stored at –80°C. For the histological examination, the samples were placed in 4% formalin in phosphate-buffered saline (PBS) for 1 week at room temperature, then routinely dehydrated in a series of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, and 100%) or xylol for 12 h, and finally embedded in paraffin.

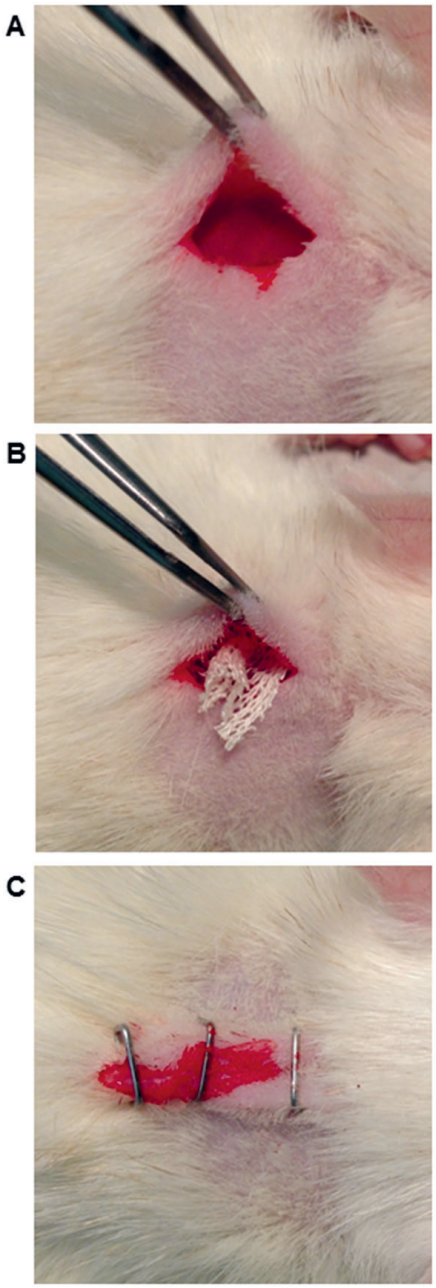

Fig. 1. Surgical approach: A – skin incision above the latissimus dorsi; B – insertion of the oxidized cellulose; C – wound closure using skin staples

Histological processing

The paraffin blocks were trimmed in serial longitudinal sections of about 3 µm using a microtome (Leica RM 2155; Leica Microsystems GmbH, Wetzlar, Germany). Some of the sections were then stained with hematoxylin/eosin (H&E) to assess the general overview of the tissue structure. Some sections were also immunostained against CD68 antibody, a marker for various cells of the macrophage lineage, including monocytes, histiocytes, giant cells, Kupffer cells, and osteoclasts; or against CD45, an antibody which distinguishes leucocytes/lymphocytes from nonhematopoietic cells.

For immunohistological (IHC) staining, the sections were first deparaffinized, rehydrated, rinsed for 10 min in tris-buffered saline (TBS) and incubated in citrate buffer pH 6.0 in a water bath at 95–99°C for 40 min. After cooling down the slides for 20 min at room temperature, the endogenous peroxidase was blocked with 0.3% H_2O_2 in darkness. Following further rinsing, the sections were incubated with the primary antibody (Table 1) in a humid chamber. The staining was performed following the instructions for the EnVision+ System-HRP (DAKO, Glostrup, Denmark), counter-stained with Mayer's acid hemalum and then cover-slipped. For negative controls, the primary antibody was replaced by PBS.

Quantitative reverse transcription polymerase chain reaction

Homogenization of the muscle samples and total RNA isolation were performed using TRIzol and QIAzol lysis reagents (Qiagen Inc., Hilden, Germany) and the RNeasy® Lipid Kit (Qiagen) according to manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) for cDNA synthesis was done in the Thermocycler TOptical Real-Time PCR (Analytik Jena AG, Jena, Germany) using 200 ng RNA and innuSCRIPT Reverse Transcriptase, innuNucleotide Mix and Random primer (Analytik Jena AG).

The following genes were quantified: collagen type 1 and type 2 (*COL1A1* and *COL2A1*); insulin-like growth factor (*IGF*) 1 and *IGF2*; vascular endothelial growth factor (*VEGF*); and myostatin (*MSTN*), a negative regulator of muscle growth. The quantification of the expression of different rat genes were performed as described previously by using the master mix contained in the innuMix qPCR Master Mix Probe (Analytik Jena AG), ×10 specific probes and primers (*IGF1*: Rn 00710306_m1; *IGF2*: Rn 00580426_m1; *VEGF*: Rn 01511602_m1; *MSTN*: Rn 00569683_m1; *COL1A1*: Rn 01463848_m1; *COL2A1*: Rn 00563954_m1; Eukaryotic 18S rRNA Endogenous Control: 4310893E; PE (Applied Biosystems, Weiterstadt, Germany) and RNase free water.[7,27]

Table 1. Details and incubation protocols of the antibodies used

Antibody	Isotype	Producer	Incubation protocol	For staining of
CD45	rat monoclonal, clone 30-F11	BD Bioscience (Heidelberg, Germany)	1:50, overnight, 4°C	lymphocytes
CD68	mouse monoclonal	Abcam (Cambridge, UK)	1:150, overnight, 4°C	macrophages

Statistical analysis

All the statistical analyses used the paired t-test and were performed using SigmaStat 3.5 software (Systat Software, Inc., San Jose, USA). Data is given as means ± standard error of the mean (SEM). The p-value <0.05 was considered statistically significant.

Results

All the animals survived the procedure without major complications, such as allergic reactions, abscesses or infections, and postoperative healing was smooth.

Degradation of oxycellulose in skeletal muscles: the results of histiocytic infiltration

In the histological sections after both 4 and 8 weeks, there were no signs of intact oxycellulose in the muscle tissues. Above the muscle tissue, a strong collection of round basophil cells was visible, which are normally not present in this area (Fig. 2A). On closer inspection, this cell collection could be identified as a marked resorptive histiocytic inflammation with an accumulation of foam cells. There were some histiocytes embedded in connective tissue that showed a foamy inside – probably an indication of captured oxycellulose particles. Furthermore, no giant cells, lymphocytes or encapsulation of the material were observed (Fig. 2,3). The area enriched in the rounded cells showed collagen accumulation (Fig. 2A). The accumulated cells were CD68-positive and thus of monocyte/macrophage origin (Fig. 4A). Nontreated control rats showed a slight positive reaction for CD68 in the epimysium (Fig. 4C).

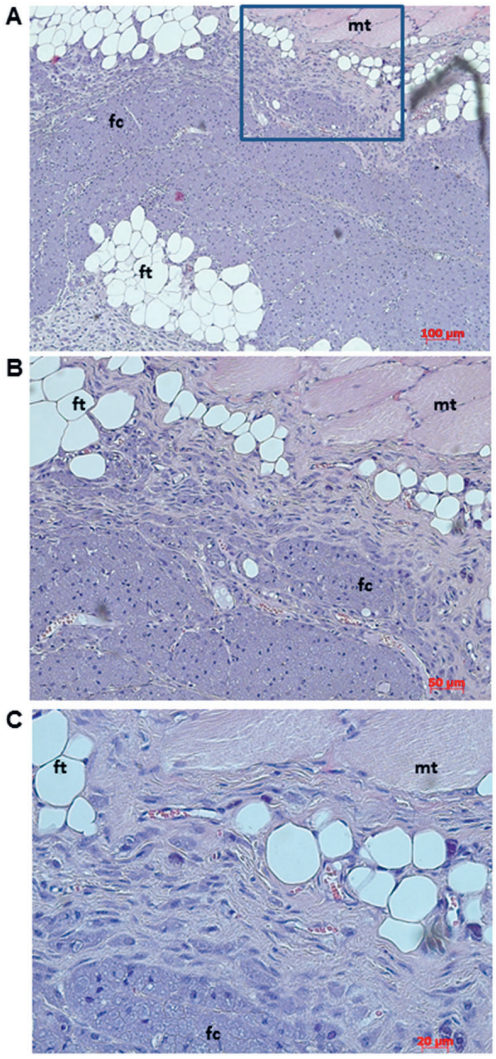

Fig. 2. Hematoxylin/eosin (H&E) staining of muscle tissue cross-section embedded in paraffin 4 weeks after the insertion of oxidized cellulose: A – overview image, magnification ×100; the blue frame marks the area of the enlarged photographs as shown in B – magnification ×200 and C – magnification ×400

fc – foam cells; mt – muscle tissue; ft – fatty tissue.

Quantitative reverse transcription polymerase chain reaction

Four weeks after oxycellulose insertion, the animals were sacrificed and 2 muscle sections were prepared: 1. treated muscle tissue and 2. untreated muscle tissue. Both samples from each rat were used for molecular-biological analyses and the intra-individual gene expression of collagens and growth factors were detected (Table 1).

In oxycellulose-treated muscle tissue, 1.9 times higher mRNA levels of *COL1A1* were found. None of the other genes tested showed any difference between muscle tissue from oxycellulose-treated and untreated rats (Table 2).

Table 2. Gene specific transcription levels in rat muscle tissue samples. The mRNA levels are given in relation to those of 18S rRNA. Means ± standard error of the mean (SEM) are given in all cases for n = 8 samples. The p-values indicate statistically significant differences between oxycellulose-treated muscle tissue and untreated tissue from the same rat; paired t-test

Gene	Treated muscle	Untreated muscle	p-value
COL1A1	0.32 ±0.092	0.62 ±0.15	p = 0.008
COL2A1	0.0057 ±0.0035	0.018 ±0.0069	NS
IGF1	0.014 ±0.0028	0.018 ±0.0028	NS
IGF2	0.013 ±0.003	0.013 ±0.0039	NS
VEGFA	0.016 ±0.0015	0.018 ±0.0033	NS
MSTN	0.021 ±0.0074	0.029 ±0.0088	NS

NS – none significant.

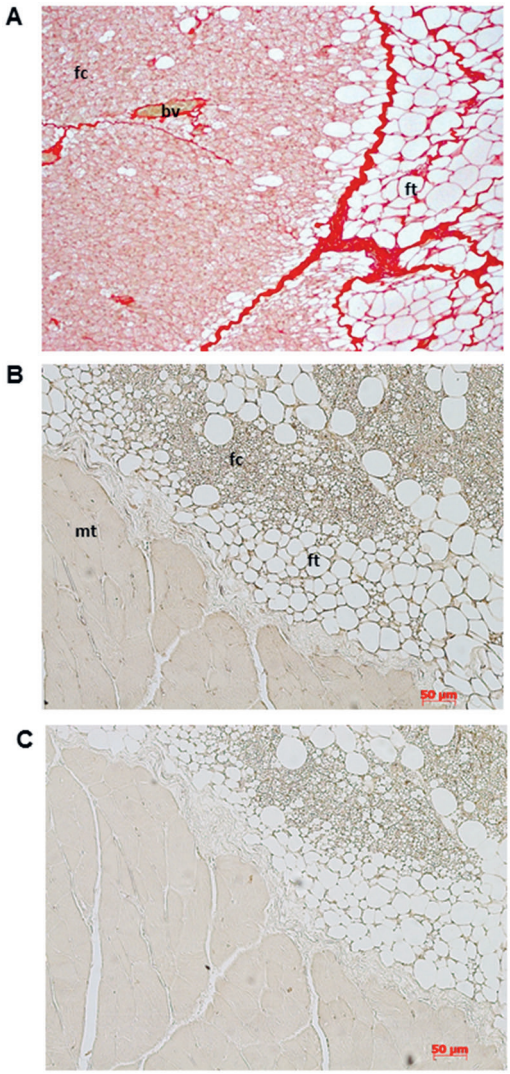

Fig. 3. A – Sirius Red staining and B – detection of CD45 antibody in muscle tissue cross-section embedded in paraffin 4 weeks after the insertion of oxidized cellulose; C – control staining with phosphate-buffered saline (PBS) instead of the primary antibody. Magnification ×200

fc – foam cells; mt – muscle tissue; ft – fatty tissue: bv – blood vessel.

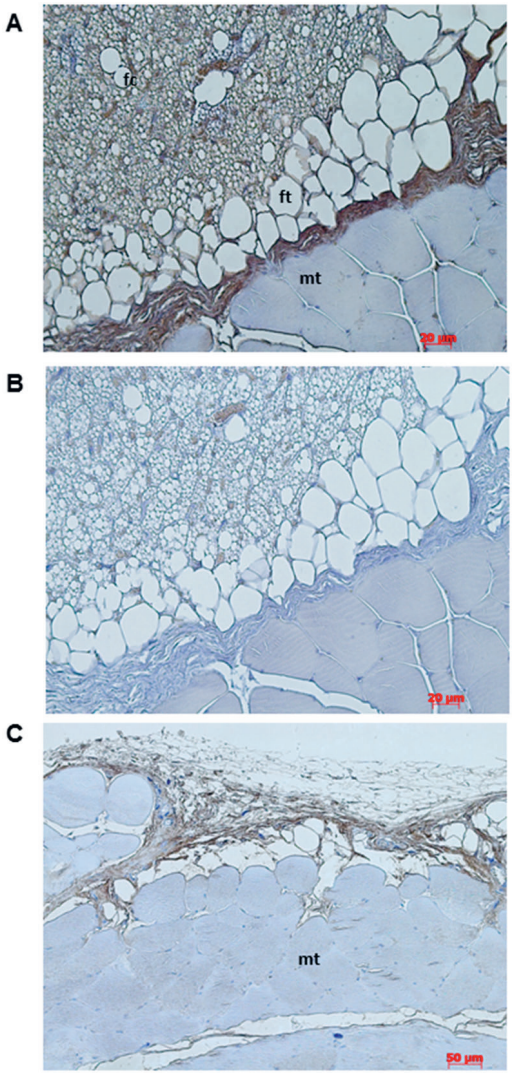

Fig. 4. Detection of CD68 antibody in muscle tissue cross-section embedded in paraffin from (A+B) oxidized cellulose-treated rats and C – untreated rats; B – control staining with phosphate-buffered saline (PBS) instead of the primary antibody. Magnification ×400 (A + B) and ×200 (C)

fc – foam cells; mt – muscle tissue; ft – fatty tissue.

Discussion

The use of plant fibers for the production of wound covering materials is becoming an important area of science. Effective wound closure, reduced treatment times and reductions in post-traumatic infection can be achieved by imbedding drugs in the fiber materials.[28] It was recently shown that treatment with flax dressing products from genetically engineered flax plants able to synthesize antioxidative compounds reduced wound exudates, wound size and fibrin levels, and increased the level of new granulation in patients with chronic venous ulceration.[13] Another genetically modified flax plant was able to produce polyhydroxybutyrate (PHB).[29] Composites from these plants have demonstrated good in vitro and in vivo biocompatibility.[7–9] A problem with the use of plant fibers is the fact that they are not biodegradable and cannot be absorbed, since the main component of the fibers is the natural polymer cellulose.[30] This problem can be circumvented by oxidizing the cellulose.

Oxidized cellulose was first introduced in 1943 and was developed by controlled oxidation of cellulose.[31] This material is widely used to control intraoperative diffuse capillary, venous and small-arterial bleeding in general surgery and breast-conserving surgery.[14–16,18,19,21–23] Furthermore, tissue regeneration after surgical exposure of periodontal defects has been shown when using oxidized cellulose meshes.[20,32] Due to the fact that oxidized cellulose is bioabsorbable within 7–14 days, this material is often left in the surgical bed.[33] It is advantageous that oxycellulose has definite and potent antipathogenic effects against a variety of organisms as well as an ability to mediate blood-platelet aggregation, deposition and activation.[34–36]

Meanwhile, there have been many other reports about the histopathologic effects of hemostatic reagents in animals and humans.[33,37–39] Rather mild side effects have been described. Oxidized cellulose applied on the epidural region of laminectomy sites in rats caused chronic inflammation, moderate or severe fibrosis and mild or moderate increased vascularity in some animals.[39] Similar results were described in an experimental study on the use of oxycellulose as filler in oncoplastic breast cancer.[22] An increase in connective tissue was confirmed by the molecular biological studies in the present work: we found that *COL1A1* mRNA expression was significantly increased in oxycellulose-treated muscle tissue compared to the untreated animals. The histological results of the study presented here showed more serious side effects: we found a persistent strong resorptive histiocytic reaction in the implantation bed of oxidized cellulose. Resorptive histiocytic inflammation is a reaction of macrophages/foam cells, which record the material in the cells. In contrast, both giant cells and lymphocytes were missing. Thus, our results are consistent with those of previous studies.[39]

It is well-known that oxidized cellulose decreases the pH value of the surrounding tissue. The lowering of the pH is the reason for the antimicrobial effect against pathogenic organisms.[34,35] However, Tomizawa showed that acidic pH also increases inflammation of the surrounding tissue and delays wound healing.[40] Recently, it was reported that the use of oxidized cellulose could cause intra-abdominal foreign-body granuloma, a more serious side effect, which is indistinguishable at first glance from a tumor.[33,37,38]

As early as 1943 it was shown that the biodegradation of oxidized cellulose depends on different factors, such as the quantity of implanted material and environmental conditions.[31] It should be noted that the size of the implanted oxycellulose used in our study (1 cm^2) was very large in comparison with the implantation site. This, among other things, may have triggered the strong inflammatory reaction. Indeed, we observed clear infiltration of mononuclear cells in the muscle tissue in the area of the implanted oxycellulose. However, no oxycellulose could be observed 4 weeks after its implantation. This implies that the filling material was absorbed by the activity of monocytes/macrophages, identified by the presence of the common CD68 antigen, which extensively infiltrated the treated muscle tissue.

The implantation site plays also an important role in the biodegradability of oxycellulose.[31] It was recently shown that the use of oxidized cellulose was characterized by diffuse fibrosis and neovascularization within the oxycellulose construct at postoperative week 20, without any inflammatory effects or encapsulation. Furthermore, macrophages were predominantly found 10 weeks after surgery, whereas fibroblasts dominate at postoperative week 20.[22] These results indicate that in the case of experimental breast-conserving surgery the oxycellulose was still detectable even after 30 weeks. In the current study, no intact cellulose fibers were found 4 and 8 weeks after insertion of the oxycellulose strips on the latissimus dorsi. A previous study reported a similar observation in the skulls of rats: no residual fibers of oxycellulose could be detected after 8 weeks. In that earlier study it was demonstrated that insertion of oxidized cellulose in bone defects resulted in decreased bone healing. The surgically created bone defects were completely filled with connective tissue with embedded foam cells. Furthermore, bone resorption processes seemed to be activated, since residual bone was resorbed.[17] The biggest difference between our new study and a study by Franceschini et al. was that in that study, no accumulation of absorbed oxycellulose could be detected in the breast tissue of the rats.[22] This could be due to the nature of the material; in our study strongly woven oxycellulose fibers were used (data not shown).

Conclusions

In conclusion, the study has shown that oxidized cellulose can cause an inflammatory response after this material is implanted in skeletal muscle tissue. Therefore, it is not recommended to leave this material in the body over a long period. However, this material could be used as an auxiliary material in the treatment of periodontal defects.

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The anatomical relation of the extracranial internal carotid artery in the parapharyngeal space

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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 proximity of the internal carotid artery to the pharyngeal wall poses a risk of injury during nasopharyngeal surgery.

Objectives. The aim of this study was to assess the distances between the extracranial internal carotid artery (ICA) and the pharyngeal wall.

Material and methods. Measurements were taken on certain levels of the pharynx using computed tomography angiography (angio-CT) scans of 97 patients. One-tailed Student's t-test for independent variables and a comparison of expected values for dependent pairs of observations were applied.

Results. The shortest distance between the ICA and the pharyngeal wall was 1.1 mm. The ICA is closer to the pharyngeal wall at the epiglottis apex level (16.46 ±0.89 mm) than to the Eustachian tube (ET) (19.8 ±0.62 mm) ($p < 0.0005$). In women, the ICA is closer to the ET (19.44 ±0.78 mm) than in men (20.17 ±0.96 mm) ($p = 0.04$). In women, the right ICA is closer to the pharyngeal wall than the left ICA at the level of the lower margin of the 2nd cervical corpus vertebra (C2) (right: 17.6 ±1.8 mm; left: 20.7 ±1.7 mm) ($p = 0.002$) and at the level of the epiglottis apex (right: 15.2 ±1.7 mm; left: 17.4 ±1.4 mm) ($p = 0.028$). The bifurcation of the common carotid artery (CCA) is higher in men (19.48 ±2.19 mm below the C2) than in women (21.82 ±1.02 mm) ($p < 0.001$). When the bifurcation is at the level of the epiglottis apex, the ICA is closer to the pharyngeal wall (12.3 ±1.69 mm) than in other cases (16.46 ±0.89 mm) ($p = 0.005$). In men, the higher the bifurcation is, the closer the ICA is to the pharyngeal wall at the level of the lower margin of the C2 ($p = 0.003$).

Conclusions. The risk of ICA incision during surgery differs between the pharyngeal levels, genders and sides of the neck. The ICA may be much closer to the pharyngeal wall than described in the literature.

Key words: common carotid artery, internal carotid artery, otorhinolaryngologic surgical procedures, Eustachian tube

Introduction

The extracranial internal carotid artery (ICA) begins as an extension of the common carotid artery (CCA). It runs in the parapharyngeal space in an S-shaped course, then bends medially and heads toward the external opening of the carotid channel on the temporal bone. The distance from the ICA to the palatine tonsil has a mean value of 10–20 mm posteriorly,whereas it is 23.5 mm from the Eustachian tube (ET).[1,2]

Cases of ICA incision during tonsillectomy and adenoidectomy followed by a severe hemorrhage have been reported in the literature.[3–5] Although many anatomical variations of this artery have already been described, only a few applicable and accurate pieces of information about the topography of the ICA have been provided.[2,6–12]

The aim of our study was to assess the distances between the ICA and the pharyngeal wall as well as to define their correlation with sex.

Material and methods

The presented work is a retrospective study, based on the computed tomography angiography (angio-CT) scans (0.625 mm resolution) of 97 patients (Table 1) performed between January 2013 and January 2015 in the Jan Mikulicz-Radecki University Teaching Hospital in Wrocław, Poland.

The scans were obtained with a LightSpeed VCT 64-slice CT scanner (GE Healthcare, Milwaukee, USA). The examined area covered the whole neck from the upper thorax to the base of the skull.

In all cases, the slice thickness was 0.67 mm, the pitch amounted to 0.9 and the average gantry rotation time was 0.5 s. The contrast agent bolus was administered using an automatic syringe. Vascular access was obtained with an 18G or 20G needle inserted into the ulnar vein. The volume of iodinated contrast agent ranged from 60 to 90 mL. The rate of contrast agent administration was 4.0–4.5 mL/s. Contrast medium administration was followed by an injection of 40 mL of physiological salt solution. Automatic bolus tracking was performed in all cases, with a peak enhancement of 150 HU; scanning was automatically initiated after that. The obtained scans were analyzed using a dedicated workstation (AW4.4; GE Healthcare). Image postprocessing techniques involved 2- and 3-dimensional reconstructions (Maximum Intensity Projection – MIP; Volume Rendering – VR).

The following distances were measured:

– the distance between the ICA ambit and the deepest visible point of the Eustachian Tube (ET) opening (Fig. 1);

– the distance between the ICA ambit and the pharyngeal wall at the level of the lower margin of the 2nd cervical vertebra (C2) (Fig. 2);

– the distance between the ICA ambit and the pharyngeal wall at the level of the epiglottis apex (Fig. 3); and

Table 1. The number, age and sex of the studied subjects

Group	Number	Mean age	SD
All	97	51.8	3.6
Women	49	50.7	5.3
Men	48	53.0	4.8
Group X	12	48.7	8.5

Group X – people with the common carotid artery bifurcation on the level of the epiglottis apex; SD – standard deviation.

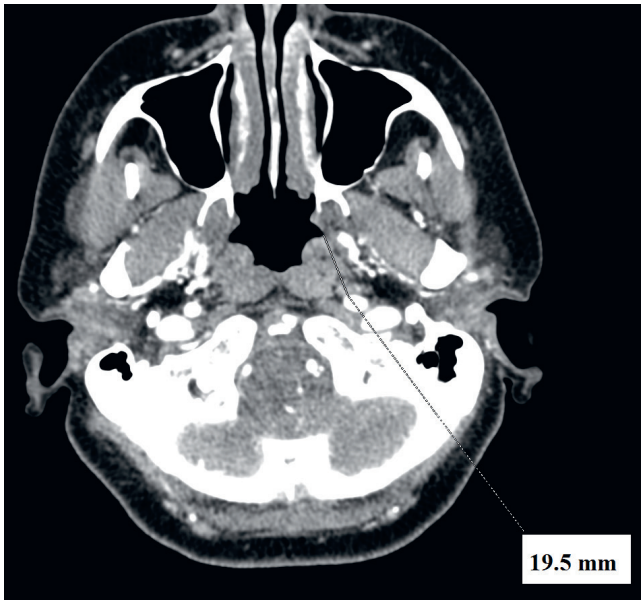

Fig. 1. Example of measuring the distance between the ICA ambit and the deepest visible point of the ET opening (left side: 19.5 mm)

ET – Eustachian tube; ICA – extracranial internal carotid artery.

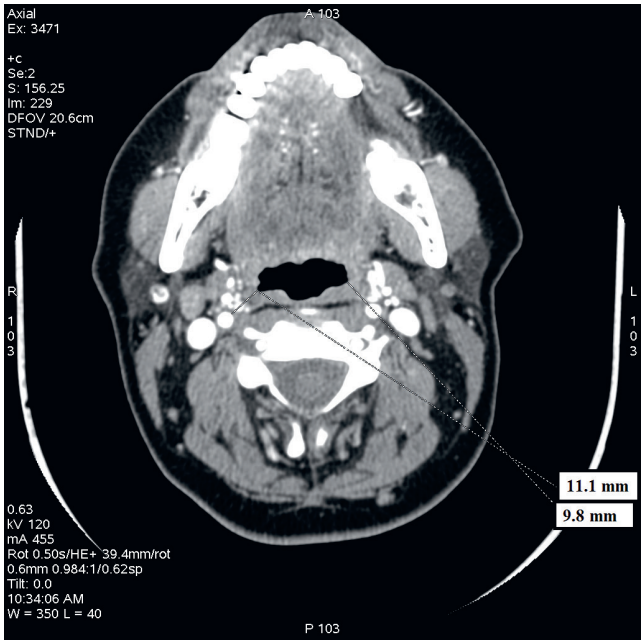

Fig. 2. Example of measuring the distance between the ICA ambit and the pharyngeal wall at the level of the C2 lower margin (right side: 11.1 mm; left side: 9.8 mm)

C2 – 2nd cervical vertebra; ICA – extracranial internal carotid artery.

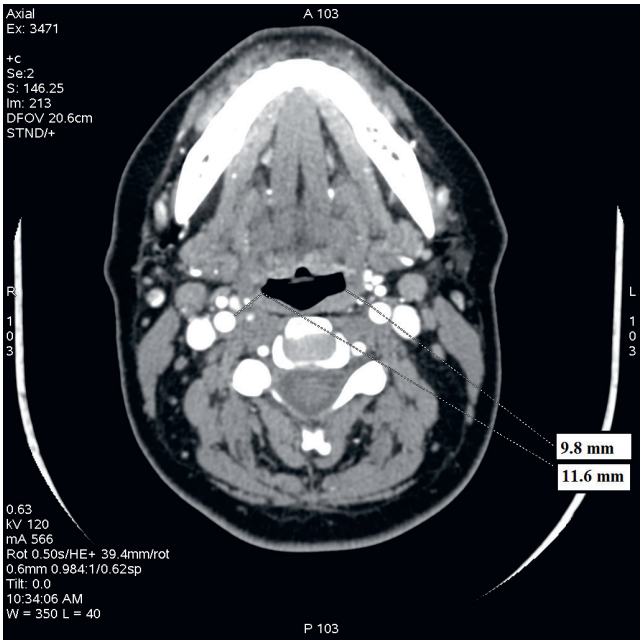

Fig. 3. Example of measuring the distance between the ICA ambit and the pharyngeal wall at the level of the epiglottis apex (right side: 11.6 mm; left side: 9.8 mm)

ICA – extracranial internal carotid artery.

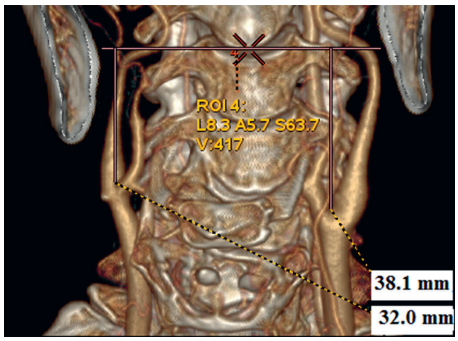

Fig. 4. Example of measuring the distance between the CCA bifurcation and the C2 lower margin (right side: 32.0 mm; left side: 38.1 mm)

CCA – common carotid artery; C2 – 2nd cervical vertebra.

– the distance between the CCA bifurcation and the lower margin of the 2nd C2 (Fig. 4).

Scans with a motion artifact, with masses that could cause dislocation of the artery or with damage to the facial bones were excluded. Only scans of good and very good opacification of the ICA were analyzed.

The values were compared using 1-tailed Student's t-test for independent variables and a comparison of expected values for dependent pairs of observations.

Results

The shortest distance found between the ICA and the pharyngeal wall was 1.1 mm (Fig. 5).

In 11 arteries, the closest distance to the pharyngeal wall along the whole course of the artery was found at Rosenmüller's fossa (mean: 3.74 ±1.2 mm; min: 1.4 mm) (Fig. 6).

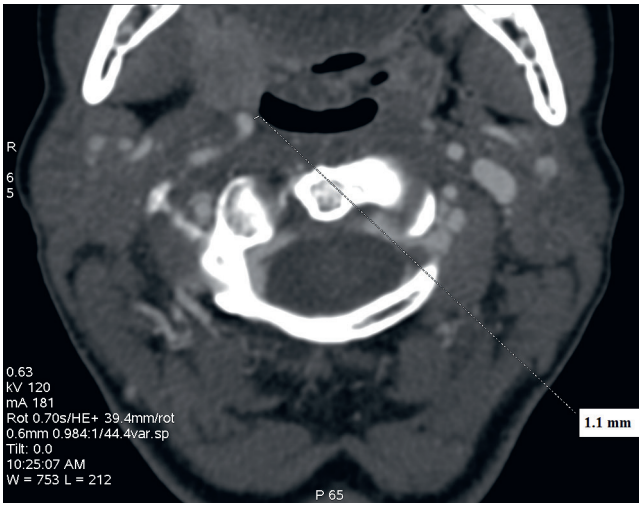

Fig. 5. The shortest distance found between the ICA and the pharyngeal wall: 1.1 mm

ICA – extracranial internal carotid artery.

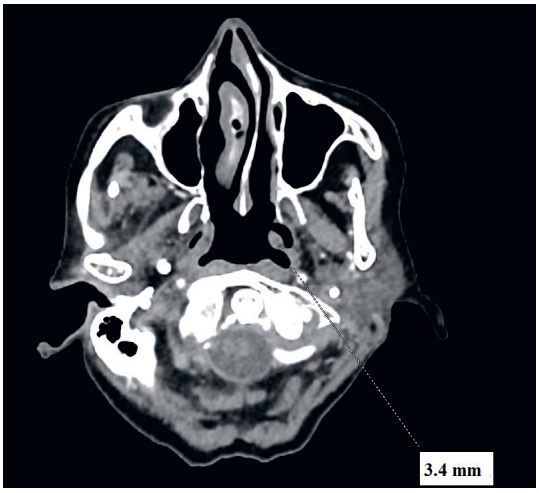

Fig. 6. Patient with a very small distance (3.4 mm) between the internal carotid artery and RF

RF – Rosenmüller's fossa.

The pharynx levels

The ICA is closer to the pharyngeal wall at the level of the epiglottis apex (16.46 ±0.89 mm) than to the ET (19.8 ±0.62 mm) ($p < 0.0005$) in both genders (Table 2). In women, the ICA is closer to the ET (19.49 ±0.78 mm) than in men (20.17 ±0.49 mm) ($p = 0.04$) (Table 2).

The side of the neck

In women, at the level of the lower margin of the C2, the distance between the ICA and the pharyngeal wall was shorter on the right side (17.6 ±1.8 mm) than on the left side (20.7 ±1.7 mm) ($p = 0.002$) (Table 3). In women, at the level of the epiglottis apex, the distance between the ICA and the pharyngeal wall was shorter on the right side (15.2 ±1.7 mm) than on the left side (17.4 ±1.4 mm)

Table 2. Distance between the ICA and the pharyngeal wall at different levels of the pharynx

Group	Mean [mm]	SD	Min [mm]	Max [mm]
A. Distance between the ICA ambit and the ET opening				
All	19.8	0.6	9.7	31.1
Women	19.4	0.8	9.7	30.5
Men	20.2	1.0	9.7	31.1
Group X	19.1	1.9	10.7	23.5
B. Distance between the ICA and the pharyngeal wall at the level of the C2 lower margin				
All	19.4	1.0	4.7	37.9
Women	19.1	1.4	4.7	35.9
Men	19.8	1.5	5.1	37.9
Group X	20.8	2.6	7.1	28.1
C. Distance between the ICA and the pharyngeal wall at the level of the epiglottis apex				
All	16.5	0.9	2.5	33.3
Women	16.2	1.1	2.5	30.6
Men	16.7	1.4	6.6	33.3
Group X	12.3	1.7	5.0	13.7

Group X – people with the common carotid artery bifurcation on the level of the epiglottis apex; C2 – 2nd cervical vertebrae; ET – Eustachian tube; ICA – extracranial internal carotid artery; SD – standard deviation.

Table 3. Distances between the right and left ICA and the pharyngeal wall at different pharynx levels in women

Type of distance	Right	SD	Left	SD
Distance between the ICA and the pharyngeal wall at the level of the C2 lower margin	17.6	1.8	20.7	1.7
Distance between the ICA and the pharyngeal wall at the level of the epiglottis apex	15.2	1.7	17.4	1.4
The minimal distance of the ICA to the pharyngeal wall along its course between the epiglottis apex and the ET	10.6	1.5	13.3	1.9

C2 – 2nd cervical vertebra; ET – Eustachian tube; ICA – extracranial internal carotid artery; SD – standard deviation.

(p = 0.028) (Table 3). The minimal distance of the ICA to the pharyngeal wall along its whole course between the epiglottis apex and the ET was smaller in women on the right side (10.55 ±1.5 mm) than on the left side (13.3 ±1.9 mm) (p = 0.015) (Table 3). Men showed no asymmetry in any measurements.

The common carotid artery bifurcation

The bifurcation of the CCA is located closer to the C2 lower margin in men (19.48 ±1.12 mm) than in women (21.82 ±1.02 mm) (p < 0.001) (Table 4). In men, the higher the CCA bifurcation is, the closer the ICA is to the pharyngeal wall at the level of the C2 lower margin (p = 0.003) (Table 4).

Table 4. Distance between the CCA bifurcation and the C2 lower margin

Group	Mean distance [mm]	SD	Min distance [mm]	Max distance [mm]
All	20.8	1.5	–15.0*	48.6
Women	21.8	1.0	3.0	48.3
Men	19.5	2.2	–15.0*	48.6
Group X	13.3	4.1	–15.0*	33.1

Group X – people with the CCA bifurcation at the level of the epiglottis apex; CCA – common carotid artery; C2 – 2nd cervical vertebra; SD – standard deviation; *15 mm above the C2 lower margin.

Group X

We found 12 patients (group X: 8 men and 4 women; mean age: 48.7 ±8.5 years) who had the CCA bifurcation at the level of the epiglottis. In group X, the distance between the ICA and the pharyngeal wall at the level of the epiglottis apex was significantly lower (12.29 ±1.68 mm) than in the rest of our study group (16.54 ±0.98 mm) (p = 0.005).

Discussion

The difference between genders

The ICA was closer to the ET in the case of women (p = 0.04), although at any other level of the pharynx, no such dissimilarity between genders was found. The ET is located near the entrance of the ICA into the external opening of the carotid artery canal in the temporal bone. As the female skull is smaller than the male one, the position

of the canal may cause moving of the ICA closer to the medial line of the body, and therefore shortening of its distance to the pharyngeal wall.[13] In the middle course of the ICA, where no difference between genders was revealed, the impact of the muscle mass and tension is diverse.

Bergin et al. reported that the distance between the ICA and the ET was shorter in women, which is in line with our study.[2] However, Jun et al. reported that at all levels of the pharynx, the distances from the ICA to the pharyngeal wall were shorter in women.[11]

Asymmetry

The distance of the ICA to the pharyngeal wall at the C2 level ($p = 0.002$), at the level of the epiglottis apex ($p = 0.028$), and the minimal distance in the course between the epiglottis apex and the ET ($p = 0.015$) are all shorter on the right side for women. An analysis of the CCA origin on both sides may provide an explanation for this difference. The right CCA starts in the bifurcation of the brachiocephalic trunk, whereas the left one begins at the aortic arch and runs in its pectoral part in an oblique direction and to the left.[1] The brachiocephalic trunk runs steeper than the aortic arch, and therefore allows the right CCA (and thus the right ICA) to branch closer to the medial line than the left ICA. The left CCA is also shifted toward the left side in its pectoral part due to the position of the heart.

There is no difference between the left and the right distance between the ICA and the ET. We assume that this is caused by the symmetry of the external carotid canal openings in the skulls of both men and women, hence the artery is forced by their position to change its position in relation to the medial line, and thus to the pharyngeal wall.[14]

Men presented no asymmetry in any of the abovementioned measurements.

The common carotid artery bifurcation

The CCA bifurcation is usually situated at the level of the upper part of the 4th cervical vertebra (C4) or the lower part of the 3rd cervical vertebra (C3). Less than 1% of people are thought to have the bifurcation at the level of the C2, although such cases have been reported in the literature.[1,15,16]

One of the examined patients had the CCA bifurcation 15 mm above the C2 lower margin; therefore, only the CCA was present in the oropharynx.

According to our study, the CCA bifurcation is closer to the C2 lower margin in men (19.48 ±1.12 mm) than in women (21.82 ±1.02 mm) ($p < 0.001$).

In men, the higher the CCA bifurcation is, the closer the ICA is to the pharyngeal wall at the level of the C2 lower margin ($p = 0.003$).

In group X, the ICA was closer to the pharyngeal wall at the level of the epiglottis apex (12.29 ±1.68 mm) than in the rest of the study group (16.54 ±0.98 mm) ($p = 0.005$). This may be caused by the widening of the CCA in the area of the bifurcation.[17]

The distance between the ICA and the pharyngeal wall at the level of the epiglottis apex is the most significant in reference to tonsillectomy, since it is very close to the level of the tonsillar fossa. If men are prone to having the CCA bifurcation higher than women, they are more exposed to the risk of fatal hemorrhage caused by an incision of the CCA. This danger is even more increased by the fact that if the ICA still does not branch at the level of the epiglottis apex, the CCA may be closer to the pharyngeal wall than the ICA would be at this level.

The levels of the pharynx

The ICA is closer to the pharyngeal wall at the level of the epiglottis apex than at the level of the ET ($p < 0.0005$). Therefore, only the measurements at the oropharynx can be taken into consideration while estimating the risk of arterial trauma during tonsillectomy, because the course of the ICA in the nasopharynx and oropharynx is divergent. Such reasoning can also be referred to the measurements made by Jun et al.[11] The mean minimal distances in that study were 17.1 ±4.1 mm at the nasopharynx, 15.8 ±4.6 mm at the oropharynx and 13.5 ±6 mm at the hypopharynx. Those distances decreased significantly downward ($p < 0.0001$).[18] However, Deutsch et al., who correlated the distance of the ICA to the ET with age and weight, formulated the conclusion that this parameter can be used to predict the distance from the tonsillar fossa to the ICA in children, whose anatomical relations in the nasopharynx are different than in adults.[10]

We discovered 11 arteries in which the minimal distance was at a very deep Rosenmüller's fossa (RF) (mean: 3.74 ±1.2 mm; min: 1.4 mm). This finding corresponds with the results of Bergin et al., who reported a possible shortening of the distance between the ICA and RF, with a minimal value of 0.2 mm.[2] This should be kept in mind while performing adenoidectomy. During this procedure, the blade of the curette is pushed over toward the posterior nasopharyngeal wall.[19]

The average distance between the ICA and the ET was 19.8 ±0.62 mm. Bergin et al. reported the mean distance to be 23.5 mm, Lien et al. found 23.1 ±3.1 mm for non-aberrant arteries and 19.6 ±2.8 mm for aberrant arteries, and for Deutsch et al., it amounted to 25 mm in children 12 years in age or 56 kg in weight.[2,10,12] The divergence probably occurs due to different measuring methods; the above-mentioned authors chose to determine the distance to the anterosuperior margin of the torus, located further from the ICA than our measuring point – the deepest part of the ET opening.

Age

We compared the mean values of the measurements in several age groups (Fig. 7). The amount of data we had did not allow us to pursue a statistical analysis of the correlations between age and distance. Nevertheless, the mean minimal distance decreased from 13.8 mm in the 14–31 age group to only 9.5 mm in the 71–86 age group. The literature suggests that the ICA may be closer to the pharyngeal wall in older people.[11,12] The prevalence of the ICA morphological variations increases with age, which can cause a decrease in the distance to the pharyngeal wall.[2,19–22]

Aberrations

It is assumed that the ICA, which is a transition zone between a vessel of the elastic type (CCA) and the muscular type (intracranial ICA), is particularly susceptible to metaplastic transformations, leading to aberrations.[5,20] Such transformations reduce the distance between the artery and the pharyngeal wall.[2,19,21,22] Pfeiffer and Ridder stated that the minimal distance between the aberrant vessels and the pharyngeal wall ranged from 0.8 to 17.9 mm (mean: 7.0 mm).[23] Kinking and coiling of the ICA did not shorten the mean distances from the ICA to the nasopharyngeal walls.[23] As the aberrations usually cause a medial curve of the ICA, it is advisable to be especially cautious while operating on patients with the specific features suggested by numerous studies as the risk factors for the presence of an aberrant ICA (Table 5).

Further studies should provide accurate data on the correlation between the position of the ICA in the parapharyngeal space with the abovementioned characteristics. It would help to estimate whether it is recommendable to perform radiological examination prior to tonsillectomy in some cases. The most accurate techniques for this purpose are believed to be time-of-flight magnetic resonance (MR) angiography or multiple detector computed tomography (MDCT).[7,22] However, CT and MR angiographies,and the much cheaper Doppler ultrasonography (USG) were also used to define the position of the neck vessels.[23–27]

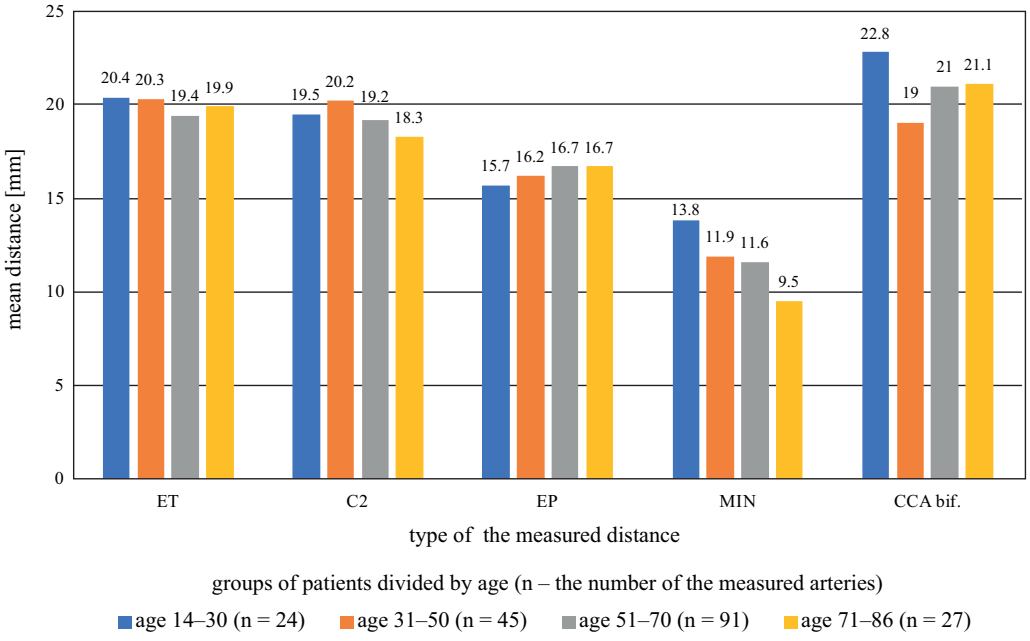

Fig. 7. Mean values of the measured distances in the chosen age groups

ET – distance between the ICA ambit and the deepest visible point of the ET tube opening; C2 – distance between the ICA ambit and the pharyngeal wall at the level of the lower margin of the 2nd cervical vertebra; EP – distance between the ICA ambit and the pharyngeal wall at the level of the epiglottis apex; CCA bif. – distance between the common carotid artery bifurcation and the lower margin of the 2nd cervical vertebra; MIN – minimal distance of the ICA to the pharyngeal wall found along its course between the Eustachian tube opening the epiglottis apex level; ICA – extracranial internal carotid artery.

Table 5. Predicted risk factors for the presence of an aberrant ICA or a decreased distance between the ICA and the pharyngeal wall

Advanced age[11,12]	Diabetes[26]
Female sex[11,12, 26]	Cerebrovascular symptoms[24 denied by 25]
Hypertension[26,27]	Cephalgia[23]
Atherosclerosis[26 denied by 25]	Chronic infection of the tonsils (local spasm and thrombosis)[5]
Cardiovascular diseases[23 denied by 25]	Cervical dysesthesia[23]
Transient ischemic attacks[23]	Cervical pain[23]
Difficulties with swallowing and speech[23]	Pharyngeal pressure, foreign body sensations, intraoral pulsations[23]
Noise perceptions, dizziness, tinnitus[23]	Glossopharyngeal neuralgia[23]

ICA – extracranial internal carotid artery.

Conclusions

In women, the ICA tends to be closer to the pharyngeal wall on the right side of the oropharynx; however, its distance to the ET is the same on both sides.

The ICA is closer to the pharyngeal wall in women than in men only at the level of the ET.

The ICA is closer to the pharyngeal wall at the level of the epiglottis apex than at the level of the ET; therefore, any measurements at the ET level are not representative of the distance between the ICA and the tonsillar fossa.

Patients with the CCA bifurcation at the level of the epiglottis apex have a shorter ICA distance to the pharyngeal wall at this level. Men have the CCA bifurcation higher than women, so they are more prone to CCA incision during surgery.

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Sport and physical activity after ankle arthrodesis with Ilizarov fixation and internal fixation

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Abstract

Background. Severe osteoarthritis (OA) of the ankle joint constitutes an important social problem.

Objectives. We used (1) the GRIMBY scale, (2) the LOWER LIMB Activity scale, (3) the UCLA (University of California Los Angeles) activity scale, (4) the VAS (visual analogue scale) ACTIVITY scale, and (5) the FAAM (foot and ankle ability measure) SPORT scale to verify whether the type of ankle joint arthrodesis stabilization affected sports and physical activity levels.

Material and methods. We carried out a prospective clinical study of 47 patients who had undergone ankle arthrodesis with Ilizarov external fixator stabilization (Group 1, n = 21) or internal stabilization with screws (Group 2, n = 26) at Orthopaedic Clinic at the Wroclaw Medical University, Poland, from 2007 to 2015. Sports and physical activity levels were measured by (1) the GRIMBY scale, (2) the LOWER LIMB Activity scale, (3) the UCLA activity scale, (4) the VAS ACTIVITY scale, and (5) the FAAM SPORT scale.

Results. A comparison between the average results of Group 1 and Group 2 on the LOWER LIMB Activity scale and the GRIMBY scale before and after surgery revealed no significant differences. In Group 1, the mean scores on the VAS ACTIVITY scale and the UCLA activity scale after treatment were higher than in Group 2. In Group 1, the mean outcome in the SPORT FAAM scale after treatment was 40; in Group 2 it was 30.06.

Conclusions. Ilizarov fixation of ankle arthrodesis is associated with better scores on the FAAM SPORT, UCLA activity and VAS ACTIVITY scales after treatment than internal fixation. The scores on the GRIMBY scale and the UCLA activity scale were significantly higher after treatment than before treatment in both groups. In this study, ankle fusion with Ilizarov fixation and internal fixation was found to be effective in the treatment of ankle arthritis. The levels of sport and physical activity were satisfactory in both groups, but the outcomes after fixation with the Ilizarov apparatus were better than after internal stabilization.

Key words: sport, physical activity, internal fixation, ankle arthrodesis, Ilizarov fixation

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Introduction

Severe osteoarthritis (OA) of the ankle joint constitutes an important social problem. It results in functional disturbances and in compensation of irregularities, which are more pronounced while moving and standing.[1–5] This leads to poorer functioning of the lower limb, lower muscle strength, deterioration in gait efficiency, and reduced mobility of joints, which reduces the physical activity and participation in sports.[1–5] Ankle arthrodesis has been the gold standard operative treatment for end-stage ankle arthritis.[4,6–19] Ankle arthrodesis can be achieved by external fixation or internal fixation.[6–8,11-13,16–20] An operation is effective when it reduces pain and improves the function of the limb.[1,2,11,20,21] Nowadays, patients tend to quickly return to work, independence and sports activities.[3,4] Ankle arthrodesis may be associated with a reduction in physical activity and sport after surgery.[2–5]

An optimal method for ankle arthrodesis should provide a good functional outcome and should enable the patient to be involved in sports and other forms of physical activity to the same or even a greater extent than prior to the procedure.[1–5]

All of the studies report sports and physical activity levels separately for each of the 2 ankle arthrodesis procedures: Ilizarov fixation and internal fixation.[2,4,5] There is no study in the literature comparing sport and physical activity level with the GRIMBY scale, the LOWER LIMB Activity scale, the UCLA (University of California Los Angeles) activity scale, the VAS (visual analogue scale) ACTIVITY scale, and the FAAM (foot and ankle ability measure) SPORT scale in both groups with Ilizarov fixation and internal fixation of ankle arthrodesis.

We hypothesized that the type of ankle arthrodesis stabilization does not affect sports and physical activity levels. Our objective was to verify whether the type of ankle joint arthrodesis stabilization affected sports and physical activity levels.

Material and methods

We carried out a retrospective clinical study of all 55 patients who had undergone ankle arthrodesis with Ilizarov external fixator stabilization (Group 1) or internal stabilization with screws (Group 2) at Orthopaedic Clinic at the Wroclaw Medical University, Poland, from 2007 to 2015. The indications of ankle arthrodesis included severe primary or secondary (post-traumatic, neurogenic, rheumatoid, or congenital) degenerative/deforming changes of the ankle joint.[22]

Patients were subjected to ankle arthrodesis with either external Ilizarov fixation or internal fixation with cannulated screws. Individuals with a poor status of the soft tissues (necrosis, inflammation, fistulas, trophic changes, scars, vascular lesions, skin lesions, or ulcers) or bones (osteoporotic, bone loss or infections), severe deformities (>15° in 1 axis or multiplanar deformities), or infection were always qualified for ankle arthrodesis with Ilizarov fixation. The remaining patients were subjected to ankle arthrodesis with either external Ilizarov fixation or internal fixation with cannulated screws. We preferred ankle arthrodesis with internal fixation in patients with a good status of the soft tissues and bones, deformities <5° and without any concomitant disease which prevented immobilization in a plaster cast, and in patients who cooperated with restricted limb weight bearing just after the surgery.[22]

The criteria for inclusion in the study consisted of: performance of ankle arthrodesis with Ilizarov external fixator stabilization or internal stabilization with cannulated screws; more than 24 months from the conclusion of the treatment; the presence of baseline values of ankle pathology etiology and demographic data in medical records; and the presence of preoperative and postoperative sport and physical activity level scores (the GRIMBY scale, the LOWER LIMB Activity scale, the UCLA activity scale, the VAS ACTIVITY scale, and the FAAM SPORT scale).

The exclusion criteria consisted of: ankle arthrodesis without Ilizarov external fixator stabilization or internal stabilization with cannulated screws; a lack of baseline values of ankle pathology etiology and/or demographic data in medical records; a lack of preoperative and postoperative sport and physical activity level scores; a follow-up period shorter than 24 months; Charcot neuroarthropathy; multiple joint or bilateral ankle injuries; and undergoing associated procedures during surgical intervention.

Patients were included in the study based on medical history, medical record analysis performed before and after treatment, and physical examination. The participants were informed about the voluntary nature of the study and they gave their consent to participate in the study. In the case of minors, consent was given by their legal guardians. The study was approved by the Wroclaw Medical University Bioethics Commission.

Between 2007 and 2015, 55 patients underwent ankle arthrodesis. Of these, 24 were treated with an Ilizarov device and 31 with internal fixation (screws). In the Ilizarov group, 1 patient (4%) was lost to follow-up during the 2 years, 1 (4%) was not included because of missing data in the patient's records and 1 (4%) was excluded because of neuropathic arthropathy, bilateral ankle injuries or associated ankle procedures at the time of the arthrodesis, leaving a total of 21 patients for analysis. In the internal fixation group, 2 patients were lost to follow-up during the 24 months (6%), 2 (6%) were not included because of missing data in their records and 1 (3%) was excluded because of neuropathic arthropathy, bilateral ankle injuries or associated ankle procedures at the time of the arthrodesis, leaving a total of 26 patients for analysis.

All patients were given perioperative antibiotics and were placed in a supine position; then a tourniquet was applied (320 mm Hg). An anterior approach centered over the ankle joint was used for ankle joint fusion. An Ilizarov

apparatus (Group 1) or cannulated screws (Group 2) were used to create compression at the ankle joint. The Ilizarov apparatus for ankle arthrodesis consisted of a proximal ring fixed to the tibia and fibula with 3 Kirschner wires, a distal ring fixed to the tibia and fibula with 2 Kirschner wires, and a U-shaped foot ring fixed to the calcaneus with 2 Kirschner olive wires and fixed to the distal part of the metatarsal bones with 1 Kirschner olive wire. All patients in Groups 1 and 2 were operated on by 3 surgeons. Patients from Group 1 (Ilizarov stabilization) started weight bearing on postoperative day 1. The minimum time of wearing the Ilizarov fixator was 9 weeks. After the Ilizarov fixator was removed, patients transitioned to a walker boot for a minimum of 6 weeks. Postoperatively, patients from Group 2 remained non-weight bearing for a minimum of 6 weeks in a cast, followed by protected progressive weight bearing in a controlled ankle motion walker (CAM walker) for the next 6 weeks. Usually, by 3 months patients had made a transition to wearing normal shoes.[22]

The patients' sports and physical activity levels were assessed using the GRIMBY scale, the LOWER LIMB Activity scale, the UCLA activity scale, the VAS ACTIVITY scale, and the FAAM SPORT scale.[20,23–26] Mean preoperative and postoperative scores have been calculated.

To verify whether the average value of the variables varied significantly, the Mann-Whitney U test and Student's t-test were used. To analyze the significance of differences between the mean values of data variables in Groups 1 and 2, we used the Mann-Whitney U test and Student's t-test. Where the use of test data required fulfillment of assumptions about normality, the Shapiro-Wilk test and Kolmogorov-Smirnov test were used to verify the hypothesis of normality. All analyses were carried out at an assumed significance level of $\alpha = 0.05$ using STATISTICA v. 10.0 software (StatSoft Inc., Tulsa, USA).

Results

There was no statistically significant difference in the demographic data for patients in Groups 1 and 2 (Table 1).

Frontal plane alignment of the ankle joint was observed in 100% of the patients from the Ilizarov group and in 75.8% from the internal fixation group. The sagittal plane alignment rates were 100% in Group 1 and 84.8% in Group 2. Ankle fusion was achieved in 100% of the patients in Group 1 and in 87.9% in Group 2.

In the group with Ilizarov fixation (Group 1), the average rate of complications was 0.62 complications per patient. In the group with internal stabilization, there were 0.58 complications per patient. In Group 1, 90.5% of the patients were very satisfied or satisfied with the treatment. In the group with internal fixation with cannulated screws (Group 2), 88.5% of the patients were very satisfied or satisfied with the treatment.

In Group 1, the median activity score on the GRIMBY scale before treatment was 3. The median GRIMBY scale

Table 1. Demographic characteristics of the patients

Variable	Group 1 – Ilizarov external fixator (n = 21)	Group 2 – internal stabilization (n = 26)	p-value
Age [years]	44 (17–65)	47 (17–67)	p = 0.24
Sex	14 (66.6%) male	17 (65.4%) male	p = 0.45
Follow-up [months]	45 (24–108)	47 (24–104)	p = 0.38
Disease diagnosis	–	–	–
primary OA	2 (9.5%)	3 (11.5%)	p = 0.29
secondary OA	–	–	–
post-traumatic	10 (47.6%)	15 (57.7%)	p = 0.12
rheumatoid	0 (0%)	1 (3.8%)	p = 0.13
congenital	4 (19%)	3 (11.5%)	p = 0.16
neuropathic	5 (23.8%)	4 (15.4%)	p = 0.21
SPORT FAAM scale score after treatment (0–100)	median 41; mean 40 (11–100)*	median 30; mean 30.06 (14–100)*	p = 0.041
GRIMBY scale score before treatment (1–6)	median 3; mean 3.23 (1–6)	median 3; mean 3.12 (1–6)	p = 0.29
GRIMBY scale score after treatment (1–6)	median 4.5; mean 4.45 (1–6)	median 4.5; mean 4.42 (1–6)	p = 0.49
LOWER LIMB Activity scale score before treatment (1–18)	median 9.5; mean 9.63 (4–18)	median 9.5; mean 9.7 (2–15)	p = 0.51
LOWER LIMB Activity scale score after treatment (1–18)	median 12; mean 12.25 (7–15)	median 12; mean 11.77 (3–17)	p = 0.19
UCLA activity scale score before treatment (1–10)	median 5; mean 4.93 (2–10)	median 5; mean 4.98 (2–9)	p = 0.41
UCLA activity scale score after treatment (1–10)	median 7; mean 6.97 (3–9)*	median 6; mean 5.97 (2–9)*	p = 0.033
VAS ACTIVITY scale score before treatment (0–10)	median 5; mean 5.13 (1–10)	median 5; mean 5 (0–10)	p = 0.32
VAS ACTIVITY scale score after treatment (0–10)	median 7; mean 6.85 (2–10)*	median 5.5; mean 5.35 (2–10)*	p = 0.043

* statistical difference between the groups ($p < 0.05$); OA – osteoarthritis; UCLA – University of California Los Angeles; VAS – visual analogue scale; FAAM – foot and ankle ability measure.

score after treatment increased to 4.5 and these values were statistically different (p = 0.041). In the group with internal stabilization, the median value of GRIMBY activity level before treatment was 3. The median GRIMBY scale score after treatment was 4.5. These values were statistically different (p = 0.045) (Table 1).

In Group 1, the median value of the LOWER LIMB Activity scale score before treatment was 9.5. The median LOWER LIMB Activity scale score after treatment was 12. These values were not statistically different (p = 0.13). In the group with internal stabilization, the median score on the LOWER LIMB Activity scale before treatment was 9.5. The median LOWER LIMB Activity scale score after treatment was 12 (Table 1). These values were not statistically different (p = 0.16).

In Group 1, the median score on the UCLA activity scale before treatment was 5. The median UCLA activity scale score after treatment was 7. These values were statistically different (p = 0.041). In the group with internal stabilization, the median score on the UCLA activity scale before treatment was 5. The median UCLA activity scale score after treatment was 6 (Table 1). These values were statistically different (p = 0.047). The UCLA activity scale values after treatment for Group 1 were significant higher than for Group 2 (p = 0.033).

In Group 1, the median score on the VAS ACTIVITY scale before treatment was 5. The median VAS ACTIVITY scale score after treatment was 7. These values were statistically different (p = 0.039). In the group with internal stabilization, the median score on the VAS ACTIVITY scale before treatment was 5. The median VAS ACTIVITY scale score after treatment increased to 5.5 (Table 1). These values were not statistically different (p = 0.34). After treatment, the group with Ilizarov stabilization (Group 1) had significantly higher VAS ACTIVITY scale scores (Table 1) (p = 0.043).

The FAAM functional outcome was significantly higher in the group with Ilizarov stabilization (Table 1) (p = 0.041).

Discussion

The method for ankle joint arthrodesis should allow for the practice of sports and physical activity after treatment at the highest possible level.

To the best of our knowledge, none of the previous studies analyzed sports and physical activity levels after ankle arthrodesis regarding the fixation method used. MacMahon et al. analyzed sports and physical activity levels in 38 patients who had undergone primary partial arthrodesis for a Lisfranc injury.[4] Preoperatively, 47.1% engaged in high-impact sports, and postoperatively, 44.8% did. Compared to the preoperative levels, the difficulty was the same in 66% and had increased in 34% of physical activities. Participation levels improved in 11%, remained the same in 64% and were impaired in 25% of physical activities. The decrease in participation or increase in difficulty of some activities suggests that some patients experienced postoperative limitations in exercise.[4]

As far as we know, none of the previous studies compared GRIMBY scale scores in patients subjected to ankle arthrodesis with external and internal fixation. Morasiewicz et al. analyzed sports and physical activity levels in 56 patients after derotational osteotomy with the Ilizarov method; the mean level of activity on the GRIMBY scale after treatment was 4.2.[3] Their findings are similar to those obtained in our present study. The mean GRIMBY scale scores after treatment increased statistically significantly in both groups.

None of the previous studies compared the values of the LOWER LIMB Activity scale score in patients after ankle arthrodesis with external and internal fixation. The mean value of the LOWER LIMB Activity scale in patients examined by Morasiewicz et al. was 11.84.[3] Their findings are similar to those obtained in the present study. In our study, patients from both groups presented with similar pre- and post-treatment values of the LOWER LIMB Activity scale score. The post-treatment values of the LOWER LIMB Activity scale score are higher than pretreatment values, but not statistically significantly.

None of the previous studies compared the values of the UCLA activity scale score in patients with external and internal fixation of ankle arthrodesis. Schuh et al. used this scale to examine 21 patients after ankle arthrodesis; the mean postoperative UCLA activity scale score in this group was 7.0.[5] According to Morasiewicz et al., the mean post-treatment UCLA activity scale score in 56 patients subjected to derotational osteotomy with the Ilizarov method was 6.18.[3] Their findings are similar to those obtained in our present study. Irrespective of fixation type, we did not observe statistically significant differences between preoperative UCLA activity scale scores. The posttreatment values of the UCLA activity scale score are higher than the pretreatment values in both groups. The average score on the UCLA activity scale after treatment was significant higher for the Ilizarov group than for the internal fixation group.

To the best of our knowledge, none of the previous studies compared the values of the VAS ACTIVITY scale in individuals with ankle arthrodesis with external and internal fixation. In the study conducted by Morasiewicz et al., mean VAS ACTIVITY scale score after treatment was 5.98, i.e., similar to our present study.[3] We observed a significant postoperative increase in the VAS ACTIVITY scale scores of patients with Ilizarov fixation; the post-treatment scores in this group turned out to be significantly better than in subjects with internal fixation of ankle arthrodesis.

As far as we know, the results of the FAAM SPORT scale in patients with external and internal fixation of ankle arthrodesis have not been thus far subjected to a comparative analysis. According to Dalat et al., the mean postoperative FAAM SPORT scale score for a group of 46 patients after ankle arthrodesis with internal fixation was 29.8; this value

was slightly lower than the one we found in our subjects.[2] In our present study, individuals with Ilizarov fixation presented with better FAAM SPORT scale scores than those subjected to internal fixation.

Patients with Ilizarov fixation had better FAAM SPORT scale scores, UCLA activity scale scores and VAS ACTIVITY scale scores after treatment than those after internal fixation. Ilzarov fixation exerts lesser impact on musculoskeletal biomechanics than in the case of internal fixation. Also, the possibility of fully loading the treated limb soon after fixation with the Ilizarov apparatus seems to be more beneficial than completely sparing the extremity after internal stabilization. The better sports and physical activity results of arthrodesis with external fixation can be related to better frontal and sagittal plane alignment of the ankle joint and higher ankle fusion rate. However, irrespective of the study group, the sports and physical activity level scores of our patients were close to those reported by other researchers.[2,5] According to some authors, the sports and physical activity level outcomes of ankle arthrodesis are mediocre.[2,4,5] In a study conducted by Chahal et al., the mean functional scores of patients subjected to ankle arthrodesis with internal fixation were below the reference values for the American population.[1] In a study conducted by Dalat et al., the overall mean athletic level after the surgery was relatively low compared to the state prior to the injury.[2] After ankle arthrodesis, the number of patients participating in sports decreased.[5] McKee et al. noticed that 64% of patients with post-traumatic deformity correction with the Ilizarov method could return to physical activity, but at a reduced level; this study did not provide a detailed description of the types of activity.[3,27]

In general, patients who had ankle joint arthrodesis can expect worse sports and physical activity levels than the general population.[4] This would indicate that they experience pain and a higher level of disability than the general population. Improper function of the lower limbs limits and even prevents participation in sports and physical activity. Worse limb function may result from instability and limitation of movement of the joints, from pain, reduced muscle strength, connective tissue scars, and increased body weight.[3] We noticed an improvement in the sports and physical activity levels of patients from both groups. The lack of a very significant improvement in sports and physical activity levels might reflect a post-arthrodesis disruption of lower extremity biomechanics. Significant asymmetry in gait and a reduced range of motion remained after ankle joint arthrodesis compared to normal.[28]

One potential limitation of our present study may stem from the fact that the FAAM SPORT scale scores were determined solely postoperatively. However, the values of all other activity scales were determined both before and after treatment. The patients in both groups were operated on by 3 different surgeons, but following the same protocol. The strong points of this study are the homogeneity of the techniques and surgical recovery in both groups. Importantly, none of the previous studies compared the sports and physical activity levels after ankle arthrodesis with 5 different activity scales.

This study was not randomized. Both groups were similar in age, etiology, gender, and follow-up time. Ilizarov arthrodesis was predominant in patients with a poor status of soft tissues and bones, severe deformities and infection. These factors, which we used as criteria for Ilizarov external fixator stabilization, could have impacted the final results. Theoretically, patients in Group 1 had more negative influences on sports and physical activity levels before surgery. Thus, postoperative sports and physical activity levels should have improved more in Group 1 than in Group 2. Ankle arthrodesis with internal fixation was performed in patients with a good status of soft tissues and bones, with deformities <5°, and without concomitant diseases or infection. Supposedly, patients in Group 2 had fewer adverse factors for sports and physical activity. Treatment with cannulated screws was less stressful. Theoretically, sports and physical activity levels after arthrodesis with cannulated screws may be less improved than Group 1.

In this paper, we compared only sports and physical activity after ankle arthrodesis with Ilizarov fixation and internal fixation; as in our other published works, we evaluated the radiological outcomes and clinical outcomes in patients after ankle arthrodesis with Ilizarov fixation and internal fixation.[22]

Ankle arthrodesis with Ilizarov fixation and internal stabilization resulted in normalization of lower limb loads. Balance after ankle arthrodesis with Ilizarov fixation is worse than with internal stabilization ankle arthrodesis.

Ilizarov fixation of ankle arthrodesis is associated with better FAAM SPORT scale scores, UCLA activity scale scores and VAS ACTIVITY scale scores after treatment than after internal fixation. The scores of the GRIMBY scale and the UCLA activity scale after treatment were significantly higher than before treatment in both groups.

In this study, ankle fusion with Ilizarov fixation and internal fixation was found to be effective in the treatment of ankle arthritis. Sports and physical activity levels were satisfactory in both groups, but the outcomes after fixation with the Ilizarov apparatus are better than after internal stabilization.

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Factors affecting mortality in children requiring continuous renal replacement therapy in pediatric intensive care unit

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Abstract

Background. Acute kidney injury (AKI) occurs in up to 30% of pediatric intensive care unit (PICU) patients and is associated with a high mortality rate.

Objectives. The objective of the study was to evaluate factors associated with the outcome and to identify the prognostic factors in children receiving continuous renal replacement therapy (CRRT).

Material and methods. This was a retrospective, single-center study, including 46 patients.

Results. Logistic regression analysis demonstrated significant effects on patient survival exerted by the percentage of fluid overload (FO%) (odds ratio (OR): 1.030; $p = 0.044$). In the group of patients with FO% <25%, the mortality was 33.3%, and in the FO% ≥25% group, the mortality was 67.9% ($p < 0.001$). The probability of death without multi-organ failure (MOF) was 13%, while with MOF it was 74%. There was no difference in the duration of hospitalization between the CRRT patients (mean: 21.9 days) and the general population of children hospitalized in PICU in the same period ($n = 3,255$; mean: 25.4 days); however, a significant difference was noted in mortality between the 2 groups of patients (54% vs 6.5%; $p < 0.001$).

Conclusions. The mortality of PICU CRRT patients is more than 8-fold higher than the mortality of the total PICU population. Coexisting MOF increases the mortality almost 6 times. The mortality of children with FO% ≥25% was more than 2-fold higher than the mortality of children with FO% <25%.

Key words: acute kidney injury, survival, anticoagulation, pediatric intensive care unit, continuous renal replacement therapy

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Introduction

Acute kidney injury (AKI) is observed in up to 30% of children admitted to pediatric intensive care units (PICUs) and is associated with a mortality rate of up to 50%.[1–3] There is evidence that the epidemiology of pediatric AKI has changed over the past few decades and that primary renal diseases are currently no longer the most common causes of AKI.[4] Nowadays, the most frequent causes of AKI in developed countries are acute tubular necrosis, nephrotoxic medications, oncological diseases, septic shock, and their common consequence – multi-organ-failure (MOF).[5] Over the past 2 decades, in many developed countries, continuous renal replacement therapy (CRRT) has become the most widely used renal support modality in critically ill children.[6] It eliminates the need for fluid restriction and allows for the provision of medications, blood products and nutrition in critically ill children.[7]

Despite the increasing use of CRRT, AKI is associated with an increased risk of mortality in critically ill patients.[8,9] Although formal and clearly defined indications for CRRT use are lacking, it is mostly employed for individual AKI and AKI associated with fluid overload (FO), MOF and sepsis.[10] Furthermore, there are still not many published reports addressing CRRT in PICUs.[6]

The main objective of the present study was to evaluate the clinical course, to analyze factors associated with the outcome and to identify prognostic factors in critically ill children receiving CRRT.

Material and methods

This was a retrospective, single-center, chart review study, including 46 patients receiving CRRT, who had been admitted to the PICU in the Department of Anesthesiology and Intensive Care, Institute of Pediatrics, Jagiellonian University Medical College, Kraków, Poland, between January 2009 and December 2016. This study was approved by the Bioethical Committee of Cracow Medical Chamber (OIL/KBL/4/2017).

Initiation, prescription and general CRRT management occurred under the guidance of the attending intensivist and consulting nephrologist. The primary indications for the initiation of CRRT were categorized as AKI, FO or MOF. Fluid overload percentage (FO%) was determined using the PICU admission weight in kilograms as the baseline weight for comparison. The FO% was defined using the method described by Goldstein et al. ([fluid in – fluid out]/[intensive care unit admission weight] × 100%), and fluid in (or out) was considered to be the amount of fluid from admission to PICU to CRRT initiation.[11] Acute kidney injury was determined by the pediatric-modified Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease score (RIFLE, modified into p-RIFLE).[2] Multi-organ failure was defined as the presence of at least 2 organs involved at any time on admission to PICU.[12] Sepsis was defined using the criteria of the International Consensus Conference on Pediatric Sepsis.[12]

A Prismaflex dialysis machine (Gambro, Barcelona, Spain) was used for CRRT in all the patients. Dual lumen catheters were used depending on the age, weight and morphology of the child, using the same criteria for the patients treated with regional citrate anticoagulation (RCA) and heparin anticoagulation (Hep-ACG).[8,13] Hollow fiber hemofilters were used depending on the body weight of the patient, according to the guidelines of the manufacturer.[14]

In all the Hep-ACG cases, predilutional continuous veno-venous hemodiafiltration (CVVHDF) was employed and unfractionated heparin (Hep) was used. In the Hep-ACG protocol, a Hep bolus was administered during the connection along with a subsequent continuous Hep infusion to achieve a post-filter activated clotting time (ACT) between 180 and 240 s. The ACT value was checked on average every 2 h within the first 12 h of CRRT, and then every 4–6 h.

The RCA procedure was conducted according to the guidelines from the study by Tolwani et al. and from the Prismaflex operator's manual.[14,15] An initial citrate concentration of 3.0 mmol/L was used for a target post-filter ionized calcium level of 0.25–0.50 mmol/L. The citrate effect was neutralized using a continuous calcium infusion of 10% calcium gluconate (or 5% in children weighing <15 kg) to maintain ionized calcium blood levels between 1.0 and 1.2 mmol/L.[15,16] Samples were taken every hour for the first 4 h and every 6–8 h afterwards. Total calcium levels were also checked at least once daily. Citrate accumulation was avoided by monitoring the patient's pH status and by total calcium/ionic calcium index (CaT/CaI) being maintained ≤2.5.

Regarding the CRRT solutions for Hep-ACG, Hemosol, or PrismaSol 2 (Gambro) as a dialyzate and Phoxilium (Gambro) or PrismaSol 2 as a predilutional substitute were used; whereas for RCA, the citrate solution Prismocitrate 18/0 (Gambro) was administered in a pre-blood pump (PBP), the dialyzate normocarbonate solution PrismOcal B22 (Gambro) was used as a calcium-free dialysis solution, and the low-flow post-filter infusion of PrismaSol 2 or Phoxillum was employed as a postdilutional substitute to avoid clotting in the deaeration chamber return line.

In both techniques, the dialyzate and substitution flow rates were initially programmed to achieve a dialysis dose of at least 35 mL/kg/h and were subsequently adjusted in an ongoing manner to meet the patients' needs. None of the filters were used for longer than 72 h. The blood flow rates (BFR) were determined according to the patients' body mass and were monitored by the pressure at the access sites.[15]

Dual lumen catheters were individually adapted to the child's morphology and body weight according to the literature recommendations.[8,13] The implantation sites of the

dual lumen catheters included the left femoral vein in 19 cases (41.3%), the right femoral vein in 13 cases (28.3%) and the right internal jugular vein in 14 cases (30.4%).

The collected data addressed the patients' demographics, underlying disease, detailed CRRT parameters, and the mode and dose of the applied anticoagulation (ACG) method.

Statistical analysis

The distribution of polytomous variables was described employing the mean value, standard deviation (SD), median value, and interquartile range (IQR). To compare the distributions, Student's t-test was used, providing the Kolmogorov-Smirnov (KS) test yielded a positive result for conformity of the empirical distribution and Gaussian distribution. In case of a lack of such conformity, the Wilcoxon rank-sum test (equivalent to the Mann-Whitney U test) was employed; this fact was indicated by marking the appropriate p-value with an asterisk (*). The sample sizes were compared by means of the χ^2 test; in the cases with low predicted frequencies, Yates's modification was employed, which was indicated by marking the appropriate p-values with 2 asterisks (**). The effect of FO% on the cumulative survival probability of the patients was analyzed employing the Kaplan-Meier curves and the Mantel-Cox test. Additionally, the univariate and multivariate analyses of significant parameters were carried out depending on ACG and the outcome. In addition, the univariable and multivariable logistic regression analyses were performed, identifying the parameters showing a significant odds ratio (OR) and describing the effect on the outcome. When only dichotomous input variables were considered, the OR was computed directly from the contingency table analysis. It should be emphasized that depending on the employed mode of analysis, the numerical assessment of the effect of a given parameter on the outcome was achieved in the form of probability of death (mortality) or OR of death (mortality OR). In all tests, the significance threshold was assumed to be 0.05, with the exception of the KS test, where a value of 0.1 was assumed to be significant. The STATISTICA v. 12 (StatSoft Inc., Tulsa, USA) and MATLAB v. 2015a (Mathworks, Natick, USA) packages were used for all calculations and for creating the graphs.

Results

The number of children requiring CRRT in particular years was as follows: in 2009 – 6 patients; 2010 – 3; 2011 – 7; 2012 – 4; 2013 – 6; 2014 – 5; 2015 – 5; and in 2016 – 10. As RCA has been used in our center since 2015, 4 patients in 2015 and 10 patients in 2016 were treated with this mode of ACG. The Hep-ACG was applied in all other cases.

A detailed characteristics of the study population is included in Tables 1 and 2. The main diagnoses in the studied population were as follows: 1. oncological diseases – 20 children (43.5%), including acute lymphoblastic leukemia – 11 (8 after bone marrow transplantation (BMT)), lymphoma – 3, solid tumors – 3, and acute myeloid leukemia after BMT – 3; 2. hematological diseases – 6 children (13%), including aplastic anemia after BMT – 3, severe combined immunodeficiency after BMT – 2 and hemophagocytic syndromes after BMT – 1; 3. nephrological diseases – 11 children (24%), including chronic kidney disease (CKD) exacerbation – 4, chronic dialysis complications – 3, rapid progressive glomerulonephritis – 1, hemolytic uremic syndrome – 2, and tubulointerstitial nephritis – 1; 4. sepsis – 3 children (6.5%), including *Neisseria meningitidis* – 2 and *Streptococcus* spp. – 1; 5. burns – 2 children (4.3%); 6. cardiovascular diseases – 2 children (4.3%); 7. rheumatic disease (systemic lupus erythematosus) – 1 child (2.2%); and 8. gastroenterological diseases – 1 child (2.2%).

There were no statistically significant differences in the ratio of the main diagnoses, neither between Hep-ACG and RCA, nor between deceased and surviving patients (apart from nephrological diagnoses in the case of deceased vs surviving patients: 1 vs 10; p = 0.001).

While comparing the sample size in children dialyzed with Hep-ACG vs RCA, no statistically significant differences were noted in gender, body mass, body height, or outcome, which means that the studied populations were mutually equivalent and relevantly representative for the current study. The patients treated with RCA demonstrated longer hospitalization in PICU, were younger, and, in view of the additional citrate flow, they received a higher dialysis dose in total than the children treated with Hep-ACG. In turn, when compared to the RCA group, the children treated with Hep-ACG were characterized by a more severe clinical status (which was evident in a higher number of vasopressor medications (VPS) at CRRT initiation and a higher absolute percentage of days with VPS administration during their PICU stay), required mechanical ventilatory support extending over a longer proportion of their PICU hospitalization and their urine output (UO) at the time of CRRT initiation was lower.

As shown in Tables 2 and 3, 46% of the patients from the study population survived. There were no significant differences between the survivors and non-survivors with respect to age, weight, height, gender, number of days spent in PICU, estimated glomerular filtration rate (eGFR) value, urea concentration, total protein, albumin concentration at the start of CRRT, day of CRRT initiation, number of hours of CRRT per patient, dialysis dose per patient, ultrafiltration rate (UFR) value per patient, or ACT value.

On the other hand, as shown in Table 2, the surviving children were subjected to CRRT mainly for AKI or FO% (as the immediate causes of CRRT initiation), required less intensive mechanical ventilatory support during their hospitalization in PICU, had fewer VPS at CRRT initiation,

Table 1. Clinical characteristics of children treated with heparin anticoagulation (Hep-ACG) and regional citrate anticoagulation (RCA)

Variable	Hep-ACG, n = 32 (69.6%) n [%]	RCA, n = 14 (30.4%) n [%]	p-value	Total, n = 46 n [%]
Surviving/deceased	12 (37.5)/20 (62.5)	9 (64.3)/5 (35.7)	NS	21 (45.6)/25 (54.5)
Males/females	16/16	11/3	NS	27/19
Patients with MOF as the main reason for CRRT implementation	24	7	NS**	31
Patients with AKI as the main reason for CRRT implementation	5	2	NS**	7
Patients with FO% as the main reason for CRRT implementation	3	5	NS**	8
Patients with mechanical ventilation at CRRT initiation	27 (84.4)	8 (57.1)	NS**	35 (76.1)
Days with VPS administration during PICU stay	406 (81.5)	273 (53.4)	<0.001	679 (67.3)
Days with diuretic administration during PICU stay	336 (67.5)	282 (55.2)	<0.001	618 (61.2)
CRRT sessions, clotted/non-clotted	27 (24.1)/85 (75.9)	34 (31.5)/74 (68.5)	NS	61 (27.7)/159 (72.3)
Number of hours of CRRT sessions, clotted/non-clotted	516 (10)/4,666 (90); 5,182 (48.6)	882 (16.1)/4,599 (83.9); 5,481 (51.4)	<0.001	1,398 (13.1)/9,265 (86.9); 10,663
Variable	**mean ±SD/median (IQR)**	**mean ±SD/median (IQR)**	**p-value**	**mean ±SD/median (IQR)**
Age [years]	10.5 ±5.4/11.7 (10.2)	6.7 ±6.8/3.8 (12.8)	0.046	9.3 ±6.0/10.3 (11.4)
Body mass [kg]	36.3 ±20.3/31.5 (31.6)	26.8 ±22.6/16.3 (35.6)	NS	33.4 ±21.2/29.5 (34.6)
Body height [cm]	133.9 ±31.0/137.0 (58.5)	111.6 ±43.6/108.5 (84.0)	NS	127.1 ±36.4/134.5 (67.0)
Number of days in PICU/patient	15.6 ±12.2/12.5 (17.0)	36.5 ±26.1/32.5 (52.0)	0.001	21.9 ±19.9/17.5 (27.0)
Percentage of days of mechanical ventilation	77.7 ±37.1/100.0 (40.0)	49.3 ±43.1/58.5 (100.0)	0.021*	69.0 ±40.7/100.0 (68.0)
Number of VPS at CRRT initiation	1.34 ±0.90/1.00 (1.00)	0.71 ±0.73/1.00 (1.00)	0.026	1.15 ±0.89/1.00 (2.00)
CRP [mg/L] at CRRT initiation	189 ±144/187 (229)	132 ±110/116 (149)	NS	172 ±136/151 (215)
Total protein [g/L] at CRRT initiation	51.4 ±10.7/51.5 (14.1)	54.5 ±12.5/56.2 (9.7)	NS	52.4 ±11.2/53.1 (15.1)
Albumin [g/L] at CRRT initiation	26.6 ±5.9/26.0 (7.8)	31.3 ±9.6/33.4 (10.1)	NS	28.1 ±7.4/28.0 (11.2)
eGFR [mL/min/1.73 m^2] at CRRT initiation	37.1 ±23.9/33.2 (27.9)	42.4 ±21.6/44.3 (29.1)	NS	38.7 ±23.1/34.7 (32.7)
Urea [mmol/L] at CRRT initiation	25.1 ±14.4/25.2 (24.0)	21.5 ±16.7/16.1 (8.8)	NS	24.0 ±15.0/20.1 (20.4)
UO [mL/kg/h] at CRRT initiation	0.75 ±0.71/0.45 (1.25)	1.54 ±1.41/1.10 (2.10)	0.014	0.99 ±1.03/0.65 (1.30)
FO% at CRRT initiation	36.7 ±32.8/26.0 (32.8)	32.6 ±29.7/29.8 (30.0)	NS	35.5 ±31.6/27.3 (32.5)
Day of CRRT initiation	5.2 ±5.3/2.5 (8.0)	6.8 ±9.9/1.5 (5.0)	NS*	5.7 ±6.9/2.0 (8.0)
Dialysis dose: QD+QS (+QC) [mL/h/kg]/patient	50.7 ±16.0/48.0 (18.6)	68.2 ±26.4/63.6 (35.7)	<0.001*	59.6 ±23.6/55.0 (31.9)

* Wilcoxon rank-sum test; ** Yates's modification of the χ^2 test; AKI – acute kidney injury; CRP – C-reactive protein; CRRT – continuous renal replacement therapy; eGFR – estimated glomerular filtration rate; FO% – fluid overload percentage; Hep-ACG – heparin anticoagulation; MOF – multi-organ failure; PICU – pediatric intensive care unit; QC – citrate flow rate; QD – dialyzate flow rate; QS – supplement flow rate; RCA – regional citrate anticoagulation; UO – urine output; VPS – vasopressors; SD – standard deviation; IQR – interquartile range; NS – not significant.

had a lower absolute percentage of days with VPS administration during their PICU stay, and had lower values of inflammatory marker (C-reactive protein (CRP)), but they maintained a significantly higher diuresis value at the time of CRRT initiation, which resulted in their mean degree of overhydration being 1.9 times lower on average. If the positive blood culture was not associated with sepsis, or with AKI and/or FO%, it also did not affect mortality in these patients. The only factor that was associated with a lower mortality was nephrological primary disease. Multi-organ failure occurred in 92% of the deceased children and it proved that the probability of death without MOF amounted to 13%, while the probability of death with MOF was 74%, thus being 5.7 times higher (mortality OR: 18.7; $p < 0.001$).

As shown in Table 2, FO% showed significant differences between the surviving and deceased children. In the group of children with FO% <25% (n = 18), mortality amounted to 33.3% (n = 6), while in the group of children with FO% ≥25% (n = 28), mortality was more than 2-fold higher, amounting to 67.9% (n = 19, $p < 0.001$). Also, the analysis of the Kaplan-Meier curves (Fig. 1) showed that the survival outcomes worsened with FO% ≥25% at CRRT initiation.

The univariable logistic regression analysis demonstrated that significant effects on patient survival were exerted by FO% (OR: 1.030; confidence interval (CI) 95%: 1.001–1.059; $p = 0.044$), CRP value at CRRT initiation (OR: 1.078; CI 95%: 1.019–1.142; $p = 0.009$) and percentage of days with mechanical ventilatory support (OR: 1.068; CI 95%: 1.026–1.112; $p = 0.001$). This denoted that each 1% increase

Table 2. Univariate analysis of clinical factors associated with mortality in the studied population

Variable	Deceased, n = 25 (54%) n [%]	Surviving, n = 21 (46%) n [%]	p-value	Total, n = 46 n [%]
Males/females	15/10	12/9	NS	27/19
Main reason of CRRT initiation – MOF	23	8	<0.001	31
Main reason of CRRT initiation – AKI	0	7	0.006**	7
Main reason of CRRT initiation – FO%	0	8	0.003**	8
Patients with mechanical ventilation – SIMV	20	9	0.009	29
Patients with mechanical ventilation – oscillation	5	3	NS**	8
Patients intubated on the day of CRRT initiation	24	11	<0.001	33
Patients with positive blood culture during PICU stay	12	5	NS	17
Patients on diuretics on the day of CRRT initiation	19	14	NS	33
Days with VPS administration during PICU stay	425 (83.3)	254 (50.9)	<0.001	679 (67.2)
Days with diuretics administration during PICU stay	368 (72.2)	250 (50.0)	<0.001	618 (61.2)
Variable	**mean ±SD/median (IQR)**	**mean ±SD/median (IQR)**	**p-value**	**mean ±SD/median (IQR)**
Age [years]	9.9 ±6.0/10.5 (12.1)	8.7 ±6.0/8.3 (11.6)	NS	9.3 ±6.0/10.3 (11.4)
Body mass [kg]	36.2 ±21.8/32.0 (34.3)	30.2 ±21.8/25.0 (34.3)	NS	33.4 ±21.2/29.5 (34.6)
Body length [cm]	132.4 ±35.7/138.0 (67.8)	120.9 ±35.7/127.5 (75.1)	NS	127.1 ±36.4/134.5 (67.0)
Number of days in PICU/patient	20.4 ±18.4/17.0 (21.5)	23.8 ±18.4/19.0 (26.3)	NS	21.9 ±19.9/17.5 (27.0)
Percentage of days of mechanical ventilation	95.5 ±16.1/100.0 (0.0)	37.5 ±16.1/32.0 (70.3)	<0.001*	69.0 ±40.7/100.0 (68.0)
Number of VPS at CRRT initiation	1.40 ±0.87/1.00 (1.00)	0.86 ±0.87/1.00 (1.00)	0.039	1.15 ±0.89/1.00 (2.00)
CRP [mg/L] at CRRT initiation	221.9 ±146.0/238.0 (236.8)	111.3 ±146.0/82.0 (149.8)	0.005	171.4 ±135.8/151.0 (215.0)
Total protein [g/L] at CRRT initiation	53.5 ±11.6/53.7 (16.6)	51.0 ±11.6/52.0 (13.0)	NS	52.4 ±11.2/53.1 (15.1)
Albumin [g/L] at CRRT initiation	28.5 ±7.5/29.0 (8.9)	27.5 ±7.5/27.0 (12.9)	NS	28.1 ±7.4/28.0 (11.2)
eGFR [mL/min/1.73 m^2] at CRRT initiation	44.6 ±21.4/45.6 (25.7)	31.7 ±21.4/28.4 (25.8)	NS	38.7 ±23.1/34.7 (32.7)
Urea [mmol/L] at CRRT initiation	22.5 ±13.1/17.0 (22.4)	25.9 ±13.1/21.0 (18.3)	NS	24.0 ±15.0/20.1 (20.4)
UO [mL/kg/h] at CRRT initiation	0.72 ±0.72/0.40 (0.95)	1.31 ±0.72/1.20 (1.42)	0.048	0.99 ±1.03/0.65 (1.30)
FO% at CRRT initiation	45.0 ±36.1/32.0 (32.0)	24.1 ±36.1/20.0 (27.0)	0.024	35.5 ±31.6/27.3 (32.5)
Day of CRRT initiation	6.6 ±7.9/3.0 (8.3)	4.6 ±7.9/1.0 (5.8)	NS*	5.7 ±6.9/2.0 (8.0)

* Wilcoxon rank-sum test; ** Yates's modification of the χ^2 test; AKI – acute kidney injury; CRP – C-reactive protein; CRRT – continuous renal replacement therapy; eGFR – estimated glomerular filtration rate; FO% – fluid overload percentage; Hep-ACG – heparin anticoagulation; MOF – multi-organ failure; PICU – pediatric intensive care unit; QC – citrate flow rate; QD – dialyzate flow rate; QS – supplement flow rate; RCA – regional citrate anticoagulation; SIMV – synchronized intermittent mandatory ventilation; UO – urine output; VPS – vasopressors; SD – standard deviation; IQR – interquartile range; NS – not significant.

in FO% increased the odds of death by 3%, each 10 mg/L increase in CRP value increased the odds of death by 7.8% and each 1% increase in the percentage of mechanical ventilation increased the odds of death by 6.8%. In the case of the other parameters analyzed, including the volume of UO, eGFR, number of days spent in PICU, and number of hours of CRRT usage, no significant effect on patient survival was demonstrated.

The multivariable logistic regression for 2 input variables, CRP (OR: 1.098; CI 95%: 1.024–1.178; p = 0.009) and UO (OR: 0.404; CI 95%: 0.163–1.000; p = 0.050), indicated that with simultaneous consideration of both factors, each 10 mg/L increase in CRP with a constant UO value caused an increase in the odds of death by 9.8%, whereas each 1 mL/kg/h increase in UO with a constant CRP value caused a decrease in the odds of death by 59.6%.

Heparin anticoagulation was used significantly more frequently and for significantly longer periods in the deceased children, as compared to the group of survivors (Table 3). As shown in Table 3, the mortality for Hep-ACG was 2.3 times higher than the mortality for RCA (0.66 vs 0.29), while the mortality OR demonstrated 4.8 times higher odds of death with the use of Hep-ACG vs RCA (the odds of death for a Hep-ACG and RCA patient were 21/11 = 1.91

Table 3. Univariate analysis of CRRT-associated factors in the deceased and surviving children in the studied population

Variable	Deceased, n = 25 n [%]	Surviving, n = 21 n [%]	p-value	Total, n = 46 n [%]
Patients on Hep-ACG/RCA	21 (84.0)/4 (16.0)	11 (52.4)/10 (47.6)	0.020	32 (69.6)/14 (30.4)
CRRT sessions on Hep-ACG/RCA	73 (56.6)/56 (43.4)	38 (41.8)/53 (58.2)	0.030	111 (50.5)/109 (49.5)
Number of hours of CRRT sessions on Hep-ACG/RCA	3,363 (52.2)/3,076 (47.8)	1,800 (42.6)/2,424 (57.4)	<0.001	5,163 (48.4)/5,500 (51.6)
Variable	**mean ±SD/median (IQR)**	**mean ±SD/median (IQR)**	**p-value**	**mean ±SD/median (IQR)**
Number of hours of CRRT/patient	257.6 ±319.0/154.0 (258.5)	201.1 ±191.9/144.0 (218.3)	NS	231.8 ±267.3/149.0 (222.0)
Filters/patient	5.2 ±5.6/4.0 (3.0)	4.3 ±4.2/2.0 (3.3)	NS*	4.8 ±5.0/3.0 (3.0)
Number of hours of a single filter work	49.9 ±24.2/66.0 (48.0)	46.4 ±26.0/59.0 (50.8)	NS*	48.5 ±25.0/62.0 (48.0)
BFR [mL/min/kg]	2.57 ±1.17/2.40 (1.55)	2.78 ±1.05/2.50 (1.30)	NS	2.67 ±1.11/2.50 (1.30)
Dialysis dose: QD+QS (+QC) [mL/h/kg]/patient	59.47 ±24.24/50.00 (33.96)	59.87 ±22.62/60.00 (21.73)	NS*	59.63 ±23.56/55.00 (31.85)
UFR [mL/h/kg]	2.62 ±1.63/2.40 (1.95)	2.40 ±1.44/2.10 (2.10)	NS*	2.53 ±1.55/2.30 (2.00)
ACT [s]	147.81 ±44.60/133.63 (47.14)	138.43 ±20.32/136.60 (24.61)	NS*	137.38 ±37.54/132.00 (41.05)
Heparin bolus [mg/kg] at the session start	0.20 ±0.12/0.18 (0.10)	0.21 ±0.10/0.21 (0.09)	NS	0.21 ±0.11/0.20 (0.09)
Heparin dose [mg/kg/h]	0.13 ±0.09/0.11 (0.13)	0.17 ±0.10/0.16 (0.08)	0.002*	0.14 ±0.10/0.13 (0.11)
Percentage of calcium compensation	73.44 ±22.58/70.00 (30.00)	70.74 ±23.87/78.00 (40.00)	NS*	72.18 ±23.19/75.00 (35.00)
Citrate dose	3.13 ±0.24/3.10 (0.20)	2.98 ±0.26/3.00 (0.30)	<0.001*	3.06 ±0.26/3.00 (0.20)
CaT/CaI	1.91 ±0.70/2.08 (0.35)	1.14 ±0.93/1.00 (1.99)	<0.001*	1.52 ±0.91/1.95 (1.17)

* Wilcoxon rank-sum test; ACT – activated clotting time; BFR – blood flow rate; CaT/CaI – total calcium/ionic calcium index; CRRT – continuous renal replacement therapy; Hep-ACG – heparin anticoagulation; RCA – regional citrate anticoagulation; QC – citrate flow rate; QD – dialyzate flow rate; QS – supplement flow rate; UFR – ultrafiltration rate; SD – standard deviation; IQR – interquartile range; NS – not significant.

Table 4. Multivariable analysis of the impact of fluid overload percentage (FO%) and applied anticoagulation (ACG) mode on the survival rate

	FO% – deceased	FO% – surviving	p-value
Hep-ACG	45.9 ±34.5	21.4 ±23.8	0.039
RCA	41.4 ±46.4	27.7 ±16.8	NS
p-value	NS	NS	–

Data presented as mean ± standard deviation (SD). ACG – anticoagulation; FO% – fluid overload percentage; RCA – regional citrate anticoagulation; NS – not significant.

and 4/10 = 0.40, respectively; thus, the mortality OR was 1.91/0.40 = 4.8). Nevertheless, the number of VPS employed at the time of CRRT initiation and the absolute percentage of days with VPS administration during the PICU stay in the Hep-ACG group were significantly higher than in the RCA group (Table 1); thus, it may be concluded that the clinical status of the children treated with Hep-ACG was generally more severe compared to the RCA group, which is a probable reason of the higher mortality. While analyzing whether the employed ACG method might possibly affect the outcome, the multivariable analysis of the effect of FO% and the applied ACG mode on the survival rate was conducted. The data presented in Table 1 demonstrates that no significant difference was found in FO% values in the children dialyzed using the Hep-ACG vs RCA methods. On the other hand, as shown by the multivariable analysis of the effect of FO% and the applied ACG mode on survival rate, the only difference in survival was observed in the population of children dialyzed with Hep-ACG, while in the population of the RCA children, no significant difference was seen in mortality (Table 4).

The surviving children required a significantly higher Hep dosage, which means that the amount of the administered Hep was not associated with mortality, while the deceased children received a significantly higher citrate dosage, despite the fact that their CaT/CaI ratio was maintained within the normal range (Table 3).

The mean and median values of FO% of the CRRT children since 2009 were high in comparison to the reported percentages in the literature.[8,9] There was no difference in FO% between the periods 2009–2012 and 2013–2016 (mean ±SD: 35.1 ±34.0 and median (IQR): 25.0 (35.2) vs mean ±SD: 35.7 ±30.4 and median (IQR): 28.8 (29.0)). In addition, there was no difference in the mean (2009–2012: 5.5 ±5.6 vs 2013–2016: 5.8 ±7.9) and median (2009–2012: 3.0 (8.0) vs 2013–2016: 2.0 (8.0)) day of CRRT initiation during the children's hospitalization in PICU over the analyzed period.

There was no difference in the duration of the PICU hospitalization between the CRRT patients (n = 46 (1.4%); mean ±SD/median (IQR): 21.9 ±19.9/17.5 (27.0)) and the rest of the PICU children hospitalized during the same period (n = 3,255; mean ±SD/median (IQR): 25.4 ±26.2/16.0 (24.0)). However, a significant difference was noted in the mortality between the 2 groups of patients (54% vs 6.5%; $p < 0.001$).

Discussion

Continuous renal replacement therapy has become the treatment of choice for supporting critically ill patients with AKI.[17] Using a revised AKI definition, recent studies have indicated that up to 10% of all children admitted to PICUs suffer from some degree of kidney injury.[3] In accordance with the data from the literature, in the present study, the highest percentage of patients requiring CRRT included children with a primary diagnosis of a hematological/oncological disorder (56.5%) and children co-diagnosed with MOF (67.4%).

In the literature, several factors have been identified as outcome predictors in the pediatric population requiring CRRT, including late CRRT initiation, hemodynamic instability, specified VPS number and dosage dependency, underlying diseases, low body weight, young age, need for mechanical ventilation, presence of MOF, and FO%.[5,11,18–25] In our center, the surviving children required less intensive mechanical respiratory support, were administered a lower number of VPS at CRRT initiation, a lower absolute percentage of days with VPS administration during their PICU stay, and lower CRP values, but they maintained a significantly higher UO value on the day of CRRT initiation, which resulted in the degree of their overhydration at the initiation of CRRT being almost 2-fold lower on average. On the other hand, in the population of non-survivors, MOF as the immediate cause of CRRT initiation was significantly more common. According to the literature, MOF occurs in 30–50% of children in PICUs and is responsible for a higher percentage of total deaths.[26,27] In a study by Hayes et al., a diagnosis of MOF was significantly associated with increased mortality, as it was present in 100% of non-survivors and in 69% of survivors.[22] Although in our center no significant differences were observed in the main diagnosis as a factor that might affect mortality, apart from purely nephrological causes, it was nevertheless demonstrated that the probability of death in the cases of concomitant MOF was almost 6 times higher.

Determining the therapeutic dose for CRRT in critically ill children remains a challenge. An analysis of a few randomized studies comparing a conventional vs intensive CRRT dose did not result in any differences in the outcomes (including kidney function and mortality) of PICU patients.[8,28] In our center, no significant differences were noted in the dialysis dose between the survivors vs non-survivors, either.

There are numerous studies which prove that higher fluid accumulation is an independent risk factor of death in CRRT patients even after adjusting for severity of illness.[19,21–23] The majority of studies indicate that a threshold of FO% equal to 20% is associated with a large increase in mortality.[8,9] In the literature addressing FO% in pediatric CRRT, the average FO% was 16.4% in survivors and 34% in non-survivors, and the survival rate was 58% in those with FO% <20% as compared to 40% in those with FO% >20%, despite comparable illness severity scores.[11,25] According to Sutherland et al., multivariable analysis suggested that each 1% increase in FO% was associated with a 3% increase in mortality OR.[21] In the current study, the mean values of FO% showed significant differences between the surviving children (24.1%) and non-survivors (45.0%). The cut-off value was assumed as FO% = 25%, which demonstrated that in the group of children with FO% <25%, the mortality was significantly more than 2-fold lower than in the group of children with FO% ≥25% (33.3% vs 67.9%). A worsening of the prognosis in the latter population of patients was also demonstrated by the analysis of the Kaplan-Meier curves (Fig. 1). In addition, the univariable logistic regression analysis, as in the study conducted by Sutherland et al., confirmed that each increase in FO% by 1% increased the mortality OR by 3%.[21]

In the current literature there are studies evaluating the efficacy and safety of RCA vs Hep-ACG in pediatric CRRT. These studies have shown that RCA is effective, provides equivalent circuit survival and decreases bleeding as compared to Hep-ACG.[29] In the studied population, treatment sessions were generally well tolerated by all patients.

Furthermore, in the literature, there are both studies reporting that there is no significant difference in mortality between the RCA (41.3%) and Hep-ACG (42.7%) groups, and studies, such as the one by Oudemans-van Straaten et al., proving that RCA appears particularly beneficial, especially after surgery, in sepsis and MOF (suggesting an interference with inflammation), reducing the overall probability of death.[13,30–32]

Although the data collected in our center suggests that the mortality in children dialyzed with Hep-ACG is more than 2-fold higher than the mortality while using the RCA mode, one should take into consideration the fact that the clinical status of children treated with Hep-ACG was in general more severe than the clinical status of

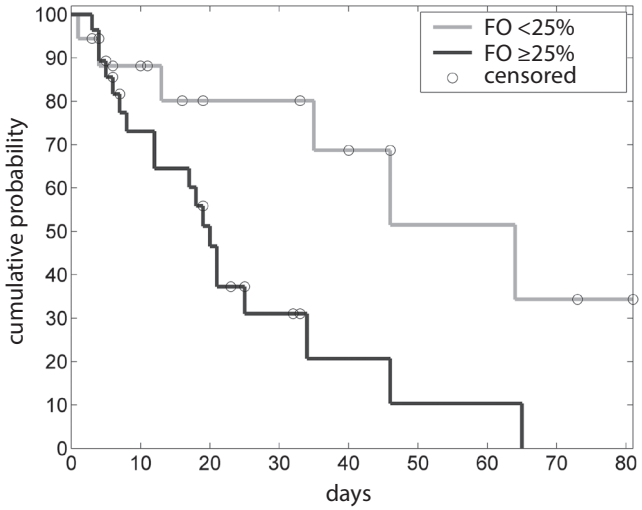

Fig. 1. Comparative Kaplan-Meier analysis for the probability of survival of the continuous renal replacement therapy (CRRT) patients depending on fluid overload percentage (FO%). The cut-off value of 25% was statistically significant (Mantel-Cox test: p = 0.012)

the RCA group, which is undoubtedly the reason of their higher mortality. However, subsequent analyses comparing Hep-ACG with RCA in detail demonstrated that a significant effect on the outcome depending on the values of FO% occurred only in the group of children dialyzed using Hep-ACG.

A crucial factor in the discrepancy between the mortality of patients treated with the particular modes of ACG might be the fact that RCA in our center has been practically used only since the beginning of 2015, and that since that time virtually all (14/15) CRRT sessions were performed using this mode of ACG, which means that patients qualified to CRRT during that time were in a better general condition, which may reflect higher vigilance and awareness of the medical staff about the need for CRRT implementation in PICU patients, as well as better acquaintance with the CRRT technique itself.

In our center, CRRT is prescribed and handled by the nephrologists in collaboration with the PICU team. Nevertheless, since the onset of this technique in our center (2009), we have not observed a decrease in the degree of FO% or a significant difference in the day of initiation of this renal replacement mode.

According to the literature, mortality in PICU patients with secondary AKI requiring CRRT is high, being estimated at 35.6–60%; CRRT is required by about 5% of all PICU patients.[4,18,19,21,22,24,25,33] Children with AKI at the time of admission are hospitalized twice as long as patients with normal renal function, while those who develop AKI during the PICU course have a 4-fold increase in the length of stay.[3] In our center, in the period 2009–2016, contrary to the data from the literature on the subject, the duration of PICU hospitalization did not significantly differ between the children who did and did not require CRRT, but the mortality in the former group was more than 8-fold higher, which confirmed the reports from the literature describing a poorer prognosis in those patients.[9]

The authors are cognizant of some limitations of the study, as it was a retrospective, observational, single-center study, where interventions could not be standardized. Nevertheless, in the present study, a detailed analysis was performed of the course of PICU hospitalization of all 46 children who were subjected to this treatment technique since the onset of CRRT use in the center. In order to achieve the maximum homogeneity of the analyzed population, in view of the differing nature of AKI in such patients, the analysis did not include patients hospitalized in the cardiac surgery intensive care unit. The study provided a detailed characterization of practically all the factors that might affect the outcome in this population of patients, which is of high importance for future improvements in the quality of care of such patients.

Conclusions

The mortality of PICU patients who require CRRT is more than 8-fold higher than the mortality of the total PICU population. Coexisting MOF as the immediate cause of CRRT introduction increases the mortality almost 6 times. Apart from the patients' general clinical condition (which is reflected by the CRP values and the need for mechanical ventilation), only FO% exerts a significant negative effect on the survival of patients requiring CRRT. The mortality of children whose FO% at the time of CRRT initiation equals or exceeds 25% is almost 2-fold higher than the mortality of children whose FO% at the time of CRRT initiation does not exceed this value.

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Trigonocephaly: Long-term results after surgical correction of metopic suture synostosis

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

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Abstract

Background. Premature closure and ossification of the metopic suture results in a triangular head shape called trigonocephaly and is characterized by a wedge-shaped forehead and frontotemporal narrowing. Untreated craniosynostosis may lead to increased intracranial pressure (ICP) and, thereby, impaired neurodevelopment. Over the last decades, its incidence has been increasing, currently making it the 2nd most common type of isolated craniosynostosis. Treatment consist of cranioplasty, which should be performed before the age of 1 year.

Objectives. The aim of this study was to evaluate the long-term surgical outcomes in children operated on for trigonocephaly.

Material and methods. The authors reviewed 30 consecutive cases of metopic synostosis treated over a 14-year period in the Plastic Surgery Department in Polanica-Zdrój, Poland. The data was evaluated using the patients' clinical records, and preoperative and postoperative photographs. The patients showed up on a follow-up visit at a median age of 9 years and were examined by an ophthalmologist and a neurologist. The surgical outcomes were evaluated according to the Whitaker classification. In 23 patients, remodeling and the advancement of fronto-orbital skull segments was performed at a median age of 18 months and in 7 milder cases, simple suturectomy or burring of the metopic ridge was sufficient.

Results. According to the Whitaker classification, results were considered good to excellent (category I and II). Only 1 patient was included into category III. None of the examined cases were included into category IV, which would require a major craniofacial procedure, duplicating or exceeding the original operation. Neurological abnormalities were found in 12 cases and vision defects in 15 cases.

Conclusions. Trigonocephaly is currently the 2nd most common type of isolated synostosis. Surgical treatment based on Tessier's and Marchac's modified methods provides good results in patients at the age of about 12 months and prevents the consequences of ICP increase. Primary neurological and behavioral disorders may occur, despite corrective surgery.

Key words: craniosynostosis, trigonocephaly, metopic synostosis

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Background

The term 'trigonocephaly' was first proposed by Welcker in 1862, who used it to describe a patient with a wedge-shaped scull combined with a cleft lip. Trigonocephaly is a congenital cranial deformation caused by premature fusion and ossification of the metopic suture. The metopic suture separates 2 frontal bones at birth and is the first skull suture to close physiologically, starting as early as at 3 months and generally being completely fused at the age of 8 months. In the case of trigonocephaly, premature synostosis takes place in the prenatal period.[1] According to Virchow's theory, it results in the characteristic cranial malformation.[2] Growth inhibition perpendicular to the synostosed suture induces frontotemporal narrowing and the shortening of anterior cranial fossa, and a compensative increase in the biparietal diameter and posterior cranial fossa. Triangular forehead deformity with protruding bony midline ridge may be accompanied by hypotelorism and ethmoid bone hypoplasia. In 55% of cases, anterior fontanel is closed prematurely (Fig. 1–3).[3]

Trigonocephaly occurs either as an isolated malformation with or without another primary defect of morphogenesis and without any particular syndrome, or as a syndromic form. It is now estimated that 10–20% of patients with trigonocephaly are affected by syndromes such as Opitz C, Say–Meyer, Frydman, Baller–Gerold, Muenke, and Seathre–Chotzen, although most metopic synostosis are nonsyndromic.[4,5] Trigonocephaly may be defined as a complex and heterogeneous condition supporting a strong genetic component accompanied by epigenetic and environmental factors affecting a child in prenatal period. There are 3 major theories defining this deformity formation. The first one assumes that cranium deformation results from genetic, metabolic or pharmacological agents. The second one considers intrauterine fetal head constraint caused by multiple gestation or uterine abnormalities. The third one considers the brain to be the main reason behind the onset of craniosynostosis.[6]

The most frequent associated malformations are limb, heart and genitourinary tract anomalies. In 38% of cases, more than 1 extracranial malformation is diagnosed. In these patients, the IQ score is considerably lower than in patients with an isolated form. Moreover, these patients reveal intellectual disability, ADHD or autism spectrum disorders more often.[7]

Trigonocephaly prevalence has increased over the recent years. The latest researches reveal that this malformation is the second most frequent isolated craniosynostosis (1:5,000) and it may account for 23–28% of all isolated cases. The male to female ratio is reported to be about 3:1 and positive family history is found in 6.8% of patients.[8–10]

The goal of surgical treatment is the improvement of the forehead shape, hypotelorism correction and anterior cranial fossa extension. The procedure aims to obtain the normal development of the intensively growing brain.

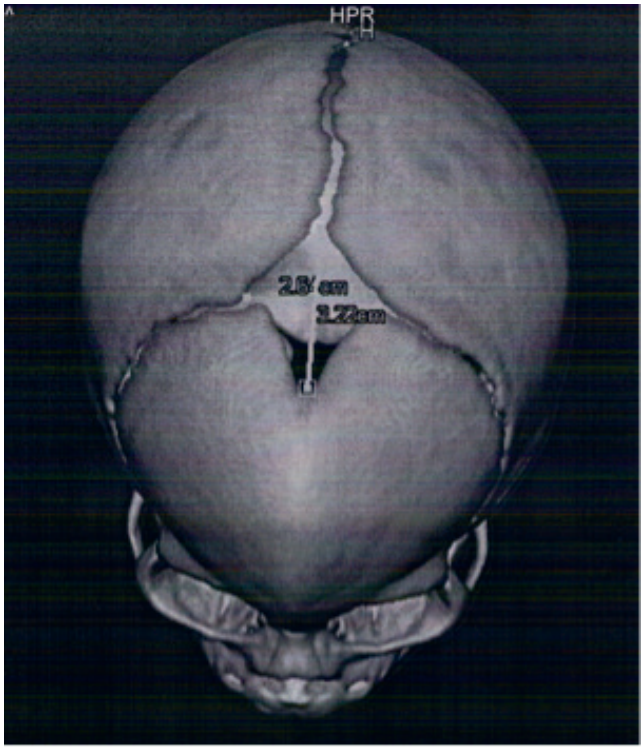

Fig. 1. Radiological features of trigonocephaly

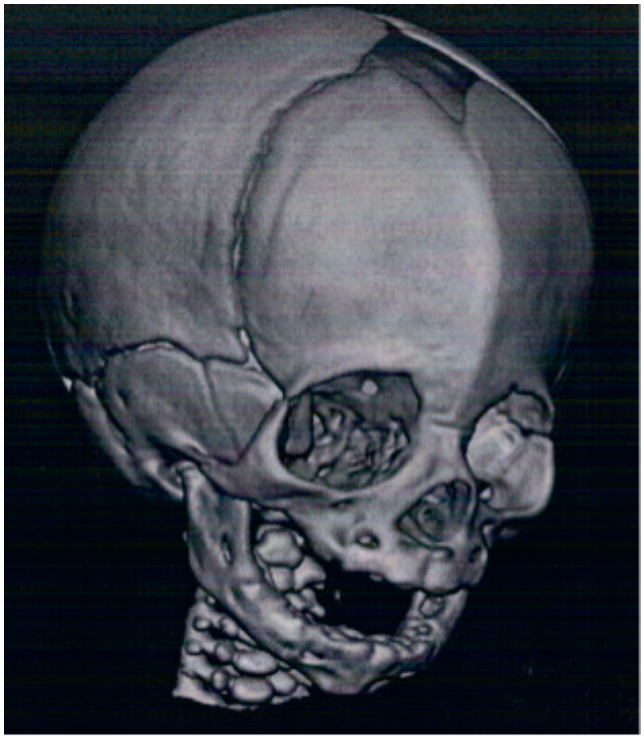

Fig. 2. Radiological features of trigonocephaly

Untreated isolated craniosynostoses may result in the increase of intracranial pressure (ICP) in 15–20% of cases.[11] In trigonocephaly, visual defects like strabismus and astigmatism are common.

The majority of surgical techniques are based on forehead remodeling and anterior cranial fossa extension

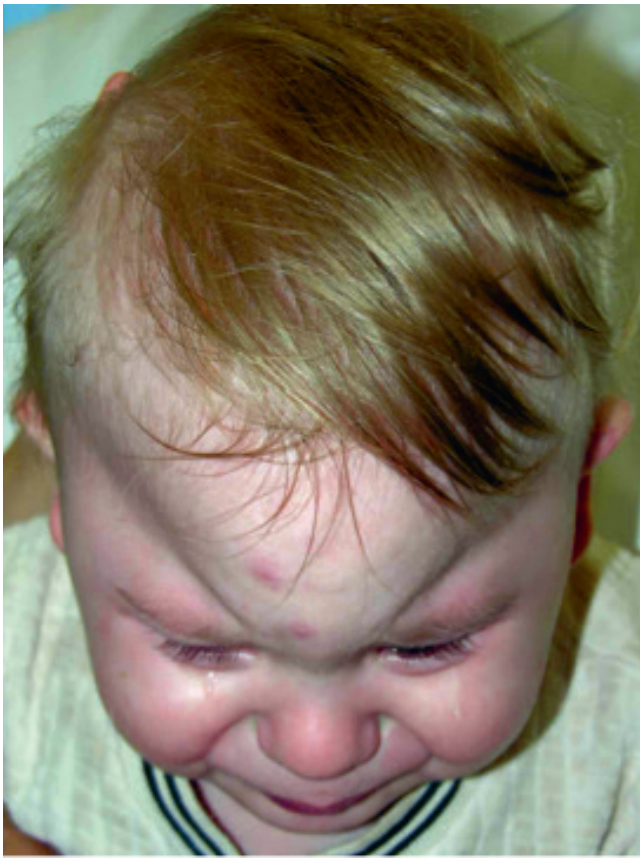

Fig. 3. Clinical features of trigonocephaly

by forehead and supraorbital bar advancement.[12] Historically, procedures consisted only of the excision of synostosed cranial sutures-strip craniectomy. The breakthrough in the treatment of craniosynostosis came in 1971, when Paul Tessier proposed a horizontal advancement of the forehead along with the supraorbital area reaching the temporal fossae, the so-called fronto-orbital advancement (FOA). The advanced and remodeled forehead was fixed in a temporal fossa and nasal root area.[13] In 1977, Marchac described cranioplasty based on bilateral cranial remodeling in infants with trigonocephaly, plagiocephaly and brachycephaly. The procedure, based on the concept of a "floating forehead", consists in advancing the supraorbital bar along with a remodeling the forehead, which was formed out of excised bones and sutured only on its lower edge. Such a floating forehead, which is not fixed to the cranial borders, leans against dura mater loosely and enables brain growth.[14]

The procedures presented above are regarded as basic treatment techniques of trigonocephaly and have undergone many modifications.[15,16] The surgery should be performed at about 12th month of life and surgical results are usually evaluated using the 4-degree Whitaker classification.[17,18]

The aim of this study was to evaluate the long-term surgical outcomes in children operated on fortrigonocephaly.

Material and methods

In years 2000–2014, in Plastic Surgery Department in Polanica-Zdrój (Wroclaw Medical University, Poland), 154 craniosynostotic patients were treated. This group of patients included 37 cases of trigonocephaly, of which 30 were analyzed in this study. Medical records were reviewed and the following data was retrospectively collected: sex, birth weight, gestation period and delivery course, age at operation, age at follow-up visit, other congenital deformities, time and type of surgical procedures, hospitalization length, treatment course, complications, and volume of transfused blood. All patients were consulted by an ophthalmologist and a neurologist, and the surgical treatment results were assessed with the Whitaker classification (Table 1).

Table 1. Whitaker classification of surgical results

Category I	No refinements or surgical revisions considered advisable or necessary
Category II	Soft-tissue or lesser bone-contouring revisions advisable apt to be performed on an outpatient basis or requiring a maximum of 2-day hospitalization
Category III	Major alternative osteotomies or bone grafting procedure advisable, i.e., orbital repositions, onlay bone grafts, being these procedures not so extensive as the original operations
Category IV	A major craniofacial procedure advisable, duplicating or exceeding the original operation

In addition, head measurements were taken, including the cranial index and head circumference. Moreover, blood for genetic test was collected and the results will be presented in a separate paper.

Surgical procedures were carried out using the Tessier's and Machac's modified method. It was based on the advancement and remodeling of the supraorbital band and forehead, leading to the enlargement of the anterior cranial fossa. Bicoronal skin incision was used to provide access. The skin was mobilized together with the galea and periosteal layer. Frontal bone and supraorbital band incision line was planned and craniotomy was carried out on the forehead and cranial vault border. After that, long supraorbital band reaching bilaterally to the temporal fossae was excised and was addressed by an wedge osteotomy on the posterior midline and lateral orbital wall in order to facilitate banding the bar in appropriate shape. Prepared osseous band was advanced by 1–1.5 cm. In order to prevent recurrence of frontal narrowing and temporal hollowing, remodeled band was supported with bone grafts in median and lateral area. The frontal bones were cut in the midline and remodeled to fit in the new shape of the supraorbital band. After that, remodeled frontal bone was stitched to the osseous band with several vicryl sutures. According to the concept of "floating forehead",

the posterior edge remained free. The skin flaps were closed tightly with mattress or simple sutures and drains were left for 2–3 days (Fig. 4–7). In mild metopic synostosis, simple synostectomy or milling the frontal midline prominence was sufficient.

Results

Among patients tested, there were 23 males (M) and 7 females (F); the M:F ratio was 3.31. Average birth weight was 3244 g, 16 patients were delivered naturally and in 14 cases cesarean section was applied. Four patients were born prematurely. In 14 cases, gestation course was uneventful. In 3 mothers, gestational infection occurred, 8 mothers took medications mainly for hypothyroidism, 1 mother took valproic acid for epilepsy, and 1 was an alcoholic. One patient was delivered as a triplet after in vitro fertilization. Mothers of 11 patients admitted smoking cigarettes during pregnancy and in the period preceding fertilization (Table 2).

In none of the patients, familial history was detected. Before the procedure, genetic tests were made in 5 patients. In 2 patients, chromosomal aberrations were found – 9p syndrome (patients No. 24 and 28). In 1 patient, 1 and 22 chromosome polymorphism was diagnosed (patient No. 14) and in 1 patient, amniotic band syndrome was observed (patient No. 11). Only 5 patients were diagnosed geneticaly before surgery and they are listed above. In 8 patients, other extracranial malformations were found, however, not as elements of any particular syndrome. More than 1 additional malformation was diagnosed in 4 patients. These abnormalities concerned mainly genitourinary tract. In 19 patients, no coexisting malformations were found (Table 3, 4).

In 23 cases, cranioplasty consisting of remodeling and advancement of fronto-orbital skull segments was performed and in 7 mild cases, simple synostectomy or burring of the frontal midline prominence was sufficient. Further analysis concerns the group of 23 cranioplasty patients.

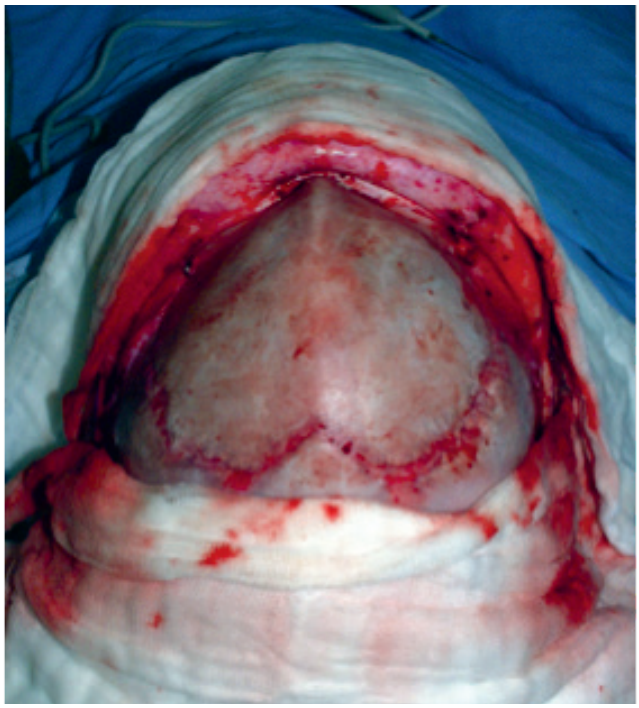

Fig. 4. Bicoronal skin incision used to provide access to the bone (skin is mobilized with the galea and periosteal layer)

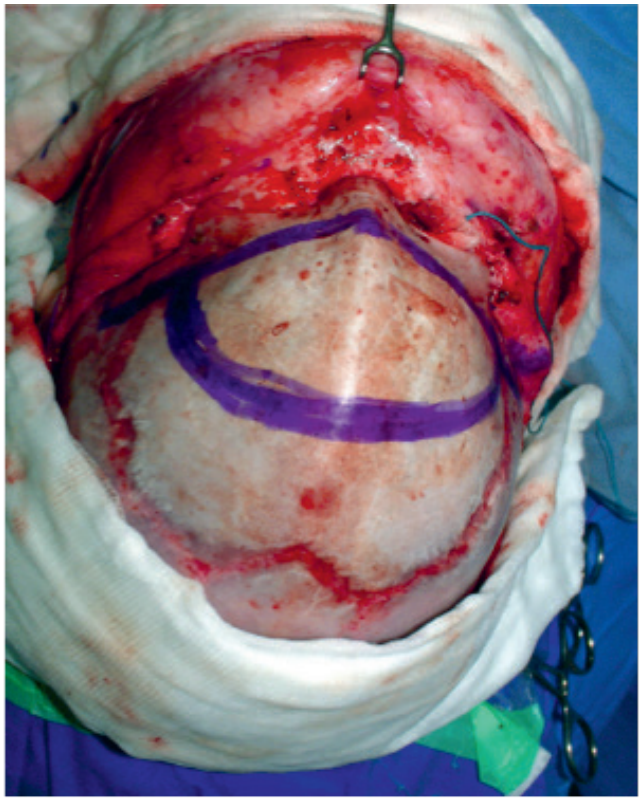

Fig. 5. Frontal bone and supraorbital band incision lines

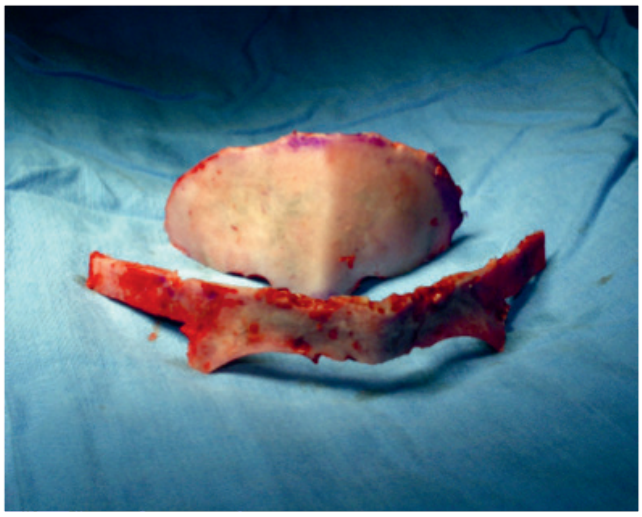

Fig. 6. Excised frontal bone and supraorbital band (before remodeling)

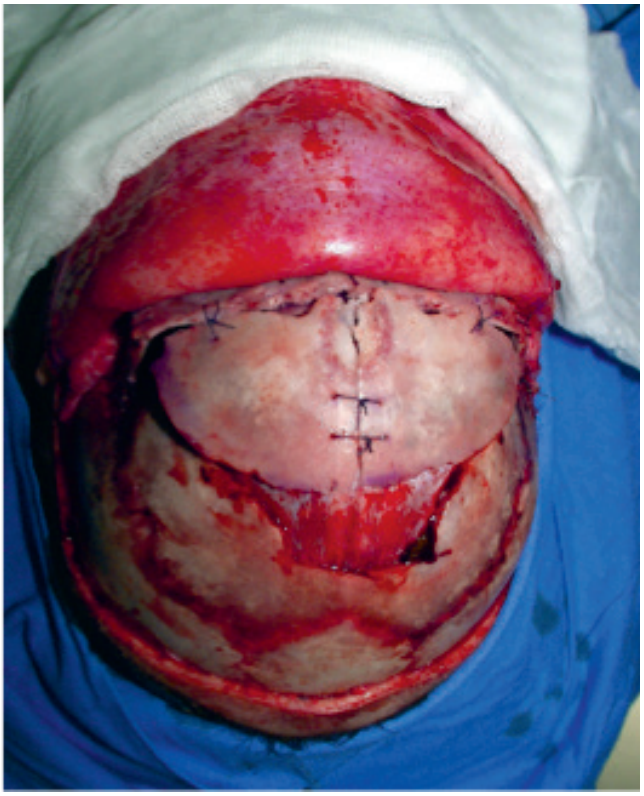

Fig. 7. Frontal bone and supraorbital band after remodeling and advancement

In our Department, patients are qualified for cranium remodeling and advancement at the age of 8–12 months. In the group of patients operated on (n = 23), average age at the surgery was 18 months. Ten patients were operated on before 12th month of life (43% of the operated patients). The youngest patient was operated on at the age of 4 months and the oldest syndromic child was operated on at the age of 84 months.

Average age of follow-up visit was 9 years. Twenty cranioplasties were performed by one of the authors of this publication. Surgical procedure average length was 3.7 h. Blood was transfused in 21 patients (mean volume: 311 mL, range: 180–750 mL). Hospitalization mean time was 5.6 days (range: 3–10 days). No complications were observed in any of 23 operated cases. In 1 patient, in the surgery site, seroma formed but it was successfully evacuated. Preoperative assessment and qualification for the procedure was based on ophthalmological, neurological and imaging (X-ray and computed tomography) examinations. In 9 patients, additional intracranial abnormalities were found such as: frontal lobes hypoplasia, pericerebral fluid space or ventricular dilatation, arachnoid cyst, septum pellucidum cyst or cavity, corpus callosum agenesis, underdevelopment of ethmoid bone, or lateral nasal sinuses. In 12 patients, variety of neurological disorders were observed (psychomotor

Table 2. Medical history data on the course of pregnancy in examined cases

Patient No.	Mother infection	Complications of pregnancy	Mother diseases	Other	Smoking cigarettes
2	urinary tract	–	–	–	–
4	boreliosis	–	–	–	–
5	–	premature contractions	–	–	–
8	–	premature contractions	–	–	yes
9	–	–	hypothyroidism	–	–
10	–	–	–	–	yes
11	–	–	hypothyroidism	–	–
12	–	premature placental abruption	–	–	yes
13	–	–	hyperthyroidism, diabetes	–	–
14	–	–	hypothyroidism	triple pregnancy by in vitro fertilization	–
15	–	–	hypothyroidism	–	–
16	–	–	–	–	yes
17	–	–	–	–	yes
18	–	–	hypertension	–	–
19	–	–	–	–	yes
20	–	–	alcoholism	–	yes
21	–	–	hypertension	–	–
22	–	premature contractions	–	–	yes
23	–	–	–	–	yes
24	intrauterine infection	–	–	–	–
26	–	–	–	–	yes
27	–	–	epilepsy	–	yes

Table 3. Additional malformations in examined patients

Patient No.	Genitourinary	Limbs	Cardiovascular	Cleft	Central nervous system	Gastrointestinal	Other
4		–	–	–	–	–	hearing disorders
10	testis hydrocele	–	–	–	–	–	–
11	ectopic kidney	–	–	cleft palate	–	–	amniotic bands
14	hypospadias	–	–	–	–	–	–
15	kidney agenesis	feet abnormalities	–	–	corpus callosum agenesis	–	–
20	testis hydrocele	–	–	–	–	–	–
21	–	–	interventricular septum aneurysm	–	–	–	–
24	hypospadias	feet abnormalities	–	–	–	sphincter stenosis	–
25	–	–	–	–	–	sphincter stenosis	–
27	hypospadias	–	–	–	–	–	–
28	reflux	feet and hands abnormalities	ASD	cleft palate	–	–	–

ASD – atrial septal defect.

Table 4. Division of patients according to Lajeunie et al. criteria

Additional malformations	Group I (n = 19)	Group IIA (n = 8)	Group IIB (n = 3)
1 malformation	0	7	0
>1 malformation	0	1	3

development disorders, muscle tone changes) and ophthalmological abnormalities were found in 7 patients. In no patient, optic nerve disc edema standing for intracranial hypertension was observed.

According to the Whitaker classification, out of 23 patients who underwent cranioplasty, 18 (78%) were qualified to category I, which means that the treatment result was satisfactory and the patient did not require further treatment. In 4 patients, minor cranial deformities were observed which needed corrective procedures and these cases were included to category II. One patient was qualified to category III due to severe bitemporal hollowing. None of our patients were included into category IV, which would require major craniofacial procedure, duplicating or exceeding the original operation (Table 5) (Fig. 8–11).

In the group of patients who had undergone cranioplasty, a follow-up visit revealed 1 or more neurological abnormalities in 12 cases, i.e., intellectual disabilities

Table 5. Whitaker classification of patients after cranioplasty

Whitaker classification category	Number of patients
I	18
II	4
III	1
IV	0

of various degrees (6 patients), frequent headaches (5 patients), ADHD (2 patients), autism symptoms (1 patient), and cerebral palsy (2 patients). An ophtalmological examination showed vision defects in 15 cases and, most frequently, they included strabismus (10 cases), astigmatism and myopia. In 1 patient, lacrimal fistula of the lower ducts was diagnosed. Average cephalic index was 78 (range: 72–86) and it was adequate to the values of mesocephalic-intermediate skull.

Discussion

Over the last decades, the incidence of trigonocephaly has been increasing, currently making it the 2nd most common type of isolated synostosis. In the Plastic Surgery Department in Polanica-Zdrój, the 1st surgery for trigonocephaly was performed in 1988. Until 2000, 11 procedures of skull remodeling in children were carried out in order to correct this malformation. In the years 2000–2014, the number of patients operated on for trigonocephaly increased to 37, which constituted 24% of all the cases treated for craniostenosis (without division into isolated and syndromic cases). Di Rocco et al. in their observations carried out on a large group of patients, including over 2,800 patients treated in 1 center in the period of 20 years, report a considerable increase in the number of patients operated on for craniosynostoses.[9] During this 20-year period the number of non-syndromic patients increased progressively, from 90 cases in 1988 to 163 in 2007. Such increase might be caused by better diagnostics and recognition of skull malformations made by pediatricians as well as by a larger number of patients admitted to the specialist unit. However, this may be also the symptom of an increased prevalence

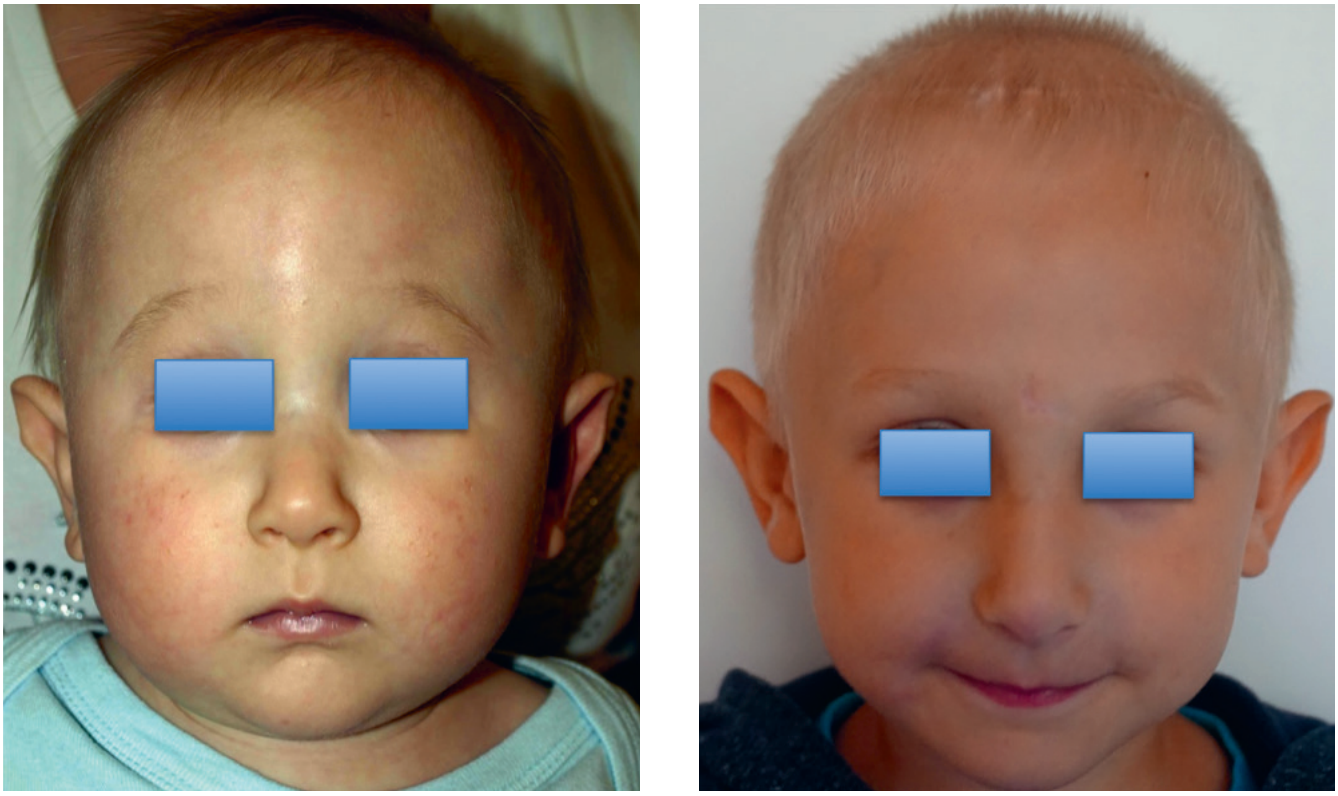

Fig. 8. Patient before the procedure (at the age of 21 months) and after the procedure at the age of 5 years

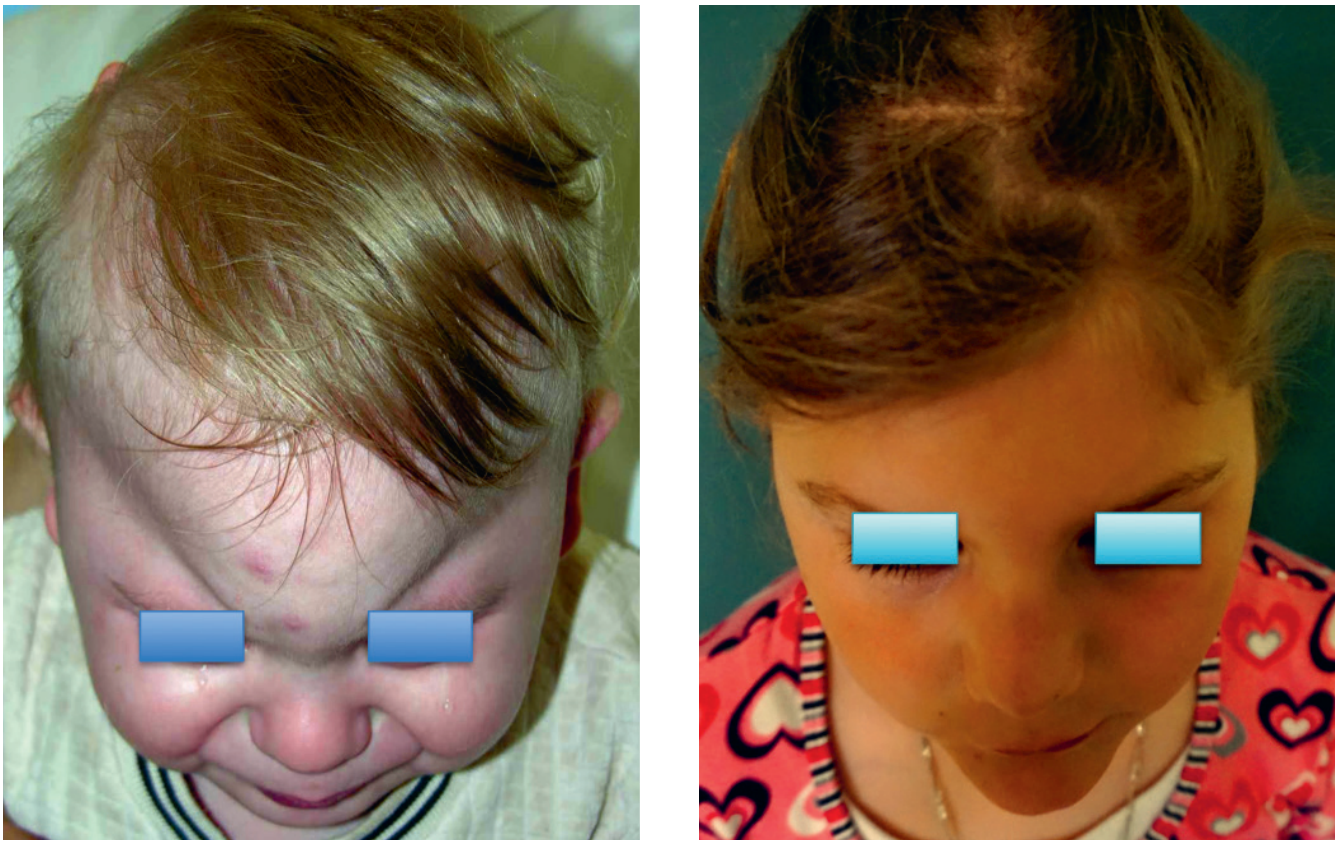

Fig. 9. Patient before the procedure (at the age of 8 months) and after the procedure at the age of 5 years

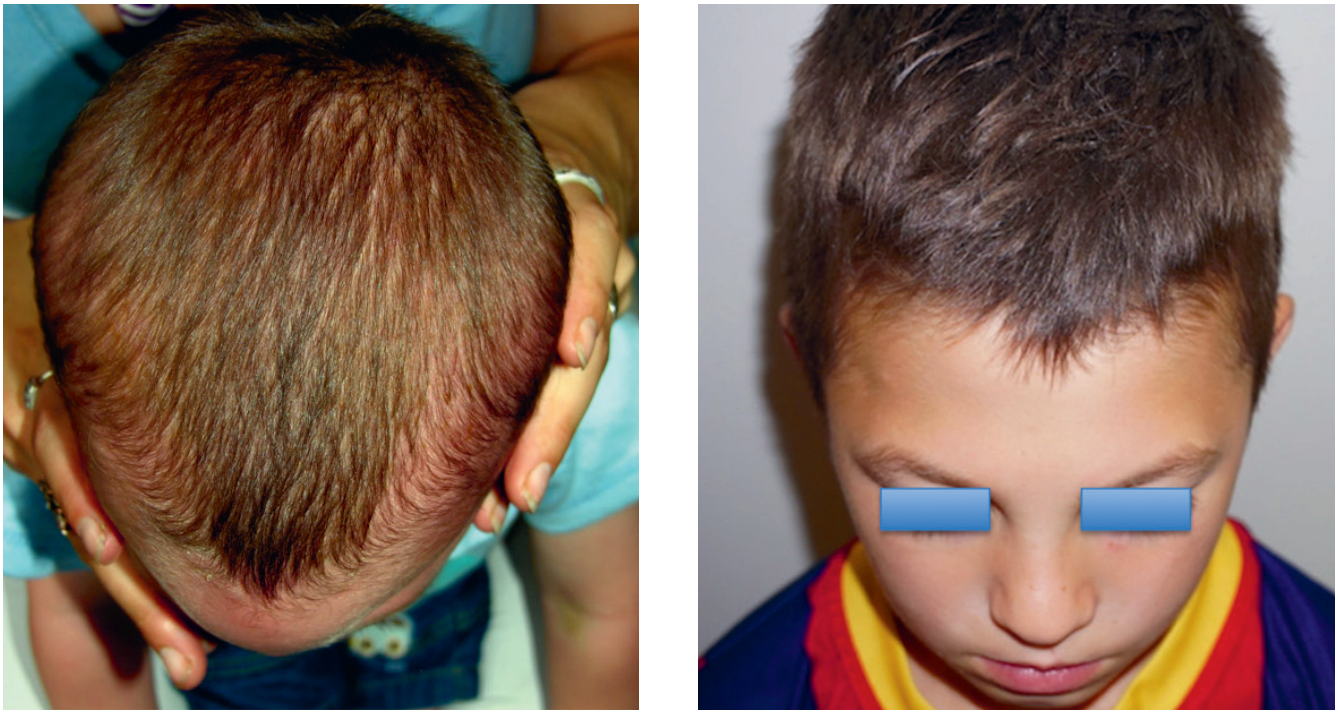

Fig. 10. Patient before the procedure (at the age of 8 months) and after the procedure (at the age of 4 years)

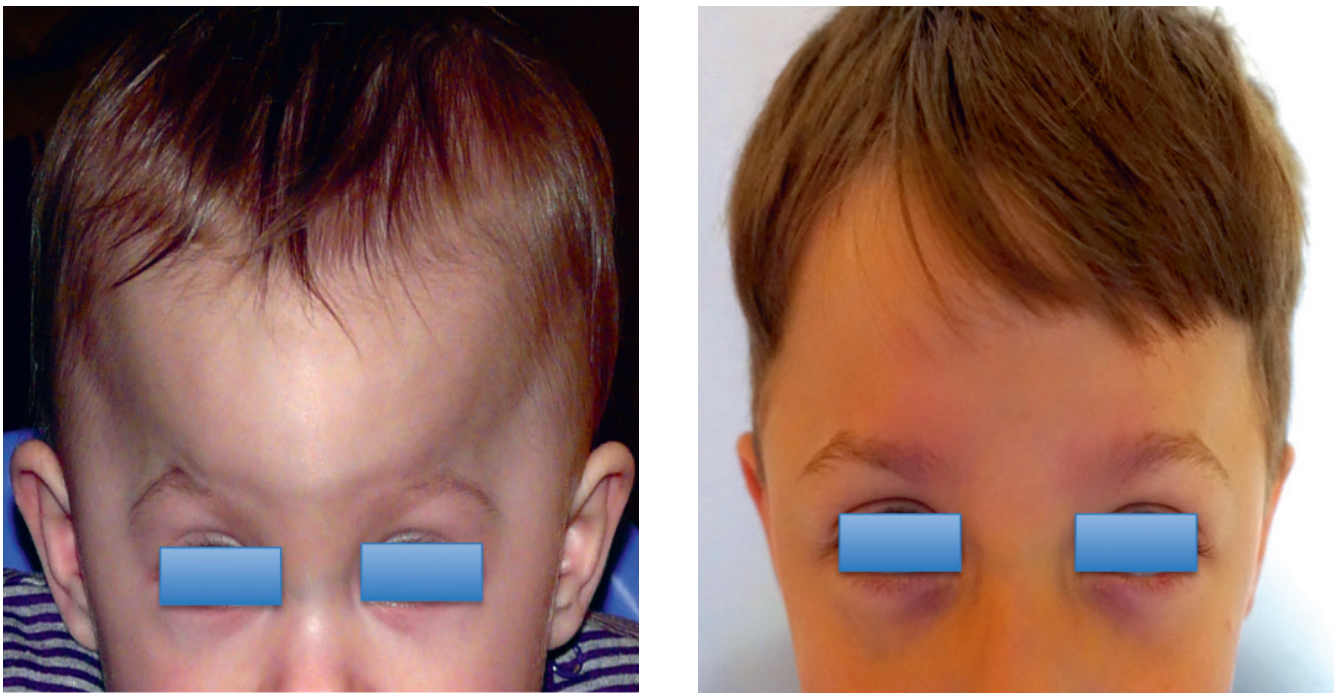

Fig. 11. Patient before the procedure (at the age of 7 months) and after the procedure (at the age of 7 years)

of cranial malformations. Trigonocephaly cases increased from 10 up to 42 cases a year and the number of scaphocephalies (the most common isolated form) from 48 up to 87 cases a year. What is interesting, the percentage of sagittal suture synostosis remained the same (53%), whereas the percentage of trigonocephaly cases increased from 11% to 25% of all nonsyndromic cases. This phenomenon was also observed by van der Meulen et al.[8] in their studies. The prevalence of metopic suture synostosis was estimated at 1:1,5000 live births, which made it the 3rd most frequent single-suture craniosynostosis, after scaphocephaly (1/4,200–1/8,500) and plagiocephaly (1/1,1000). The percentage of trigonocephaly in publications discussing big groups of patients (>100 craniosynostosis patients) amounted to 3–10% of all operated cases in 1960s and 1970s. The pan-European study concerning

the years 1997–2006, in which 3,240 patients were operated on in 7 participating units, showed a significant increase in the absolute number as well as of the percentage of trigonocephaly cases (23%). Lajeunie et al. in their analysis of 237 trigonocephaly patients, observed a considerable predominance of male cases (M:F = 3,3:1).[4] In our group of patients, this ratio was identical.

Trigonocephaly is often accompanied by a malformation involving other systems. Taking into account the division proposed by Lajeunie et al.,[19] our patients may be divided into the following groups: group I (n = 19) – patients with isolated malformation, and group II (n = 11; containing 2 subgroups: IIA – patients with additional malformations without any diagnosed syndrome (n = 8), and IIB – patients with a recognized syndrome (n = 3)). Only in 1 patient from group IIA, more than 1 additional malformation was found, whereas this kind of abnormality was found in all patients from group IIB (Table 4). Among these cases, genitourinary system abnormalities (n = 6) and limbs malformations (n = 3) were predominant.

The reasons for trigonocephaly formation have not been clearly recognized yet; however, in accordance with 3 theories concerning disease etiology, genetic, metabolic or pharmacological factors may be considered, as well as fetal head intrauterine constraint or brain internal malformations.[3] Patients in whom these factors might have appeared during fetal development, are listed in Table 2.

In our Department, before the surgery, a fundoscopy is performed to exclude increase of ICP and this symptom was not observed in any patient. Intracranial pressure increase is the most important factor which negatively influences brain development in the case of craniosynostosis. Disproportionate cranial cavity resulting from premature cranial suture synostosis may cause an increase in ICP, which is more common in the case of craniosynostoses involving many sutures as well as in syndromic cases (30–40%). What is more, its prevalence is correlated with the patient's age at the time of surgery: the older the child, the bigger the probability of ICP increase.[20] In the majority of isolated craniosynostoses, ICP is doubled after 1 year of life and in the case of scaphocephalies, it is quadrupled. Optic nerve disc edema is a symptom of this process and it may result in nerve compression and loss of vision. Study results imply that edema develops at values >15 mm Hg.[21] Tuite et al. tested fundoscopy efficacy in ICP diagnosis and reported that optic nerve edema was correlated with increased ICP in 87% of patients in whom ICP was greater than 15 mm Hg.[22] At the same time, in a group of patients younger than 8 years, edema directly suggests ICP increase, but the absence of this sign does not indicate lack of ICP increase. Hence, it cannot be excluded in patients treated in our Department. In patients with isolated craniosynostosis, ICP increase takes place in about 15–20% of cases, and in trigonocephaly it is even rarer – 8–17% of cases.[23]

In trigonocephaly, vision defects, such as primary strabismus or astigmatism, may occur. They are caused by deformities in the orbital, frontal and zygomatic areas, as well as abnormal attachments of oculomotor muscles, which forces affecting the eyeball are changed.[24] The vision defects mentioned above were observed in our material as well.

In the group of children with trigonocephaly operated on after the 1st year of life, Bottero et al.[25] distinguished 52% of cases with psychomotor disorders. In the group of children operated on before the 1st year of life, this percentage was significantly lower and comprised 22.6% of such cases. A higher risk of developmental disorders occurred in patients with additional extracranial abnormalities as well as in patients in whom the value of frontal stenosis was higher.

Nowadays, it is generally accepted that the surgery should be carried out before the 1st year of life.[26] In our center, patients are qualified for the procedure of skull remodeling for aesthetic or medical reasons at the age of 8–12 months. Mild malformations, which require milling or suture excision only, can be operated on at an older age. In some patients, however, surgical intervention is significantly delayed. This may be due to a late diagnosis made by a pediatrician or a neonatologist and delayed registration for the surgery in a specialist center. Sometimes, parents postpone their decision to operate their child because of the risky and extensive procedure.

The aim of surgical intervention is to excise prematurely fused suture and correct the associated deformities of the calvaria with remodeling and advancement of the forehead and supraorbital band. This enables the enlargement of the anterior cranial fossa and appropriate brain development as well as hypotelorism correction.[27] Tessier and Marchac have largely contributed towards the development of trigonocephaly surgical treatment. At present, their methods are considered basic and many modifications have been implemented. Surgeries performed in our center are also based on these methods and their results are satisfactory.

In 5 cases, our patients required additional corrections mainly due to temporal hollowings, which are common complications in trigonocephaly treatment. Theories on its etiology focus either on bone or soft tissues damages during surgical procedures. Atrophy of the superficial temporal fat pad or temporal muscle could be another explanation for the appearance of these depressions. Van der Meulen et al.[28] supported the theory that it is not the surgical technique but rather intrinsic synostotic bones defect that reduces the potential of osseous growth and is responsible for such a deformity. In turn, Oh et al.[29] suggested that the incorrect surgical technique resulting in making the frontal bone too short creates a lack of bone in the temporal region. These conclusions are supported by a series of cases in which bitemporal depression disappeared after the application of bone grafts in the temporal areas.

Wes et al.[30] in their retrospective studies of trigonocephaly treatment carried out on a large group of patients

(n = 147), found that with protracted time to follow-up visits, secondary deformations percentage increased. These deformations included bitemporal depressions and orbits lateral walls retrusion. They also observed a larger number of patients qualified to categories III and IV according to the Whitaker classification (independent factor in patients followed up after 5 years). In this study, almost 50% of patients were followed up after 5 years and as much as 36% of them were included to category III. In another study carried out on 60 patients, similar results were elicited and 85% of patients were qualified to category I in Whitaker classification.[31] In our own study, though 17 patients registered for follow-up visits at the age over 5 years, the results were assessed as excellent and good (category I – 78%).

Mortality and complication rates in trigonocephaly surgeries are low and dura mater tears, subgaleal hematomas, postoperative infections, and cerebrospinal fluid leakage are the most prevalent ones.[32] In our own study, only in 1 patient the complication appeared in the form of seroma, which was evacuated 10 days after the surgery.

In order to decrease blood loss during surgery, endoscopic procedures proposed by Jimenez et al. were applied.[33] This method was implemented in the 1990s and it was based on a simple suturectomy with the use of an endoscope. In the majority of patients operated on with the use of this method, surgery time was reduced to 52 min and blood loss to 26 mL. These operations were performed before the 4th month of life and postoperative treatment required helmet molding therapy for about 7 months. This method is restricted to young infants, preferably before the 4th month of life. In older patients with severe and persistent malformations of the skull, results do not prove satisfactory.

The choice of surgery method depends on the severity of the malformation and the patient's age. Metzler et al.[34] compared the FOA method with the tilt procedure, which is characteristic for leaving medial and lateral bone attachments of fronto-orbital segment in the area of frontonasal and frontozygomatic sutures and vascularized from trochlear, supraorbital and superficial frontal vessels. The tilt procedure carries benefits when applied in younger infants, as this group has malleable osseous segments, allowing greenstick fracture. The degree of surgical correction achievable using the tilt would be well-applied in cases of mild and moderate metopic synostosis. In severe cases, the larger magnitude of movement possible with FOA may allow for distinct 3D correction and overcorrection.

Apart from quite obvious characteristics of a triangle skull shape in all of our patients, 9 of them revealed additional malformations in preoperative imaging study. Many radiologically detected abnormalities in the cerebral region are associated with craniosynostoses – mainly syndromic ones. They may be divided into 3 categories. The 1st one is general brain malformation strictly connected with mechanical compression and skull deformation. The 2nd one concerns Chiari I malformation and is also caused by compression and posterior cranial fossa reduction, especially in premature synostosis of lambdoid suture. The 3rd one is related to the white matter abnormalities. They range from ventriculomegaly and hydrocephalus to callosal anomalies or agenesis, hypoplasia or absence of the septum pellucidum, paucity or dysplasia of temporal white matter, distortions of the cerebral cortex, as well as pyramidal or hippocampus hypoplasia. Usually, these abnormalities were considered secondary to cranial growth inhibition. Tokumaru et al. found abnormalities within the hippocampus, corpus callosum and septum pellucidum to be primary abnormalities of brain development.[35] This theory may be confirmed by the occurrence of neurological and behavioral disorders, despite corrective surgery. Fibroblasts growth factor mutations, which are the most common reason for syndromic craniosynostoses, play an important role in both cranium and brain development.[36]

Trigonocephaly is associated with a relatively high level of neurodevelopmental problems. These children frequently reveal speech and language development delay, as well as cerebral function disorders connected with frontal lobes dysfunction (ADHD, autism).[37,38] In our own study, autism was diagnosed in 1 and ADHD was found in 2 patients. The total number of patients with neurological disorders was 12. Hence, the question is whether surgical treatment performed before the 1st year of life can prevent developmental and neurological disorders, which can be the result of primary brain dysfunction and not cranial suture synostosis. Additionally, our observations prove that some of our patients who had been diagnosed with neurological disorders before the surgery did not reveal them anymore after the procedure, and some revealed them after surgery. This may confirm the presumption that central nervous system lesions are primary and they occur regardless of the patient's age at the time of the surgery.

Conclusions

Early pediatric diagnostics makes it possible for the corrective surgery of the skull to be performed in the 1st year of life. Surgical treatment based on Tessier's and Marchac's modified methods provides good results in patients at the age of about 12 months. The procedure performed at this age prevents consequences from ICP increase. Primary neurological and behavioral disorders may occur despite corrective surgery.

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The association between neurological diseases, malignancies and cardiovascular comorbidities among patients with bullous pemphigoid: Case-control study in a specialized Polish center

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Abstract

Background. Bullous pemphigoid (BP) is the most common autoimmune bullous disease associated with higher mortality and coexisting comorbidities. The strongest relationship has been reported with neurological diseases (NDs) but the particular type of ND differed depending on the study. There are some doubts on the prevalence of other comorbidities.

Objectives. The aim of this study was to compare the incidence of various comorbidities in a cohort of BP patients with controls.

Material and methods. A cohort of 218 patients (137 females, 81 males, aged 76.2 ±11.6 years) with newly diagnosed BP who were hospitalized at a specialized center in Poland in the years 2000–2014 was included in this retrospective study. The controls consisted of 168 sex- and age-matched individuals. Univariate and multivariate logistic regression analyses were performed to assess the association between the groups studied.

Results. At least 1 ND was present in 33.5% of BP patients vs 11.3% of controls. A strong association between the incidence of NDs and BP was found (OR = 3.76; 95% CI = 2.13–6.65; $p < 0.001$), especially for dementia (20.6% vs 2.9%, OR = 7.89; 95% CI = 2.99–20.85; $p < 0.001$). Surprisingly, BP patients with ND were older than the BP patients without ND (79.2 vs 74.7 years), and similarly for dementia (81.08 vs 74.90 years). The same was observed in comparison with controls. Arterial hypertension, among other comorbidities, was a strong independent factor associated with BP (OR = 2.17; 95% CI = 1.35–3.49; $p < 0.001$). Malignancies were observed more frequently in BP patients than in controls (12.8% vs 9%) but such association was significant in univariate analysis only.

Conclusions. Neurological diseases, particularly dementia, had a significant association with BP. A strong relationship with arterial hypertension and weak relationship with malignancies were noted. Thus, for appropriate medical care, patients with BP need accurate screening for dementia and control of comorbidities with interdisciplinary management.

Key words: comorbidities, neurological diseases, bullous pemphigoid, internal diseases

Introduction

Bullous pemphigoid (BP) is the most common autoimmune bullous disorder caused by autoantibodies directed against BPA2 (BP180) and BPA1 (BP230) proteins located in the basement membrane zone (BMZ). Clinically it is characterized by tense bullae, urticarial skin lesions and pruritus, primarily affecting elderly individuals, with risk increasing with age.[1,2] The worldwide incidence ranges from 6.6 to 42.8 new cases per million people yearly while in Poland the BP incidence is estimated at 7 per million inhabitants per year. Bullous pemphigoid is a potentially fatal disease with 1-year and 3-year mortality rates assessed at 22.4% and 39.5%, respectively, in Polish BP patients.[3,4]

In recent years, many epidemiological studies have investigated the coexistence of BP with various comorbidities. In fact, the most intense research has been focused on neurological diseases (NDs), showing that BP patients are more likely to have any type of ND.[2,5–7] However, the particular types of ND among BP patients differed depending on the study and some results were even conflicting.[1,2,5,6,8–13] Some data found a higher prevalence of stroke, dementia, Parkinson's disease, epilepsy, and multiple sclerosis in BP.[1,2,5,6,8–14] Other studies have also identified psychiatric diseases such as schizophrenia, personality or unipolar/bipolar disorders as a risk factor for BP.[6,15] Moreover, some studies have referred to the incidence of malignancies in BP patients, with some showing a higher proportion in BP patients.[16–19] In spite of this, the debate is ongoing and this relationship remains controversial. There are several publications in the literature that have reported a higher prevalence of arterial hypertension in BP patients. The fact is that this disease may play an important role in BP patients, especially since major drugs used to treat hypertension such as diuretics, ACE inhibitors or spironolactone can provoke BP.[6,13,20–22] Among other comorbidities, single studies have demonstrated the association of BP with diabetes.[23–25]

To confirm or reject the abovementioned discrepancies, the aim of this study was to assess the relationship between BP and selected comorbidities. The groups of NDs (dementia, stroke, Parkinson's disease, epilepsy, multiple sclerosis, and depression), cardiovascular diseases (arterial hypertension, arrhythmia, coronary artery disease, heart failure, and thromboembolic syndrome), malignancies, and diabetes mellitus were included.

Material and methods

The patients were diagnosed and treated in the Department of Dermatology and Immunodermatology at the Medical University of Warsaw (Poland) in the period 2000–2014. The retrospectively-assessed group consisted of 386 persons (218 BP patients and 168 controls). The BP diagnosis was based on clinical features and positive direct immunofluorescence (DIF) of perilesional skin (linear IgG and/or C3 deposits along the BMZ). Additionally, indirect immunofluorescence (IIF) detecting circulating anti-BMZ antibodies and/or examination of salt split skin and/or enzyme-linked immunosorbent assay (ELISA) for anti-BP180 NC16a (MESACUP BP 180 ELISA kit; Medical&Biological Laboratories, Nagoya, Japan) were performed. In the group studied, almost all BP patients were positive for circulating autoantibodies (in 95.7%).

The controls were randomly selected from patients admitted in the same period due to other dermatological diseases such as eczema, drug eruptions, erysipelas, herpes zoster, leg ulcers, urticaria, atopic dermatitis, scabies, erythema multiforme, lichen planus, or pityriasis rubra pilaris. Subjects with dermatological diseases characterized by systemic symptoms (e.g., connective tissue disorders, psoriasis, lymphoma) were not included to the control group. The diagnosis of particular additional diseases was based on a reliable anamnesis or medical documentation including the results of appropriate tests. If any uncertainty was present, a cardiologist and/or a neurologist was asked for a medical consultation to confirm or reject a diagnosis.

Approval from the local Ethics Committee of the Medical University of Warsaw was obtained before starting the study.

Statistical analysis

The patients with BP and controls were compared using either the Student's t-test or the Wilcoxon test according to the parameters of distribution assessed with the Kolmogorov-Smirnov test. Variables with a normal distribution are presented as a mean followed by standard deviation (SD). Variables not showing the normal distribution are presented as a median with range values. For categorical variables, the differences between groups were compared using the exact Fisher test. All tests were double-sided. Correlations were evaluated using Spearman correlation coefficients. Crude and adjusted odds ratios (OR) and a 95% confidence interval (95% CI) were estimated using univariate and multivariate logistic regression analyses for both groups. An association model was constructed using BP diagnosis as the dependent variable. Values of $p < 0.05$ were considered statistically significant. Analyses were performed using a statistical software package (STATISTICA v. 12; StatSoft Inc., Tulsa, USA).

Results

Among the cohort of 218 BP patients, 137 (62.8%) were female and 81 (37.2%) were male. In the 168 controls there were 101 (60.1%) females and 67 (39.9%) males. Mean age at BP diagnosis was 76.2 ±11.62 years, median 78 years (range: 33–100 years); and mean age for the controls was 75 ±10.92 years, median 77 years (range: 33–92 years). The BP patients and controls were age- and gender-matched (Table 1).

Table 1. Univariate and multivariate regression analysis of comorbidities influencing bullous pemphigoid and controls

Variable	BP patients n = 218 [%]	Controls n = 168 [%]	p-value	Univariate analysis in BP patients		Multivariate analysis in BP patients			
				[a]OR (95% CI)	p-value	[b]OR (95% CI)	p-value	[c]OR (95% CI)	p-value
Gender: female	137 (62.8)	101 (60.1)	0.60	–	–	–	–	–	–
Age [years]	76.23 ±11.62	75.03 ±10.92	0.30	–	–	–	–	–	–
Any neurological disease	73 (33.5)	19 (11.3)	<0.001	3.95 (2.26–6.89)	<0.001	3.67 (2.05–6.55)	<0.001	3.76 (2.13–6.65)	<0.001
Dementia	45 (20.6)	5 (2.9)	<0.001	8.48 (3.27–21.9)	<0.001	7.86 (2.94–21.03)	<0.001	7.89 (2.99–20.85)	<0.001
Stroke	28 (12.8)	9 (5.3)	0.01	2.60 (1.19–5.69)	0.01	2.12 (0.91–4.94)	0.08	2.18 (0.95–4.98)	0.06
Parkinson's disease	11 (5)	3 (1.7)	0.1	2.92 (0.80–10.69)	0.1	3.03 (0.74–12.32)	0.11	–	–
Multiple sclerosis	1 (0.5)	1 (0.6)	1.0	0.77 (0.04–12.50)	0.85	1.05 (0.06–17.92)	0.97	–	–
Epilepsy	1 (0.5)	1 (0.6)	1.0	0.77 (0.04–12.50)	0.85	0.86 (0.05–14.46)	0.91	–	–
Arterial hypertension	166 (76.1)	100 (59.2)	<0.001	2.17 (1.40–3.37)	<0.001	2.16 (1.31–3.73)	0.002	2.17 (1.35–3.49)	0.001
Stable coronary artery disease	82 (37.6)	48 (28.5)	0.06	1.51 (0.98–2.33)	0.06	1.29 (0.77–2.15)	0.32	–	–
Myocardial infarction	16 (7.3)	12 (7.1)	1.0	1.03 (0.47–2.23)	0.94	0.84 (0.34–2.08)	0.71	–	–
Thromboembolic events	13 (5.9)	4 (2.3)	0.13	2.6 (0.83–8.15)	0.1	2.51 (0.71–8.80)	0.14	–	–
Arrhythmias	46 (21.1)	26 (15.4)	0.18	1.46 (0.86–2.48)	0.16	1.40 (0.79–2.50)	0.24	–	–
Malignancies	32 (14.6)	13 (7.7)	0.03	2.05 (1.03–4.60)	0.03	1.74 (0.84–3.65)	0.13	1.92 (0.94–3.96)	0.07
Diabetes mellitus	47 (21.5)	33 (19.6)	0.70	0.64 (0.68–1.85)	0.64	0.97 (0.54–1.72)	0.91	–	–

[a]crude OR (95% CI), [b]adjusted OR (95% CI) to age and gender, analyzed to all variables, [c]adjusted OR (95% CI) analyzed to significant values; BP – bullous pemphigoid; OR – odds ratio.

Neurological diseases

At least 1 ND was found in 73 BP patients (33.5%) and in 19 controls (11.3%). The significant association between BP and the incidence of NDs was confirmed in multivariate regression analysis (OR = 3.76, 95% CI = 2.13–6.65, p < 0.001) (Table 1). Thirteen BP patients (6%) had 2 NDs in comparison with none in the controls. The mean age of BP patients with ND was 79.2 vs 74.7 years in patients without ND. The same was observed in comparison with controls (Table 2). The most frequent ND in BP was dementia, which was observed in 45 (20.6%) patients, followed by stroke in 28 (12.8%), Parkinson's disease in 11 (5%), epilepsy in 1 patient, and multiple sclerosis in 1 other patient. In the controls, stroke was the most frequently observed ND in 9 (5.3%) subjects, followed by dementia in 5 (2.9%), Parkinson's disease in 3 (1.7%), epilepsy in 1, and multiple sclerosis in 1 individual. The BP patients had a significantly increased risk for dementia (OR = 8.48, 95% CI = 3.27–21.90, p < 0.001) and stroke (OR = 2.60, 95% CI = 1.19–5.69, p = 0.01) assessed by univariate analysis, whereas only dementia remained statistically significant in multivariate regression analysis (OR = 7.89, 95% CI = 2.99–20.85, p < 0.001) (Table 1). A statistically higher age for BP patients with dementia vs without was observed (81.1 vs 74.97 years, p = 0.001) (Fig. 1).

Table 2. Age of BP patients and controls with and without selected comorbidities

Variable	Age		p-value
	BP patients	controls	
Neurological disease			
yes	79.21 ±7.50	76.15 ±9.85	0.14
no	74.73 ±12.99	74.89 ±11.07	0.91
Dementia			
yes	81.08 ±7.32	73.80 ±16.42	0.07
no	74.97 ±12.21	75.07 ±10.78	0.93
Stroke			
yes	77.67 ±7.89	74.77 ±7.89	0.34
no	76.02 ±12.08	75.05 ±11.08	0.44
Arterial hypertension			
yes	78.00 ±10.22	75.88 ±10.08	0.10
no	70.59 ±13.95	73.79 ±12.01	0.18
Malignancies			
yes	77.81 ±9.87	72.15 ±10.88	0.09
no	75.96 ±11.90	75.27 ±10.92	0.58

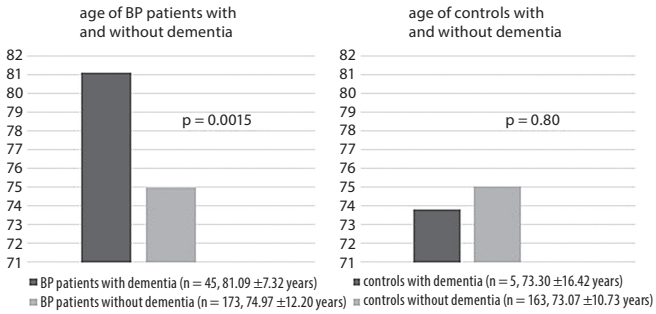

Fig. 1. Age of bullous pemphigoid (BP) patients and controls with and without dementia

Malignancies

A history of malignancies was recognized in 32 BP patients (14.6%) and 13 controls (7.7%). This relationship with malignancy occurrence in BP was statistically significant for univariate analysis only (OR = 2.05, 95% CI = 1.03–4.60, p = 0.03). The BP patients and controls had a history of similar types of cancers, and the most common ones in BP patients and controls were also the most common in the general population (such as lung, prostate, breast, ovarian, and colon cancers as well as lymphoma or leukemia).

Other comorbidities

Out of all examined comorbidities, at least 1 comorbidity was present in 199/218 (91.3%) of BP patients and in 132/168 (78.6%) of controls. In BP patients and controls, arterial hypertension was the most frequent comorbidity (in 76.1% and 59.2% subjects, respectively). Arterial hypertension was independently associated with BP in multivariate regression analysis (OR = 2.17, 95% CI = 1.35–3.49, p = 0.001). No association with diabetes mellitus among patients and controls was found. Chronic various drug intake was noted in 96% of BP patients. The most frequently prescribed drugs in BP were ACE-inhibitors, furosemide, calcium channel blockers, aspirin, proton pump inhibitors, and statins.

Discussion

Association with neurological diseases

Our case-control study has shown a remarkably higher incidence of NDs in BP patients (33.5% vs 11.3% in controls). This is consistent with other studies published so far, where ND rates ranged from 23% to 73.8% in BP patients and from 4% to 24.2% in controls.[2,5,7–14,23,26,27] Additionally, our results have indicated that Polish BP patients were approx. 4 times more likely to suffer from any type of ND as compared to other individuals. This high value is also comparable with the results of a recently-published first meta-analysis covering 14 case-control or population-based studies from Europe (Czech Republic, France, Italy, Portugal, Denmark, Spain, and the UK), the US and Asia (Taiwan and Malaysia).[14] In this meta-analysis, BP patients have been estimated to be 5 times more likely to have ND than the general population.[14]

Moreover, similarly to previously published studies, we were not able to demonstrate that all NDs assessed separately had a higher association with BP. The reasons for these discrepancies between various studies may be explained by the different age of BP patients, relatively small patient groups in some evaluations, different selection of control groups, environmental factors, and finally, low morbidity for certain NDs such as epilepsy or multiple sclerosis. Also, the retrospective nature of the studies discussed may have influenced these differences.

In fact, we have shown that dementia was the only ND significantly associated with BP as demonstrated using multivariate regression analysis. Occurring in 20.6%, dementia in our BP patients remained in the middle range of percentages in comparison to previously published studies, where the frequency oscillated from 5.3% to 42.7%.[1,2,5,6,8–13,26,27] It is also important to note that, based on data from PubMed, dementia was independently associated with BP in all of the 13 case-control or population-based studies evaluating the relationship of this entity among BP patients confirmed by multivariate regression analysis, although the incidence of dementia varied considerably and ranged from 2.5 to 12.9 times more than in the general population.[1,2,5,6,9–12,14] Our BP patients faced a 7.86-fold increased risk of dementia, similarly to English patients with BP.[5]

Surprisingly, we observed a much older age of BP patients with dementia compared to BP patients without this condition (a difference of 8 years, p = 0.0015). The same was true when compared to the control group (a difference of 8 years, p = 0.07). In the literature, the data on BP patients' age with dementia is very limited. Only Langan et al., in a large group of 868 patients and 3,453 controls, noted a higher proportion of elderly BP patients with dementia compared to healthy controls with dementia.[1]

Interestingly, similarly to dementia, our BP patients with any type of ND were also older than BP patients without ND (a difference of 5 years). In the literature, several studies have evaluated the mean age of BP patients with any type of ND, showing that French, German and Iranian populations of BP patients with ND were much older than those without ND (a difference of 4, 7 and 17 years, respectively).[7,11,28] The significance of the abovementioned findings is difficult to explain, especially as one could expect the opposite results. It seems that these unexpected results need confirmation in subsequent observations.

In our BP patients, the profile of other NDs was 12.8% for stroke, 5% for Parkinson's disease, less than 1% for epilepsy, and less than 1% for multiple sclerosis, being the closest to the French group of BP patients.[8] According to the literature, the prevalence of stroke in BP is estimated at between 7.7% to 44.4%, Parkinson's disease is reported between 2.3% and 17.9% of BP patients, the frequency of epilepsy varies from 2% to 11.1% and the frequency of multiple sclerosis is estimated at between 0% and 5%.[1,2,5,6,8–11,13–15,26–28]

We identified a relationship between BP and stroke, but the association was lost in the multivariate regression analysis. In fact, 12 case-control or population-based studies out of 15 which evaluated the prevalence of stoke in BP using multivariate regression analysis showed no significant association of this disorder among BP patients.[1,2,5,6,8–11,13–15,26,27]

Our study did not show greater incidences of multiple sclerosis, Parkinson's disease or epilepsy among BP patients either. Although the relationship between multiple sclerosis and BP is the strongest among all the NDs, probably due to the autoimmune nature of both disorders, studies of American and Iranian populations of BP patients did not

show an increased risk of multiple sclerosis, similarly to our study.[2,11] Congruous observations for Parkinson's disease or epilepsy and BP can be noted too. Until now, in 4 case-control studies, an increased relationship with Parkinson's disease was revealed[1,2,6,10,12] but the results of 5 others did not confirm this observation with multivariate regression analysis.[2,5,10,13] Likewise, several studies have investigated the prevalence of epilepsy in BP and in 3 of them a positive association was observed,[1,10,15] while the English, Malaysian and Iranian studies did not note such an association at all.[5,11,13]

The interaction between NDs and BP might play a crucial role in the explanation of the pathological mechanisms in BP. In fact, both cutaneous BP antigens BPA2 and BPA1 (neuronal isoform) are found in the central nervous system. It has been suggested that alterations of the blood–brain barrier in the course of NDs may expose the neuronal BP antigens leading to an autoimmune response which results in the development of BP. A recent study by Messingham et al. proved that patients with various forms of dementia and Parkinson's disease even without BP had BPA2 antibodies detected in 23% and 37% of cases, which may confirm this hypothesis.[29] Such patients have been suggested to represent a pre-disease state in which, firstly, neuronal immunoreactivity against BP180 is manifested, and later, as a result of some additional triggers, cutaneous autoimmunity is developed.[30]

Association with malignancies

In contrast to NDs, the relationship between BP and malignancies has been under debate for many years. The results of our study showed a positive association between BP and malignancy occurrence, but various case-control studies have revealed the opposite results so far. Based on data from studies published in PubMed, the incidence of malignancies among BP patients ranged from 5.8% to even 21.3%. However, in some of these studies, no association was observed as compared to the control group. For example, a large English epidemiological study which included many cases of BP patients did not detect a higher risk of cancer in these patients in contrast to the general population.[16] In another study carried out by Lindelof et al. in Swedish BP patients, no association between BP and malignancies was found.[31] Unlike the abovementioned studies, some others have suggested an increased frequency of malignancies in BP patients.[17,18,19,23] Studying a German population of BP patients (1,743 patients), Schulze et al. found that BP patients face a 2.5-fold higher risk of different hematological malignancies, with a percentage of 6.7% in BP patients.[19] Moreover, Ogawa et al. investigated a large Japanese population of BP patients (1,113 patients) and found malignant diseases in 5.8% of patients compared with 0.61% among controls.[18] In addition, a case-control study of the Danish population disclosed a slightly higher incidence of tumors in BP patients (14%), but no statistical analysis was conducted to clarify these findings.[23] There are also numerous single case reports that have determined the co-existence of certain carcinomas. These cases were analyzed jointly by Balestri et al., showing that cancers of the prostate, breast, stomach and colon, as well as lymphoma and leukemia were observed the most often in BP patients.[32]

Some authors have emphasized that the coexistence of BP and malignancies should not be found surprising as they result from the high rate of malignancies in this elderly patient population. The open question is if there is a common pathophysiological pathway of BP and malignancies. So far, several hypotheses have been proposed to explain this concomitance. The first and main theory suggests that antibodies against tumor-specific antigens may cross-react with the BMZ. Another one indicates that a tumor could secrete a hormone-like substance destroying BMZ, which causes the production of anti-BP antibodies. Another hypothesis considers the role of an external factor, e.g., a virus, which could be tumorigenic and lead to BMZ destruction at the same time.[32]

Association with cardiovascular diseases

Finally, our data demonstrated the significant association between BP and arterial hypertension revealed by multivariate regression analysis. The prevalence of hypertension appears to be around 30–45% of the general population, with a steep increase with ageing.[33] In both groups studied, arterial hypertension was the most common comorbidity, however it seems to be more frequent in BP patients. Our results were consistent with recently published studies carried out by Kremer et al., who found that arterial hypertension was the most frequent comorbidity in BP (in 64% of patients studied, $p = 0.04$).[21] In studies by Kwan et al. and Cai et al., arterial hypertension was also the most frequent out of all comorbidities (in 62.8% and 59.3%, respectively).[13,20] In another study concerning BP patients, multi-morbidity was observed in 84% with the highest percentage for arterial hypertension.[22] On the other hand, Jedlickova et al. did not find differences in cardiovascular disease incidences among patients with BP and controls.[7]

Nevertheless, a certain role in the pathogenesis of BP is attributed to drug consumption, especially agents used to treat hypertension. We suppose that positive association with arterial hypertension occurrence in our patients might also be related to antihypertensive drugs, which in some cases could certainly contribute to inducing or exacerbating BP.[23] Similarly, in our BP patients with arterial hypertension, the intake of at least 1 antihypertensive drug was noted; therefore, the potential influence of prescribed treatment on BP course cannot be excluded either.

Limitations

Our single-center study includes some limitations. The first is the retrospective nature of our evaluation.

Moreover, the hospitalized patients with other dermatological conditions serving as controls in this study might contribute to a certain selection bias.

Conclusions

Our study on a relatively large group of BP patients demonstrated a significantly higher prevalence of various NDs, especially dementia. Surprisingly, the BP patients with any NDs, presented were older than the BP subjects without these disorders. Among other common comorbidities, arterial hypertension and other malignancies typical for the general population were the ones most associated with BP occurrence. For appropriate medical care, patients with BP need accurate screening, especially for dementia and any neurological abnormalities. Interdisciplinary management of BP patients including dermatologists, neurologists, cardiologists, and general practitioners is strongly required.

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Do barbed sutures with different surface textures have different effects on adhesion formation and histological features? An experimental blinded study in an animal model

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Abstract

Background. The obstetrics and gynecology literature has expanded in recent years to include clinical trials assessing the use of barbed sutures. The difficulty of intracorporeal suturing continues to be a barrier to a wider use of laparoscopy. Although the use of barbed sutures has been shown to ease the process of laparoscopic suturing considerably, concerns have been raised regarding a potentially increased risk of adhesions or inflammation as a result of their use.

Objectives. The aim of this study was to determine whether differences in surface textures, resulting from the variations in the geometric configurations of barbs, lead to differences in intra-abdominal adhesion formation.

Material and methods. A total of 27 non-pregnant female Wistar Hannover rats, weighing 200–250 g, with intact uteri were used as an adhesion formation model. The rats were randomly assigned to 3 groups: barbed suture group 1, barbed suture group 2 and control group (no intracorporeal suture). A 2-centimeter vertical incision was performed on the anti-mesosalpingeal side of one of the uterine horns. The incision on the uterine horn was reapproximated with a running suture, entailing 3 needle punctures and left untied at one end. Six weeks after the operation, intra-abdominal adhesion formations were investigated both clinically and histopathologically.

Results. Clinical adhesion scores and histopathological parameters in both the barbed suture groups were statistically significantly higher than in the control group ($p < 0.05$). There was no significant difference between the barbed suture groups regarding the adhesion scores.

Conclusions. The 2 types of barbed sutures with different surface textures, used for myometrial closure, form a similar profile with respect to postoperative adhesion formation.

Key words: adhesion formation, barbed suture, knotless suture, myometrial closure, smooth suture

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Introduction

Since their first clinical use on human cadavers in 1967, barbed sutures have been employed in many areas, including general surgery, cosmetic surgery, orthopedic surgery, and gynecological surgery. At present, 3 types of barbed sutures present on the market are approved by the US Food and Drug Administration (FDA): Quill SRS bidirectional barbed sutures (Quill™ Self-Retaining System; Angiotech Pharmaceuticals, Vancouver, Canada), V-Loc unidirectional barbed sutures (V-Loc™ Absorbable Wound Closure Device; Covidien Ltd., Mansfield, USA) and Stratafix unidirectional and bidirectional barbed sutures (Stratafix™ Knotless Tissue Control Device; Ethicon Inc., Somerville, USA).[1] These suture materials have sharp barbs that diffuse wound tension equally throughout the suture line by attaching tightly to each millimeter of tissue.[2] Currently, the barbs on the surface of the sutures available on the market are placed on a monofilament thread in 3 different geometries and configurations.[2,3]

The obstetrics and gynecology literature has expanded in recent years to include clinical trials assessing the use of barbed sutures. Recent meta-analyses have shown that the use of barbed sutures, which ease intracorporeal suturing considerably during laparoscopic surgery, decreases the total duration of operations, the time spent on suturing and the estimated blood loss when compared with conventional sutures.[4,5] Nevertheless, despite the many potential advantages of barbed sutures, some case reports have shown these new sutures to be associated with a higher risk of small bowel obstruction following myomectomy, vaginal cuff closure and sacrocolpopexy. Such adverse events might occur due to a potentially increased risk of adhesion formation or inflammation, resulting from the entrapment of barbs in the neighboring tissue.[6–9] Adhesions can hamper the functions of abdominal organs, leading to infertility, chronic pelvic pain or increased morbidity.[10]

The aim of this study was to determine whether differences in surface textures, resulting from the variations in the geometric configurations of the barbs lead to differences in intra-abdominal adhesion formation. We compared 2 types of commercial barbed sutures with respect to clinical adhesion scores and histopathologic features in a rat animal model.

The study protocol was prepared in accordance with the National Institutes of Health (NIH) guidelines and approved by the Institutional Animal Care and Use Committee at the University of Health Sciences, Bağcılar Training and Research Hospital, Istanbul, Turkey (ethics committee registration No. 2016/111, dated September 28, 2016).

Material and methods

Study animals

A total of 27 non-pregnant female Wistar Hannover rats (9 rats allocated to each of the 3 groups), weighing 200–250 g, with intact uteri were used as an adhesion formation model. The rats were numbered sequentially and kept in standard plastic cages with sawdust-covered floors, in a room without windows, at a constant temperature of 21 ±1°C, 55–60% constant humidity, in a controlled environment with 12-hour cycles of darkness and light. The rats were given standard rat food and water ad libitum.

Experimental groups

The groups were assigned as follows:

- barbed suture group 1: animals sutured using Stratafix™ Knotless Tissue Control Device, a synthetic absorbable monofilament suture with a single-angle barb design with 8 barbs/cm and 1 helix/5.08 mm (Fig. 1, A1);
- barbed suture group 2: animals sutured with V-Loc™ Absorbable Wound Closure Device, a synthetic absorbable monofilament suture with a dual-angle barb design with 20 barbs/cm and 1 helix/1.52 mm (Fig. 1, B1); and
- control group: no intracorporeal suturing.

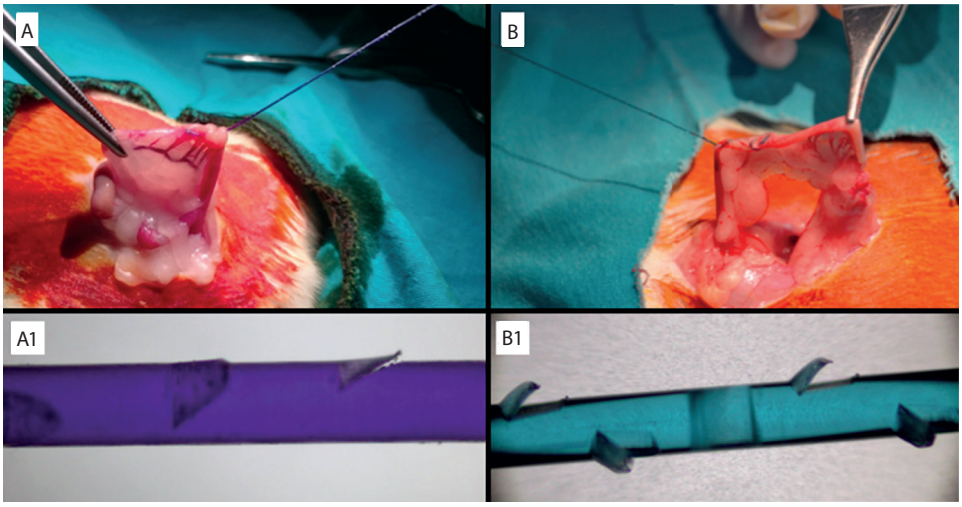

Fig. 1. Barbed suture group 1 (A, A1); barbed suture group 2 (B, B2). A1 – A single-angle barb design with 8 barbs/cm and 1 helix/5.08 mm; B1 – A dual-angle barb design with 20 barbs/cm and 1 helix/1.52 mm.

Surgical procedure

All the rats were anesthetized with a single intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar; Eczacıbaşı, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun; Bayer Turk, Istanbul, Turkey). Each rat was assigned a number. The type of suture to be used and the uterine horn to be sutured were determined by assigning a closed envelope to each rat. Before surgery, the abdomen was shaved and antisepsis was obtained using a 10% povidone-iodine solution. Sterile powder-free surgical gloves were used during all procedures. The researcher (T.K.) made a 5-centimeter abdominal midline vertical incision to expose the uterine horns.

The animal model to test for intraperitoneal adhesions has previously been used by Api et al.[9] In the control group, the abdominal cavities were entered as previously described and closed without performing any further surgical procedures. In the study groups, a 2-centimeter incision was made with a No. 15 scalpel on the anti-mesosalpingeal side of one of the uterine horns to mimic a myomectomy wound. The incision on the uterine horn was reapproximated with a running suture, entailing 3 needle punctures and left untied at one end. The free end of the barbed suture was cut at the same level as the tissue, without leaving any protruding parts. No adhesion barriers were left in the peritoneal cavity. All the uterine horn procedures were performed by the same researcher (T.K.). Blood and fibrin were removed by rinsing the surgical site with a physiological serum solution after confirmation of hemostasis, and the abdominal wall was closed with simple interrupted sutures using 3-0 polyglactin 910 sutures (Vicryl®; Ethicon Inc.). The operation times were limited to about 10 min for each rat to prevent drying of the tissue with room air. After the operations, the rats were housed separately.

Macroscopic examination

After the completion of the 6-week recovery period, all the animals were sacrificed using pentobarbital 250 mg/kg, and second-look laparotomies were performed. During the second-look operation, the researchers examined all the possible locations for intra-abdominal adhesions – under the laparotomy incision, between the intestinal loops, at the uterine horns, etc. – by careful manipulation with a fine tissue holder. Visible intra-abdominal adhesions were identified and scored according to the adhesion scoring system devised by Leach et al.,[11] by researchers blinded to the previous allocation of the suture type in each animal (S.P. and E.O.).

The extent of adhesions involving the uterus was scored as follows: 0 = no uterine adhesions; 1: 1–25% involvement; 2: 26–50% involvement; 3: 51–75% involvement; and 4: 76–100% involvement. Adhesions were further scored during gross examination to determine the severity as follows: 0: no adhesion; 1: filmy avascular adhesions; 2: vascular or opaque adhesions; and 3: a cohesive attachment of the uterine horns to each other or to other abdominal organs. The degree of adhesion formation was evaluated with the following adhesion scores: 0: no adhesion; 1: the adhesion could be separated from tissue with gentle traction; 2: the adhesion could be separated from tissue with moderate traction; and 3: separation required a sharp dissection. The sum of the 3 parameters was used as the total score for each group (Fig. 2).

Histopathological examination

After completing the scoring, the adhesions were also examined histopathologically (by 2 histopathologists blinded to the groups), and graded for the presence of inflammation and fibrosis, using grading scales previously published by Hooker et al.[12] For this purpose, the uterine horns sutured with barbed sutures were excised, and then fixed in a 10% buffered formalin solution for 24 h. After fixation, routine tissue-processing procedures were performed and the sampled tissues were embedded in paraffin. The paraffin wax blocks were sectioned (4 μm) using a microtome (Leica RM 2125RTS; Leica Biosystems, Nussloch, Germany). The prepared sections were stained with hematoxylin-eosin (H&E) stain for inflammatory cell reaction and with Masson's trichrome stain for collagen fibers. An experienced histopathologist blinded to which uterine horn had been sutured examined the tissue sections under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan). Inflammation was scored as follows: 0: no inflammation; 1: presence of giant cells, occasional lymphocytes and plasma cells; 2: presence of giant cells, plasma cells, eosinophils, and neutrophils; and 3: presence of many inflammatory cells and microabscesses. The amount of fibrosis was scored as: 0: no fibrosis; 1: minimal, loose fibrosis; 2: moderate fibrosis; and 3: florid, dense fibrosis.

Statistical analysis

Descriptive and comparative statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) v. 19.0 (SPSS Inc., Chicago, USA). The results were expressed as median, mean ± standard error of the mean (SEM) and 95% confidence interval (95% CI). The Kruskal–Wallis test and Wilcoxon rank-sum test were used to compare the differences in scores among the groups, and the Bonferroni adjustment was used. For inter-rater reliability, the results of the observations scored by S.P. and E.O. were statistically analyzed by Cohen's kappa test, and a κ-value of 80% was considered the minimum acceptable inter-rater agreement.

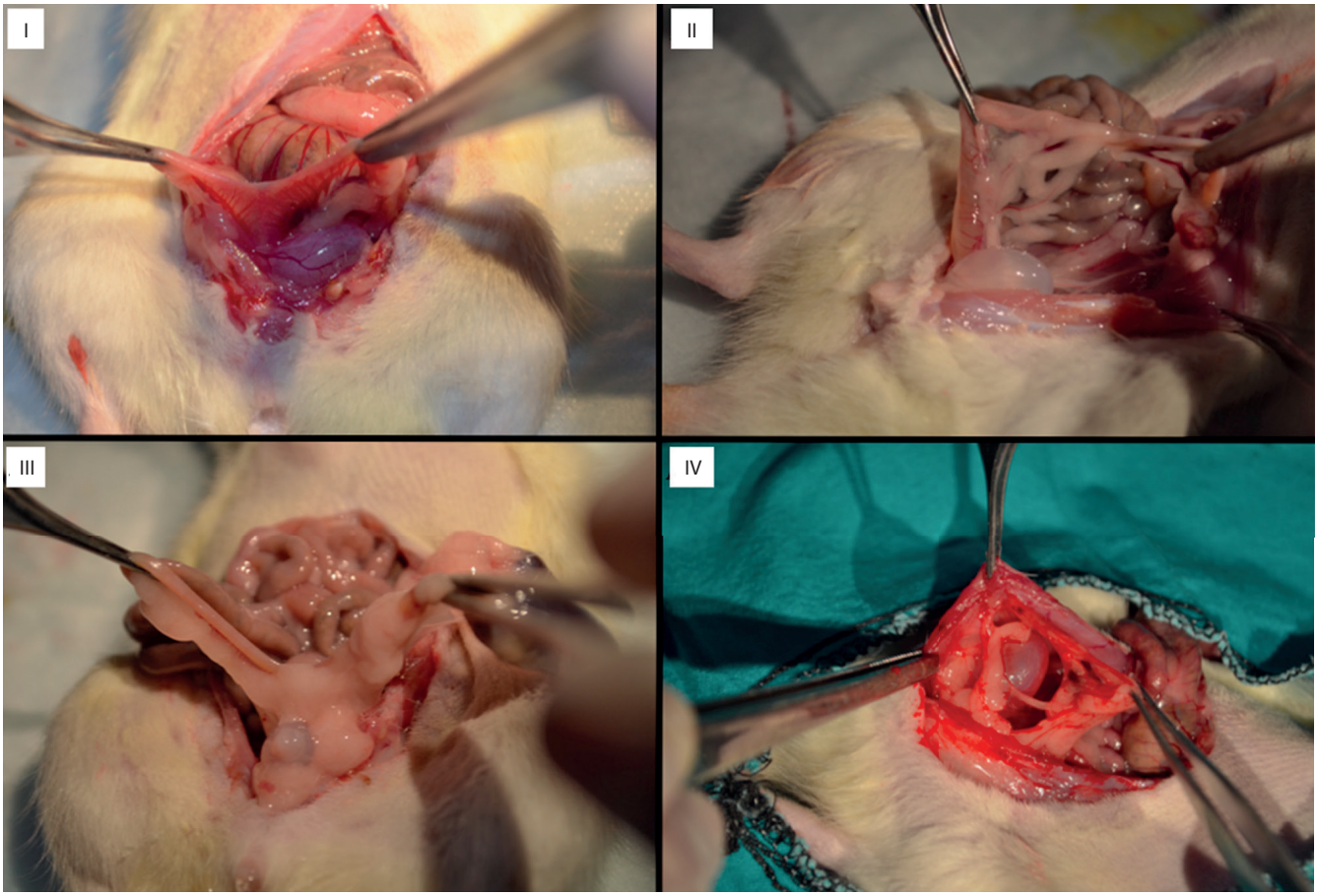

Fig. 2. Macroscopic view of rat uterine horns with various degrees of adhesion formation during the second-look laparotomy: I – the uterine horns without adhesion formation; II – filmy adhesion on the right horn; III – dense adhesion between the uterine horns; IV – a very dense attachment of uterine horns to each other or to other abdominal organs

Results

The surgical procedures on all 27 animals were performed successfully without any complications. All laparotomy areas were intact and no deaths occurred. The histopathological parameters (collagen deposition and inflammatory cell reaction) and clinical adhesion scores (extent, severity, degree, and total adhesion scores) of the groups are summarized in Table 1. Statistically significant differences were present between all the 3 groups in both clinical adhesion scores and histopathological parameters. A subgroup analysis showed that clinical adhesion scores and histopathological parameters in both barbed suture groups were statistically significantly higher than in the control group ($p < 0.05$). The inflammatory cell response and collagen deposition were higher in barbed suture group 2 than in barbed suture group 1; however, this difference was not statistically significant ($p > 0.05$) (Fig. 3). Similarly, there was no significant difference between the barbed suture groups in their adhesion scores ($p > 0.05$) (Fig. 4).

Table 1. Comparison of clinical and histological adhesion scores among the 3 groups

Parameter	Barbed suture group 1	Barbed suture group 2	Control group	p-value
Clinical adhesion score*				
Extent score	3 (2.55 ±0.33; 1.91–3.19)	3 (2.60 ±0.23; 2.15–3.05)	1 (1.11 ±0.30; 0.53–1.69)	<0.01
Severity score	2 (1.77 ±0.22; 1.34–2.20)	2 (1.88 ±0.26; 1.38–2.38)	1 (0.75 ±0.23; 0.30–1.20)	0.01
Degree score	2 (1.88 ±0.35; 1.20–2.56)	2 (1.77 ±0.27; 1.25–2.29)	0 (0.55 ±0.24; 0.08–1.02)	0.01
Total score	6 (6.22 ±0.32; 5.60–6.84)	6 (6.33 ±0.37; 5.61–7.05)	2 (2.44 ±0.60; 1.27–3.61)	<0.01
Histological features				
Collagen deposition	2 (1.88 ±0.20; 1.49–2.27)	2 (2.00 ±0.23; 1.55–2.45)	0 (0.44 ±0.17; 0.11–0.77)	<0.01
Inflammatory cell reaction	2 (2.11 ±0.26; 1.61–2.61)	2 (2.22 ±0.22; 1.79–2.65)	1 (0.77 ±0.22; 0.34–1.20)	<0.001

* Values are presented as median (mean ± standard error of the mean (SEM), 5^{th}–95^{th} percentile, 95% confidence interval (95% CI)).

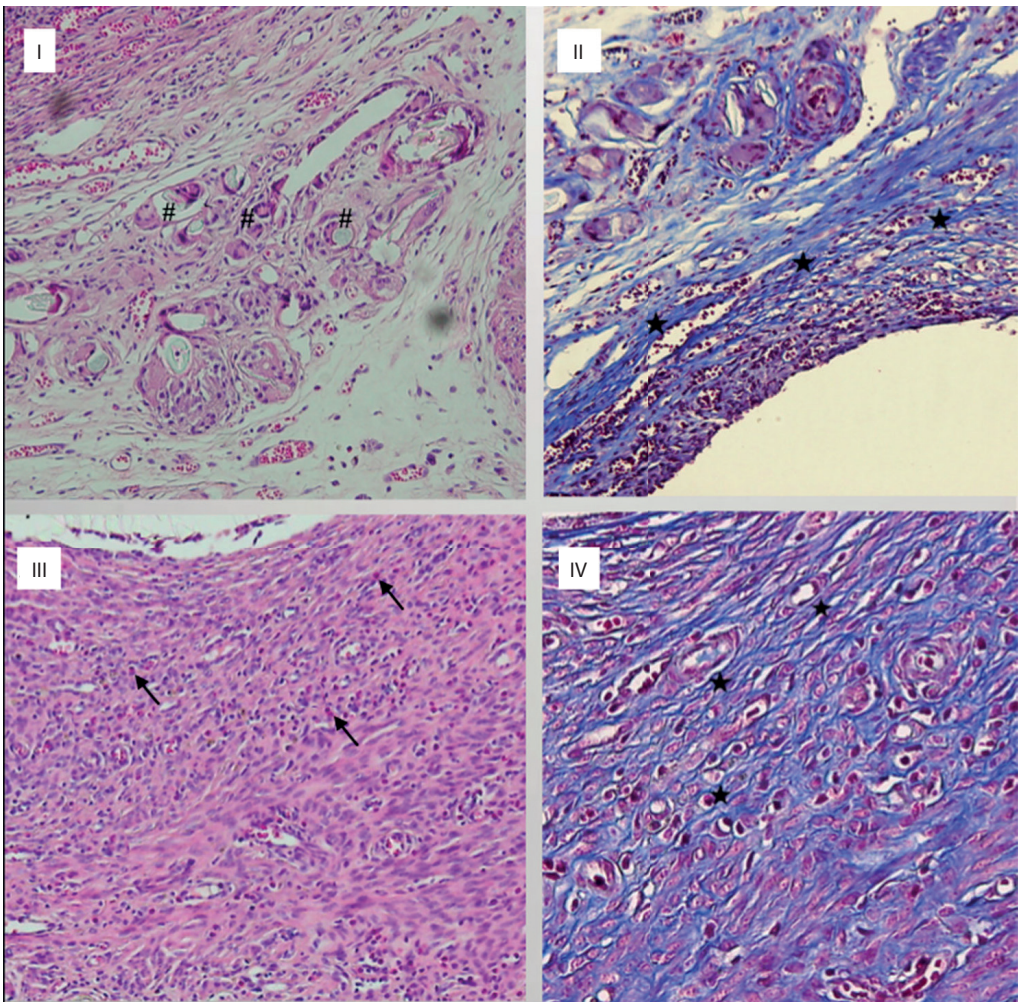

Fig. 3. Representative tissue specimens of hematoxylin-eosin (H&E) staining (I, III) and Masson's trichrome staining (II, IV) (original magnification ×20 and ×40); I, II – barbed suture group 1; III, IV – barbed suture group 2

--> polymorphonuclear leukocytes; # – foreign-body type giant cell; * – collagen deposition.

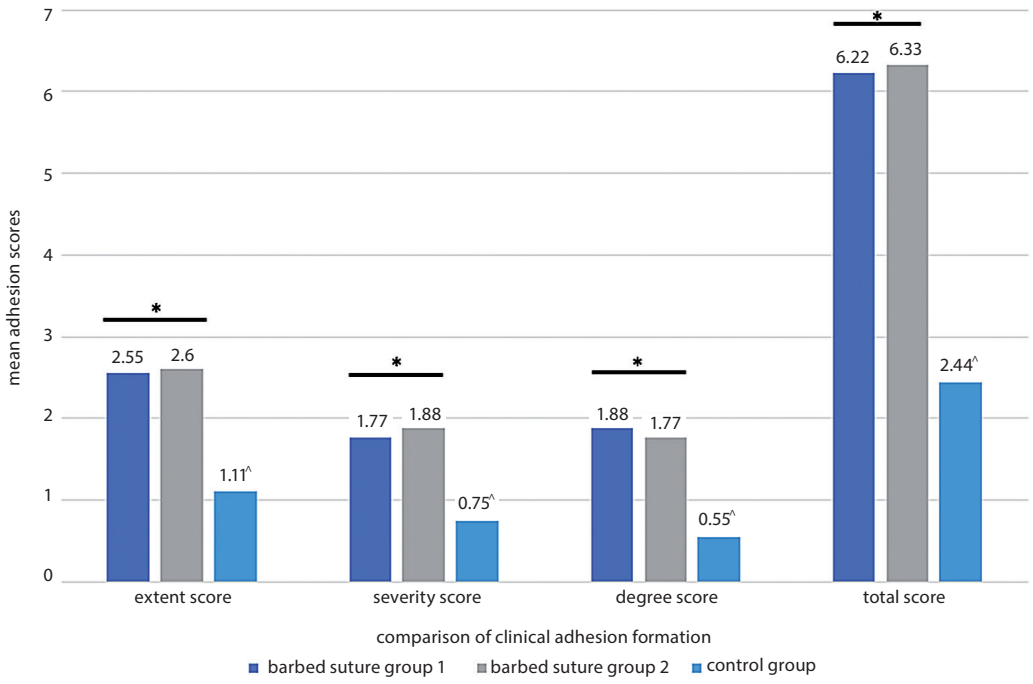

Fig. 4. Comparison of mean the clinical adhesion scores among the 3 groups during the second-look operation, 6 weeks after the first operation, in a myomectomy wound model of 27 rats

* no significant difference between the barbed suture groups in clinical adhesion scores ($p > 0.05$); ^ clinical adhesion scores in both barbed suture groups were statistically significantly higher than in the control group ($p < 0.05$).

The inter-rater reliability values for clinical adhesion scores were 0.80 kappa for extent, 0.94 kappa for severity and 0.84 kappa for degree ($p < 0.001$). Inter-rater reliability for histopathological parameters were 0.94 kappa for collagen deposition and 0.89 kappa for inflammatory cell reaction.

Discussion

In this study, the effects of different surface textures of barbed sutures on adhesion formation in a rat uterine horn model were investigated both clinically and histopathologically. The results of the present study demonstrated that different surface textures of barbed sutures and various configurations of barbs cause adhesion formation of a comparable extent, severity and degree.

The difficulty of intracorporeal suturing continues to be a barrier to a wider use of laparoscopy. Although the use of barbed sutures has been shown to ease the process of laparoscopic suturing considerably, concerns have been raised regarding a potentially increased risk of adhesions or inflammation as a result of their use.[13] Adhesions can cause loss of function of abdominal organs and lead to infertility, chronic pelvic pain, bowel obstruction, and significant morbidity.[10] Suture materials can act as foreign bodies within the abdomen, provoking inflammatory reactions that lead to peritoneal adhesion formation. Contrary to the chronic process of adhesion formation, small bowel obstruction can occur acutely and may be related to the anchoring of sharp barbs onto the neighboring tissue rather than to the presence of a foreign material within the abdomen. Many previous studies have compared the effects of different suture characteristics on adhesion formation.[14,15] However, to our knowledge, the relative outcome differences between the 2 types of barbed sutures with different barb geometries, barb numbers and helicity have not previously been studied.

In 2 studies conducted by Einarsson et al., a bidirectional polydioxanone barbed suture (Quill) and a polyglactin 210 standard suture (Vicryl) were compared with respect to the amount of adhesion formation.[15,16] According to the results of those studies, while the adhesion percentage was 52.2% and the median adhesion score was 4.0 (0–10) for cases in which barbed sutures were used, the same parameters for cases in which standard sutures were used were 43% and 0 (0–9), respectively. Barbed sutures were found to induce a similar degree of inflammatory reaction as compared to standard sutures; however, their adhesion scores were 10% higher than those of standard sutures. Nevertheless, this 10% difference in adhesion scores between the 2 types of sutures did not reach statistical significance. In a study by Api et al., V-loc barbed sutures were found to provoke less inflammatory tissue response, but a statistically significantly higher amount of clinical adhesion formation than polyglactin 910 (Vicryl) sutures.[9] Similar results were observed when V-loc and Vicryl sutures were compared for cystotomy repair in rats.[17] Different results in the adhesion scores reported by Einarsson et al. and Api et al. when barbed sutures (Quill and V-loc) were compared with standard sutures (Vicryl) may not only be a result of the different methodologies used in the studies (for example, the type of animals used – rat vs ewe, the type of instrument used for the incision over the horns – scalpel vs harmonic scalpel, or the follow-up period after the surgeries – 6 weeks vs 3 months), but also of the different surface textures of the sutures.[9,14,15] These histological features and the differences in the degree of adhesions may be explained by the mechanical effect of the barbed suture material on intra-abdominal peritoneal surfaces. Surface textures of barbed sutures may cause the entanglement of adjacent tissues by creating a rough environment on the peritoneal surface, which may consequently produce adhesions.[9] The 2 barbed suture brands with different surface textures that were used in the present study were not found to cause a difference either in clinical adhesion scores or in histological parameters.

Researchers have attributed the different biometric behavior patterns between various barbed sutures to differences in the surface designs and diameters of sutures. In a similar study with a similar intent, Jordan et al. aimed to determine whether 2 different barbed sutures behaved differently biomechanically ex vivo.[3] With this purpose, they compared barbed sutures on pig flexor tendons with respect to the maximum load (ultimate tensile strength) and displacement. V-loc was found to have similar displacement patterns as Stratafix, but it had a higher tensile strength. Similar results were reported in studies in the field of esthetic surgery.[14]

The present study has some noteworthy strengths. The methodology used to assess and compare the risk of adhesion formation connected with using barbed sutures is different compared to previous studies, in which subjects were used as their own controls; in our study, the study groups and the control group comprised different subjects. This is a simple but significant methodological difference, because the adhesion conditions measured in each subject may not necessarily be the adhesion potential rendered by the suture material at one circumscribed location of the whole abdominal environment (especially with barbed sutures, which are hypothesized to form a rough peritoneal surface entangling the neighboring structures to cause adhesions); hence, in principle, we believe the subjects should not serve as their own controls. The other strength of the present study was that 2 researchers blinded to the study groups assessed the degree of adhesion formation intraoperatively. Consequently, underestimation or overestimation of the degree of adhesions as a result of photographic evaluation of specimens postoperatively were prevented.

However, some limitations inherent to animal studies are also present in this study. Most importantly, it is not certain to what extent the results of this study are transferrable to human subjects. Myomectomy was not performed in our rat model; instead, an imitation of it was performed by making an incision over one of the uterine horns. It may not be possible to determine to what extent a simple incision and closure may evoke an adhesion response similar to that of a myomectomy. Certainly, human studies will be required to retest our results.

In our study, the control group served as a sham group to distinguish the effects of laparotomy and anesthesia.

Conclusions

To date, there is no final consensus on the ideal suture material to be used for myometrial closure. An ideal suture material would provide adequate tensile strength during the healing process, cause minimal foreign-body reaction and would be easy to use. The current clinical literature has shown the performance of absorbable barbed sutures to be equivalent to conventional absorbable smooth sutures for soft tissue reapproximation in gynecology.[18,19] Future studies are necessary to determine the association between different types of barbed sutures and adhesion formation in humans and to evaluate the long-term tensile strength in myometrial defects repaired with different types of barbed sutures.

In conclusion, the results of the present study indicate that these 2 types of barbed sutures with different surface textures have a similar profile regarding postoperative adhesion formation when used for myometrial closure.

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Association of coronary artery disease with toll-like receptor 4 genetic variants: A meta-analysis

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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. Toll-like receptor 4 (TLR4) plays an important role in the formation of coronary atherosclerotic plaque and the pathogenesis of coronary artery disease (CAD).

Objectives. The aim of the study was to conduct a meta-analysis assessing the relationship between 2 common genetic variants in the *TLR4* gene (rs4986790 and rs4986791) and susceptibility to CAD.

Material and methods. A systematic search of Web of Science, Embase, Scopus, PubMed, and Wanfang Med Online was undertaken. Case-control studies assessing the association of rs4986790 and rs4986791 with CAD risk were included. The odds ratio (OR) and 95% confidence interval (CI) were used as the metric of choice for the evaluation of risk.

Results. The literature search generated 427 studies, of which 14 met the inclusion criteria, for a total of 13,927 participants. Our meta-analysis revealed a significant association between rs4986791 and CAD risk in Asians using the dominant model (CT + TT vs CC: OR = 0.35, 95% CI = 0.21–0.56, $p < 0.001$), heterozygote contrast (CT vs CC: OR = 0.32, 95% CI = 0.19–0.57, $p < 0.001$) and allele contrast (T vs C: OR = 0.38, 95% CI = 0.25–0.58, $p < 0.001$). No significant association between rs4986791 and CAD was observed among Caucasians. For rs4986790, the results provided no evidence of an association with CAD risk.

Conclusions. Our analysis suggests that rs4986791 is negatively associated with CAD risk in Asians but not in Caucasians. No association between rs4986790 and CAD risk was found.

Key words: polymorphism, coronary artery disease, meta-analysis, toll-like receptor 4

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Coronary artery disease (CAD) is the leading cause of death and disability worldwide.[1] It presents in 2 main forms: myocardial infarction (heart attack) and angina. The 2013 Global Burden of Disease Study estimated that almost 30% of all deaths worldwide were caused by CAD.[2] Although much of the risk of CAD is explained by conventional risk factors, a great deal remains unexplained. Epidemiological studies have suggested that genetic factors are involved in the pathogenesis of CAD.[3] A number of studies have looked at associations between polymorphic variants in candidate genes and CAD.[3] One potential candidate gene system is the toll-like receptor (TLR) family.

Toll-like receptors are transmembrane proteins expressed on immune cells. Toll-like receptor 4 is a well-characterized TLR family member with a leucine-rich extracellular domain and an intra-cellular domain with strong similarity to the interleukin 1 (IL-1) receptor.[4] Toll-like receptor 4 is involved in the adaptive and innate immune responses by binding to microbial or endogenous molecules such as lipopolysaccharide (LPS), heat shock proteins and fibronectin.[4] Toll-like receptor 4-ligand complexes activate signal transduction pathways via an enzymatic cascade, leading to increased pro-inflammatory cytokine expression.[5] The *TLR4* gene is located on chromosome 9q32-q33.[6] Genetic variants within TLR4 would alter TLR4 expression and thus increase or decrease the risk of CAD. Many epidemiological studies have assessed the association of 2 common single-nucleotide polymorphisms (SNPs) in the *TLR4* gene – rs4986790 and rs4986791 – with CAD risk. However, owing to insufficient statistical power and various clinical and methodological factors, the findings remain inconsistent. We aimed to summarize the current evidence by systematically reviewing the literature and performing a meta-analysis.

Material and methods

Search strategy

This meta-analysis adhered to the guidelines for systematic reviews of genetic association studies.[7] A literature search was implemented in the online databases Web of Science, Embase, Scopus, PubMed, and Wanfang Med Online to search for case-control studies evaluating the relationship of the TLR4 polymorphisms rs4986790 and rs4986791 with susceptibility to CAD. The search was limited to studies published between 1990 and 2017. Search terms included "genetic variant", "polymorphism", "coronary heart disease", "coronary artery disease", "toll-like receptor 4", and "susceptibility". Electronic database searches were supplemented with manual searches of the references of all relevant publications and review articles. The search and selection of studies were conducted by 2 researchers; disagreements were resolved by discussion until a consensus was reached.

Eligibility criteria

The studies included were required to meet all of the following conditions: 1. involving human subjects; 2. published in peer-reviewed journals in Chinese or English; 3. employing a case-control design; 4. no overlap with other studies (if there was an overlap with another study, we included the study with the largest sample size); and 5. investigating the relationship between TLR4 polymorphisms rs4986790 and/or rs4986791 and the risk of CAD. Case status was defined as having a diagnosis of CAD confirmed with coronary angiography. We did not specify the Hardy-Weinberg equilibrium (HWE) as an inclusion criterion. Specific exclusion criteria included animal studies, familial studies and studies including only cases. The reason for excluding a study during the full-text screening was recorded.

Data extraction and quality assessment

The following data was extracted from each eligible study using a pre-made extraction form: the last name of the first author, country of origin, ethnicity, year of publication, diagnostic criteria, disease type, case and control sample size, and genotype counts for the cases and controls. Two researchers independently extracted data and reached consensus on all the items. The quality assessment of the studies was conducted according to the Newcastle-Ottawa Scale (NOS).[8]

Statistical analysis

We calculated unadjusted odds ratios (ORs) with corresponding confidence intervals (CIs) from the raw genotype frequency data. For groups with 0 events, we added 0.5 to each cell. Meta-analyses were carried out to investigate the association between CAD risk and the TLR4 polymorphisms in terms of allele contrast, heterozygote contrast, homozygote contrast, recessive model, and dominant model. The allele contrast compared the number of rare alleles with the number of common alleles in the cases and controls. The heterozygote contrast compared the number of heterozygotes with that of common homozygotes. The homozygote contrast compared the number of rare homozygotes with the number of common homozygotes. In the recessive model, we compared rare homozygotes with individuals carrying common alleles. In the dominant model, we compared individuals carrying rare alleles with individuals who were homozygous for common alleles. The degree of between-study heterogeneity was assessed using the I^2 statistic, and the significance of this statistic was assessed using Cochran's Q test. A p-value <0.10 or I^2 >50% indicated a significant statistical heterogeneity across studies,[9] allowing for the use of a random-effects model to estimate the combined effect.[10] In addition to the overall analysis,

which included all the available data, a subgroup analysis for each ethnic group was also performed. Sensitivity analyses were performed to investigate the impact of each study on the pooled OR. Publication bias was appraised with visual inspection of funnel plots, with asymmetry assessed formally using Egger's and Begg's tests. Stata software v. 12.0 (StataCorp LLC, College Station, USA) was used for all the statistical analyses.

Results

Characteristics of the eligible studies

The literature search resulted in a total of 427 potentially relevant citations that were screened at the first review stage. Of these, 198 were duplicates and were removed, leaving 229 studies for the screening of abstracts. Thirty-one studies were read in full and 17 studies were excluded. Ultimately, 14 case-control studies were included in the meta-analysis.[11–24] Figure 1 presents a flow chart of the retrieved and excluded studies with the reasons specified. The eligible studies included populations from China, Croatia, France, Germany, Ireland, Italy, Mexico, Norway, Russia, Turkey, the USA, and the UK. The sample sizes in the 14 studies ranged from 240 to 4,868. The characteristics of the studies included are summarized in Table 1.

Data synthesis

Tables 2 and 3 present the pooled ORs in detail. Seven studies including 6,886 cases and 2,682 controls dealt with the rs4986791 variant.[16,17,19,21–24] The combined analyses of all the eligible studies produced no evidence of an association between rs4986791 and CAD risk using the dominant model (OR = 0.85, 95% CI = 0.59–1.23; p = 0.391), the recessive model (OR = 0.80, 95% CI = 0.46–1.39; p = 0.424), heterozygote contrast (OR = 0.85, 95% CI = 0.59–1.23; p = 0.385), homozygote contrast (OR = 0.76, 95% CI = 0.44–1.32; p = 0.333), or allele contrast (OR = 0.87, 95% CI = 0.61–1.23; p = 0.415) (Table 2, Fig. 2). However, the subgroup of Asian populations showed a strong association using the dominant model (OR = 0.35, 95% CI = 0.21–0.56; p < 0.001), heterozygote contrast (OR = 0.32, 95% CI = 0.19–0.57; p < 0.001) and allele contrast (OR = 0.38, 95% CI = 0.25–0.58; p < 0.001) (Table 2, Fig. 2). We did not find a significant association between rs4986791 and CAD risk in Caucasians under any of the comparison models (Table 2, Fig. 2). There was evidence of heterogeneity among these studies (I^2 = 78.9%, p < 0.001) (Table 2). The sensitivity analyses showed that the results remained unchanged after removing each study in turn (Fig. 3).

Thirteen case-control studies with 8,762 cases and 4,712 controls provided results on associations between rs4986790 and CAD risk.[11–18,20–24] We did not find evidence

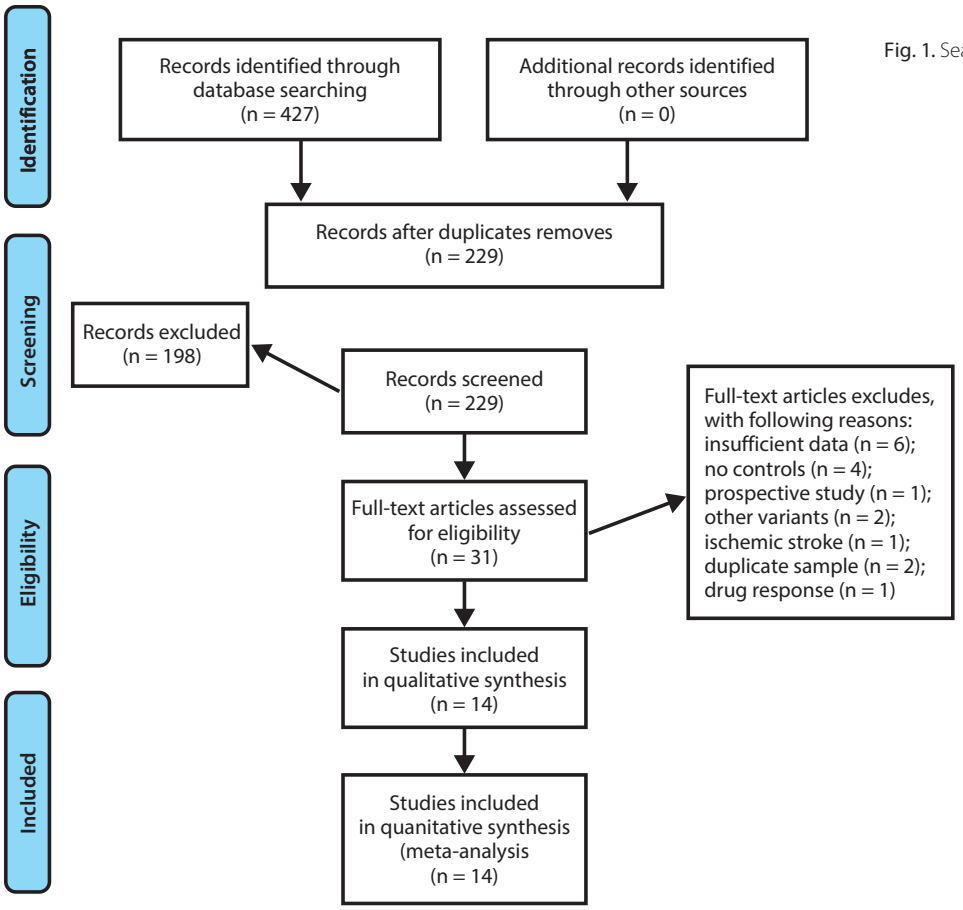


Fig. 1. Search strategy flow

Table 1. Characteristics of individual studies included in the meta-analysis

Author	Year	Ethnicity	Country	Cases	Controls	Control origin	Data for polymorphisms	Genotyping method	NOS score
Ameziane	2003	Caucasian	France	183	216	hospital	rs4986790	TaqMan allelic discrimination test	7
Balistreri	2004	Caucasian	Italy	105	182	hospital	rs4986790	Allele-specific PCR	6
Morange	2004	Caucasian	France and UK	247	490	population	rs4986790	Allele-specific PCR	8
Zee	2005	Caucasian	USA	370	695	not specified	rs4986790	ABI Assay-by-Demand allelic discrimination method	7
Hamann	2005	Caucasian	Germany and UK	388	163	not specified	rs4986790	PCR	7
O'Halloran	2006	Caucasian	Ireland	1598	386	population	rs4986790 and rs4986791	Allele-specific PCR	7
Koch	2006	Caucasian	Germany	3657	1211	hospital	rs4986790 and rs4986791	Allele-specific PCR	7
Nebel	2007	Caucasian	Germany	606	323	not specified	rs4986790	TaqMan SNP Genotyping Assay	7
Wang	2009	Asian	China	156	172	hospital	rs4986791	PCR-RFLP	8
Džumhur	2012	Caucasian	Croatia and Norway	120	120	hospital	rs4986790	TaqMan SNP Genotyping Assay	7
Martínez-Ríos	2013	Latin Americans	Mexico	457	283	hospital	rs4986790 and rs4986791	TaqMan Genotyping Assay	7
Golovkin	2014	Caucasian	Russia and UK	702	300	hospital	rs4986790 and rs4986791	TaqMan SNP Genotyping Assay	6
Guven	2015	Caucasian	Turkey	300	150	hospital	rs4986790 and rs4986791	Real-time PCR using hybridization probes	6
Li	2017	Asian	China	167	180	hospital	rs4986790 and rs4986791	DNA sequencing	7

NOS – Newcastle-Ottawa scale; PCR – polymerase chain reaction; PCR-RFLP – PCR-restriction fragment length polymorphism; SNP – single nucleotide polymorphism.

Table 2. Meta-analysis of associations between rs4986791 and CAD risk

Comparison	Subgroup	Number of studies	Test of association			Test of heterogeneity		Test of publication	
			OR	95% CI	p-value	I^2	p-value	p-value for Begg's test	p-value for Egger's test
CT + TT vs CC (dominant)	all	7	0.85	0.59–1.23	0.391	78.9	<0.001	0.548	0.573
	Caucasians	4	1.09	0.78–1.52	0.622	69.9	0.019	NA	NA
	Asians	2	0.35	0.21–0.56	<0.001	0.0	0.676	NA	NA
TT vs CT + CC (recessive)	all	7	0.80	0.46–1.39	0.424	0.0	0.622	0.260	0.202
	Caucasians	4	1.20	0.56–2.57	0.648	0.0	0.733	NA	NA
	Asians	2	0.47	0.19–1.15	0.096	0.0	0.769	NA	NA
CT vs CC	all	7	0.85	0.59–1.23	0.385	77.2	<0.001	0.368	0.513
	Caucasians	4	1.07	0.75–1.52	0.710	71.5	0.015	NA	NA
	Asians	2	0.32	0.19–0.57	<0.001	0.0	0.752	NA	NA
TT vs CC	all	7	0.76	0.44–1.32	0.333	0.0	0.541	0.260	0.227
	Caucasians	4	1.20	0.56–2.57	0.642	0.0	0.747	NA	NA
	Asians	2	0.42	0.17–1.03	0.057	0.0	0.760	NA	NA
T allele vs C allele	all	7	0.87	0.61–1.23	0.415	79.3	<0.001	0.764	0.643
	Caucasians	4	1.10	0.81–1.48	0.547	66.4	0.030	NA	NA
	Asians	2	0.38	0.25–0.58	<0.001	0.0	0.615	NA	NA

CAD – coronary artery disease; CI – confidence interval; NA – not applicable; OR – odds ratio.

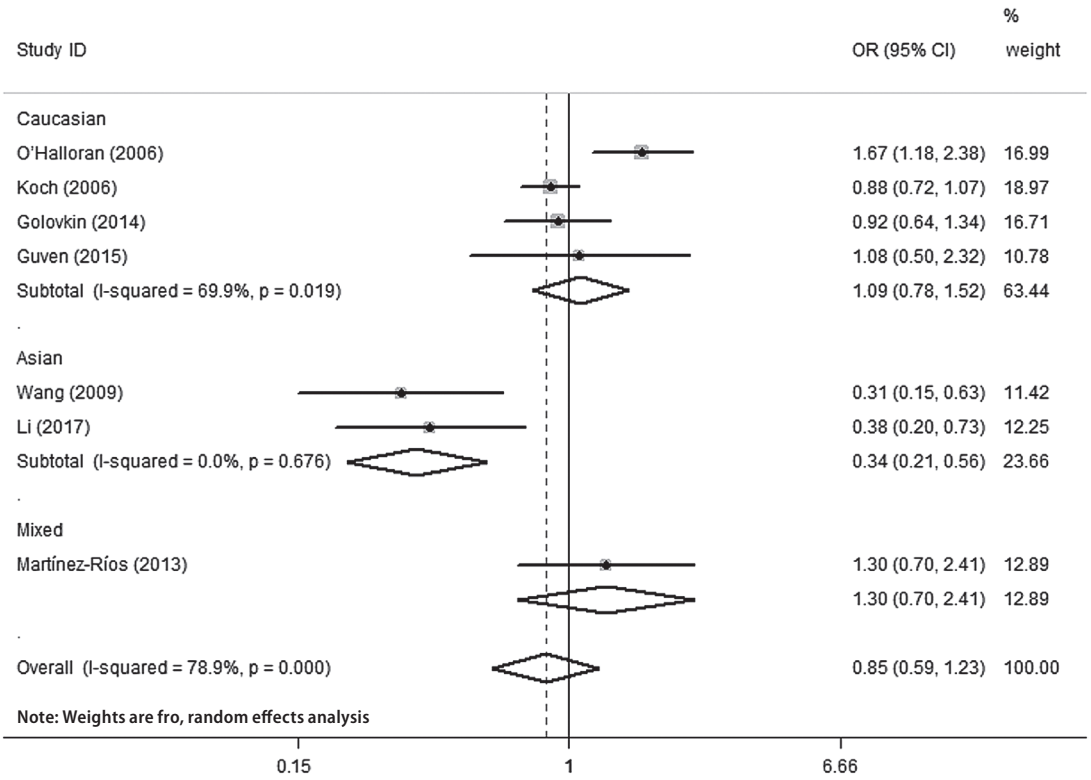
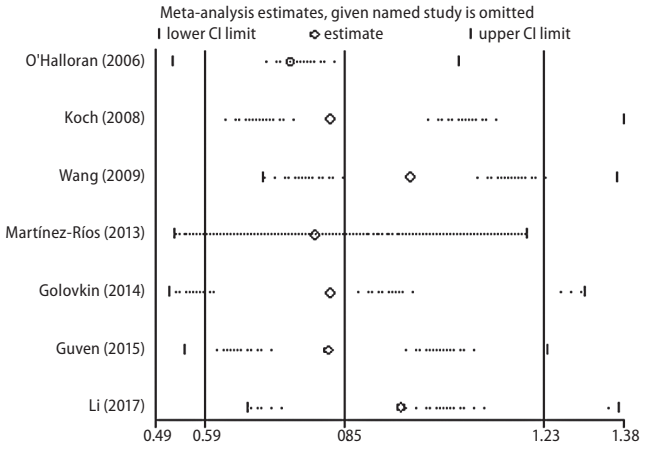


Fig. 2. Forest plot for included studies examining the association of rs4986791 with CAD risk under the dominant model

Table 3. Meta-analysis of associations between rs4986790 and CAD risk

Comparison	Subgroup	Number of studies	Test of association			Test of heterogeneity		Test of publication	
			OR	95% CI	p-value	I^2 (%)	p-value	p-value for Begg's test	p-value for Egger's test
AG + GG vs AA (dominant)	all studies	13	0.94	0.76–1.17	0.591	58.1	0.004	0.200	0.519
	Caucasians	11	0.91	0.73–1.15	0.445	64.0	0.002	NA	NA
GG vs AG + AA (recessive)	all studies	13	1.16	0.61–2.20	0.656	0.0	0.947	1.000	0.726
	Caucasians	11	1.12	0.54–2.12	0.532	0.0	0.824	NA	NA
AG vs AA	all studies	13	0.94	0.76–1.16	0.566	55.3	0.008	0.127	0.500
	Caucasians	11	0.91	0.73–1.14	0.420	61.4	0.004	NA	NA
GG vs AA	all studies	13	1.10	0.61–1.99	0.693	0.0	0.934	1.000	0.727
	Caucasians	11	1.09	0.60–1.97	0.654	0.0	0.892	NA	NA
G allele vs A allele	all studies	13	0.95	0.78–1.17	0.643	59.1	0.004	0.246	0.533
	Caucasians	11	0.93	0.74–1.16	0.494	64.9	0.001	NA	NA

CAD – coronary artery disease; CI – confidence interval; NA – not applicable; OR – odds ratio.



of an association between rs4986790 and CAD risk using the dominant model (OR = 0.94, 95% CI = 0.76–1.17; p = 0.591), the recessive model (OR = 1.16, 95% CI = 0.61–2.20; p = 0.656), heterozygote contrast (OR = 0.94, 95% CI = 0.76–1.16; p = 0.566), homozygote contrast (OR = 1.10, 95% CI = 0.61–1.99; p = 0.693), or allele contrast (OR = 0.95, 95% CI = 0.78–1.17; p = 0.643) (Table 3, Fig. 4). Subgroup analyses by ethnicity did not identify any associations of this variant with CAD risk in Caucasians (Table 3,

Fig. 3. Sensitivity analysis for included studies assessing the association of rs4986791 with CAD risk under the dominant model

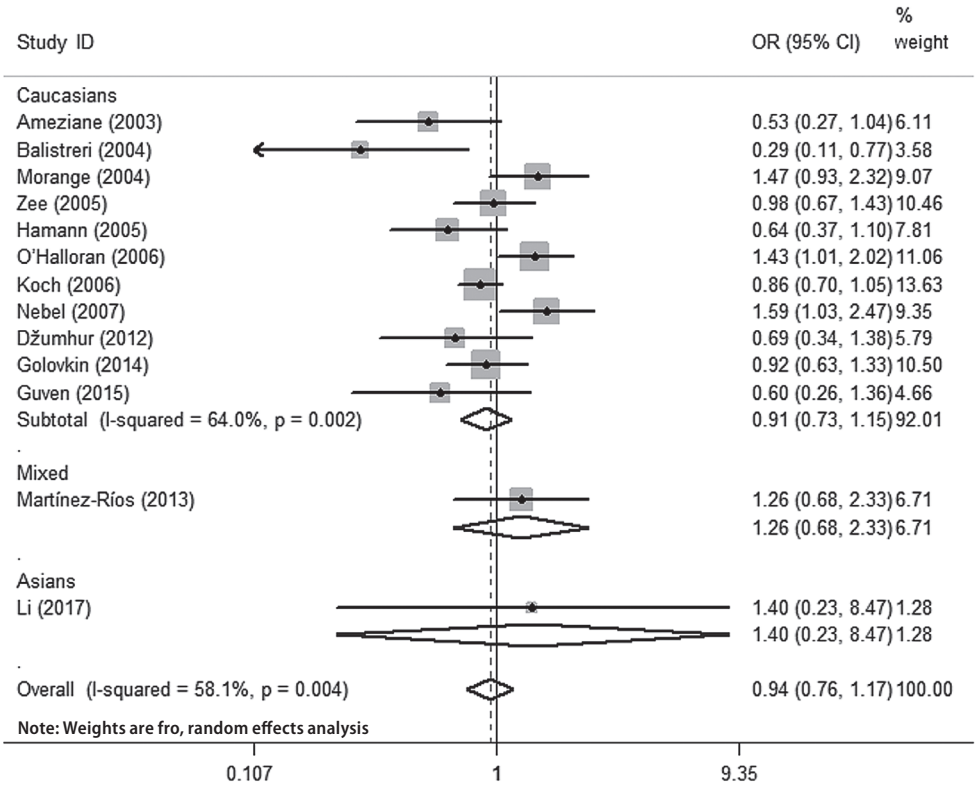


Fig. 4. Forest plot for included studies examining the association of rs4986790 with CAD risk under the dominant model

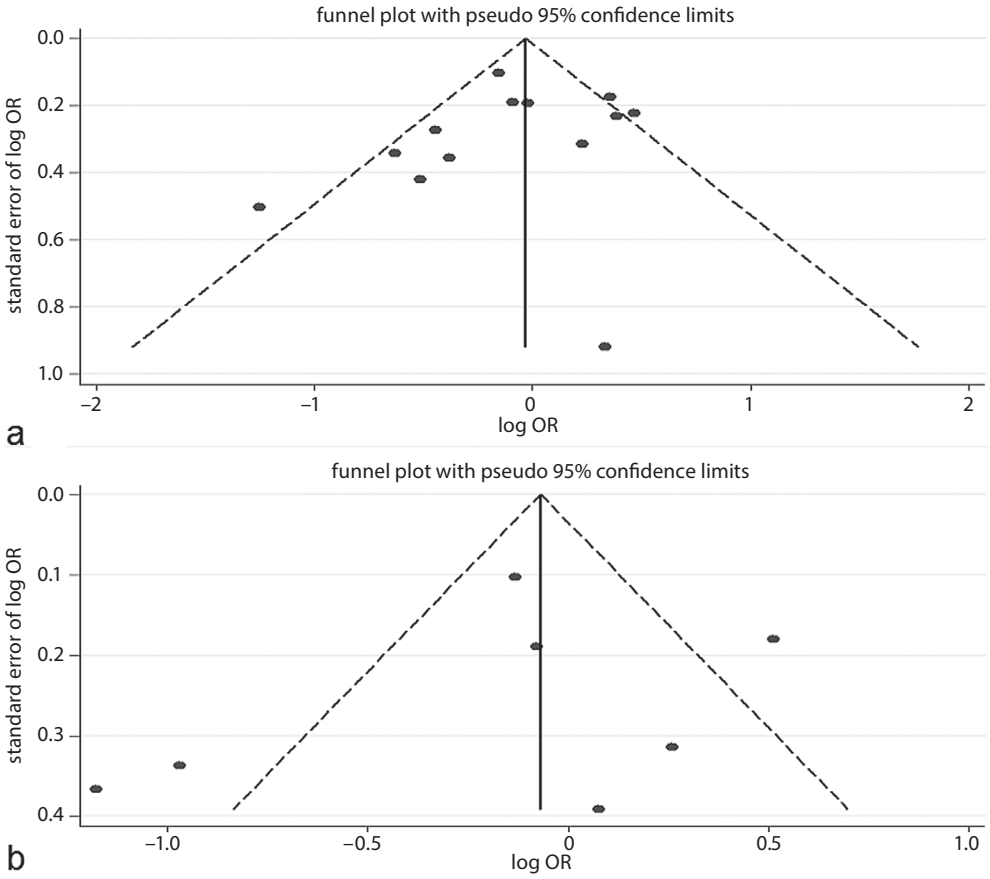


Fig. 5. (a) Funnel plot for included studies assessing the association of rs4986790 with CAD risk under the dominant model. (b) Funnel plot for included studies assessing the association of rs4986791 with CAD risk under the dominant model

Fig. 4). Since the number of studies performed in Asians and Latin Americans was limited, we did not conduct ethnicity-specific analyses in these ethnic groups. Moderate between-study heterogeneity was found among the studies (Table 3). The results of the sensitivity analysis for rs4986790 were virtually unchanged (not shown).

Publication bias

Publication bias was evaluated by performing funnel plots and Egger's and Begg's tests under all models. For rs4986790 and rs4986791, the funnel plots were symmetrical (Fig. 5). Egger's and Begg's tests showed no evidence of publication bias (Table 2,3).

Discussion

Toll-like receptors constitute a major subgroup of pattern recognition receptors, responding to inter- and intracellular molecules typically associated with pathogens. To date, 10 functional human TLRs (TLR1-10) have been identified, among which TLR4 is a prominent member.[4] Toll-like receptor 4 is expressed by a variety of immune and non-immune cells, including macrophages, neutrophils, endothelial cells, smooth muscle cells, and cardiac myocytes.[5] It is activated by bacterial LPS or a number of endogenous ligands that are formed in pathological conditions.[4] Activated TLR4 signals through the canonical nuclear factor-κB (NF-κB) pathway, resulting in the production of pro-inflammatory cytokines such as IL-1β, IL-6 and tumor necrosis factor α (TNF-α).[25] Toll-like receptor 4 has been shown to play an important role not only in the formation of atheromatous plaque, but also in the deterioration of the coronary arteries.[25] Toll-like receptor 4 is necessary for oxidized low-density lipoprotein-induced macrophage differentiation into foam cells.[26] Animal studies have demonstrated that apolipoprotein E-deficient mice additionally lacking TLR4 are resistant to atherosclerosis.[27] In addition, mice lacking macrophage TLR4 expression have been found to have reduced atherosclerotic lesion size when fed low-fat diets.[28] Human studies have demonstrated that expression levels of TLR4 in circulating monocytes and coronary plaques were significantly elevated in acute coronary syndrome (ACS) patients.[29–31] Increased expression of TLR4, but not TLR2, has been observed in ruptured human coronary atherosclerotic plaque, suggesting that TLR4 plays a critical role in plaque instability.[30] All of the above findings imply that the *TLR4* gene may be a candidate marker for susceptibility to CAD.

In this meta-analysis, we combined data from published case-control studies to assess the relationship between 2 variants in the *TLR4* gene – rs4986791 and rs4986790 – and CAD risk. The results suggest that the rs4986791 variant is protective against CAD in Asian populations but not in Caucasians. We did not find evidence of an association between the rs4986790 variant and susceptibility to CAD.

The rs4986791 variant, also known as Thr399Ile, is a functional polymorphism characterized by cytosine/thymine transition at nucleotide 1196, leading to a threonine (Thr) for isoleucine (Ile) substitution at amino acid 399 in the protein chain.[6] In the vicinity of the mutation area, rs4986791 causes conformational changes, decreases the expression level of TLR4, reduces the binding efficiency of TLR4 with its ligands and affects the interactions of TLR4 with downstream signaling proteins.[32–34] Since TLR4 is involved in the formation of atheromatous plaque and CAD pathogenesis, rs4986791 may reduce the risk of CAD by downregulating the expression level of TLR4 and modifying its functions. The results of our subgroup analyses for rs4986791 indicate that the association between rs4986791 and the risk of CAD may depend on the ethnicity of the study population. It is noteworthy that this is the first meta-analysis assessing the relationship between rs4986791 and CAD risk.

In addition to rs4986791, we investigated the association between rs4986790 and CAD risk, finding no evidence of association. The results for rs4986790 were in line with those of 2 prior meta-analyses.[35,36] However, we could not exclude the possibility that rs4986790 along with other factors may have a synergistic effect on CAD risk. Boekholdt et al. found that among patients with coronary atherosclerosis, cardiovascular events, including myocardial infarction, were significantly decreased when statins were administered to carriers of the rs4986790 G allele.[37] Similar findings were obtained by Holloway et al.[38] These results suggest that rs4986790 modified the efficacy of statins in preventing cardiovascular events. Interestingly, lifestyle-related risk factors like smoking may interact biologically with rs4986790 to alter the risk of CAD. A study by Edfeldt et al. found that a synergistic interaction between smoking and rs4986790 genotypes significantly affected the risk of CAD, implying that smoking was of special concern in the determination of rs4986790-mediated risk modification.[39]

Our meta-analysis has several limitations. First, only commonly investigated TLR4 variants assessed in ≥3 studies could be included in the pooled analyses. Besides rs4986790 and rs4986791, the association between CAD and other TLR4 genetic variants, including rs11536889, rs10116253 and rs10983755, have also been evaluated by genetic studies.[40] However, due to the limited published data, we were unable to include these variants in the present meta-analysis. Second, although Egger's and Beggs' tests and funnel plots did not reveal any evidence of publication bias, we could not exclude the possibility that some case-control studies obtaining negative results might not be published in peer-reviewed journals. Third, we could not exclude the possibility that several of the statistically significant associations from the eligible studies might be false-positive results. However, this is unlikely in our review, because we strictly selected the studies and evaluated their quality using the NOS scale.

Conclusions

In summary, this meta-analysis provides a comprehensive evaluation of the existing literature on the relation between 2 common genetic variants in TLR4 genes and

the risk of CAD. The results suggest that the rs4986791 variant is negatively associated with CAD in Asians but not in Caucasians. There is no evidence of an association between the rs4986790 variant and CAD risk.

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Age is the main determinant of glycated hemoglobin levels in a general Polish population without diabetes: The NATPOL 2011 Study

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Conflict of interest
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Abstract

Background. Measurements of glycated hemoglobin (HbA1c) in non-diabetics can identify subjects who are at increased risk for future cardiovascular (CV) events. There is no consensus agreement whether the addition of HbA1c improves the CV risk prediction.

Objectives. The objective of this study was to assess mean values of HbA1c levels in a representative sample of general, diabetes mellitus (DM)-free Polish population, and its subgroups, and to identify important covariants.

Material and methods. HbA1c was measured in blood samples collected from 1,868 participants (males/females (M/F) 901/967, age: range 18–74, mean 44.03 years) of NATPOLL 2011 study without previously and newly diagnosed DM. Univariate and multivariate analyses of HbA1c level in relationship to age, body mass index (BMI), waist circumference (WC), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting plasma glucose (FPG), lipids, creatinine, C-reactive protein (CRP), gender, and smoking status were performed.

Results. Mean HbA1c level was 5.46 ±0.31% in the entire population and significantly higher levels were found in subjects with male gender, hypertension, fasting hyperglycemia, abdominal obesity, and higher BMI values but not in smokers. Univariate analysis revealed numerous significant correlations of HbA1c with the highest values correlation coefficient values for age ($r = 0.55$), FPG ($r = 0.43$), WC ($r = 0.36$), and BMI ($r = 0.36$). The best, final multivariate model explained 40% of HbA1c variance and the most important covariant was the age, explaining approx. 50% of R^2, followed by FPG and BMI.

Conclusions. HbA1c in non-diabetic level is associated with certain CV risk factors, mainly with age. Since known risk factors explain less than a half of HbA1c variance, the inclusion of HbA1c into the assessment may increase the performance of algorithms predicting CV risk.

Key words: glycated hemoglobin, age, lipids

According to different algorithms for cardiovascular (CV) risk, type 2 diabetes mellitus (DM2) is considered an equivalent of coronary heart disease (CHD). Also, patients with diabetes, when compared to non-diabetic subjects, have a higher prevalence of CHD, a greater extent of coronary ischemia, and are more likely to have a myocardial infarction (MI) and silent myocardial ischemia.

In the general population, there is even a larger group of subjects who do not meet the diagnostic criteria for diabetes but are affected by different degrees of dysglycemia, including impaired fasting glucose or impaired glucose tolerance. Measurements of glycated hemoglobin (HbA1c) can identify subjects who are at increased risk not only for the future development of DM2,[1,2] but also for CV events.[3–6]

In a meta-analysis, HbA1c was found to be the best marker of dysglycemia and CV risk in subjects without DM2.[7] In the INTERHEART study, the association between HbA1c and the history of MI was stronger in non-diabetics than in patients with DM2.[8] Mechanisms underlying this association remain unclear and there is no consensus agreement whether addition of HbA1c level as a diagnostic tool improves the risk prediction for cardiovascular disease (CVD).[9]

Material and methods

A detailed design of the NATPOL 2011 Survey has been reported elsewhere.[10] Briefly, the NATPOL 2011 survey was designed as a cross-sectional representative observational study to assess the prevalence of main atherosclerotic CVD risk factors in Poland. It was carried out on a representative sample of Polish residents aged 18–79 years. The participants were randomly selected in bundles, in a stratified, proportional draw performed in 3 stages. The response rate among respondents who were invited and eligible for the study was equal to 66.4%. Finally, 2,413 subjects (1,245 females and 1,168 males) participated in the survey. Subjects with previously diagnosed diabetes, with HbA1c level >6.5 % and incomplete biochemical measurements were excluded from the current analysis. Therefore, the final analysis was performed in n = 1,868 subjects (male/female 901/967, mean age 44.03 ±16.28 years) and the comparisons between the subgroups with and without the following: hypertension, fasting hyperglycaemia, abdominal obesity and according to body mass index (BMI) and current smoking status were performed.

The Institutional Ethics Committee at the Medical University in Gdańsk (Poland) approved the study protocol; all participants provided written informed consent.

The examination was performed by 234 well-trained nurses who lived in or close to the randomly selected geographical bundles. Participants were examined during 2 visits at subjects' homes. The examination of an individual subject comprised the following components: completing the questionnaire, taking blood pressure readings and anthropometric measurements (weight, height, waist circumference (WC)), and collecting blood and urine samples.

The questionnaire was completed during the 1st visit. Only selected items of the questionnaire were used for the following substudy including age, history of diabetes and hypertension, antihypertensive and statin use, and smoking.

Blood pressure readings were taken 3 times during the 1st and the 2nd visit using fully automated oscillometric blood pressure measuring device (A&D UA 767 A&D Company, Tokyo, Japan). Mean values of 2nd and 3rd measurements from 2 visits were used for the analysis. Hypertension was diagnosed if during both visits mean systolic blood pressure (SBP) was ≥140 mm Hg and/or mean diastolic blood pressure (DBP) was ≥90 mm Hg or if the patient was taking hypertensive drugs over the past 2 weeks due to an earlier diagnosis of hypertension.

Anthropometric measurements used in the following substudy included weight, height and WC. Weight was measured with the subject shoeless and dressed in light clothes (without outer garments – jackets, coats, etc.), using approved personal electronic scales, with accuracy to the nearest 0.1 kg. Height was measured using a portable personal measuring device with accuracy to the nearest centimeter, and WC using a tailor's tape measure, with an accuracy to the nearest 0.5 cm. Overweight was defined as BMI 25.0–29.9 kg/m^2, obesity as BMI ≥30 kg/m^2 and abdominal obesity as WC ≥102 cm in men and WC ≥ 88 cm in women.

Blood and urine samples were taken from subjects at the 2nd visit, after 10. to 12-hour fasting. Frozen plasma and serum samples were transported to the central laboratory, where blood and urine analyses were carried out.

Routine blood tests: fasting plasma glucose (FPG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), serum triglycerides (TG), plasma creatinine, serum C-reactive protein (CRP), and urine albumin and creatinine were measured on the Architect c8000 chemistry analyzer (Abbott Laboratories, Chicago, USA). Glucose was measured using the hexokinase method. Serum cholesterol was measured with the enzymatic method, using cholesterol esterase and cholesterol oxidase; serum HDL-C was measured with the direct method using Accelerator Selective Detergent (Abbott Laboratories, Chicago, USA) with accelerated non-HDL-C oxidation and HDL-C dissolving. Serum triglycerides were measured by the enzymatic method using glycerol kinase and glycerol phosphate oxidase. Low-density lipoprotein cholesterol (LDL-C) was calculated using Friedewald formula. If TG concentration was >350 mg/dL, the Friedewald formula was not used.

Hypercholesterolemia was defined as TC ≥190 mg/dL (4.9 mmol/L) or taking statins. Fasting hypergylcemia was defined as fasting glucose ≥100 mg/dL (≥5.6 mmol/L). Plasma creatinine was measured using Jaffe method, method and CRP in serum using immunoturbidimetric method.

Serum insulin was measured on the Architect i2000sr, immunochemistry analyzer (Abbott Laboratories) using chemiluminescence immunoassay. Urine albumin was measured by the immunoturbidimetric method and creatinine using the Jaffe method. Albumin–creatinine ratio (ACR) was calculated on the basis of urine albumin and urine creatinine measurement.

HbA1c level was measured in ethylenediaminetetraacetic acid (EDTA) whole blood samples using turbidimetric inhibition immunoassay on the Cobas Integra 800 analyzer (Roche Diagnostics, Mannheim, Germany). The statistical analysis of the data was performed using R package v. 3.0.2 (The R Foundation, Vienna, Austria).

We compared mean values of HbA1c in both genders, in subjects with and without the following: hypertension, impaired fasting glucose and current smoking status. Due to the skewness, ACR values were log-transformed. We used the student's t-test or analysis of variance (ANOVA) and χ^2 test, where appropriate. All results were presented as means ± standard deviation (SD). Significant p-value was set at $p < 0.05$.

We performed univariate analysis calculating correlation coefficients of HbA1c with the following quantitative parameters: age, BMI, WC, SBP, DBP, FPG, insulin, lipids, creatinine, CRP, and ACR using Pearson's method.

For the multivariate analysis, we included all quantitative parameters mentioned above, and additionally gender and smoking habit, removing variables that highly correlated with the others ($r > 0.6$) namely DBP, WC and LDL-C. The selection of variables for the final model was performed using "leaps" R package applying all subsets regression method. We calculated standardized beta coefficients "QuantPsyc" R package) together with corresponding significance and R^2 for all variables selected for the final model using the "relaimpo" R package with "lmg", "last", "first", and "Pratt" algorithms.

Table 1. Characteristics of all participants and men vs women

Parameter	All subjects	Men	Women	p-value
	mean ±SD or n [%]	mean ±SD or n [%]	mean ±SD or n [%]	
Number of patients	1868	901 (48.2)	967 (51.8)	–
Age [years]	44.03 ±16.28	43.34 ±15.66	44.69 ±16.82	0.0725
BMI [kg/m^2]	26.2 ±4.8	26.9 ±4.5	25.5 ±5.0	<0.0001
Normal/low BMI	810 (43.4)	303 (33.6)	507 (52.4)	<0.0001
Overweight	687 (36.8)	394 (43.7)	293 (30.3)	<0.0001
Obesity	371 (19.9)	204 (22.6)	167 (17.3)	<0.0001
WC [cm]	90.35 ±13.92	96.5 ±12.22	84.62 ±12.93	<0.0001
Abdominal obesity, n	1104 (59.1)	537 (59.6)	567 (58.6)	0.7062
SBP [mm Hg]	127.1 ±17.8	131.5 ±16.6	123.1 ±17.9	<0.0001
DBP [mm Hg]	79.8 ±9.9	80.9 ±10.3	78.8 ±9.5	<0.0001
Hypertension, n	551 (29.5)	299 (33.2)	252 (26.1)	<0.0001
Glucose [mg/dL]	90.69 ±11.81	92.83 ±11.94	88.69 ±11.33	<0.0001
Glucose [mmol/L]	5.04 ±0.66	5.16 ±0.66	4.93 ±0.63	<0.0001
Insulin [mLU/L]	8.25 ±5.44	8.49 ±6.24	8.03 ±4.55	0.0761
HbA1c [%]	5.46 ±0.31	5.47 ±0.31	5.44 ±0.31	0.0280
HbA1c [mmol/mol]	36.2 ±3.4	36.3 ±3.4	35.0 ±3.4	0.0280
Hyperglycemia, n	368 (19.7)	232 (25.7)	136 (14.1)	<0.0001
TC [mg/dL]	199.9 ±41.3	198.7 ±42.4	200.9 ±40.15	0.2425
LDL-C [mg/dL]	125.9 ±34.4	125.9 ±34.7	125.9 ±34.12	0.9699
HDL-C [mg/dL]	50.3 ±13.1	46.0 ±12.6	54.3 ±12.35	<0.0001
TG [mg/dL]	120.4 ±80.8	138.2 ±99.5	103.8 ±53.24	<0.0001
CRP [mg/dL]	2.77 ±5.63	3.02 ±7.05	2.54 ±3.86	0.0735
Plasma creatinine [mg/dL]	0.82 ±0.17	0.9 ±0.15	0.75 ±0.15	<0.0001
ACR [mg/g]	15.67 ±187.88	12.74 ±65.39	18.39 ±253.42	0.5028
logACR [mg/g]	0.78 ±0.35	0.72 ±0.36	0.84 ±0.33	<0.0001
Current smoking, n	516 (27.6)	281 (31.2)	235 (24.3)	0.0011

BMI – body mass index; SBP – mean systolic blood pressure; DBP – mean diastolic blood pressure; TC – total cholesterol; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; TG – triglycerides; ACR – albumin/creatinine ratio; WC – waist circumference; CRP – C-reactive protein; SD – standard deviation.

Results

Characteristics of the population studied are presented in Table 1. Mean HbA1c level value was 5.46 ±0.31% (36.2 ±3.4 mmol/mol) and was significantly higher in men than in women (Table 1). HbA1c level was higher in subjects with hypertension 5.61 ±0.31% vs 5.39 ±0.29% (37.8 ±3.4 mmol/mol vs 35.4 ±3.2 mmol/mol, p < 0.001) than without; higher in fasting hyperglycemia – 5.69 ±0.32% (38.7 ±3.5 mmol/mol) than in subjects with normal fasting glucose – 5.40 ±0.28% (35.5 ±3.1 mmol/mol, p < 0.001); and higher in subjects with abdominal obesity than without – 5.54 ±0.31% vs 5.34 ±0.27% (37.1 ±3.4 mmol/mol vs 34.9 ±3.0 mmol/mol, p < 0.001). Significant differences in HbA1c were observed in subjects with normal/low BMI, overweight and obesity: 5.34 ±0.26% vs 5.50 ±0.30% vs 5.62 ±0.31%, respectively (34.9 ±2.8 mmol/mol vs 36.6 ±3.3 mmol/mol vs 37.9 3.4 mmol/mol, p < 0.001). No significant difference in HbA1c was observed in smokers vs non-smokers.

Univariate analysis revealed numerous significant correlations of HbA1c with other covariants including age (r = 0.55) (Fig. 1), fasting glucose (r = 0.43), WC (r = 0.36), BMI (r = 0.36), SBP (r = 0.28), LDL-C (r = 0.26), total cholesterol (r = 0.23), and DBP (r = 0.21). Correlation coefficients for insulin, creatinine, ACR, triglycerides, and HDL-C, although significant, were below 0.2.

The best final multivariate linear regression model was selected with all subsets regression procedures and included following variables independently associated with HbA1c levels: age, FPG, BMI, TC, HDL-C, ACR, TG, and gender (Table 2). Albumin–creatinine ratio and gender were not significant correlates. The final model explained 40.25% of HbA1c variance. The most important covariant of HbA1c, irrespective of the method of assessment of the relative importance of variables selected to the final model, was age, followed by FPG and, in the majority of methods, BMI (Fig. 2). Relative R^2 for the other covariants was negligible, below 10%.

Table 2. Multivariate associations between HbA1c and clinical/biochemical parameters in the final model expressed as adjusted beta coefficients

Parameter	beta	p-value
Age	0.40	<0.001
Gender	–0.02	ns
FPG	0.26	<0.001
BMI	0.09	<0.001
TC	0.10	<0.001
HDL-C	–0.13	<0.001
TG	–0.05	<0.05
logACR	0.02	ns

FPG – fasting plasma glucose; BMI – body mass index; TC – total cholesterol; HDL-C – HDL cholesterol; TG – triglycerides; ACR – albumin/creatinine ratio; ns – not significant; significant p-value <0.05.

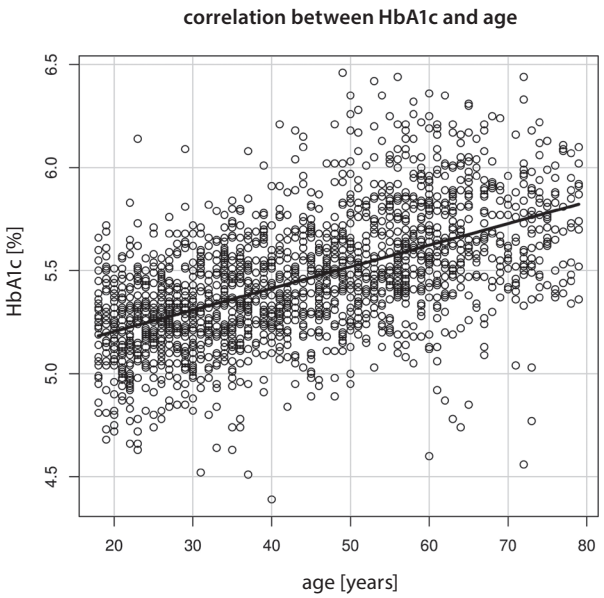

Fig. 1. Correlation between HbA1c and age

Discussion

Our study revealed that the mean HbA1c level in a representative sample of the Polish population without previously diagnosed diabetes and with non-diabetic range of HbA1c was similar to other non-diabetic population studies.[6,11] HbA1c levels were higher in subjects with certain well-known CV risk factors, namely male gender, obesity, abdominal obesity, hypertension, and hypercholesterolemia but not in current smokers.

Univariate analysis revealed numerous significant correlations with other covariants, with highest r values found for age and also for FPG, WC and BMI. It should be noted that the correlation between FPG and HbA1c was not very high and similar to that found in the Dutch general population.[12] In the large Finnish METSIM study, the respective r coefficient in non-diabetic men was even lower – 0.207. On the other hand, our analysis, when compared with METSIM study, revealed a 2-fold higher r coefficients for HbA1c levels and age, BMI and SBP.[11]

In our study, age, glucose, BMI, total cholesterol, HDL-C, and TG were significant and independent determinants of HbA1c level. In contrast to other studies, other covariants including CRP[11] and smoking[6] were not included into the model. The main determinant of HbA1c variance in our participants was age, which explained half of the variance of HbA1c in the model, similarly to the METISM study. The relative R^2 determined by FPG was approx. 25%, as reported in METSIM study (24.7%).[11]

In METSIM study, age, FPG, CRP, genetic risk score, and smoking were the most important determinants of the variance in HbA1c among participants without DM2, explaining 12–14% of variance in HbA1c, whereas insulin secretion and insulin sensitivity indices explained only <2% of variance.[11] In a Dutch study performed in non-diabetic

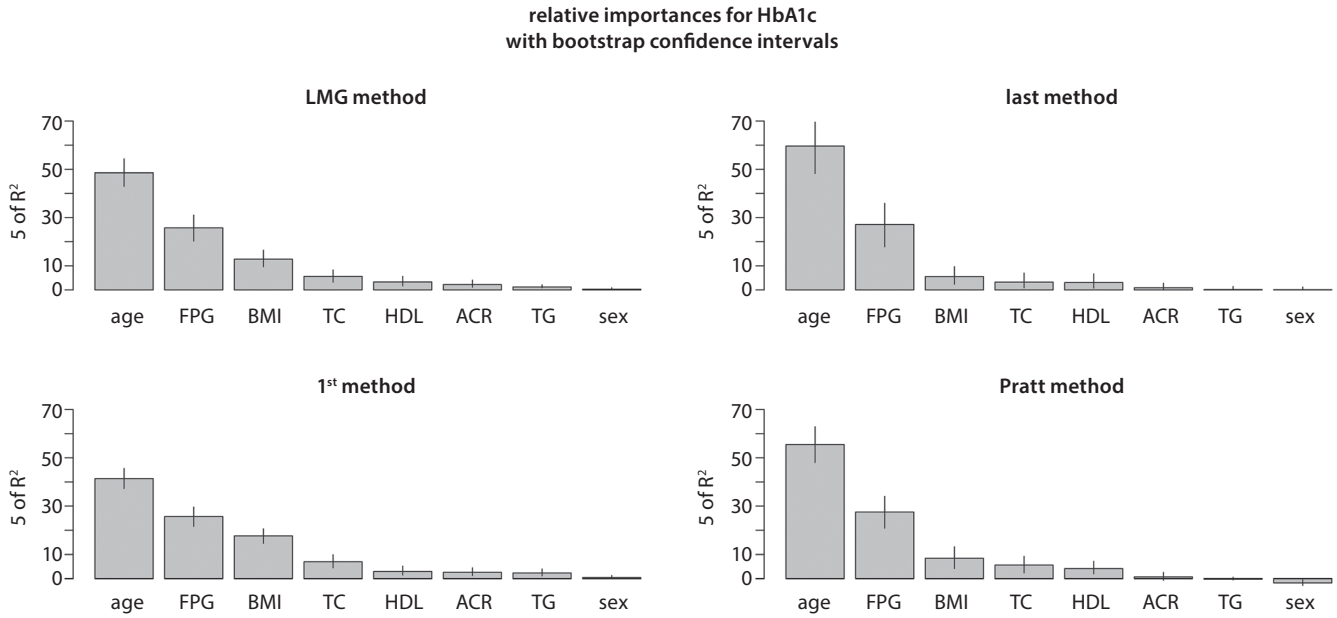

Fig. 2. Relative importance for HbA1c expressed as % of R^2 for the final model calculated using different methods

adults, regression model that included age, gender, BMI, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, current smoking, and alcohol consumption explained only 26% of HbA1c variance.[6]

Our model predicted HbA1c levels more precisely, explaining 40% of the variance of HbA1c compared with 14–26% of the variance in other studies.[6,11] Differences between our and other studies may be explained by the lower mean age of our subjects, resulting in lower incidence of concomitant CV risk. Other studies have shown that variability in HbA1c may be contributed to age, ethnicity, smoking, anemia, and genetic factors.[13–16] Apart from age and, to a minor extent FPG and BMI, the influence of other variables in our model was small, almost negligible.

Of note, multivariate regression models including traditional and non-traditional risk factors/markers explain only 40% of variance of HbA1c. Therefore, the physiological link between HbA1c and CV risk in non-diabetic subjects remains unexplained, thus suggesting that HbA1c may be an independent CV risk factor. Prediabetes is a well-known CV risk factor and HbA1c should be considered a useful independent CV risk factor also in the diabetes-free population.

The studies on the possible determinants of HbA1c levels in non-diabetic populations are difficult to compare due to methodological differences, including diagnosis of diabetes, selection of the potential covariates and statistical methodology.

Several limitations of our study must be mentioned. We did not perform oral glucose tolerance test (OGTT) and the participants had only 1 FPG measurements; therefore, to minimize the possibility of including patients with DM to the analysis, we excluded the participants with HbA1c ≥ 6.5% as in other studies.[17] However, post-load glucose measurement did not improve the prediction of HbA1c.[11]

Conclusions

HbA1c in non-diabetic level is associated with some CV risk factors, mainly with age. Since known risk factors explain less than half of its variance, the inclusion of HbA1c into risk assessment may increase the performance of algorithms predicting CV risk.

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Matrix metalloproteinase-3 levels in relation to disease activity and radiological progression in rheumatoid arthritis

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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. Rheumatoid arthritis (RA) is a chronic inflammatory and systemic disease of unknown etiology that primarily affects synovial joints and involves progressive destruction around the joints. Inflammation starting in the joint synovium causes the destruction of cartilage, bone and other adjacent tissues with pannus formation.

Objectives. The aim of this study was to evaluate serum matrix metalloproteinase-3 (MMP-3) levels and their clinical and radiological significance in patients with rheumatoid arthritis.

Material and methods. The study included 59 patients with RA and 30 healthy controls. Serum MMP-3 levels were measured using the enzyme-linked immunosorbent assay (ELISA) method. Patients with a Disease Activity Score 28 (DAS28) $\leq$3.2 were categorized as having lower disease activity, while a DAS28 score >3.2 indicated patients with moderate/high disease activity. Additionally, the patients were divided into 2 groups in terms of disease duration: early RA (disease duration $\leq$2 years) and established RA (disease duration $\geq$2 years). Functional disability was evaluated using the Health Assessment Questionnaire (HAQ) and Nottingham Health Profile (NHP). Radiographs were scored using modified Larsen scoring.

Results. Serum MMP-3 levels in patients with RA were significantly higher than in controls ($p = 0.001$). Serum MMP-3 levels were correlated with laboratory and clinical parameters of disease activity, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), DAS28, and HAQ score; the exceptions were rheumatoid factor (RF) and cyclic citrullinated peptides (CCP). The serum MMP-3 levels of RA patients with moderate/high disease activity were found to be significantly higher than those of the patients with low disease activity ($p < 0.001$). However, MMP-3 levels were found to be similar in both established and early RA patients ($p = 0.927$). Additionally, the modified Larsen scores, which indicate structural damage, correlated significantly with serum MMP-3 levels ($p = 0.001$).

Conclusions. These results indicate that serum MMP-3 levels may be used as an indicator for structural damage such as erosions in the early stages of the disease, and to monitor disease activity.

Key words: rheumatoid arthritis, matrix metalloproteinase 3, disease activity, modified Larsen score

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Rheumatoid arthritis (RA) is a chronic inflammatory and systemic disease of unknown etiology that primarily affects synovial joints and involves progressive destruction around the joints. Inflammation starting in the joint synovium causes the destruction of cartilage, bone and other adjacent tissues with pannus formation. Progressive joint deformations restrict mobility, and as a result, the patients' quality of life declines significantly.[1–3] According to current guidelines, disease activity is the baseline in the treatment of RA; treatment is regulated on the basis of the "treat-to-target" (T2T) strategy, the aim of which is remission or low disease activity.[4] However, this strategy has limitations in preventing structural damage.[5] Radiological progression has been observed in some RA patients during the course of remission; this shows the limitations of the main disease activity indicators used for estimating disease progression.[6] Since the common disease activity indicators were not specific for arthritis, inflammatory cytokines, destructive enzymes, collagen and non-collagen breakdown products of cartilage, as well as new multi-biomarker scores, including a few biomarkers, were quickly developed to assess structural damage in RA.[7,8]

Matrix metalloproteinases (MMP) are important members of the extracellular proteinase family. Their most important task is to decompose extracellular matrix components (ECM). They are known to be involved in many physiological and pathological processes. Matrix metalloproteinase-3 (MMP-3) is a proteinase generated by synovial fibroblasts and chondrocytes in joints.[9] Active MMP-3 can accelerate joint damage in RA by degrading aggregate nucleoprotein, cartilage-associated protein, fibronectin, and collagen types IV, VII, IX, and XI.[10] Serum MMP-3 has been studied as an indicator of disease activity in RA. A few studies have shown that serum MMP-3 levels increase in patients with RA and that disease activity is positively correlated with synovial MMP-3 expression; in addition, it has been shown that serum MMP-3 is elevated in some RA patients in remission or with low disease activity.[11,12]

The aim of this study was to investigate the association between serum MMP-3 levels and disease severity measured with disease activity, functional capacity and structural damage evaluated using conventional radiography.

Material and methods

Patient population

The study included 59 patients (43 females and 16 males) with a diagnosis of RA who had been monitored for at least 1 year. All of the patients met the 2010 American College of Rheumatology (ACR) criteria for an RA diagnosis.[13] Any change (except dose changes) in treatment with disease-modifying antirheumatic drugs (DMARDs) in the previous 3 months was a reason for exclusion. Other exclusion criteria were diagnoses of autoimmune diseases other than RA; acute and chronic infections; malignancies; or serious pulmonary, hepatic, kidney, or endocrinological diseases. Moreover, smokers, patients with hypertension (arterial blood pressure ≥140/90 mm Hg or on anti-hypertensive drugs) and hypercholesterolemia, as well as those aged below 20 and above 70 years were excluded from the study. We also enrolled 30 healthy controls. The study was approved by the local ethics committee and written informed consent forms were obtained from all the participants.

Clinical evaluations

The severity of pain, fatigue and the patients' and physicians' global assessment of disease activity were assessed on a visual analogue scale (VAS). No change or addition is needed, because the assessment criteria are universal. The duration of morning stiffness was noted in minutes. Disease activity was evaluated using the Disease Activity Score 28 for Rheumatoid Arthritis (DAS28).[14] Patients with DAS28 scores ≤3.2 were evaluated as having lower disease activity, while DAS28 scores >3.2 indicated patients with moderate/high disease activity.[15] Additionally, patients were divided into 2 groups depending on disease duration: early RA (duration ≤2 years) and established RA (duration ≥2 years). Functional disability was evaluated using the Health Assessment Questionnaire (HAQ) and Nottingham Health Profile (NHP).[16]

Laboratory evaluations

The results of routine laboratory tests were recorded (erythrocyte sedimentation rate (ESR), blood chemistry, whole blood cell count, and urinalysis). Rheumatoid factor (RF) and C-reactive protein (CRP) levels were measured using nephelometric methods.

Matrix metalloproteinase-3 assay

Venous blood samples were drawn from the patients and healthy controls and centrifuged at 3,000 rpm for 10 min. The sera were kept at –20°C until analysis. Serum MMP-3 was studied with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Human Total MMP-3 ELISA Kit; Aviscera Bioscience Inc., Santa Clara, USA) in accordance with the manufacturer's instructions. The results were expressed in ng/mL.

Radiological evaluation

Standard plain hand radiographs of the patients taken within the previous 6 months were examined. Structural damage in the hand radiographs was evaluated by a radiologist who was blinded to the patients' clinical and laboratory data. The radiographs were scored using modified Larsen scoring. In this scoring system, 24 areas in both hands are scored (0 to 5 points for each area, with total scores of 0–120 points).[17]

Statistical analysis

The statistical analysis of the study was performed using SPSS for Windows, v. 20.0 IBM Corp., Armonk, USA). The results of the study were evaluated using parametric and nonparametric statistical methods for data with normal and non-normal distribution, respectively. Intergroup comparisons were performed using the independent samples t-test for parametric values and the Mann-Whitney U test for nonparametric values. Correlations between parameters were evaluated using Spearman's correlation coefficient. In statistical evaluations, $p < 0.05$ was accepted as the level of significance. The results were expressed as mean ± standard deviation (SD).

Results

The mean disease duration was 9.0 ±7.5 years (range: 1–30 years) (Table 1). Rheumatoid factor was positive in 49 patients (83.0%) and anticyclic citrullinated peptide (anti-CCP) were positive in 45 patients (76.3%).

There were 49 patients (83.1%) using corticosteroids and the average prednisolone dose was 6.61 ±4.42 mg/day (range: 2–16 mg/day). There were 10 patients (16.9%) using only methotrexate, 28 patients (47.5%) taking 1 or more DMARDs in addition to methotrexate, 6 patients (10.2%) using a biological agent in addition to methotrexate, and 15 patients (24.4%) taking only DMARDs other than methotrexate (such as leflunomide, hydroxychloroquine or sulfasalazine).

Serum MMP-3 levels were significantly higher in the patients with RA than in the controls ($p = 0.001$) (Table 1).

Similarly, the serum MMP-3 levels in the RA patients with moderate/high disease activity were found to be significantly higher than in the patients with low disease activity ($p < 0.001$) (Table 2). However, MMP-3 levels were found to be similar in both established and early RA patients ($p = 0.927$) (Table 3).

Serum MMP-3 levels were correlated with all of the clinical and laboratory parameters of disease activity except RF and anti-CCP. Furthermore, there was a statistically significant correlation between structural joint damage (modified Larsen scores) and serum MMP-3 levels (Table 4).

Serum MMP-3 levels were similar in the patients taking both methotrexate and a biological agent and the patients taking DMARDs other than methotrexate. No statistically significant difference was identified between these 2 treatment groups in terms of DAS28 score, ESR, CRP, RF, and anti-CCP levels.

In this study, the cut-off value of serum MMP-3 was determined as 18.73 ng/mL. The sensitivity and specificity of serum MMP-3 were found to be 93.2% and 82.8%, respectively.

Table 1. Comparisons of demographic, clinical and laboratory parameters among the RA patients and the healthy control group

Parameters	Rheumatoid arthritis group (n = 59)	Control group (n = 29)	p-value
Age [years]	45.3 ±8.6 (22–70)	43.3 ±12.6 (22–70)	0.371
Female/male	43/16	21/8	0.963
Duration of the disease [years]	9.0 ±7.5	–	–
Morning stiffness [min]	54.58 ±64.55	–	–
Pain [0–100 mm VAS]	40.68 ±22.73	–	–
Fatigue [0–100 mm VAS]	45.08 ±16.33	–	–
Patients global [0–100 mm VAS]	40.68 ±19.92	–	–
Physicians global [0–100 mm VAS]	20.85 ±16.19	–	–
Number of the swollen joints (0–28)	1.03 ±2.30	–	–
Number of the tender joints (0–28)	4.36 ±3.51	–	–
ESR [mm/h]	33.86 ±25.15	11.90 ±6.85	0.001
CRP [g/dL]	2.57 ±2.72	0.33 ±0.17	0.001
RF [IU/mL]	117.20 ±162.46	7.99 ±3.40	0.001
Anti-CCP [IU/mL]	407.3 ±411.3	10.1 ±2.9	0.001
Serum MMP-3 [ng/mL]	88.39 ±63.11	14.24 ±5.54	0.001
DAS28	3.94 ±1.30	–	–
HAQ20 (0–3)	1.37 ±0.71	–	–
Modified Larsen Score (0–120)	38.56 ±30.60	–	–

VAS – Visual Analogue Scale; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; RF – rheumatoid factor; anti-CCP – anti-cyclic citrullinated peptide; DAS28 – Disease Activity Score 28; HAQ20 – Health Assessment Questionnaire 20.

Table 2. Comparisons of patients with moderate/high disease activity and those with low disease activity

Parameters	Moderate/high disease activity group (n = 32)	Low disease activity group (n = 27)	p-value
Age [years]	53.44 ±11.88	50.37 ±12.02	0.304
Female/male	19/13	24/3	0.011
Duration of the disease [years]	8.13 ±7.64	10.04 ±7.33	0.195
Prednisolone [mg/day]	7.59 ±4.40	5.44 ±4.23	0.035
Methotrexate [mg/week]	7.56 ±5.19	6.53 ±4.69	0.308
ESR [mm/h]	48.91 ±24.63	16.04 ±8.93	0.001
CRP [g/dL]	4.05 ±2.89	0.81 ±0.82	0.001
Serum MMP-3 [ng/mL]	122.07 ±61.30	48.48 ±36.80	0.001

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein.

Table 3. The data for early and established rheumatoid arthritis

Parameters	Early RA group (<2 years) (n = 13)	Established RA group (>2 years) (n = 46)	p-value
Age [years]	46.3 ±13.3	53.6 ±11.1	0.087
Female/male	8/5	35/11	0.297
Serum MMP-3 [ng/mL]	111 ±66.3	81.97 ±61.38	0.927
Anti-CCP [IU/mL]	446.4 ±417.2	396.3 ±413.5	0.530
RF [IU/mL]	91.06 ±75.2	124.5 ±179.5	0.841
ESR [mm/h]	37.1 ±27.7	32.9 ±824.6	0.833
CRP [g/dL]	1.7 ±1.5	2.8 ±2.9	0.336
DAS28	4.37 ±1.86	2.75 ±0.42	0.426
HAQ20 (0–3)	1.66 ±0.85	1.20 ±0.65	0.179
Modified Larsen Score (0–120)	36.9 ±28.9	39.20 ±31.36	0.927

Anti-CCP – anti-cyclic citrullinated peptide; RF – rheumatoid factor; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; DAS28 – Disease Activity Score 28; HAQ20 – Health Assessment Questionnaire 20.

Table 4. Spearman's correlation coefficients (r) for serum MMP-3 levels and various clinical and laboratory parameters in patients with rheumatoid arthritis

Parameters	Serum MMP-3	
	p-value	r
Age	0.402	0.111
Period of disease	0.360	–0.121
Morning stiffness	**<0.001***	**0.696**
Pain level	**<0.001***	**0.642**
Number of swollen joints	**<0.001***	**0.706**
HAQ score	**<0.001***	**0,688**
ESR	**<0.001***	**0.462**
CRP	**<0.001***	**0.464**
RF	**0.019***	**0.304**
Anti-CCP	0.229	0.159
DAS28	**<0.001***	**0.674**
Modified Larsen score	**<0.001***	**0.706**

HAQ – Health Assessment Questionnaire; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; RF – rheumatoid factor; anti-CCP – anti-cyclic citrullinated peptide; DAS28 – Disease Activity Score 28; numbers marked in bold indicate a statistically significant difference.

Discussion

Rheumatoid arthritis is one of the most common autoimmune diseases, affecting approx. 1% of the world population. Early diagnosis and treatment of RA is very important in order to prevent damage to joint tissues and disability. In patients with typical symptoms, a diagnosis can typically be made easily in the 1st year of the disease. Nevertheless, most of the time clinical symptoms are not explicit in the earliest stages of the disease. A long time may pass from the onset of symptoms to the diagnosis in patients with atypical progression. Therefore, specific and sensitive serologic tests are necessary for a quick diagnosis. The most common autoantibodies in the ACR 2010 criteria for a diagnosis of rheumatoid arthritis are RF and anti-CCP. Rheumatoid factor is an autoantibody with high sensitivity but it is not sufficiently specific to RA; it can be found in healthy individuals and also accompanies various diseases, which means it is not an ideal autoantibody for a diagnosis.

As Porto et al. wrote: "The progression of RA brings an evolutionary potential to varying degrees of joint damage and functional disability. Thus, special attention should be [paid] to the identification of poor prognostic indicator parameters, because ideally the definition of therapeutic intensity level should be based on the reliable predictors of severity. It is already known that some features when present, are associated with a worse outcome of the disease, such as the presence of high titer rheumatoid factor, smoking and HLA-DRB1".[18,19]

Porto et al. continued: "Regarding the prognostic role of anti-CCP, its association with disease activity and functional capacity has still not been clarified, although many studies suggest that these antibodies are associated with more severe and erosive disease,[20,23] especially in cases of initial RA.[24,27] It is worth noting the methodological heterogeneity of the studies that analyzed the association of anti-CCP with structural damage. Although most studies have made use of conventional radiography as an evaluation tool, different radiographic score systems were used".[19]

A study by Kumagai et al. showed that – in addition to RF – anti-CCP, anti-agalactosyl IgG antibodies and MMP-3 could be diagnostic indicators in RA.[28] With 81% sensitivity and 92.4% specificity, anti-CCP autoantibodies were identified as being more valuable than RF for the early diagnosis of RA. Joint damage progression was found to be faster in MMP-3-positive patients. Accordingly, MMP-3 that correlates strongly with CRP was determined to be very useful in assessing joint damage and RA disease activity.[28] In another study, serum MMP-3 and CRP levels showed a strong correlation. In this study, it was reported that the sensitivity of the MMP-3 and anti-CCP combination was 83.3% in early RA diagnosis.[29] In the present study, serum MMP-3 sensitivity was found to be 93.2% and its specificity was 82.8% in RA diagnosis. This result shows that MMP-3 is a useful marker in RA diagnosis despite the limited number of patients in the study. Furthermore, in parallel with the aforementioned studies, this study found a strong positive correlation between serum MMP-3 level and CRP values. Matrix metalloproteinase 3 is not commonly found in synovium and serum under normal conditions, but growth factors can be easily induced by cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α).[30] The positive correlation between CRP and MMP-3 found in this study shows that pro-inflammatory

cytokines such as IL-1 and TNF-α, which are responsible for the production of acute phase reactants, also stimulate MMP-3 production.

One of the strongest indicators of long-term prognosis in rheumatoid arthritis is the progressive structural joint damage. The progressive cartilage and bone damage in rheumatoid arthritis are considered dependent on the intensive secretion of MMP enzymes.[31] In their study, Taylor et al. determined that synovial liquid MMP-3 levels could be several hundred times higher than serum MMP-3 levels in RA patients, and that there was a strong correlation between serum and synovial liquid MMP-3 levels.[32] Kobayashi et al. indicated that serum and synovial liquid in late RA patients had higher MMP-3 levels than in osteoarthritis (OA) patients when they compared the 2 groups functionally and radiographically.[33] Similarly, Klimiuk et al. compared the serum and synovial liquid samples of RA and OA patients and found that RA patients had higher MMP-3 levels than OA patients in all circumstances.[34] As in these studies, serum MMP-3 levels of the RA patients in our study were significantly higher than those of the healthy control individuals. This confirms that MMP-3 is released by inflammatory cells and clarifies why serum MMP-3 levels are high in patients with an elevated inflammatory cell load and intensive inflammation.

It has been reported in a few studies that high MMP-3 levels could be the most important independent indicator of radiological progression.[35,36] Young-Min et al. reported that MMP-3 levels are superior to traditional indicators in foreseeing radiographical progression.[37] Our results concur with these studies, showing a positive correlation between MMP-3 levels and the modified Larsen score of the RA patients.

Identifying the number of sore joints (among a total of 28 joints) is one of the most important criteria used in the evaluation of disease activity in rheumatoid arthritis, and is considered one of the most reliable. Scores concerning sore joints generally comply with other clinical criteria. Radiological findings are indicators of joint damage in RA, and the progression rate can be considered a disease activity criterion.[38,39]

Several studies in the available literature assert that serum MMP levels could reflect joint damage along with disease activity in RA and can be helpful in diagnosing the disease in the early stage.[34,40–43] In this study, a statistically significant positive correlation was identified between MMP-3 and DAS28, ESR, CRP, and clinical disease parameters (swollen and sensitive joints, pain level, HAQ, NHP, the duration of morning stiffness, and asthenia). This shows that MMP-3 can reflect disease activity effectively.

Some studies have indicated that serum MMP-3 levels could be affected by the DMARDs, biological agents and corticosteroids often used in RA treatment. Serum MMP-3 levels decreased with the use of non-biological DMARDs and therefore, serum MMP-3 levels could reflect response to the treatment.[44–46] It was stated in another study that testing MMP-3 levels is a simple and non-invasive method for evaluating the efficiency of antirheumatic drug therapy, especially biological agents, and for monitoring synovial inflammation.[47]

In this study, no significant difference was determined in terms of serum MMP-3 levels between the patient group taking methotrexate and a biological agent and the patients taking other DMARDs (sulfasalazine, leflunomide, hydroxychloroquine). This may result from the repression of MMP-3 levels by corticosteroids, biological agents and methotrexate in patients examined in this study.

The main limitation of this study is its cross-sectionality. Moreover, the limited number of patients, the absence of a patient control group and the examination of MMP-3 levels only in serum are other limiting factors of this study.

This study has shown that the serum MMP-3 levels of the RA patients increased and that disease activity was correlated with clinical and laboratory parameters. Accordingly, it can be concluded that increases in MMP-3 levels in RA patients may indicate progression of joint erosion as a result of synovial inflammation development. Serum MMP-3 levels can be used to assess joint erosion progression in the early stage of RA as an indicator of disease activation and to monitor patients' response to treatment.

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Assessment of apelin, apelin receptor, resistin, and adiponectin levels in the primary tumor and serum of patients with esophageal squamous cell carcinoma

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Abstract

Background. Disturbances in adipokine secretion are associated with the risk of cancer growth and progression.

Objectives. The aim of the study was to evaluate the mRNA expression and protein levels of apelin, the apelin receptor, resistin, and adiponectin in the tumor tissues of surgically treated esophageal squamous cell carcinoma (ESCC) patients. Concentrations of serum adipokines were assessed in relation to ESCC progression.

Material and methods. The study group consisted of 53 patients with ESCC and 27 controls. In the ESCC group, 27 patients were surgically treated and 26 were treated with palliative procedures. RT-PCR and ELISA tests were used to measure the mRNA expression and protein level of adipokines in tissues and their concentration in serum.

Results. We found that mRNA expression and protein concentrations of apelin, the apelin receptor and resistin were significantly higher in tumor tissue than in control tissue. The protein concentration of apelin were significantly increased in the tumors of patients with lymph node metastasis ($p < 0.005$). Circulating levels of apelin, the apelin receptor and resistin were significantly higher in the cancer patients than in controls ($p < 0.05$ for all). The concentration of serum apelin receptor significantly decreased in patients with stage IV cancer, the presence of lymph node or distant metastasis ($p < 0.05$).

Conclusions. Apelin may participate in lymphangiogenesis and the progression of ESCC. The apelin receptor is intensely produced in the early stage of cancer development and it may take part in the carcinogenic processes of ESCC.

Key words: adipokines, apelin, esophageal squamous cell carcinoma, apelin receptor

Adipokines are proteins secreted by the adipose tissue. They play an important role in energy homeostasis, lipid and glucose metabolism and insulin resistance[1], and may act as autocrines, paracrines or endocrines.[2,3] Several clinical studies have demonstrated a significant relationship between unbalanced adipokine secretion and the prevalence of endometrial, renal, colon, or gastrointestinal cancer.[1,4–7] Adipocytes are important components of the tumor microenvironment and adipocyte-derived adipokines promote cancer growth, progression and angiogenesis.[4,7]

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers, with rapid tumor growth and early metastasis to the lymph nodes.[8] It has been suggested that abundant adipose tissue surrounding the esophagus at the adventitia influences the development of esophageal cancer.[9] Recent studies have demonstrated the role of adipokines as new markers for predicting the stage of biological aggressiveness and prognosis of ESCC patients' survival.[7,8,10,11]

Apelin and the apelin receptor are expressed not only in adipose tissue, but also in the human heart, liver, lung, gastrointestinal tract, and other organs.[12] It has been reported that the apelin/apelin receptor system stimulates blood endothelial cell growth and may play a significant role in cancer angiogenesis and lymphangiogenesis.[12,13] The possible involvement of apelin and the apelin receptor has not been evaluated in ESCC.

Resistin, like the apelin/apelin receptor system, promotes the growth, differentiation and migration of endothelial cells. It has been observed that elevated levels of serum resistin are associated with the progression of lung, colon, gastric, or oral cancer.[2,8,14,15] In contrast, adiponectin inhibits endothelial cell proliferation and migration and stimulates cell apoptosis.[7]

In our previous study, we showed that a high resistin concentration in the serum of gastroesophageal cancer patients is associated with the cachexia syndrome and the presence of distant metastases, while low serum levels of adiponectin reflect adipose tissue wasting in these patients.[10] Our results were in agreement with earlier immunohistochemical studies concerning high levels of resistin and apelin in tumors of gastroesophageal cancer patients.[7,8,10,11] However, the importance of resistin and adiponectin has not been fully elucidated in ESCC.

There is no data concerning quantitative analyses of apelin, apelin receptor, resistin, and adiponectin concentrations in ESCC tumors. Therefore, in the present study, we evaluated the mRNA expression and protein levels of apelin, apelin receptor, resistin, and adiponectin in tumor tissues obtained from surgically treated ESCC patients. The association between protein levels of adipokines in tumors and disease progression was also investigated. Concentrations of serum apelin, apelin receptor, resistin, and adiponectin were measured in relation to ESCC progression.

Material and methods

Patient characteristics

The study population consisted of 80 individuals: 53 cancer patients with histopathologically confirmed squamous cell carcinoma of the esophagus and 27 healthy subjects. The ESCC patients (36 men and 17 women, mean age 58.8 ±8.8 years) were admitted to the Department of Gastrointestinal and General Surgery of Wroclaw Medical University (Poland) between 2010 and 2015 for curative resection of esophageal tumors or for palliative procedures. Patients with any systemic illness were not included in the study. Preoperative evaluation included a physical examination and imaging techniques, such as ultrasonography, computed tomography and/or magnetic resonance. Finally, resection of the esophagus was carried out in 27 patients, and palliative methods (argon plasma coagulation supplemented with mechanical dilation of the tumor) were used in 26 patients. The patients' demographic, clinical and pathological data is given in Table 1.

The Union for International Cancer Control (UICC) TNM staging system (7th edition) was used for clinical and pathological staging. There were 2 patients with stage I cancer,

Table 1. Characteristics of esophageal squamous cell carcinoma (ESCC) patients in whom studies of tissue samples (n = 27) and/or serum samples (n = 53) were performed. Descriptive data was expressed as median (min–max) or number (%)

Parameter	Studies of tissue samples in 27 patients	Studies of serum samples in 53 patients
Age [years]	59 (42.0–74.0)	59 (39.0–88.0)
Age ranges [years]		
<60	14 (51.9)	27 (50.9)
≥60	13 (48.1)	26 (49.1)
Gender		
male	17 (63.0)	36 (67.9)
female	10 (37.0)	17 (32.1)
BMI [kg/m^2]	20.4 (17.3–24.5)	20.5 (16.4–24.5)
Cachexia		
yes	7 (25.9)	20 (37.7)
no	20 (74.1)	33 (62.3)
Stage of disease (TNM staging system)		
I	2 (7.4)	2 (3.8)
II	9 (33.3)	9 (17.0)
III	16 (59.3)	16 (30.2)
IV	0 (0.0)	26 (49.0)
Primary tumor progression (T)		
1	1 (3.7)	1 (1.9)
2	8 (29.6)	8 (15.1)
3	15 (55.6)	15 (28.3)
4	3 (11.1)	29 (54.7)
Lymph node metastasis (N)		
N0	14 (51.9)	14 (26.4)
N1	13 (48.1)	39 (73.6)
Distant metastasis (M)		
M0	27 (100.0)	27 (51.0)
M1	0 (0.0)	26 (49.0)

9 with stage II, 16 with stage III, and 26 patients with stage IV. In the ESCC patients, the cachexia syndrome was defined as involuntary weight loss exceeding 5% of the patient's previous baseline body weight during a 3-month period.

Blood samples were collected after overnight fasting from the 53 ESCC patients prior to any treatment. From the 27 patients who underwent esophagectomy (17 men, 10 women, mean age 59.7 years), samples of the tumor and matched non-tumor tissue were collected for the analysis of mRNA and protein expression.

Serum from 27 apparently healthy blood donors (16 men, 11 women, mean age 55.9 ±3.6 years) from the Regional Blood Donation and Treatment Center (Wrocław, Poland) was used as a reference in the analysis of circulating adipokines. The control group was age- and gender-matched to the study group ($p > 0.05$).

Ethical considerations

The study was planned according to the ethical standards detailed in the Declaration of Helsinki, as revised in 1983. The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University (Poland). Informed consent was obtained from all the subjects.

Collection and preparation of samples

Fresh specimens of tumor and normal mucosa, the latter taken approx. 10 cm from the tumor, were collected after the resection and divided into 2 sets. The 1st, subsequently used for transcriptional analysis, was soaked in RNAlater® stabilizing solution (Ambion Inc., Austin, USA) and stored at –80°C; the 2nd, subsequently used for protein analysis, was rinsed with 0.9% NaCl and stored at –45°C.

Tissue samples for RNA extraction (30–40 mg) were homogenized in TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, USA) using a Fastprep 24 Homogenizer (MP Biomedicals, Santa Ana, USA) and total RNA was extracted using the phenol-chloroform method. The isolated RNA was purified using an RNeasy Mini Kit (Qiagen Inc., Germantown, USA) with DNase treatment in accordance with the manufacturer's instructions. The purified RNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Its purity was assessed by calculating 260/280 and 260/230 ratios. RNA integrity was evaluated using the Experion platform incorporating LabChip microfluidic technology and Experion RNA StdSens analysis kits (Bio-Rad Laboratories, Hercules, USA).

To determine the protein concentration, the tissue samples were homogenized in 10 mM Tris-HCl with 150 mM KCl and 1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4 buffer (proportion 1:2 w/v) using a FastPrep-24 homogenizer (MP Biomedicals) for 2 min at 4.0 m/s. The homogenates were centrifuged at 14,500 × g for 10 min at 6°C and supernatants were collected and stored at –45°C.

The blood samples were clotted (30 min, room temperature) and centrifuged at 1500 × g for 10 min at room temperature. The serum obtained was stored at –45°C.

Analytical methods

The concentrations of adipokines in the tissue homogenates and serum were determined in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Apelin and apelin receptor concentrations were determined using human ELISA kits (MyBioSource Inc., San Diego, USA). The sensitivity of the apelin assay was 2.63 pg/mL, while intra- and inter-assay coefficients of variation (CV) were <10% and <12%, respectively. The sensitivity of the apelin receptor assay was 1.0 ng/mL, and both the intra- and inter-assay CVs were less than 15%. Resistin and adiponectin concentrations in the serum and tissue homogenates were measured using ELISA kits provided by R&D Systems (Abingdon, UK). The sensitivity of the resistin assay was 0.026 ng/mL, and the intra- and inter-assay CVs were 3.8–5.3% and 7.8–9.2%, respectively. The sensitivity of the adiponectin assay was 0.246 ng/mL, while the intra- and inter-assay CVs were 2.5–4.7% and 5.8–6.9%, respectively.

In the case of the tissue samples, adipokine levels were adjusted to total protein level, measured using the Bradford method with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Bovine serum albumin was used for standard curve preparation. Concentrations were expressed as nanograms or micrograms of adipokine per grams of total protein content.

Adipokine mRNA expression levels were determined using reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The amount of 0.5 µg of RNA was transcribed using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer's instructions. Negative transcription controls were performed and tested for all samples. All the incubation steps were carried out in a C1000 Thermal Cycler (Bio-Rad Laboratories).

The qPCR reaction mixture consisted of 2 µL of cDNA (diluted 1:5), 10 µL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories), 1 µL of each 10 nM forward and reverse target-specific primer, and 6 µL of water. A list of the primers used in this study (provided by GeneSys Sp. z o.o., Wrocław, Poland) is presented in Table 2. The reactions were conducted in triplicate using the CFX96 RT-PCR system (Bio-Rad Laboratories) with the following cycling conditions: 95°C for 30 s, 95°C for 5 s and 61°C for 5 s, 40 cycles. The specificity of the product was confirmed with melting curve analysis (60–90°C with a fluorescence reading every 0.5°C) and with electrophoresis in a high-resolution agarose (SeaKem LE agarose; Lonza Ltd., Basel, Switzerland) in Tris/Borate/EDTA (TBE) buffer with SYBR Green (Lonza Ltd.) detection. For each sample, the expression of toll-like receptors (TLRs) was examined and normalized to the *GAPDH* reference gene, and relative induction was calculated using the $2^{(-\Delta\Delta Ct)}$ method.[16]

Table 2. qPCR primer sequences used to detect apelin, apelin receptor, resistin, and adiponectin

Gene	Primer sequence
Apelin	forward: CAG GGA GGT CGG AGG AAA T reverse: ACC AAT CTA TGG AGG AGA CAT AAC C
Apelin receptor	forward: ACT TCC GCA AGG AAC GCA TCG A reverse: ACA GCG TCT TCA CCA GGT GGT A
Resistin	forward: TGG AGT GCC AGA GCG TCA CCT reverse: ACT GGC AGT GAC ATG TGG TCT C
Adiponectin	forward: CAG GCC GTG ATG GCA GAG ATG reverse: GGT TTC ACC GAT GTC TCC CTT AG

Statistical analysis

All the data was analyzed using STATISTICA v. 13.0 software (StatSoft Inc., Tulsa, USA) and MedCalc v. 16.8.4 software (MedCalc Software, Ostend, Belgium). The distribution of the data was tested with the Shapiro-Wilk normality test. Descriptive data were presented as medians and min–max values (for quantitative variables) and as numbers of observations and percentages (for qualitative variables). Independent samples were analyzed using the Mann-Whitney U test, the Kruskal-Wallis analysis of variance and the post-hoc Dunn test. Paired samples were tested using the Wilcoxon test. Frequency analyses were conducted with the Fisher's exact test. Spearman's rank correlation coefficients (ρ) were calculated to evaluate associations between pairs of variables. P-values <0.05 were considered statistically significant.

Results

Protein concentrations of apelin, apelin receptor, resistin, and adiponectin in the primary tumors of cancer patients

The protein expression of apelin, apelin receptor, resistin, and adiponectin in tumor tissue and adjacent normal mucosa are presented in Table 3. Apelin, apelin receptor and resistin levels were significantly higher in tumor tissue than in the corresponding control tissue ($p < 0.05$), whereas adiponectin concentration was non-significantly lower in the primary tumors than in control tissue ($p = 0.058$).

Tumor concentrations of adipokines were evaluated in relation to demographical, clinical and pathological parameters. Apelin levels significantly increased in the patients with lymph node metastases (median for pN0: 862.3 (701.9–1084.7) ng/g protein vs median for pN1: 1165.7 (794.2–1535.7) ng/g protein, $p = 0.005$) (Fig. 1). Correlations between other study parameters were not found.

Pairwise comparison of apelin, apelin receptor, resistin, and adiponectin mRNA expression between the primary tumors and normal mucosa of cancer patients

An analysis of apelin, apelin receptor and resistin mRNA expression showed upregulated levels of these adipokines in tumor tissue as compared to normal tissue: 4.0-fold for apelin ($p < 0.0001$), 3.4-fold for the apelin receptor ($p = 0.0008$) and 4.4-fold for resistin ($p < 0.0001$). Adiponectin mRNA expression was 2.2-fold lower in the tumors than in the control tissue ($p = 0.0255$) (Table 4).

No correlations were found between the mRNA and protein expression of adipokines in tumor tissues.

Circulating levels of apelin, apelin receptor, resistin, and adiponectin in cancer patients

Serum apelin, apelin receptor, resistin, and adiponectin concentrations were measured in the group of 53 stage I–IV ESCC patients and in the 27 healthy controls, and the results are presented in Table 5. The median concentrations of serum apelin, apelin receptor and resistin were significantly higher in the cancer patients than in the controls ($p < 0.05$ for all). There was no significant difference between the ESCC group and the control group in terms of serum adiponectin levels.

Relationships between demographic, clinical or pathological parameters and concentrations of serum adipokines in the ESCC patients are shown in Table 6. There were

Table 4. Apelin, apelin receptor, resistin, and adiponectin mRNA expression in the tumor tissue of ESCC patients. Gene expression was normalized to control tissue and analyzed using the Wilcoxon test

Gene expression	Median (min–max)	p–value
Apelin	4.11 (0.13–36.75)	<0.0001*
Apelin receptor	2.16 (0.20–98.38)	0.0008*
Resistin	9.85 (0.47–86.10)	<0.0001*
Adiponectin	0.23 (0.00–6.06)	0.0255*

*statistically significant at $p < 0.05$

Table 3. Pairwise comparison of tumor and normal tissue apelin, apelin receptor, resistin, and adiponectin levels in surgically treated ESCC patients. Data was analyzed using Wilcoxon test

Variable	Tumor tissue, median (min–max)	Control tissue, median (min–max)	p-value
Apelin [ng/g protein]	938.4 (701.9–1535.7)	851.9 (427.9–1398.6)	0.001*
Apelin receptor [μg/g protein]	240.0 (84.2–833.9)	47.1 (4.5–446.7)	<0.0001*
Resistin [μg/g protein]	7.2 (1.0–37.2)	1.0 (0.2–2.9)	<0.0001*
Adiponectin [μg/g protein]	64.6 (39.4–122.6)	78.3 (37.5–163.4)	0.058

statistically higher levels of serum apelin in the patients with cachexia (p = 0.006). Levels of serum apelin tended to be significantly higher in patients at higher TNM stages (p = 0.052). Concentrations of serum apelin receptor significantly decreased in ESCC patients with stage IV cancer (p = 0.0065) and the presence of lymph node or distant metastases (p = 0.005 and p = 0.004, respectively). No differences were observed between study parameters and concentrations of serum resistin and adiponectin in the cancer patients.

We observed a positive correlation between resistin protein expression in the tumors and in serum (ρ = 0.48, p = 0.011) (Fig. 2).

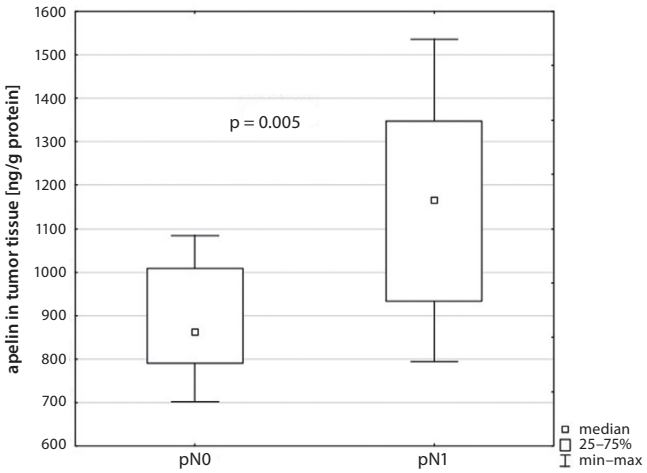

Fig. 1. Protein levels of apelin in tumor tissues of ESCC patients without (pN0) and with (pN1) lymph node metastases

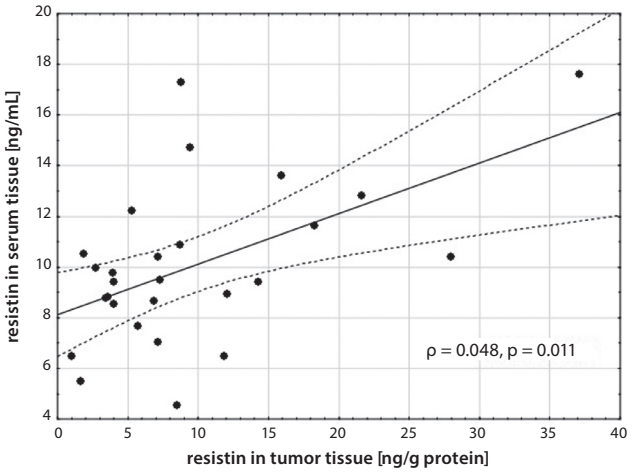

Fig. 2. The positive correlation between resistin levels in tumors and its concentration in the serum of ESCC patients. The data was demonstrated using Spearman's rank correlation coefficient (ρ)

Discussion

It has been demonstrated that apelin can influence cancer development and progression, and the apelin/apelin receptor system may play a crucial role in tumor angiogenesis.[12] However, there have been no studies of the clinical and pathological relevance of apelin and its receptor in ESCC.

To the best of our knowledge, this is the first study that has demonstrated increases in apelin and apelin receptor mRNA expression and protein levels in the tumors of ESCC patients. A similar result was reported in non-small cell lung cancer (NSCLC) tumors, where overexpression of apelin mRNA was shown by immunohistochemistry. A positive correlation between apelin mRNA and protein expression in human NSCLC specimens was reported.[13] Picault et al. have shown high levels of apelin and the apelin receptor in colon adenoma and adenocarcinoma.[17] They suggest that the apelin/apelin receptor system may participate in the growth of colon cancer through stimulation of anti-apoptotic pathways. Based on suggestions from previous research, we hypothesize that a high frequency of apelin and apelin receptor expression on the gene and protein levels might be significantly associated with ESCC development. Our results also showed significantly higher concentrations of serum apelin and apelin receptor in the cancer patients than in healthy controls, which additionally suggests the important role of these adipokines in the process of ESCC carcinogenesis.

Our investigation of associations between clinicopathological parameters and protein expression of apelin and apelin receptor levels in ESCC tumors revealed a significant relationship between high apelin levels and lymph node metastases. We did not find a similar correlation in the case of apelin receptor concentrations. Clinical and experimental studies by Berta et al. found that apelin might be a proangiogenic factor in NSCLC, and the apelin/apelin receptor system might induce lymphangiogenesis.[13,18] A study by Heo et al. confirmed that apelin is an independent prognostic factor for lymph node metastasis in oral squamous cell carcinoma patients.[19]

In vitro studies by Sorli et al. showed that apelin is a mitogenic factor for endothelial cells, and the overexpression of apelin receptor mRNA during the formation of new blood vessels has an influence on vascular network intensity in tumors.[20] They also reported that overexpression of apelin and apelin receptor stimulate tumor growth, and that this increase is related to earlier initiation of tumor development. They suggest that the observed effect is independent of the influence of tumor cells, and apelin could act on tumor neoangiogenesis via a paracrine action on the endothelial cells of the host vessels. In addition, Kalin et al. have shown that apelin and the apelin receptor are upregulated in microvascular proliferations during tumor angiogenesis in malignant gliomas.[21]

These reports led us to formulate the suggestion that the highest levels of apelin in the tumors of patients with lymph node metastases might confirm the paracrine effect of the apelin/apelin receptor system on vascular and lymphatic endothelial cells and the activation of neoangiogenic and lymphangiogenic processes in ESCC.

In our previous research, no significant differences between clinicopathological parameters and apelin concentrations in the serum of gastroesophageal cancer patients were found. In the present study, we observed a significant increase of serum apelin in the ESCC patients with cachexia syndrome and a tendency toward an increase in serum apelin levels in patients with advanced stages of the disease. This suggests that apelin may contribute to cachexia development through its participation in the systemic inflammatory response.

Although concentrations of serum apelin receptor were significantly higher in the ESCC patients than in the healthy controls, we found decreases in circulating apelin receptor in relation to the cancer patients' tumor stage progression, lymph node and distant metastases. This may suggest that the apelin receptor is intensely produced in the early stage of cancer development and may possibly take part in the carcinogenesis processes in ESCC. Further studies on larger groups of patients with stage I and II disease are necessary to evaluate the role of serum apelin receptor in ESCC.

Our study is the first to demonstrate that resistin mRNA and protein expression were significantly higher in tumor tissue than in normal mucosa in ESCC patients. These results are consistent with our previous study, where we found significant increases in resistin levels in the tumor tissue of gastroesophageal cancer patients.[10] Although no significant associations between the clinicopathological parameters and resistin protein expression in ESCC tumor tissue were found, other studies have reported that resistin plays an important role in tumor growth, neoangiogenesis and metastasis, and also in the differentiation and migration of endothelial cells in cancers.[4,7,21–25]

Table 5. Concentration of serum apelin, apelin receptor, resistin, and adiponectin in esophageal squamous cell carcinoma (ESCC) patients and healthy controls. Data was analyzed using the Mann-Whitney U test

Variable	ESCC patients (n = 53), median (min–max)	Control group (n = 27), median (min–max)	p-value
Apelin [pg/mL]	746.7 (314.4–1160.7)	570.0 (340.0–960.0)	0.036*
Apelin receptor [ng/mL]	15.8 (3.1–55.0)	7.2 (4.8–9.4)	<0.0001*
Resistin [ng/mL]	9.4 (4.5–17.6)	7.2 (1.9–14.3)	0.0007*
Adiponectin [µg/mL]	9.6 (2.1–18.7)	9.9 (4.1–17.2)	0.831

* statistically significant at $p < 0.05$

Table 6. Relationships between demographic, clinical and pathological parameters and concentrations of serum adipokines in esophageal squamous cell carcinoma (ESCC) patients (n = 53). Data was analyzed using the Mann-Whitney U test (when 2 independent samples were compared), the Kruskal-Wallis test (when 3 independent samples were compared) and post-hoc Dunn's test (for intragroup comparison)

Parameter	Apelin [pg/mL]		Apelin receptor [ng/mL]		Resistin [ng/mL]		Adiponectin (µg/mL)	
	median (min–max)	p-value	median (min–max)	p-value	median (min–max)	p-value	median (min–max)	p-value
Age:								
<60 (n = 27)	698.9 (320.8–1117.8)	0.319	14.8 (4.0–54.4)	0.810	9.5 (5.7–17.3)	0.999	8.8 (2.1–16.8)	0.083
>60 (n = 26)	809.3 (314.4–1160.7)		17.2 (3.1–55.0)		9.0 (4.5–17.6)		10.5 (5.5–18.8)	
Gender:								
male (n = 36)	779.9 (314.4–1160.7)	0.457	15.4 (4.0–54.4)	0.668	9.6 (5.7–17.6)	0.336	9.5 (2.1–16.8)	0.112
female (n = 17)	648.9 (388.3–1001.8)		16.6 (3.1–55.0)		8.9 (4.5–14.3)		9.9 (5.5–18.8)	
Cachexia:								
yes (n = 20)	907.6 (565.4–1160.7)	0.006*	14.4 (8.3–30.4)	0.657	9.5 (5.7–14.7)	0.539	10.5 (5.5–16.8)	0.882
no (n = 33)	671.7 (314.4–1107.4)		16.6 (3.1–55.0)		9.3 (4.5–17.6)		9.5 (2.1–18.8)	
pTNM:								
I + II (n = 11)	577.9 (388.3–1083.4)	0.052	24.9 (3.1–55.0)A	0.007*	9.9 (4.5–17.3)	0.853	9.5 (6.8–14.2)	0.865
III (n = 16)	828.9 (320.8–1160.7)		16.8 (4.0–36.2)		9.4 (6.5–17.6)		10.2 (3.1–16.8)	
IV (n = 26)	722.8 (314.4–949.8)		13.1 (5.2–32.8)A		9.2 (5.7–15.6)		9.5 (2.1–18.8)	
pT:								
1 + 2 (n = 9)	577.9 (395.7–1027.8)	0.404	24.9 (3.1–55.0)	0.074	10.0 (4.5–17.3)	0.869	9.5 (7.3–14.2)	0.632
3 (n = 15)	790.5 (320.8–1160.7)		15.8 (4.0–30.4)		9.4 (5.5–14.7)		10.0 (4.5–16.8)	
4 (n = 29)	746.7 (314.4–1015.6)		13.8 (5.2–36.2)		9.3 (5.7–17.6)		9.3 (2.1–18.8)	
pN:								
0 (n = 14)	660.4 (388.3–1083.4)	0.237	25.3 (3.1–55.0)	0.005*	9.9 (4.5–17.3)	0.864	9.5 (5.5–16.8)	0.538
1 (n = 39)	805.5 (314.4–1160.7)		14.4 (4.0–36.2)		9.3 (5.7–17.6)		9.9 (2.1–18.8)	
pM:								
0 (n = 27)	756.6 (320.8–1160.7)	0.251	21.3 (3.1–55.0)	0.004*	9.5 (4.5–17.6)	0.587	9.9 (3.1–16.8)	0.978
1 (n = 26)	722.8 (314.4–949.8)		13.1 (5.2–32.8)		9.2 (5.7–15.6)		9.5 (2.1–18.8)	

* statistically significant at $p < 0.05$; A – post-hoc test for I + II vs IV, $p = 0.0065$.

The present study demonstrated significantly higher concentrations of serum resistin in the ESCC patients than in the; however, no relationships between serum levels of resistin and clinicopathological parameters were found. Increases in serum resistin concentration were observed in patients with colon, gastric, esophageal, and endometrial cancer,[6,8,14,26] or in the saliva of patients with oral cavity squamous cell carcinoma.[15]

Our study demonstrated significantly lower adiponectin mRNA expression in the tumors than in the control mucosa of the ESCC patients. We observed no significant differences between the clinicopathological parameters and protein levels in tumors or serum concentrations of adiponectin. These results are in opposition to some earlier studies. For example, Guo et al. used immunohistochemistry and western blot analyses to establish that adiponectin expression was significantly lower in tumor tissue than in adjacent non-malignant epithelial tissue in tongue squamous cell carcinoma.[27] Nakajima et al. and Yildrim et al. also found significantly lower concentrations of serum adiponectin in esophageal cancer patients than in healthy controls.[8,11]

Circulating levels of adiponectin decreased with tumor stage progression. Similar results have been reported in other malignancies, such as gastric and gastrointestinal cancers.[2,6,14,28,29] and in tongue squamous cell carcinoma,[27] head and neck squamous cell carcinoma[30] or non-small cell lung cancer.[3,31] Our previous study on gastroesophageal cancer also showed significantly lower levels of serum adiponectin in patients with metastases to the lymph nodes and/or distant regions of body and in patients with cachexia syndrome.[10]

However, it has been suggested in earlier studies that decreases in adiponectin levels cannot be associated with cachexia processes but rather with mechanisms of cancer progression.[7,28] This implies that decreases in adiponectin levels may possibly play a role in cancer cell invasion and metastasis. On the other hand, increases in serum adiponectin levels have been found in cachectic patients with gastrointestinal cancer[29] and in patients with head and neck squamous cell carcinoma.[32] Reductions in overall survival for men with ESCC who had increased concentrations of serum adiponectin have also been demonstrated.[33] Therefore, further research is necessary to clarify the role of adiponectin in ESCC.

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Antiviral immunity in chronic lymphocytic leukemia measured by anti-rubella antibody

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Abstract

Background. Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia in Caucasian populations, is characterized by a decrease in anti-infective immunity. Clinical evidence of antiviral immunity decrease is the reactivation of herpes virus in the form of skin lesions. In Europe, rubella infection is common and creates lifelong persistence of IgG antibodies.

Objectives. The aim of our study was to determine whether hemagglutination inhibition (HAI) rubella test can be used to determine antiviral immunity in CLL patients.

Material and methods. The titers of the HAI test against rubella were examined in a group of 26 healthy subjects, 7 subjects with herpes labialis infection and 56 patients with CLL, among which 9 patients were co-infected with herpes virus.

Results. Statistical tests have shown differences between groups and a significant decrease of the titers of the test in patients with CLL, compared with healthy persons and the herpes group compared with other persons.

Conclusions. Our results indicate a significant decrease of antiviral immunity in patients with CLL and persons with herpes-type skin lesions. Simultaneously, relying on our previous studies, we also suggest that the result of this test may be an important indicator of antiviral immunity in patients with CLL.

Key words: chronic lymphocytic leukemia, herpes, antiviral immunity, rubella HAI test

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Background

First line of anti-infectious defense in the innate immune system is composed of receptors that recognize pathogens, which belong to 3 families with a known and recognized function: Toll-like receptors (TLRs), RiG-I – like receptors (RLRs) and nucleotide binding-oligomerization domain (NOD)-like receptors. The early antivirus response is associated with the production of interferons by epithelial cells of the respiratory and digestive systems, dendritic cells that migrate to the lymph nodes, where they stimulate differentiation and activation of lymphocytes T and B.[1] During childhood, the specific antivirus response is generated, which can be measured by appropriate (depending on the kind of virus) methods. Physiological antivirus response may be modified – generally decreased by the neoplastic diseases in the organism.

One of the most common viruses which the organism contacts during childhood, either due to infection or vaccination, is rubella. According to the World Health Organization (WHO), it is estimated that about 94–98% of the European community contracted the rubella virus, a statistic which can be established with tests using antibodies IgG or IgM against rubella. Neutralizing body titer with the use of agglutination of blood cells under the influence of antibodies has been determined as a test with high specificity.[2] Hemagglutination inhibition (HAI) method has been recognized by the WHO as the reference rubella antibody method, and the National Committee for Clinical Laboratory Standards (NCCLS) Subcomittee on Rubella Serology in 1992 recommends the use of HAI test as a reference method to establish a calibration standard for other rubella methods.[3] At the same time, in the evaluation of infection risk, the very low titer of this test is taken into account.[4] Rubella can produce images of skin lesions similar to measles and the diseases may be differentiated by the simultaneous execution of antibodies against measles and rubella, specific to the recent infection.[5]

The descriptions of rubella infection in patients with chronic lymphocytic leukemia (CLL) are not present in the available literature sources.

On the other hand, the clinical exponent of decreased antiviral immunity is the occurrence of herpes infection, demonstrating reactivation of the virus occurring commonly in the organism.[6] It may manifest in the form of a cold sore, considered a trivial viral infection, and in relatively more severe forms as a disseminated form, related to the course of nerves, herpes zoster or herpes genitalis. In the retrospective analysis of 125 skin lesions found in 40 persons from among photographically analyzed 750 patients with CLL, beside the most frequently mentioned cancers, other amendments included varicella zoster in 6 and herpes simplex in 3 patients.[7] In some cases of CLL, herpes virus infection has a rare view of the changes.[8,9] This infection occurs also in humans generally considered to be healthy, usually in the autumn and winter.

Objectives

The aim of the present study was to determine whether among previously untreated patients with CLL there is a decrease of antiviral immunity measured using antibodies IgG against rubella with HAI method and thus whether this test can be used to evaluate the antiviral immunity.

Material and methods

Study was conducted in the group of 56 patients with CLL and included 47 patients (20 women and 27 men) without symptoms of herpes and 9 CLL patients (4 women and 5 men) with symptoms of herpes, aged 36–78 years (median 64 years), previously untreated, with duration of the disease ranging from 1 month to 60 months. According to Rai classification, 13 persons were in the 1st stage of the disease, 29 in the 2nd, 11 – in the 3rd, and 3 in the 4th clinical stage.

The control group consisted of 33 persons. Twenty-six of them, aged 25–65 years, were healthy persons. Seven, aged 32–67 years, were previously healthy persons, who had herpes. Development of rubella was not observed in any of them.

IgG antibodies against rubella inhibiting hemagglutination were tested using the HAI test called Rubenosticon manufactured by Organon (Oss, the Netherlands).[10]

Test results were compared following the reduced and normal HAI test titer result using Pearson's χ^2 test with or without Yates's correction and Fisher's test. The relative risk was also established using odds ratio (OR) calculations with 95% confidence interval (95% CI).

Any aspect of the present study that involved patients was conducted with the patient's informed consent and ethical approval from the Ethic Committee of Wroclaw Medical University, Poland.

Results

In the control group of healthy persons, the test titer was 1:128 to 1:64 in 25 patients. Only in 1 healthy patient (3.8%), test titer was reduced to 1:32.[10] For 7 persons previously considered to be healthy who had herpes, test titer was reduced in all persons.

Hemagglutination inhibition test titer against rubella was significantly reduced in the group of CLL patients. Values within normal range were present only in 19 persons (40.4%). For 9 persons with CLL and coexisting herpes infection, test titer in all of them was reduced, except for the case of 1 person. In the group of all 56 CLL patients, normal test titer occurred in 20 patients (35.7%), and among 33 healthy persons it occurred in 26 persons (78.8%), which is statistically significantly ($p < 0.005$) less frequently than in the group of healthy persons, where

it occurred in 26 persons (78.8%). The risk of decreased immunity in the group with CLL was 6 times higher (OR = 6.68). Also, CLL group without herpes statistically significantly differed with lower frequency of test values within normal range, compared with the control group ($p = 0.001$) and the risk of decreased immunity was 5.5 times higher (OR = 5.47). The control group of healthy persons statistically significantly differed from persons with herpes and all CLL patients with herpes ($p < 0.005$). Comparison of all tested persons without herpes to all persons with herpes showed a statistically significant difference in the reduction of test titer for patients with herpes and 10-fold risk of reduced antiviral immunity measured with decreased test titer.

Research results are presented in Table 1 and the results of group comparisons, depending on the test titer, are presented in Table 2.

Table 1. Anti-rubella hemagglutination inhibition (HAI) test titers in patients with chronic lymphocytic leukemia (CLL) and in control without/with coexisting herpes infection

Test titer	CLL	CLL + herpes	Control	Control + herpes
Reduced	28	8	1	7
1:4	1	–	–	–
1:8	6	2	–	3
1:16	9	3	–	3
1:32	12	3	1	1
Normal	19	1	25	0
1:64	10	1	8	–
1:128	9	–	17	–
Subtotal	47	9	26	7
Total	56		33	

Table 2. Comparison between groups due to anti-rubella hemagglutination inhibition (HAI) test titer

Compared group	χ^2 test result	Exact Fisher's test result	OR (95% CI)
t CLL vs t C titer <1:64 titer <1:128	 $p < 0.005$ $p = 0.001$	 $p < 0.001$ $p = 0.001$	 6.686 (2.502–17.768) 5.919 (1.973–18.228)
CLL vs t C	$p = 0.001$	$p = 0.001$	5.474 (2.008–14.842)
CLL vs CLLh	$p = 0.193$	$p = 0.136$	0.184 (0.008–1.692)
CLL vs CLLh+Ch	$p = 0.082$	$p = 0.064$	0.211 (0.029–1.158)
CLL+C vs CLLh+Ch	$p = 0.001$	$p = 0.001$	0.094 (0.014–0.487)
C vs Ch	$p < 0.005$	$p < 0.005$	0.007 (0.000–0.166)
C vs CLL h+Ch	$p < 0.005$	$p < 0.005$	0.006 (0.001–0.086)

C – control; CLLh – chronic lymphocytic leukemia with coexisting herpes infection; Ch – control with coexisting herpes infection; OR – odds ratio; CI – confidence interval; CLL – chronic lymphocytic leukemia.

Discussion

Chronic lymphocytic leukemia is a disease in which antibacterial, antifungal and antiviral immunity impairment is a consequence of primary immunodeficiency resulting from B lymphocytes dysfunction, which is the reason of hypogammaglobulinemia, inadequate response to the vaccination and complex deficiency of number and function of B lymphocytes, as well as incorrect structure and function of NK cells, neutrophils, monocytes, and complement system.[11,12] Secondary immune defects result from damage of bone marrow function and treatment-related toxicity. Significant damage of antiviral immunity is usually caused by treatment with purine analogues and with the use of monoclonal antibodies.[13,14] It is difficult to anticipate which patient will develop the infection. Its occurrence is usually associated with hypogammaglobulinemia and duration of illness. Among numerous risk factors, analyzed by Borthakur et al. in cytomegalovirus (CMV) infections complicating CLL, the reduction of albumins in patients below 3.0 g/dL proved to be significant.[15] According to the current regulations based on the study of resistance to rubella, hemagglutination assay is preferred and test titer ≥1:8 protects against rubella.[3]

Our previous pilot studies, involving 145 patients with various diseases of the hematopoietic lymphoid tissues, revealed a reduced titer in 7 of 12 of patients with multiple myeloma and in 8 of 15 of patients with CLL – therefore in more than 50% of persons with lymphoproliferative neoplasms. This proved that in many patients test titer is reduced in relation to the titer obtained in healthy persons.[10]

Our research concerning diseases from the plasma cell clonal diseases involving 60 patients have shown a reduction of test titer in 37 persons, i.e., in 61.6% of patients, mostly in IgG myeloma, because in 25/37 of patients, there were no statistically significant differences between the average concentrations of immunoglobulins of IgG, IgA and IgM class compared to the group with normal and reduced test titer. Statistically significant differences occurred, however, in the group of patients with myeloma of IgA class, in which patients with reduced test titer exhibited a higher concentration of IgA and a lower concentration of IgG compared with patients with normal test titer. Among 4 persons with plasma cell clonal diseases, disseminated infections of herpes zoster and low test titer have been reported.[16] Unexpectedly, low test titer (lower than 1:32) was observed in previously healthy persons who had herpes, same as for healthy persons. Therefore, this titer (1:32) was adopted as a threshold value of the positivity of the test. Current analyzed research results of 56 patients with CLL before treatment revealed test titers in herpes infections both in patients with CLL (CLLh) and in persons considered to be healthy (Ch) in connection with results obtained in previous studies, which allows us to conclude that HAI rubella test may be an important indicator of humoral, especially antiviral, immunity

in the group of patients with hematological neoplasms. Among patients with these blood cancers, this evaluation is particularly difficult due to the interference of many factors resulting from both disease and therapy. Although HAI test is not currently used in detecting the incidence of rubella, it may be a valuable indicator for the evaluation of the degree of reduction of antiviral immunity. Patients with reduced titer of this test should be special care patients, requiring more frequent control for the presence of virus infections.

Conclusions

Our results indicate a very significant decrease of antiviral immunity in patients with CLL and persons with herpes-type skin lesions. We believe that drawing attention to other uses of a test which is obsolete and now useless for the diagnosis of infection against rubella may be an important contribution to research on a practical method of determining the degree of impairment of antiviral immunity.

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Chronic and oxidative stress association with total count of endothelial microvesicles in healthy young male plasma

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Abstract

Background. Chronic and oxidative stress promotes injury to the endothelium. This happens early in the disease and novel biomarkers describing the rate of the damage may be important in early diagnostics and prevention. Microvesicles are shed from endothelial cells in response to oxidative stress, inflammation, coagulation, and angiogenesis. Their increased level in plasma could reflect the state of the endothelium.

Objectives. The objective of this study was to test the association between oxidative and chronic stress markers, atherosclerosis risk factors and endothelial microvesicle (EMV) count in peripheral blood.

Material and methods. The study included 81 males, aged 25–55 years and apparently healthy. Venous blood samples were labeled with anti-CD144-FITC, anti-CD105-BV421, anti-CD42a-PerCP, anti-CD62e-PE, anti-CD31-APCy7, and anti-CD61-APC (BD Biosciences, San Jose, USA), and tested using a BD LSR Fortessa cytometer (BD Biosciences). Events were gated on forward and side-scattered light parameters. Malondialdehyde (MDA) and cortisol concentrations were measured using high-performance liquid chromatography (HPLC).

Results. Four populations of EMV expressing a combination of $CD105^+$, $CD31^+$, $CD144^+$, and CD62e with $CD42a^-$ or $CD42a^+$ markers were examined. We found correlations between MDA concentration and hair cortisol and a total count of $CD144^+$ microvesicles, and weak correlations with diastolic blood pressure (DBP) ($p = 0.003$, $r = 0.324$) and systolic blood pressure (SBP) ($p = 0.016$, $r = 0.267$), especially with the microvesicles carrying CD62e. There was a median difference of $CD105^+$ microvesicle count between smoking ($n = 13$) and non-smoking ($n = 68$) individuals. A predictive model showed an association between $CD144^+$ microvesicle counts with cortisol and MDA concentrations and waist circumference.

Conclusions. In conclusion, our data and predictive model showed that the total counts of microvesicle populations were associated with stress-related parameters – cortisol and MDA concentrations; expression of CD62e in various populations of EMV and the ratio of $CD144^+$ to $CD105^+/CD62e^+$ were associated with increased DBP and SBP, and also with total cholesterol concentration in healthy young male population.

Key words: flow cytometry, oxidative stress, atherosclerosis, dyslipidemia, microvesicles

Introduction

Cardiovascular disease remains one of the main causes of death worldwide. Oxidative stress is implicated in atherosclerosis pathogenesis and caused by the production of excess reactive oxygen species (ROS)[1]. Reactive oxygen species are formed naturally and act as regulators of various cell functions and biological processes. Uncontrolled production of ROS is implicated in vascular injury.[2] Endothelial dysfunction is known to be independently associated with cardiovascular mortality.[3]

Malondialdehyde (MDA) concentration in blood plasma is proposed as an effective oxidative stress biomarker. Most of this substance is produced during the reaction process of decomposition of products of lipid peroxidation and it helps to evaluate oxidative stress level in vascular system.[4] On the other hand, cortisol concentration in hair could be used as a biomarker for chronic stress.[5]

Many cells, such as endothelial cells, white blood cells, platelets, and red blood cells, release microvesicles. These microvesicles are non-nuclear vesicles of various sizes between 100 nm and 1000 nm, and they carry inside a variety of proteins and genetic material that may influence various intra- and inter-cellular processes. They also have specific membrane receptors that could help to regulate cellular response to the adjacent environment.

Microvesicles are shed from endothelial cells in response to various stimuli, such as oxidative stress, inflammation, coagulation, and angiogenesis.[6] So far, the precise relationship of microvesicles with cell signaling systems is not known, but it is already clear that these are not just residual particles.[7] They could be viewed as mediators in various signaling pathways in response to stimuli. It is a promising idea to use microvesicle count in routine clinical practice as an early diagnostic biomarker. Endothelial microvesicles (EMV) may indicate atherosclerosis and its clinical manifestation – ischemic heart disease.[8]

Information regarding their importance in early atherosclerosis is still inconsistent, especially in relation to phenotypes of EMV.

The aim of this study was to examine the different populations of EMV and their association with atherosclerosis risk factors and chronic and oxidative stress markers in a young healthy male population.

Material and methods

Subjects

The study group consisted of 81 males aged 25–55 years of age and apparently healthy. Patients with any history of cardiac and chronic diseases, or prior stroke or venous thromboembolism, or under antithrombotic and antihypertensive treatment were excluded from the study. We did not include female subjects in our study, as males usually have earlier onset of disease than their female counterparts.[9] This study design also allowed us to have a more homogenous population and more representative results, since biomarkers between sexes are slightly different. Informed and signed consent was obtained from all the subjects and the study protocol was approved by the local ethics committee of Vilnius University, Lithuania (permission No. 158200-15-807-319). The study was performed in accordance with the Helsinki Declaration. Signed informed consent forms were archived.

We measured the weight, waist circumference and height of the individuals, and collected data about smoking habits. We also used Beck depression inventory to measure their depression level. Laboratory tests, including complete blood count, concentration of C-reactive protein (CRP), glucose, total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were performed using routine techniques.

To better show the association with smoking habits, we also decided to split individuals into 2 groups based on current smoking: 1. smoking group (n = 13); 2. non-smoking group (n = 68).

Derivatization and high-performance liquid chromatography method for the determination of malondialdehyde in serum

The concentration of MDA from serum was measured using a method published by Khoschsorur et al.[10] The sample was prepared by cleaning it and derivatizing analyte with thiobarbituric acid (TBA) into detectable form, i.e., the MDA-TBA adduct. Malondialdehyde concentration was determined using a Shimadzu Nexera X2 UHPLC system (Shimadzu Corp., Kyoto, Japan). Chromatographic separation was achieved using an Agilent Poroshell 120 EC–C18 (2.7 µm, 3.0 × 100 mm; Agilent Technologies, Palo Alto, USA) reversed phase column with 40:60 (v/v) 50 mM phosphate buffer (pH 6.8) and methanol as mobile phase. The flow rate was 0.4 mL min^{-1}. Fluorimetric detection was performed with excitation at 515 nm and emission at 553 nm. The data was collected and processed using the LabSolutions software (Shimadzu Corp.).

Extraction and analysis of cortisol in human hair

The concentration of cortisol from hair was accomplished using an extraction method published by Raul et al.[11] and De Palo et al.[12] Hair samples were washed twice in 5 mL isopropanol. A 50 mg of each sample were finely cut and incubated in 1.5 mL of Sorenson's buffer, pH 7.6, for 16 h at 40°C, in the presence of 10 ng of 6-α methylprednisolone as internal standard. The incubation medium was centrifuged and the supernatant was transferred to SPE

Table 1. Summary of endothelial biomarkers for flow cytometry analysis of endothelial microvesicles

Marker	Function	Origin
CD144	VE-cadherin; responsible for angiogenesis and intercellular communication	endothelial cells
CD105	endoglin; important for angiogenesis	endothelial cells, macrophages
CD31	platelet endothelial cell adhesion molecule (PECAM-1); important for angiogenesis and integrin activation; associated with endothelial cell apoptosis	platelets, endothelial cells, monocytes, granulocytes, and B-cells
CD62e	E-selectin; adhesion molecule, participates in inflammation and recruiting leukocytes	activated endothelial cells
CD42a	glycoprotein IX; it forms a 1-to-1 noncovalent complex with glycoprotein Ib, a platelet surface membrane glycoprotein complex that functions as a receptor for the von Willebrand factor	platelets
CD61	integrin subunit β-3; integrins are known to participate in cell adhesion as well as cell-surface mediated signaling	platelets

Discovery DSC-18 column (Sigma-Aldrich, St. Louis, USA), which was previously equilibrated (3 mL MeOH followed by 1.5 mL of water). The succeeding steps were the following: washing with 0.5 mL of water followed by 0.5 mL of acetone/water (1:4, v/v), 0.25 mL of hexane and elution with 1.5 mL of diethyl ether. The finally obtained eluate was evaporated to dryness with nitrogen stream and the dry residue was resuspended using 100 µL of acetonitrile/water (1:1, v/v). Cortisol concentration was determined by a Shimadzu Nexera X2 UHPLC system (Shimadzu Corp.). A 10-microliter volume of the extract was injected onto the Zorbax Eclipse XDGB–C8 column (5.0 µm, 4.6 × 150 mm; Agilent Technologies). Each 20-min chromatographic run was carried out in a binary mobile phase of acetonitrile and deionized water (40:60, v/v). The flow rate was 1.0 mL min^{-1}. The UV absorbance was measured at 245 nm. Data was collected and processed using the LabSolutions software (Shimadzu Corp.). Each sample was analyzed in triplicates.

Blood sampling and preparation of platelet-poor plasma

Blood samples were collected into tubes containing lithium heparin after night fasting. Tubes were centrifuged for 10 min at 3,000 g relative centrifugal force (RCF) within 1 h from collection to obtain platelet-free plasma. Three-quarters of the recovered plasma was transferred to a sterile tube and centrifuged again at 15,000 g RCF for 30 min in an angle head rotor to create a microvesicle pellet. The resulting pellet was reconstituted with 1 mL of serum-free freezing medium (Biological Industries, Beit HaEmek, Israel) containing dimethyl sulfoxide (DMSO) and analyzed using flow cytometry. We adhered to all health and safety procedures when handling clinical samples.

Flow cytometry

Samples were run on a BD LSR Fortessa II flow cytometer (BD Biosciences, San Jose, USA). Particle size gates were defined based on forward- and side-scattered light with polystyrene microbeads of known size. We used standard microbeads from Megamix-Plus (Bio-Cytex, Marseille, France) to determine the place of microvesicles smaller than 1 µm on a dot plot. All samples were labeled with anti-CD144-FITC, anti-CD105-BV421, anti-CD42a-PerCP, anti-CD62e-PE, anti-CD31-APCy7, and anti-CD61-APC (BD Biosciences). All events were gated on a forward- vs side-scattered light dot plot according to their intensity (size). Each analysis included 1,000,000 events.

When constructing the panel of cellular markers for this study, we selected antigens that are highly expressed by endothelial cells to achieve the most precise microvesicle labeling. We took into account the specificity of every marker and its sensitivity in relation to EMV. A summary of the chosen markers is presented in Table 1.

We analyzed the size and granularity of EMV by forward-scattered light set to a logarithmic scale and side-scattered light set to a logarithmic scale. As shown in Fig. 1, the population of EMV was first identified according to their light-scattering characteristics, representing their relative size. The populations were analyzed for CD144, CD105, CD31, CD61, CD42a, and CD31 expression (Fig. 1).

We identified 4 distinctive EMV populations having these markers: 1. $CD105^+$, $CD42a^-$ and $CD61^-$; 2. $CD144^+$, $CD42a^-$ and $CD61^-$; 3. $CD144^+$, $CD42a^+$ and $CD61^-$; and 4. $CD31^+$, $CD42a^-$ and $CD61^-$. Furthermore, in each population we determined the percentage of EMV carrying CD62e.

Cell culture

In order to be sure that we gated on genuine EMV, we performed flow cytometry of EMV in cultured human endothelial cells. Human umbilical vein endothelial cells (HUVEC; Lonza, Basel, Switzerland) extracted from pooled donors were cultivated in complete endothelial cell growth media (EGM-2 BulletKit; Lonza) under standard cell culture conditions (37°C, 5% CO_2). Cells of passage 2–3 were used when 90% confluent. Endothelial microvesicles were generated by growing cells in growth media conditioned with cyclophilin A (Abcam, Cambridge, UK). We formulated 4 different solutions with different cyclophilin A concentrations of 10 ng/mL, 20 ng/mL, 30 ng/mL, and 60 ng/mL, and incubated the cells for 24 h. As a negative

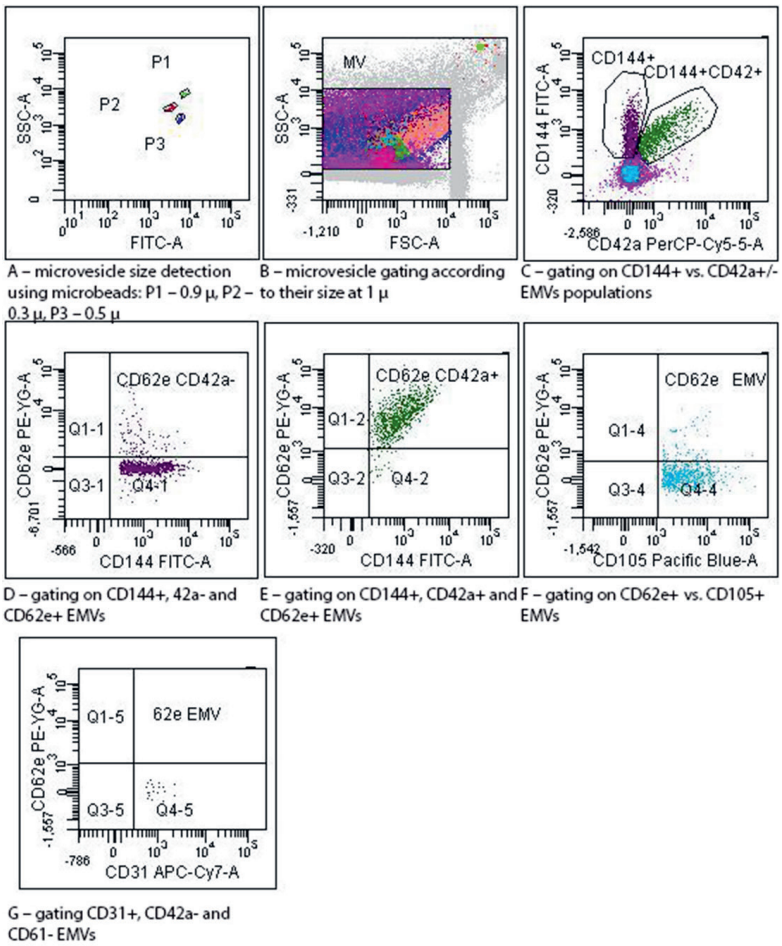

Fig. 1. Flow cytometric analysis of endothelial-derived microvesicles from plasma

A – microvesicle size detection using microbeads: P1 – 0.9 µm, P2 – 0.3 µm, P3 – 0.5 µm; B – microvesicle gating according to their size at 1 µm; C – gating on CD144$^+$ vs CD42a$^{+/-}$ EMVs populations; D – gating on CD144$^+$, CD42a$^-$ and CD62e$^+$ EMVs; E – gating on CD144$^+$, CD42a$^+$ and CD62e$^+$ EMVs; F – gating on CD62e$^+$ vs CD105$^+$ EMVs; G – gating on CD31$^+$, CD42a$^-$ and CD61$^-$ EMVs.

control, we used cells cultivated in the same conditions without cyclophilin A. Supernatant from cell cultures was collected and then centrifuged at 15,000 g RCF for 30 min to yield a pellet of microvesicles. The pellet was re-suspended in 1 mL serum-free freezing medium containing DMSO and analyzed with flow cytometer using our panel of antibodies.

Statistical analysis

The Shapiro-Wilk test was used for normal distribution of continuous variables. Results are presented as means and standard deviations (SD) or as medians (Me) and interquartile ranges (IQR) depending on variable distribution. Correlations between 2 continuous variables were performed using Pearson's correlation coefficients; otherwise the Spearman's correlation coefficients were used. Differences in means between normally distributed continuous variables were found using a t-test. The Wilcoxon rank-sum test (Mann-Whitney U test) was used to find median difference between non-normal distributed continuous variables. A multiple linear regression analysis was employed to evaluate the influence of several independent variables (waist circumference, hair cortisol and MDA concentrations, total count of EMV positive for CD144 and CD42a and total count of EMV positive for CD31) on CD144$^+$, CD42a$^-$ and CD61$^-$ microvesicle count. The significance level for the test was set at 0.05. Statistical analysis was carried out using R statistical software v. 1.0.136 and SPSS v. 21 (IBM Corp., Armonk, USA).

Results

Clinical characteristics

Demographic and clinical data are displayed in Table 2. Diastolic blood pressure (DBP), heart rate and MDA concentration were distributed normally.

Total counts of populations of microvesicles were quantified using flow cytometry and expressed as microvesicles/µL. The characteristics of all 4 populations of microvesicles are presented in Table 3.

Association of atherosclerosis risk factors with CD144$^+$ or CD105$^+$ or CD31$^+$ and CD42a$^-$ or CD42a$^+$, and CD61$^-$ endothelial microvesicles populations

The total count of EMV carrying CD105$^+$, CD42a$^-$ and CD61$^-$ had a weak correlation with systolic blood pressure (SBP) ($r = 0.23$; $p = 0.036$) (Fig. 2). We also compared

Table 2. Demographic and clinical characteristics of the population studied. The data values are presented as means and standard deviations (SD) or as medians and interquartile range (IQR) depending on variable distribution

Variables	Median (IQR) or means (SD)
Age [years]	interval: 25–55 median (IQR): 33 (16)
CRP [mg/L]	median (IQR): 0.52 (0.78)
LDL-C [mmol/L]	median (IQR): 3.06 (0.93)
HDL-C [mmol/L]	median (IQR): 1.19 (0.32)
TG concentration [mmol/L]	median (IQR): 1.17 (0.93)
SBP [mm Hg]	median (IQR): 130 (13)
DBP [mm Hg]	means (SD): 80.11 (0.99)
Heart rate [bpm]	means (SD): 71.00 (1.42)
Total cholesterol [mmol/L]	median (IQR): 4.99 (1.24)
Glucose [mmol/L]	median (IQR): 5.21 (0.51)
Waist circumference [cm]	median (IQR): 88 (17)
BMI [kg/m^2]	median (IQR): 24.25 (4.41)
Malondialdehyde concentration	means (SD): 99.6 (2.78)
Hair cortisol concentration	median (IQR): 19.82 (37.03)

CRP – C-reactive protein; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; TG – triglyceride; SBP – systolic blood pressure; DBP – diastolic blood pressure; BMI – body mass index.

medians of total count of CD105$^+$ EMV between the groups of non-smokers and smokers. In the group of non-smokers, the median of the total count of CD105$^+$, CD42a$^-$ and CD61$^-$ microvesicles was 10.11 microvesicles/μL and in the group of smokers it was 64.78 microvesicles/μL (p = 0.04). Smoking was not an independent risk factor since we also found a difference of medians of TG concentration (p = 0.048) and CRP concentration (p = 0.005) between the groups of smokers and non-smokers. The total count of these EMV also correlated with cortisol concentration in hair (r = 0.25, p = 0.03), but had no correlation with MDA concentration in blood serum.

Externalization of CD62e$^+$ in this EMV population was associated with TG level, as the percentage of CD62e$^+$ microvesicles had a weak but statistically significant correlation with TG concentration (r = 0.28, p = 0.015). Unlike CD105$^+$ microvesicles, these microvesicles correlated with MDA concentration in blood serum (r = 0.27, p = 0.01), indicating the relationship of externalization of CD62e in this microvesicle subset and oxidative stress. Other characteristics had no significant correlation.

The total count of CD144$^+$ and CD61$^-$ microvesicles correlated with MDA concentration (r = 0.27, p = 0.02) and cortisol concentration in hair (r = 0.29, p = 0.01). Since during our research we found 2 subsets of these microvesicles, one positive for CD42a and another not, we analyzed these 2 subsets separately. The total count of CD144$^+$, CD42a$^-$ and CD61$^-$ microvesicles correlated with both MDA concentration (r = 0.34, p = 0.003) and cortisol concentration in hair (r = 0.34, p = 0.03). Hair cortisol concentration also correlated with the CD144$^+$, CD42a$^+$ and CD61$^-$ subset (r = 0.33, p = 0.03). It could indicate that only CD42a$^-$ EMV could have an association with oxidative stress. The expression of CD62e in this population was, however, associated with high DBP (p = 0.025, r = 0.25), with the total cholesterol level (p = 0.048, r = 0.28) (Fig. 2) and also with the depression level (p = 0.003, r = 0.33). We measured the depression level as a continuous variable. It has to be mentioned that depression had no association with hair cortisol or MDA concentrations. Externalization of CD62e in both subsets also did not correlate with either MDA or hair cortisol concentrations.

Table 3. Characteristics of the populations of microvesicles presented as medians (Me) and interquartile range (IQR)

Microvesicle populations	Microvesicle count [microvesicles/μL] Median and quartiles (Q)	CD62e expression [%] Median and quartiles (Q)
CD105$^+$, CD42a$^-$ and CD61$^-$	min = 0 Q1 = 1.9 Me = 15.52 Q3 = 76.16 max = 1507.53	min = 0 Q1 = 0 Me = 1.6 Q3 = 4.95 max = 25
CD144$^+$, CD42a$^-$ and CD61$^-$	min = 24.3 Q1 = 139.14 Me = 308.6 Q3 = 418.75 max = 896.74	min = 0 Q1 = 2.35 Me = 5.8 Q3 = 8.3 max = 16.2
CD144$^+$, CD42a$^+$ and CD61$^-$	min = 90.73 Q1 = 200.11 Me = 274.42 Q3 = 394.95 max = 975.57	min = 0.9 Q1 = 12.75 Me = 50.7 Q3 = 97.15 max = 100
CD31$^+$, CD42a$^-$ and CD61$^-$	min = 0 Q1 = 0 Me = 1.15 Q3 = 2.8 max = 17.91	–

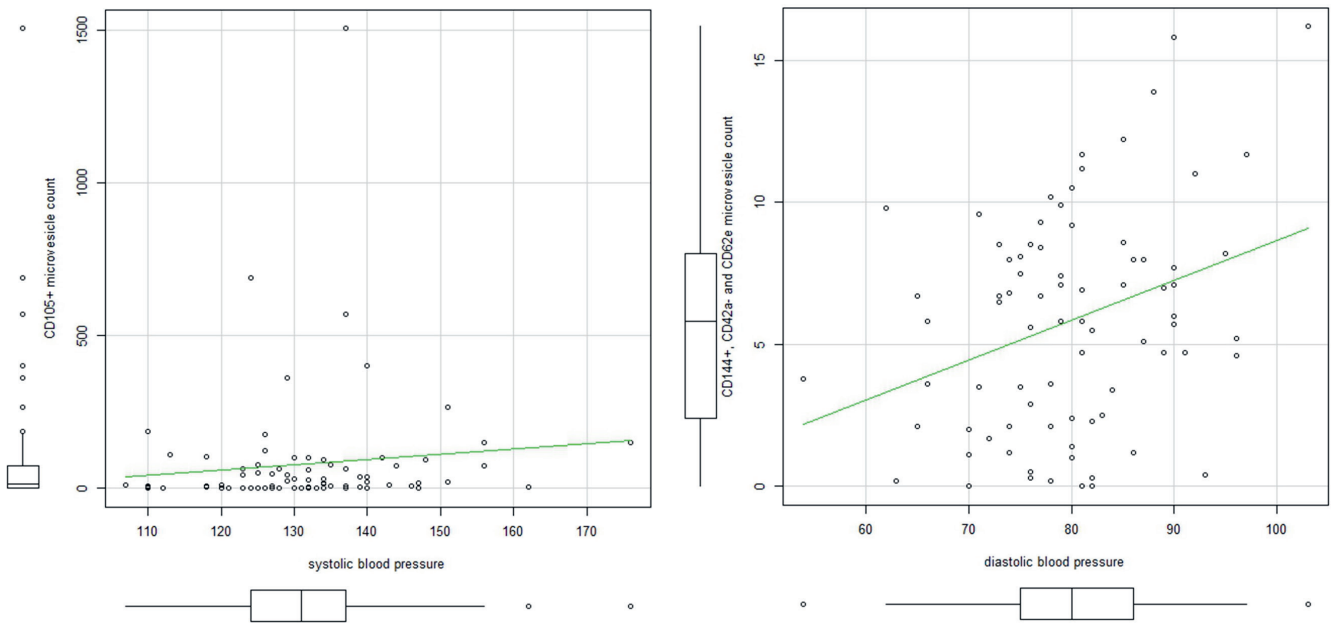

Fig. 2. Correlation of EMV carrying CD144, CD62e or CD105 with SBP and DBP. Marginal boxplots in this graph represent the lower quartile (Q1), median (Q2) and upper quartile of the sample data (horizontal boxplots represents blood pressure, vertical – total EMV count). The ends of the whiskers represent the lowest datum still within 1.5 IQR of the lower quartile and the highest datum still within 1.5 IQR of the upper quartile. Outliers have not been excluded and are shown here as dots

Table 4. Correlations between different populations of endothelial microvesicles (EMV)

Populations of EMV	$CD105^+$, $CD61^-$ and $CD42a^-$	$CD31^+$, $CD61^-$ and $CD42a^-$	$CD144^+$, $CD61^-$ and $CD42a^+$
$CD144^+$, $CD61^-$ and $CD42a^-$	r = 0.26, p = 0.017	r = 0.35, p = 0.001	r = 0.67, p < 0.001
$CD144^+$, $CD61^-$ and $CD42a^+$	–	r = 0.5, p < 0.001	–

Total count of $CD31^+$, $CD61^-$ and $CD42a^-$ had an association with age (p = 0.017, r = 0.29).

Other investigated risk factors such as body mass index (BMI), CRP concentration, glucose concentration, heart rate and dyslipidemia had no significant associations with numbers of endothelial microvesicles. Hair cortisol concentration was not associated with MDA concentration. On the other hand, both of them correlated with total cholesterol concentration (r = 0.24, p = 0.04 for hair cortisol and r = 0.39, p < 0.001 for MDA concentration) and waist circumference (r = 0.25, p = 0.02 for MDA concentration and r = 0.33, p = 0.003 for hair cortisol). Hair cortisol was associated with CRP (r = 0.24, p = 0.04) and DBP (r = 0.26, p = 0.02) and MDA concentration had correlation with LDL-C (r = 0.33, p = 0.003) and BMI (r = 0.24, p = 0.03). This was an expected result, since BMI correlated with LDL-C (r = 0.28, p = 0.01), but there was no correlation between CRP and DBP indicating hair cortisol concentration as an independent variable.

Relation between the microvesicle populations

The 4 different EMV populations were interrelated. There was a strong correlation between those carrying $CD105^+$, $CD42a^-$ and $CD61^-$ and those carrying $CD144^+$, $CD42a^-$ and $CD61^-$ (r = 0.5, p < 0.001). Furthermore, the count of $CD105^+$ microvesicles weakly correlated with $CD144^+$ and $CD42a^+$ microvesicles (r = 0.29, p = 0.009), indicating a connection between these 3 populations. Details of correlations between populations of EMV are presented in Table 4.

We also calculated ratios between different subsets of EMV and found that a higher ratio between subset of microvesicles carrying CD144 and subset of microvesicles carrying both CD105 and CD62e correlated with total cholesterol concentration (r = 0.29, p = 0.02) and high LDL-C concentration (r = 0.25, p = 0.042).

Regression analysis of the association between total count of $CD144^+$ and $CD42a^-$ endothelial microvesicles, and chronic and oxidative stress markers

Multiple variable analyses were conducted to examine the relationship between EMV carrying CD144 and not expressing CD42a and CD61 antigens on their membrane, and potential predictors, such as waist circumference, hair cortisol and MDA concentrations, total count of microvesicles carrying both CD144 and CD42a, and

Table 5. Regression model characteristics using CD144$^+$, CD42a$^-$ and CD61$^-$ microvesicle count as dependent variables, and waist circumference, hair cortisol and malondialdehyde (MDA) concentrations, total count of endothelial microvesicles (EMV) positive for CD144 and CD42a and total count of EMV positive for CD31 as independent predictors

Variable	Unstandardized coefficient	p-value	Significance	95% confidence intervals
Hair cortisol concentration	–0.36	0.17	0.038	–0.71; –0.02
Waist circumference	3.76	1.42	0.009	0.93; 6.59
MDA concentration	–1.32	0.62	0.039	–2.56; –0.07
Total count of CD144$^+$, CD42a$^+$ and CD61$^-$ populations	0.59	0.08	<0.001	0.42; 0.75
Total count of CD31$^+$, CD42a$^-$ and CD61$^-$ populations	13.11	4.27	0.003	4.60; 21.63

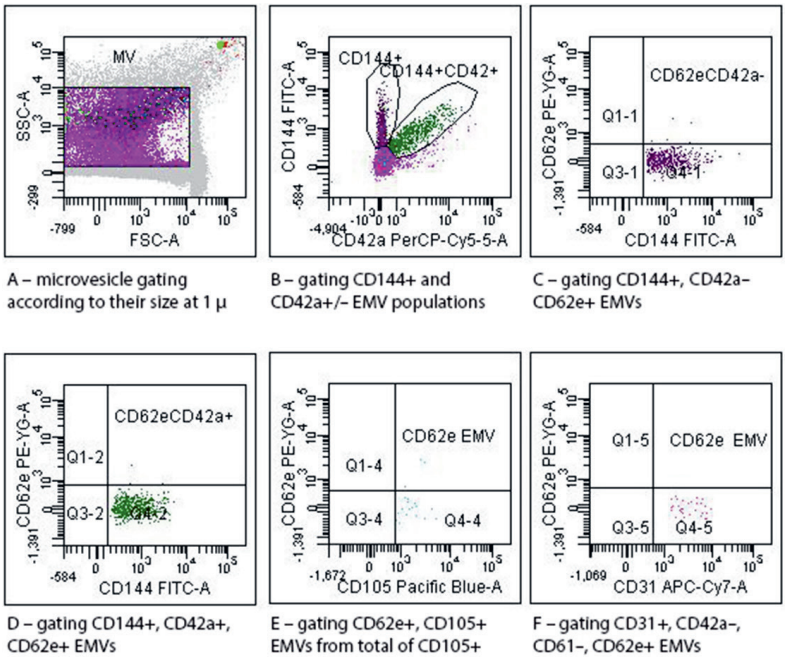

Fig. 3. Detection of EMV using flow cytometry in supernatant of endothelial cells without cyclophilin A

A – microvesicle gating according to their size at 1 µm; B – gating on CD144$^+$ and CD42a$^{+/-}$ EMV populations; C – gating on CD144$^+$, CD42a$^-$ and CD62e$^+$ EMV populations; D – gating on CD144$^+$, CD42a$^+$ and CD62e$^+$ EMV populations; E – gating on CD62e$^+$ and CD105$^+$ EMV populations from total of CD105$^+$ EMVs; F – gating on CD31$^+$, CD42a$^-$, CD61$^-$, and CD62e$^+$ EMV populations.

the total count of microvesicles carrying CD31. Waist circumference, total count of microvesicles carrying CD144 and CD42a, and microvesicles carrying CD31 positively correlated with the criterion, indicating that those with higher counts of CD144$^+$ microvesicles tend to have a larger waist, higher CD144$^+$, CD42a$^+$ and CD61$^-$ microvesicle count, and a higher CD31$^+$ microvesicle count. Hair cortisol and MDA concentrations negatively correlated with the criterion stating that higher hair cortisol and MDA concentrations are associated with lower CD144$^+$, CD42a$^-$ and CD61$^-$ microvesicle count. The multiple regression model with all 5 predictors produced $r = 0.55$ and $p < 0.001$. As it can be seen in Table 5, waist circumference and the total count of microvesicles carrying CD144 and CD42a had a significant positive regression weights, indicating that subjects with larger waist circumference and a higher total count of microvesicles carrying CD144 and CD42a were expected to have a higher count of CD144$^+$, CD42a$^-$ and CD61$^-$ EMV after controlling for the other variables in the model. Adding smoking as a predictor improved the model further ($r = 0.58$, $p < 0.001$), indicating that smoking individuals should have higher microvesicle counts.

Testing endothelial microvesicles in cell culture

We observed 4 different populations of EMV in vitro, which corresponded to results in vivo (Fig. 3,4). Treated with cyclophilin A, endothelial cells released more EMV than those that grew in resting conditions by a maximum of 2-fold. We have to note that cells grown in higher than 10 ng/mL cyclophilin A concentration released less EMV than those grown in non-cyclophilin A-treated media (negative control). It is possible that there were fewer cells in those concentrations due to the fact that cyclophilin A is a strong apoptosis agent.[13] There was no significant difference between the results of experiments in vivo and in vitro.

Discussion

Using different endothelial markers in this study, we detected 4 different EMV populations and found association between cortisol concentration in hair, MDA

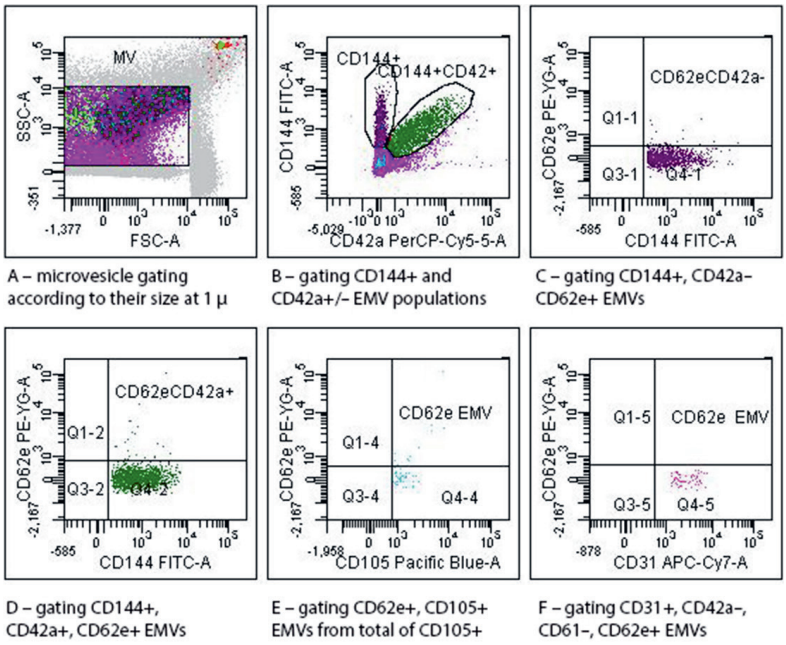

Fig. 4. EMV detection using flow cytometry in supernatant of cyclophilin A-affected endothelial cells

A – microvesicle gating according to their size at 1 µm; B – gating on CD144$^+$ and CD42a$^{+/-}$ EMV populations; C – gating on CD144$^+$, CD42a$^-$ and CD62e$^+$ EMV populations; D – gating on CD144$^+$, CD42a$^+$ and CD62e$^+$ EMV populations; E – gating on CD62e$^+$ and CD105$^+$ EMV populations from total of CD105$^+$ EMVs; F – gating on CD31$^+$, CD42a$^-$, CD61$^-$, and CD62e$^+$ EMV populations.

concentration in blood and total counts of microvesicle populations. Also, the levels of CD62e-positive microvesicles were higher in individuals with cardiovascular risk factors. There are studies that used markers mentioned in this study to research diabetes,[14] neurodegeneration and ischemic stroke,[15] and chronic heart failure.[16] These studies identified different microvesicle populations that appeared to have different associations with disease prediction. To improve our results, we used all of these markers to better describe EMV and their populations.

Studies have shown the association of microvesicles positive for CD144 with high SBP.[17] Microvesicles carrying CD144$^+$ and CD41a$^-$ or CD31$^+$ and CD41$^-$ were found to be associated with ischemic stroke and significantly correlated with stroke intensity.[18] In our study, we found an association between the expression of CD62e in this population of these microvesicles and DBP, but not SBP. Interestingly, we detected 2 subsets of CD144$^+$ microvesicles, one carrying a thrombocytic marker CD42a and another not carrying it. To check our results, we compared our findings in vivo with findings in vitro by investigating microvesicles collected from the cell culture. The release of both subsets of CD144+ microvesicles (containing and lacking CD42a) from healthy and damaged cells indicates that the endothelium release of microvesicles of a bi-lineal origin is possible. There is 1 clinical study where microvesicles of a bi-lineal origin carrying both CD62e and CD41 biomarkers were observed in patients having essential thrombocytemia.[19] This study concluded that bi-lineal microvesicles suggested ongoing endothelial activation, since their number was associated with increased levels of mature von Willebrand factor (vWF). When analyzing for association of MDA concentration ($r = 0.29$, $p = 0.01$) and hair cortisol concentration with total counts of microvesicle populations expressing CD144, we found that only the total count of CD144$^+$, CD42a$^-$ and CD61$^-$ microvesicles had a significant correlation with both MDA and cortisol concentration in hair. This does not prove to be true for the CD144$^+$, CD42a+ and CD61$^-$ subset. These findings indicate that only CD42a$^-$ EMV could have a connection with oxidative or chronic stress. Since CD42a$^+$ microvesicles had no association with either clinical or laboratory markers, they could have no importance as a disease biomarker. A weak correlation with depression level could indicate a possible connection of this marker with the psychological state of an individual, but there was no association with hair cortisol concentration. In comparison, CD144$^+$ and CD42a$^-$ EMV was associated with depression level and hair cortisol concentration. The regression model does contain both subsets of CD144$^+$ microvesicles and it would seem that an increase of CD42a$^+$ subset also increases CD42a$^-$ subset in oxidative environment. Increased hair cortisol and MDA concentration according to the model indicated lower CD144$^+$ microvesicle count. This could be interpreted as a negative correlation between oxidative stress and CD144$^+$ microvesicle count, including both CD42a$^+$ and CD42a$^-$ microvesicles. Studies show that at least in cell culture, extracellular vesicles could communicate protective messages in oxidative stress.[20]

Another investigated marker in our study was CD105 (endoglin). It belongs to a family of angiogenesis, stimulating adhesion molecules, which are found mainly on the endothelial cell membrane. It is related to revascularization and has been found to be able to influence inflammation in the arterial wall.[21] CD105 is highly expressed in unstable plaques and increased expression of this molecule could have a negative prognostic value.[22] Our data showed that an increased total count of CD105$^+$ EMV correlated positively with high SBP, but not DBP, as it was in the case of CD144$^+$ microvesicles. There was also a difference

in the medians of the total count of these microvesicles between smokers and non-smokers. In 1 study, an association between smoking and increased levels of CD62e positive microvesicles was found in healthy volunteers.[23] Our study showed that non-activated microvesicles carrying CD105, but not CD144, were associated with smoking. We also found that the percentage of CD105-positive EMV carrying CD62e had a weak positive correlation with high blood TG concentration. Interestingly, the total count of microvesicles carrying CD144 had no association with the total cholesterol or LDL-C concentrations, but their total count ratio with those EMV having CD105 and CD62e on their membrane did. Some studies show that the $CD62e^+$ to $CD31^+$ microvesicles ratio was higher in healthy controls compared to metabolic syndrome patients and that biomarkers of biomechanical stress (NT-proBNP) and inflammation (hs-CRP, osteoprotegerin) were associated with a decreased $CD62E^+$ to $CD31^+$/annexin V^+ ratio.[24] It is known that elevated CD62e-positive microvesicles can be associated with endothelial activation.[25] These results suggest that activated EMV could be more sensitive markers for endothelial dysfunction determination.

The association between MDA concentration and $CD105^+$ and $CD62e^+$ microvesicles was also evident, adding to the fact that there exists a link between oxidative stress and externalization of CD62e in $CD105^+$ EMV. This connection with MDA concentration was also detected between $CD144^+$, $CD42a^-$ and $CD61^-$ microvesicle population, indicating an interesting link between the total count of $CD144^+$ microvesicles and $CD105^+$ as well as $CD62e^+$ microvesicles in oxidative stress. It is possible that both of these populations of microvesicles are associated with oxidative stress.

According to the literature, $CD31^+$ microvesicles are possibly associated with endothelial cell apoptosis, while $CD144^+$ microvesicles are possibly associated with endothelial cell activation.[26] In our study, we only found a weak correlation between these microvesicles and age. It was not a surprising result, since the investigated individuals were relatively healthy. On the other hand, when evaluating regression model, the total count of $CD31^+$ microvesicles correlated positively with the total counts of $CD144^+$, $CD42a^-$ and $CD61^-$ populations. It suggests that the increase of $CD31^+$ microvesicles causes also an increase of $CD144^+$ microvesicles. This data could be interpreted as a possible connection between endothelial activation and apoptosis in oxidative stress and should be tested in diseased population.

In conclusion, our data shows that the total counts of microvesicle populations were associated with stress-related markers – hair cortisol and blood serum MDA concentrations. Non-activated EMV count (i.e., not carrying CD62e) had no association with any of atherosclerosis risk factors except for CD105-positive microvesicles, which showed association with SBP. Expression of CD62e in various populations of EMV and ratio of $CD144^+$ to $CD105^+$/$CD62e^+$ microvesicles were associated with increased LDL-C concentration as well as total cholesterol concentration in healthy young male population. The data suggests that $CD144^+$ microvesicles have an association with chronic and oxidative stress markers and could be useful as a biomarker for early diagnostics of endothelial disorders.

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Serum asymmetric dimethylarginine and nitric oxide levels in Turkish patients with acute ischemic stroke

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Abstract

Background. Nitric oxide synthase (NOS) is present in the brain and cerebral arteries and it enables the synthesis of nitric oxide (NO), which plays a critical role in brain perfusion. Asymmetrical dimethylarginine (ADMA) is an endogenous NOS inhibitor.

Objectives. The aim of this study was to evaluate serum ADMA levels, which are an indicator of endothelial dysfunction of the renal functions in patients with acute ischemic stroke, and to determine whether there is a possible correlation between ADMA and NO levels and the L-arginine-to-ADMA ratio.

Material and methods. Fifty-two patients (22 male and 30 female; mean age: 75.2 ±10.1 years) with a diagnosis of acute ischemic stroke in the first 24 h post-stroke and 48 healthy individuals (controls; 13 male and 35 female; mean age: 60.1 ±7.92 years) were included in this study. The risk factors recorded and evaluated were age and gender of the patients, serum lipid levels, serum ADMA levels, nitrate-to-nitrite ratios, L-arginine, L-arginine-to-ADMA ratios, sedimentation rate, C-reactive protein (CRP), urea and creatinine levels, and glomerular filtration ratio (eGFR).

Results. The mean serum ADMA level was 0.48 ±0.23 μM for the patients and 0.36 ±0.18 μM for the controls. The mean NO level was 2.78 ±0.59 μM for the patient group and 4.49 ±2.84 μM for the controls. The ADMA levels for the patient group were significantly higher than for the control group ($p = 0.011$); the NO levels for the patients were significantly lower than for the controls ($p < 0.001$). The logistic regression method demonstrated that ADMA and NO levels may be independent risk factors for the patient group, and the receiver operating characteristic (ROC) curve analysis showed that both of these variables were discriminative risk factors.

Conclusions. An increased serum level of the NOS inhibitor ADMA was found to be a possible independent risk factor for ischemic stroke.

Key words: nitric oxide, asymmetrical dimethylarginine, asymmetrical dimethylarginine/arginine levels, arginine, acute ischemic stroke

Introduction

Nitric oxide synthase (NOS), a requirement for the synthesis of nitric oxide (NO), which plays a critical role in the control of brain perfusion, is located in the brain and the walls of the cerebral arteries.[1–3] Asymmetrical dimethylarginine (ADMA) is a competitive endogenous inhibitor of endothelial NOS, which decreases endothelial NO synthesis and leads to the loss of NO bioavailability.[3] Age, diabetes mellitus (DM), hypertension (HT), carotid arterial intima-media thickness, hyperlipidemia, hyperhomocysteinemia, obesity, inflammation, and sickle cell disease have all been found to be associated with increased ADMA blood levels.[1,4] In addition, ADMA has been suggested to be an independent marker of acute stroke and transient ischemic attack.[5–7]

Increased ADMA concentration has been found to be associated with a more rapid renal disease progression and mortality via reducing NO bioavailability, which results in inflammation and oxidative stress – typical features of renal disease progression.[8–10] Asymmetrical dimethylarginine is also considered to be a uremic toxin, so it is also possible that ADMA contributes to progressive renal dysfunction by functionally impairing the integrity of the glomerular filtration barrier, promoting proteinuria, interstitial and glomerular fibrosis, and oxidative stress.[11,12] Although ADMA is attributed to impaired renal function, it is not yet clear whether ADMA is a marker or a risk factor for renal disease progression.

In this study, we aimed to investigate the relationship between serum levels of ADMA and NO and the L-arginine-to-ADMA ratio (indicators of endothelial dysfunction) in patients with acute ischemic stroke, and to discuss the possible confounding effect of renal function on ADMA and NO levels.

Material and methods

Ethics statement

This study was approved by the Ethical Committee of Ankara Numune Training and Research Hospital, Turkey. Informed consent was obtained from each participant before the study.

Study population

A total of 52 patients who were admitted to the Department of Neurology of Ankara Numune Training and Research Hospital, Turkey, between December 2009 and May 2010 with a diagnosis of acute ischemic stroke in the first 24 h post-stroke and 48 healthy individuals were included in the study.

The diagnosis of ischemic stroke was based on the patient's history, clinical examination, computed tomography (CT) examination, and magnetic resonance imaging (MRI). Hypertension was defined according to the current World Health Organization (WHO) criteria and/or the use of antihypertensive drugs. Stroke severity on admission was assessed using the National Institutes of Health Stroke Scale/Score (NIHSS). An NIHSS of 0–1 was considered mild, 2–8 was moderate, and ≥9 was severe.[13] Patients with hemorrhagic stroke, coronary artery disease, mitral fibrillation, kidney or liver disease, rheumatological or collagenous disease, those with a diagnosis of cancer, fever or infection, those receiving anticoagulation treatment, and those who had undergone vascular surgery in the previous 3 months were excluded from this study.

Methods

Blood samples were drawn from the patients on the morning of the 1st day of admission to the Department of Neurology into 10 mm tubes with red caps, not containing gel (BD Vacutainer; Becton, Dickinson and Co., Franklin Lakes, USA). After at least 30 min of incubation, the specimen was centrifuged at 1,500 × g for 10 min, and then lipid profiles and urea, creatinine and C-reactive protein (CRP) levels were determined using commercial kits (Beckman Coulter DXC 800; Beckman Coulter, Inc., Brea, USA). A fibrinogen test was performed using a tube containing citrate and a Siemens CA 7000 device (Siemens Healthcare Diagnostics, Marburg, Germany), and the erythrocyte sedimentation rate (ESR) was determined using a tube containing citrate and a Berkhun SDM 100 device (Blood Testing Equipments; Mediko Dardanel, Canakkale, Turkey). The serum samples to be used in determining ADMA, NO and arginine levels were stored at –80°C until analysis.

Serum ADMA and arginine levels were measured on the same day, after all the samples were collected by using an Applied Biosystems MDS SCIEX API 3200 LC-MS/MS system device (Applied Biosystems, Concord, Canada) in the ESI-positive mode and an Agilent Eclipse XDB–C18 column (Agilent Technologies, Palo Alto, USA). According to this method, the intra-day coefficient of variation (CV) and inter-day CV were 3.9% and 6.2%, respectively. Nitric oxide level was determined using a Cayman (780001) Nitrate/Nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, USA). Briefly, the first step is the conversion of nitrate to nitrite-utilizing nitrate reductase. The next step is the addition of Griess reagent, which converts nitrite into a deep purple azo compound. The photometric measurement of the absorbance due to this chromophore accurately determines the NO_2 concentration. The intra-assay CV was 3.4% and the inter-assay CV was 2.7%.

Statistical analysis

The data from the study was analyzed with SPSS software, v. 18.0 (SPSS, Inc., Chicago, USA). The conformity of continuous variables to a normal distribution was

tested with the Shapiro-Wilk test. The descriptive statistics of continuous variables were expressed as mean ± standard deviation (SD). The presence of a statistically significant differences between the groups in terms of continuous variables was examined with Student's t-test for parametric variables and with the Mann-Whitney U test for non-parametric variables. The analysis of variance (ANOVA) was used to analyze the differences among group means and their associated procedures. The presence of a correlation among the groups was assessed with Pearson's and Spearman's rho tests. The logistic regression method was performed to demonstrate whether ADMA and NO may be risk factors for stroke and the receiver operating characteristic (ROC) curve analysis was done to check whether these parameters may be discriminative factors for stroke.

Results

Fifty-two patients with acute ischemic stroke were included in this study; 22 were males and 30 were females. The mean age of the patients was 75.27 ±10.05 years. Thirty-seven patients had HT, 14 had DM and 13 had atrial fibrillation.

The ADMA levels were significantly higher in the patient group than in the control group (0.46 ±0.13 μM vs 0.40 ±0.11 μM, respectively) and the NO levels were significantly lower in the patient group than in the controls (2.78 ±1.59 vs 4.34 ±2.70, respectively) ($p < 0.05$).

There was no statistically significant difference between the serum L-arginine-to-ADMA ratio in the patient group and in the control group ($p = 0.494$). The ADMA, CRP, urea, creatinine, and the glomerular filtration ratio (eGFR) levels, and ESR were statistically significantly higher in the patient group than in the controls ($p < 0.05$). The levels of NO, high-density lipoprotein (HDL)-cholesterol and triglycerides were statistically significantly lower in the patient group than in the controls ($p < 0.05$). There was no statistically significant difference between the L-arginine, total cholesterol, low-density lipoprotein (LDL)-cholesterol, or fibrinogen levels, or the L-arginine-to-ADMA ratio in patients compared to the control group ($p > 0.05$) (Table 1).

There was no statistically significant difference in the ADMA, NO or L-arginine levels, or the L-arginine-to-ADMA ratio among the different stroke subgroups (mild, moderate or severe, as determined by NIHSS) (Table 2).

There was a negative correlation between the NO levels and age ($r = -0.251$), and a positive correlation between the NO levels and eGFR ($r = 0.223$) (Fig. 1). In addition, there was a positive correlation between ADMA and ESR ($r = 0.202$), and between ADMA and creatinine ($r = 0.224$), and there was a negative correlation between ADMA and eGFR ($r = -0.216$): the 95% confidence interval (CI) eGFR lower and upper values were 77.81 and 90.0, respectively (Fig. 1).

According to the ROC curve analysis (Fig. 2), the NO and ADMA levels were found to be discriminative parameters in the patient group. According to the model, serum NO and ADMA levels may be risk factors for the patient group: the odds ratios (OR) and 95% CI of the risk factors were 1.58 (1.189–2.098) and 1.26 (0.943–1.875), respectively.

Table 1. Demographic characteristics and biochemistry parameters of patients and controls

Parameters	Controls (n = 48)	Patients (n = 52)	p-value
Age [years]	60.10 ±7.92	75.20 ±10.10	<0.05
Gender (M/F)	13/35	22/30	0.610
ADMA [μM]	0.40 ±0.11	0.46 ±0.13	0.005
Nitrate-to-nitrite ratio [μM]	4.34 ±2.70	2.78 ±1.59	0.000
L-arginine [μM]	67.40 ±5.90	69.30 ±8.65	0.529
L-arginine-to-ADMA ratio	201.45 ±63.4	220.50 ±72.30	0.681
Total cholesterol [mg/dL]	189.10 ±37.41	184.15 ±45.99	0.576
HDL-cholesterol [mg/dL]	43.21 ±10.91	37.32 ±10.82	0.010
LDL-cholesterol [mg/dL]	117.81 ±28.60	120.43 ±38.25	0.714
Triglycerides [mg/dL]	164.57 ±117.97	119.10 ±68.45	0.022
ESR [mm/h]	15.55 ±10.84	28.31 ±20.36	0.000
CRP [mg/dL]	0.54 ±0.14	1.66 ±2.64	0.008
Fibrinogen [mg/dL]	350.81 ±53.80	371.13 ±93.85	0.319
Urea [mg/dL]	25.57 ±7.29	39.77 ±22.80	0.000
Creatinine [mg/dL]	0.66 ±0.16	1.03 ±0.52	0.000
eGFR [mL/min/1.73 m^2]	100.30 ±24.04	71.66 ±30.00	0.000

ADMA – asymmetrical dimethylarginine; CRP – C-reactive protein; eGFR – glomerular filtration ratio; ESR – erythrocyte sedimentation rate; HDL – high-density lipoprotein; LDL – low-density lipoprotein; NO – nitric oxide; nitrate-to-nitrite ratio is a measure of NO levels.

Table 2. ADMA, NO, L-arginine, and L-arginine-to-ADMA ratios in subgroups of stroke according to NIHSS scores

Parameters	Control (n = 48)	Mild stroke (n = 3)	Moderate stroke (n = 40)	Severe stroke (n = 9)	p-value
ADMA [μM]	0.40 ±0.11	0.45 ±0.06	0.46 ±0.14	0.50 ±0.14	0.329
Nitrate-to-nitrite ratio [μM]	4.34 ±2.70	2.09 ±0.16	2.92 ±1.71	2.39 ±1.18	0.732
L-arginine [μM]	67.40 ±5.90	65.3 ±1.81	69.60 ±6.55	69.20 ±7.19	0.481
L-arginine-to-ADMA ratio	201.45 ±63.40	147.66 ±23.35	241.80 ±74.60	150.50 ±45.00	0.959

ADMA – asymmetrical dimethylarginine; NIHSS – National Institutes of Health Stroke Scale/Score; NO – nitric oxide; nitrate-to-nitrite ratio is a measure of NO levels.

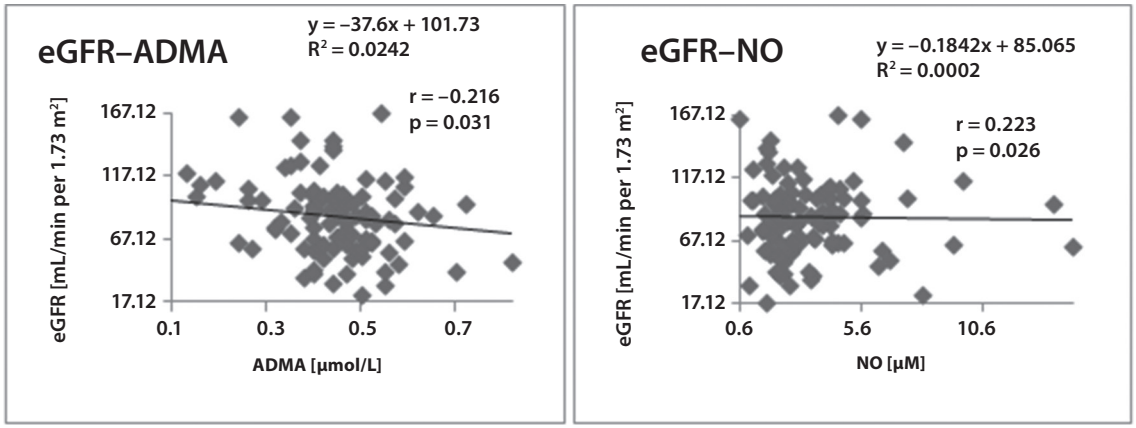

Fig. 1. eGFR–ADMA correlation (left), eGFR–NO correlation (right)

ADMA – asymmetrical dimethylarginine; eGFR – glomerular filtration ratio; NO – nitric oxide.

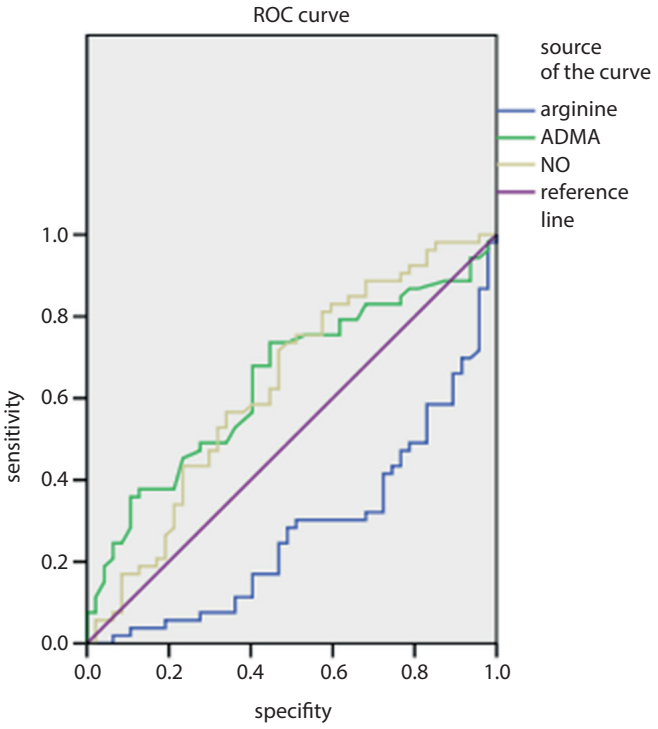

Fig. 2. ROC curve analyzing the discriminative parameters in patients with acute ischemic stroke

ADMA – asymmetrical dimethylarginine; NO – nitric oxide; ROC – receiver operating characteristic. Diagonal segments are produced by ties.

Discussion

Risk factors for stroke, such as HT, DM, smoking, hyperlipidemia, and hyperhomocysteinemia, are associated with endothelial dysfunction. The inhibition of endothelial NO plays an important role in the athero-thrombotic process.[14] Asymmetrical dimethylarginine, a post-translationally modified form of arginine,[15] is an endogenous inhibitor of NO synthase and is associated with atherosclerotic diseases.[14]

In this study, serum ADMA levels were increased and NO levels were decreased in patients who had suffered from an acute ischemic stroke as compared to controls. An increased serum ADMA level was determined to be an independent risk factor for ischemic stroke.

Although studies have examined the relationship between ADMA levels and coronary heart disease (CHD), HT, chronic renal failure, and hypercholesterolemia, studies that have investigated the relationship between ADMA levels and stroke are scarce. Yoo and Lee found that serum ADMA levels were significantly higher in 52 patients with stroke than in 36 healthy controls.[7] In our study, with a similar sample size to Yoo and Lee's study population, ADMA levels were also significantly higher in the patients with acute ischemic stroke than in the controls (p = 0.005).

Prospective clinical studies support the hypothesis that plasma ADMA concentration increases with ischemic

stroke risk factors and in patients with ischemic stroke. Mamatha et al. found significantly higher serum ADMA levels in stroke patients than in controls in a study that included 201 stroke patients and 217 controls ($p < 0.001$).[16] Increased plasma concentrations of ADMA have been reported to be an independent risk factor for ischemic stroke, after correcting for ischemic stroke risk factors (age, alcohol abuse, smoking, HT, DM, low serum HDL-cholesterol and homocysteine).[5] In our study, there was no correlation between ADMA and ischemic stroke risk factors (age, DM, HT, serum cholesterol, and serum triglycerides). In a similar study, Nishiyama et al. found that serum ADMA concentrations were significantly higher in 50 stroke patients and 116 individuals with vascular risk factors than in controls with no vascular risk factors.[5] Asymmetrical dimethylarginine concentrations were found to be associated with vascular risk factors and were suggested as a marker of a future ischemic stroke.[16]

Elevated ADMA levels have been demonstrated in some clinical conditions and in the presence of conventional cardiovascular risk factors, such as high blood pressure, serum cholesterol levels, serum triglycerides, gestational DM, insulin resistance, and smoking.[16–21] Furthermore, an association between ADMA and non-conventional cardiovascular risk factors, such as elevated levels of homocysteine, CRP, and the vascular cell adhesion molecule (VCAM) has been demonstrated.[22–24]

Acutely decreased NO levels result in vasoconstriction, an increased production of free radicals, platelet aggregation, and leukocyte adhesion on endothelial surfaces; these processes may in turn aggravate cerebral ischemia.[25] This finding suggests a mechanism for the pathogenesis of ischemic stroke.

In our study, we found significantly lower NO levels in the stroke patients compared to the controls ($p = 0.000$), suggesting cerebral ischemia due to acute ischemic stroke. Cerebrospinal fluid (CSF) ADMA levels were found to increase in parallel with stroke severity in a study by Brouns et al.[25] In our study, serum ADMA levels seemed to be higher in patients with more severe strokes; however, the association was not statistically significant ($p = 0.329$).

A recent study has suggested that renal function may have prognostic value for long-term survival in stroke patients and for the occurrence of cardiovascular events after an acute cerebral event.[26] In our study, a positive correlation was found between ADMA and creatinine levels ($r = 0.224$), and a negative correlation was observed between ADMA and eGFR ($r = -0.216$). Some methylarginines are excreted by the renal route. All symmetrical dimethylarginine (SDMA) is excreted by the renal route, whereas ADMA and N^G-monomethyl-L-arginine (L-NMMA) are metabolized extensively. The most important step in ADMA metabolism is its breakdown into citrulline and dimethylamine by dimethylarginine dimethylaminohydrolase (DDAH). The lack of SDMA measurements is another limitation of this study.

In conclusion, increased serum levels of the NOS inhibitor ADMA and decreased levels of NO may be independent risk factors for ischemic stroke. The small sample size in the stroke subgroups may be another limitation of this study; further studies with more participants should strengthen the findings of the study. Decreased NO levels cause vasoconstriction and may be important in the pathogenesis of ischemic stroke. Additional comprehensive studies are needed to validate ADMA and NO as routine risk factors of stroke.

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The effects of simvastatin on cellular viability, stemness and osteogenic differentiation using 3-dimensional cultures of stem cells and osteoblast-like cells

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Conflict of interest
None declared

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Abstract

Background. Simvastatin has been reported to increase the therapeutic effects of many kinds of stem cells by increasing the number of those cells. However, the effects of simvastatin on the differentiation potential of stem cells have not been clearly determined.

Objectives. The aim of the study was to evaluate the effects simvastatin has on cellular viability, stemness and osteogenic differentiation using 3-dimensional cell spheroids of stem cells and osteoblast-like cells.

Material and methods. Three-dimensional cell spheroids were fabricated using concave silicon elastomer-based microwells in the presence of simvastatin at concentrations of 1 μM and 10 μM. Qualitative cellular viability was determined with a confocal microscope, and quantitative cellular viability was evaluated using a cell-counting assay kit. The expression of stem cell surface markers was tested. A quantitative real-time polymerase chain reaction (qRT-PCR) was performed to evaluate the expression of collagen I and RUNx2. Alkaline phosphatase activity and alizarin red S staining were used to assess osteogenic differentiation.

Results. The spheroids formed well in the concave silicon elastomer-based microwells, and the application of simvastatin caused no significant morphological changes. No significant changes in cellular viability were noted with the addition of simvastatin on days 1, 3 and 5. Secretion of the vascular endothelial growth factor (VEGF) was observed on day 1 and remained stable throughout the culture period. Expression of the CD90 surface marker was seen on day 7. The addition of simvastatin caused a statistically significant increase in the expression of collagen I and RUNX2. It also caused decreases in alkaline phosphatase activity and alizarin red S staining.

Conclusions. The study clearly showed that the application of simvastatin enhanced collagen I and RUNX2 expression; however, this did not lead to increases in alkaline phosphatase activity or alizarin red S staining.

Key words: gingiva, simvastatin, stem cells, cell differentiation, cellular spheroids

Simvastatin is a chemical modification of lovastatin, a rate-limiting enzyme of the cholesterol synthesis pathway.[1,2] Simvastatin has been reported to promote osteoblastic activity and inhibit osteoclastic activity.[3] Previous reports have shown that simvastatin positively affects the differentiation and mineralization of osteoprecursor cells through the estrogen receptor pathway by increasing estrogen receptor-α expression.[4] A combination of simvastatin and bone morphogenetic protein-2 enhanced the differentiation of osteoprecursor cells through the bone morphogenetic protein pathway by enhancing pSmad1/5/8 expression.[5]

The effects of simvastatin on mesenchymal stem cells (MSCs) have been observed in previous studies.[6–9] Simvastatin has been reported to increase the therapeutic effects of nucleus pulposus stem cells by increasing the number of those cells.[8] It has also been shown to induce enhanced proliferation of cultured neural stem cells.[7] Conversely, simvastatin has been reported to negatively modulate bone marrow MSC proliferation in a dose-dependent manner and to regulate the expression of proliferation-related genes.[6] One report showed that simvastatin both directly interfered with the proliferation of stem cells and promoted cell death.[9] Moreover, the effects of simvastatin on the differentiation potential of stem cells have not been clearly determined. Recently, 3-dimensional culture systems using stem cells have been applied to cell therapy.[10] These stem-cell spheroids maintained the viability and stem-cell characteristics of stem-cell surface-marker expression and differentiation potential.[11] In a previous study, cell spheroids from gingival cells and osteoprecursor cells were shown to be a promising strategy for stem-cell therapy.[12] The current study was therefore performed to evaluate the effects of simvastatin on cellular viability, stemness and osteogenic differentiation using 3-dimensional cell spheroids of stem cells and osteoblast-like cells.

Material and methods

Formation of cell spheroids with human gingiva-derived stem cells and osteoblast-like cells

Stem cell spheroids were formed in concave silicon elastomer-based microwells (StemFit 3D; Micro Fit Co. Ltd., Seongnam, South Korea) with a diameter of 600 μM. Gingiva-derived stem cells (GMSCs) and osteoblast-like cells (MC3T3-E1 cells; American Type Culture Collection, Manassas, USA) were plated in a 1:1 ratio (5×10^5 cells/well), cultured in an alpha-minimal essential medium (α-MEM; Gibco, Grand Island, USA) with a total volume of 1.5 mL, and then supplemented with 15% fetal bovine serum (Gibco), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Sigma-Aldrich, St. Louis, USA), 200 mM of L-glutamine (Sigma-Aldrich), 10 mM of ascorbic acid 2-phosphate (Sigma-Aldrich), 38 μg/mL of dexamethasone (Fujifilm Wako Pure Chemical Corp., Osaka, Japan), and 2 mg/mL of glycerophosphate disodium salt hydrate (Sigma-Aldrich). The culturing was performed in the presence of simvastatin (Sigma-Aldrich) at concentrations of 0 μM (the untreated control group), 1 μM and 10 μM with dimethyl sulfoxide (DMSO) used as a dissolving agent. The media were changed every second day. The GMSCs were obtained from the mandibular area of a healthy female participant undergoing second-stage implant surgery, using a previously reported method.[13] The study design was reviewed and approved by the Institutional Review Board of Seoul St. Mary's Hospital (College of Medicine, Catholic University of Korea, Seoul, South Korea; approval No. KC11SISI0348), and informed consent was obtained from the patient. On days 1, 3 and 5, inverted microscopy (Olympus Corporation, Tokyo, Japan) was used to evaluate the morphology of the stem cells being tested.

Determination of cell viability

The viability of the spheroids was qualitatively analyzed by an assay using the Live/Dead Kit (Molecular Probes, Eugene, USA). The spheroids were washed twice with the growth media, followed by suspension in 2 mL of α-MEM (containing 1 μL of 4 mM calcein acetoxymethyl ester working solution and 4 μL of 2 mM ethidium homodimer-1) for 30 min at room temperature. The spheroids stained with calcein acetoxymethyl ester and ethidium homodimer-1 were observed under a confocal laser microscope (LSM800 w/Airyscan; Carl Zeiss, Oberkochen, Germany) on days 3 and 5 (n = 3).

Quantitative cell-viability analyses were performed on days 1, 3 and 5. Using a Dojindo Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan), we added 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8) to the cultures, and the spheres were incubated for 1 h at 37°C. Viable cells were identified through the assay, which relies on the ability of mitochondrial dehydrogenases to oxidize WST-8 into a formazan product. The spectrophotometric absorbance of the samples was measured at 450 nm using a microplate reader (BioTek Instruments Inc., Winooski, USA) (n = 3).

Evaluation of the secretion of human VEGF for paracrine effects

The 3-dimensional systems were used to determine the amount of human vascular endothelial growth factor (VEGF) on days 1, 3 and 5 using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine ELISA; R&D Systems, Inc., Minneapolis, USA). All the reagents and samples were prepared according to the manufacturers' recommendations. The absorbance levels at 450 nm and 570 nm were measured, and the differences were used as the values for the paracrine effects (n = 2).

Evaluation of maintenance of stemness

Approximately 1.5×10^5 GMSCs and 1.5×10^5 osteoblast-like cells were incubated with specific fluorescein isothiocyanate-conjugated mouse monoclonal antibodies for human CD90, CD 73 and CD 34 (BD Biosciences, San Jose, USA) (n = 2). A flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences).

Total RNA extraction and quantification using RT-PCR

The cell spheroids were harvested on day 7. Total RNA was isolated using a GeneJET RNA Purification Kit (ThermoFisher Scientific Inc., Waltham, USA), and the quantities were determined with an ND-2000 spectrophotometer (ThermoFisher Scientific Inc.) using ratios of absorbance at 260 nm and 280 nm. The sense and antisense primers were based on GenBank. The primer sequences were as follows: RUNX2 Forward 5' – AAT GAT GGT GTT GAC GCT GA – 3'; Reverse 5' – TTG ATA CGT GTG GGA TGT GG – 3'; Collagen I Forward 5' – TCA TGG CCC TCC AGC CCC CAT3'; and Reverse 5' – ATG CCT CTT GTC CTT GGG GTT C – 3'. β-actin served as a housekeeping gene for normalization. The mRNA expression was detected with real-time polymerase chain reaction (RT-PCR) using SYBR Green Real-Time PCR Master Mixes (Enzynomics Inc., Daejeon, South Korea) according to the manufacturer's protocol. The quantitative RT-PCR experiments were conducted 3 times.

Alkaline phosphatase activity assays and alizarin red S staining

Cell spheroids that had been grown on culture plates with an osteogenic medium were harvested on day 14. Alkaline phosphatase activity assays were performed with a commercially available kit (BioVision Inc., Milpitas, USA). The cells were suspended again with an assay buffer, sonicated and then centrifuged to remove insoluble material. The supernatant was mixed with a p-nitrophenylphosphate substrate and incubated at 25°C for 60 min. The optical density of the resultant p-nitrophenol at 405 nm was determined spectrophotometrically (n = 3).

On days 7 and 14, the spheroids were washed twice with phosphate-buffered saline (PBS) (Welgene, Gyeongsan, South Korea), fixed with 4% paraformaldehyde and rinsed twice with deionized water. The cultures were then stained with alizarin red S for 30 min at room temperature. To remove nonspecifically-bound stains, the cultures were washed 3 times with deionized water. A morphological evaluation was then performed using a Leica DM IRM inverted microscope (Leica Camera AG, Wetzlar, Germany) (n = 3).

Statistical analysis

The data is presented as means ± standard deviations (SD). A test of normality was conducted and a one-way analysis of variance (ANOVA) with a post hoc test were performed to determine the differences between the groups. The analysis was done using SPSS v. 12 software for Windows (SPSS Inc., Chicago, USA), and the level of significance was $p < 0.05$.

Results

Evaluation of cell morphology and viability

On day 1, the spheroids were properly formed in concave silicon elastomer-based microwells (Fig. 1A). The addition of simvastatin caused no significant changes in the morphology on day 1 (Fig. 1B,C). The morphological results for days 3 and 5 are shown in Fig. 1D–I. In general, the shapes of the cells in the experimental groups were similar to those in the control group (0 μM).

Using a confocal microscope, the cellular viability was determined using live/dead assays on days 3 and 5, as shown in Fig. 2 and 3, respectively. For day 3, most of the cells in the spheroids emitted green fluorescence and had a round morphology (Fig. 2). Similarly, most of the cells in the spheroids emitted green fluorescence, but an increase in red fluorescence was noted at the higher dose of simvastatin (10 μM). The CCK-8 results for days 1, 3 and 5 are shown in Fig. 4. In terms of quantitative results, no significant changes in cellular viability were noted with the addition of simvastatin on days 1, 3 and 5.

Secretion of human VEGF from spheroids and expression of stem cell markers

The results clearly showed that VEGF secretion was observable on the 1st day, and that stable VEGF secretion occurred throughout the culture period (Fig. 5). A decrease in VEGF secretion was noted with the addition of simvastatin (1 μM) on day 3. A statistically significant decrease in secretion was noted with the addition of simvastatin (1 μM and 10 μM) on day 5 ($p < 0.05$).

Expression of the CD90, CD73 and CD34 surface markers was seen on day 7 (Fig. 6). The percentage of CD90 was 89.7% for the untreated control group (0 μM), 86.0% for the 1 μM group and 77.1% for the 10 μM group. The percentage of CD73 was 83.5% for the untreated control group (0 μM), 82.9% for the 1 μM group and 65.9% for the 10 μM group. The percentage of CD34 was 1.4% for the untreated control group (0 μM), 1.1% for the 1 μM group and 3.2% for the 10 μM group.

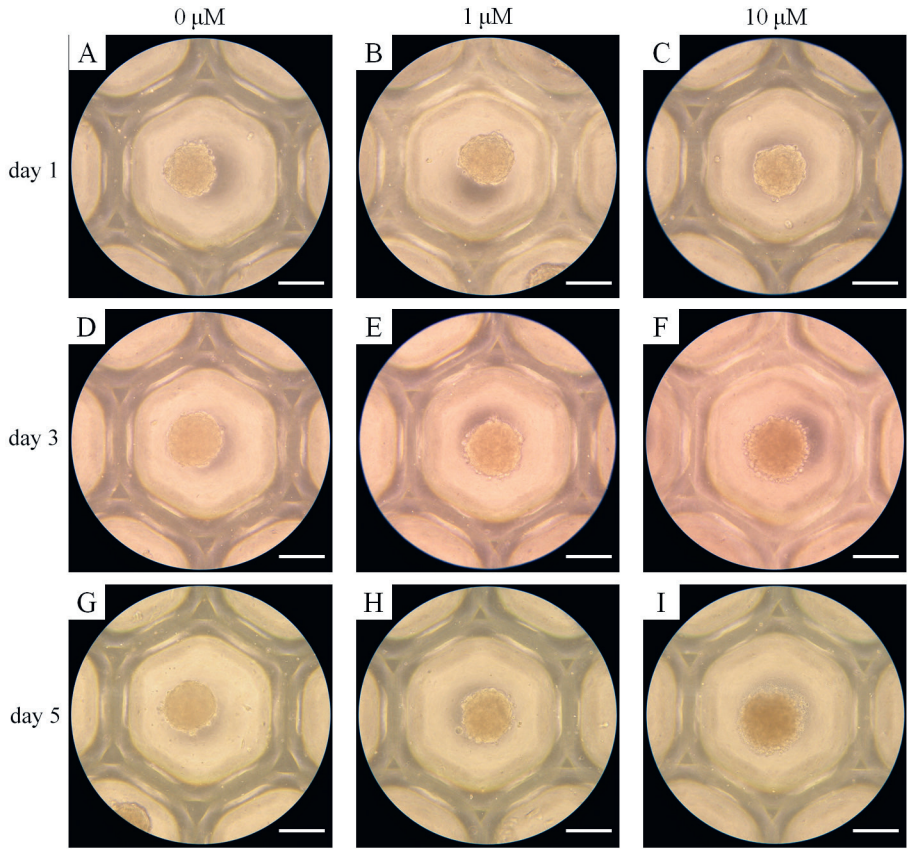

Fig. 1. Morphology of cell spheroids cultured in osteogenic media, the simvastatin 1 μM group and the simvastatin 10 μM group

(A) Morphology of the cell spheroids on day 1 for the untreated control group (original magnification ×200)
(B) Morphology of the cell spheroids on day 1 for the simvastatin 1 μM group (original magnification ×200)
(C) Morphology of the cell spheroids on day 1 for the simvastatin 10 μM group (original magnification ×200)
(D) Morphology of the cell spheroids on day 3 for the untreated control group (original magnification ×200)
(E) Morphology of the cell spheroids on day 3 for the simvastatin 1 μM group (original magnification ×200)
(F) Morphology of the cell spheroids on day 3 for the simvastatin 10 μM group (original magnification ×200)
(G) Morphology of the cell spheroids on day 5 for the untreated control group (original magnification ×200)
(H) Morphology of the cell spheroids on day 5 for the simvastatin 1 μM group (original magnification ×200)
(I) Morphology of the cell spheroids on day 5 for the simvastatin 10 μM group (original magnification ×200)
The scale bar indicates 200 μM.

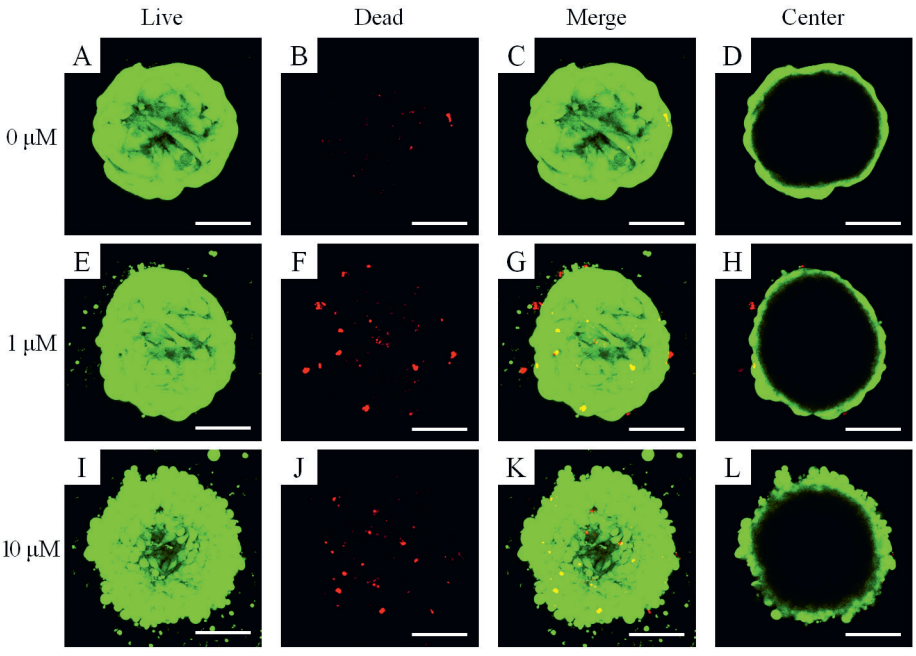

Fig. 2. Qualitative results of cellular viability under a confocal microscope on day 3

(A) Live image of the untreated control group on day 3 (original magnification ×100)
(B) Dead image of the untreated control group on day 3 (original magnification ×100)
(C) Merged image of the untreated control group on day 3 (original magnification ×100)
(D) Central image of the untreated control group on day 3 (original magnification ×100)
(E) Live image of the simvastatin 1 μM group on day 3 (original magnification ×100)
(F) Dead image of the simvastatin 1 μM group on day 3 (original magnification ×100)
(G) Merged image of the simvastatin 1 μM group on day 3 (original magnification ×100)
(H) Central image of the simvastatin 1 μM group on day 3 (original magnification ×100)
(I) Live image of the simvastatin 10 μM group on day 3 (original magnification ×100)
(J) Dead image of the simvastatin 10 μM group on day 3 (original magnification ×100)
(K) Merged image of the simvastatin 10 μM group on day 3 (original magnification ×100)
(L) Central image of the simvastatin 10 μM group on day 3 (original magnification ×100)
The scale bar indicates 100 μM.

Validation of mRNA expression using RT-PCR

The quantitative RT-PCR results for the mRNA levels of collagen I and RUNX2 are shown in Fig. 7. The addition of simvastatin produced statistically significant increases in the expression of collagen I and RUNX2. For the untreated control group, the 1 μM group and the 10 μM group, the relative expressions of collagen I were 100.0 ±1.7%, 164.1 ±2.4% and 898.4 ±1.2%, respectively ($p < 0.05$), and the relative expressions of RUNX2 were 100.0 ±1.2%, 91.5 ±0.6% and 150.1 ±0.9%, respectively ($p < 0.05$).

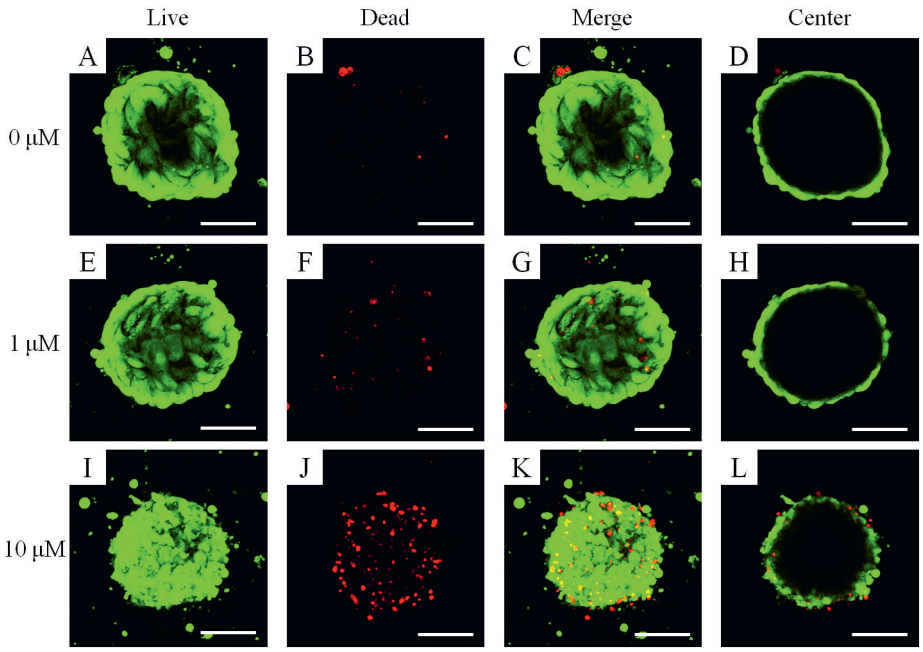

Fig. 3. Qualitative results of cellular viability under a confocal microscope on day 5

(A) Live image of the untreated control group on day 5 (original magnification ×100)
(B) Dead image of the untreated control group on day 5 (original magnification ×100)
(C) Merged image of the untreated control group on day 5 (original magnification ×100)
(D) Central image of the untreated control group on day 5 (original magnification ×100)
(E) Live image of the simvastatin 1 μM group on day 5 (original magnification ×100)
(F) Dead image of the simvastatin 1 μM group on day 5 (original magnification ×100)
(G) Merged image of the simvastatin 1 μM group on day 5 (original magnification ×100)
(H) Central image of the simvastatin 1 μM group on day 5 (original magnification ×100)
(I) Live image of the simvastatin 10 μM group on day 5 (original magnification ×100)
(J) Dead image of the simvastatin 10 μM group on day 5 (original magnification ×100)
(K) Merged image of the simvastatin 10 μM group on day 5 (original magnification ×100)
(L) Central image of the simvastatin 10 μM group on day 5 (original magnification ×100)
The scale bar indicates 100 μM.

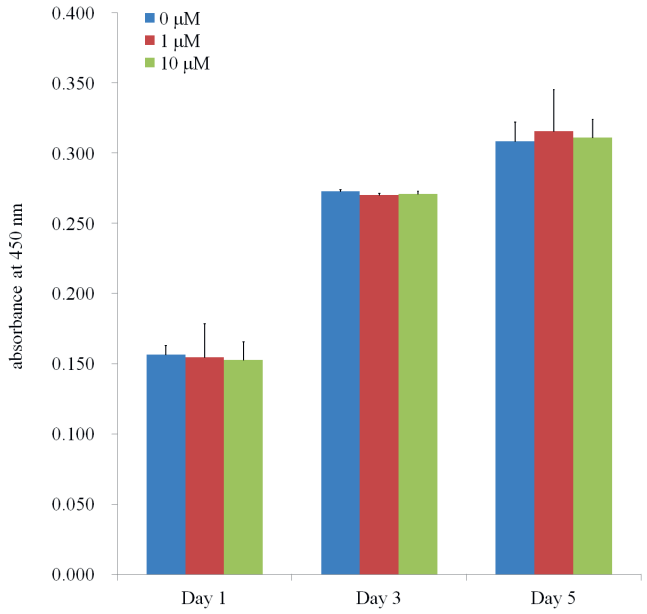

Fig. 4. Cellular viability on days 1, 3 and 5 using CCK-8. No significant changes in cellular viability were noted with the addition of simvastatin on days 1, 3 and 5

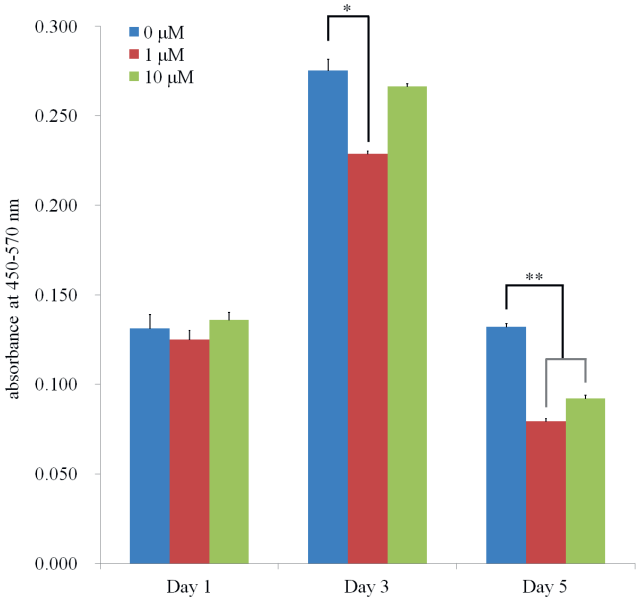

Fig. 5. Secretion of VEGFs from cell spheroids on days 1, 3 and 5. A decrease in VEGF secretion was noted with the addition of simvastatin (1 μM) on day 3. A statistically significant decrease in secretion was noted with the addition of simvastatin (1 μM and 10 μM) on day 5

Alkaline phosphatase activity assays and alizarin red S staining

Alkaline phosphatase activity decreased on day 14 after treatment with simvastatin, as shown in Fig. 8. The relative values on day 14 for the untreated control group, the 1 μM group and the 10 μM group were 100.0 ±1.6%, 49.3 ±0.4% and 25.8 ±0.0%, respectively.

Mineralized extracellular deposits were evaluated using alizarin red S staining on days 7 and 14 (Fig. 9) and were found to be equivalent in all 3 groups. An increase in mineralized deposits was noted between day 7 and day 14. Alizarin red S staining decreased with the addition of simvastatin.

Discussion

This report discusses the effects of simvastatin on cellular viability, stemness and osteogenic differentiation using 3-dimensional cell spheroids of stem cells and osteoblast-like cells. The addition of simvastatin clearly enhanced

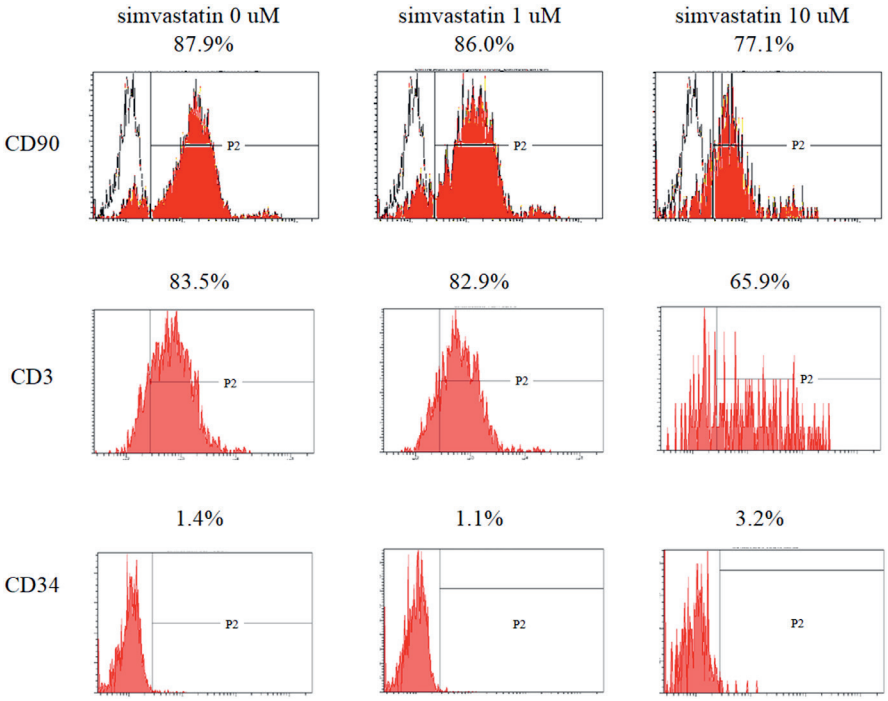

Fig. 6. Evaluation of stem cell surface marker expression using CD90, CD73 and CD34 on day 7. The percentage of CD90 was 89.7% for the untreated control group (0 μM), 86.0% for the 1 μM group and 77.1% for the 10 μM group. The percentage of CD73 was 83.5% for the untreated control group (0 μM), 82.9% for the 1 μM group and 65.9% for the 10 μM group. The percentage of CD34 was 1.4% for the untreated control group (0 μM), 1.1% for the 1 μM group and 3.2% for the 10 μM group

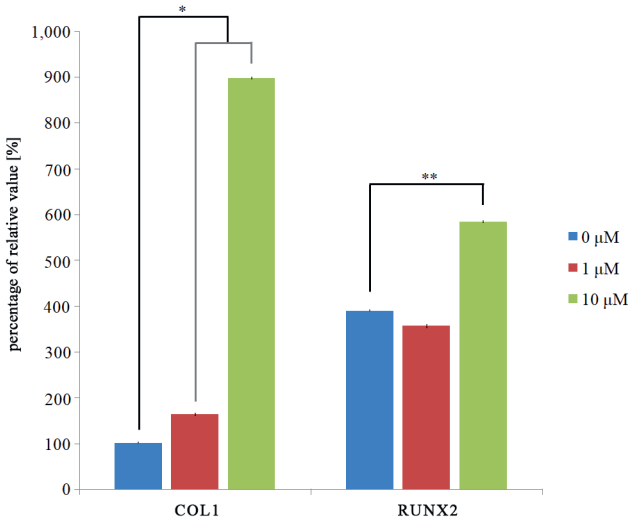

Fig. 7. Quantitative real-time polymerase chain reaction results for collagen I and RUNX2 expression on day 7. The addition of simvastatin produced statistically significant increases in the expression of collagen I and RUNX2. For the untreated control group, the 1 μM group, and the 10 μM group, the relative expressions of collagen I are 100.0 ±1.7%, 164.1 ±2.4% and 898.4 ±1.2%, respectively, and the relative expressions of RUNX2 are 100.0 ±1.2%, 91.5 ±0.6% and 150.1 ±0.9%, respectively

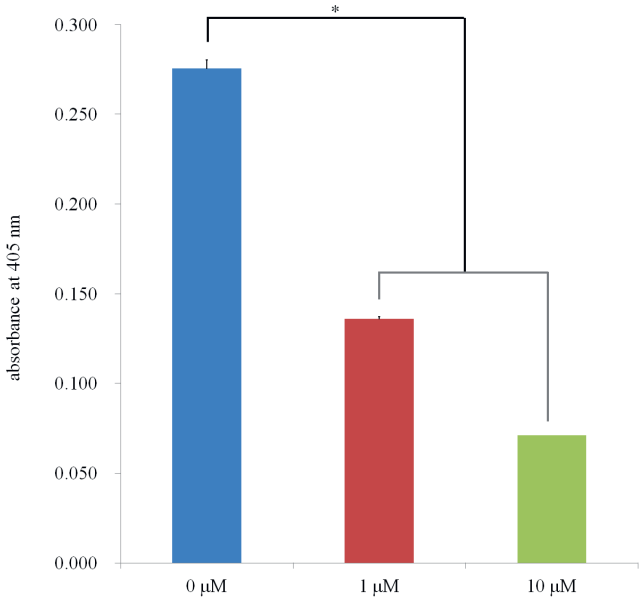

Fig. 8. Alkaline phosphatase activity on day 14. The relative values on day 14 for the untreated control group, the 1 μM group and the 10 μM group are 100.0 ±1.6%, 49.3 ±0.4% and 25.8 ±0.0%, respectively

collagen I and RUNX2 expression, but it did not lead to increased alkaline phosphatase activity or increased alizarin red S staining.

Simvastatin has been shown to increase therapeutic effects by enhancing stem cell function.[8] Simvastatin induces increased pluripotency and protects against cellular senescence in MSCs.[6] It also improves the migration of bone marrow-derived MSCs via the PI3K/AKT pathway,[14] and enhances the neurogenesis of cultured neural stem cells and elevates Notch-1 protein expression.[7] Simvastatin exhibits increased dentin sialoprotein and calcium deposition, and it has been suggested that it can promote odontoblastic differentiation in dental pulp stem cells.[15] Moreover, previous reports have shown that simvastatin stimulates osteogenic effects in bone marrow-derived MSCs.[16] It has also been shown that it enhances the osteogenic differentiation of stem cells without affecting their immunosuppressive properties.[17] Additionally, simvastatin increases bone morphogenetic protein-2 gene expression on titanium surfaces that have been sandblasted with large

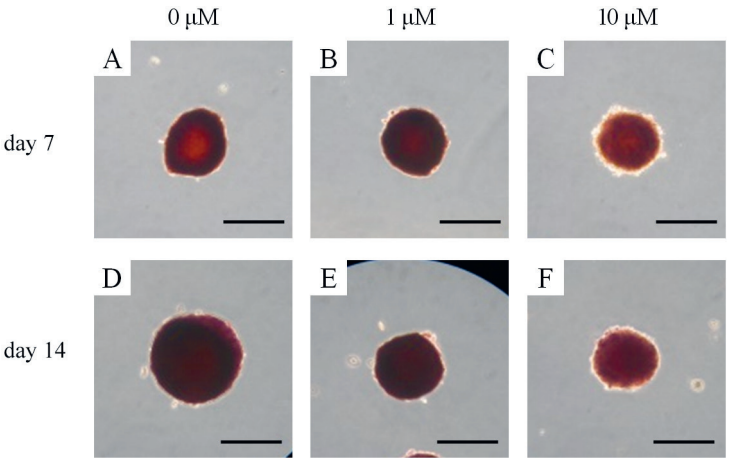

Fig. 9. Mineralization assay using alizarin red S staining

(A) Alizarin red S staining results on day 7 for the untreated control group (original magnification ×200)
(B) Alizarin red S staining results on day 7 for the simvastatin 1 μM group (original magnification ×200)
(C) Alizarin red S staining results on day 7 for the simvastatin 10 μM group (original magnification ×200)
(D) Alizarin red S staining results on day 14 for the untreated control group (original magnification ×200)
(E) Alizarin red S staining results on day 14 for the simvastatin 1 μM group (original magnification ×200)
(F) Alizarin red S staining results on day 14 for the simvastatin 10 μM group (original magnification ×200)
The scale bar indicates 100 μM.

grit and etched with acid.[18] To add to these observations, the present study showed that simvastatin enhanced collagen I and RUNX2 expression.

This report discusses the effects that simvastatin has on the viability and differentiation of cells at predetermined concentrations (1 μM and 10 μM). Previous studies have shown that simvastatin at concentrations of 0.1 μM and 1 μM can be used for the differentiation of osteoblast-like cells[4]. In another report, 0.01 μM and 0.1 μM concentrations of simvastatin were used for the osteoblastic differentiation of osteoblast-like cells.[19] In a previous report, 1 μM concentration of simvastatin was applied to bone marrow-derived MSCs[17]; simvastatin significantly decreased the proliferation of the MSCs and notably enhanced osteogenic differentiation.[20] The present study clearly proved that at 1 μM and 10 μM concentration, simvastatin had significant effects on a 3-dimensional culture model. Variations in doses could be used to achieve optimal effects based on the culture system, the duration of the culture period and the type of cells.[4,19]

Various scaffolds have been utilized for simvastatin use.[15,21–24] A hydrogel composed of gelatin-poly(ethylene glycol)-tyramine has been used as an efficient simvastatin delivery vehicle to trigger osteogenic differentiation.[21] Calcium phosphate composite scaffolds containing simvastatin-loaded poly(lactic-co-glycolic acid) microspheres have been fabricated, and MSCs have been seeded onto the scaffolds.[22] Simvastatin has been incorporated into the micelles of gelatin grafted with L-lactic acid oligomers, and enhanced alkaline phosphatase activity was achieved.[15] Simvastatin has been incorporated into mesoporous hydroxyapatite microspheres; this system led to superior bone regeneration from sustained release.[23] It has also been incorporated into hydroxyapatite in poly(ε-caprolactone)/polylactic acid polymeric scaffolds for osteogenic bioengineering.[24]

Simvastatin-stimulated osteogenesis is mediated by estrogen receptor α.[16] It has also been noted that simvastatin enhances the Rho/actin/cell rigidity pathway, contributing to osteogenic differentiation of MSCs; both keeping the actin cytoskeletons intact and enhancing cell rigidity is crucial in simvastatin-induced osteogenesis.[25] The current study added the observation that simvastatin was involved with the RUNX2 pathway.

In this study, cell viability was evaluated using a CCK-8 assay. CCK-8 is based on mitochondrial enzyme activity and quantification of the formazan generated.[12,26] Alkaline phosphatase activity is used as an early marker of osteogenesis.[27] Alizarin red S staining is used to evaluate the quantity of calcium deposits after matrix maturation.[28] There may be limitations for applying these assays to spheroid cultures because they were initially dedicated for 2-dimensional cultures.

This study clearly showed that the application of simvastatin enhances collagen I and RUNX2 expression but does not lead to increased alkaline phosphatase activity or increased alizarin red S staining.

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