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Preoperative and postoperative risk factors in laparoscopic cholecystectomy converted to open surgery

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Abstract

Background. Laparoscopic cholecystectomy (LC) is nowadays the gold standard in the surgical treatment of cholelithiasis and gallbladder diseases. But sometimes it may be inevitable to convert it to open surgery to safely end the procedure.

Objectives. In this study, we aimed to investigate the risk factors for conversion to open surgery from LC.

Material and methods. The records of patients that underwent LC in Malatya State Hospital (Malatya, Turkey) between January 2013 and May 2014 were prospectively examined. One hundred and forty-five patients were involved in this study. The patients were divided into 2 groups: LC patients and patients converted to open surgery. For the patients in both groups, the preoperative age, gender, body mass index (BMI), disease history, previous abdominal operations, and preoperative laboratory findings were recorded, as well as the fact if the abdominal ultrasonography (US) and endoscopic retrograde cholangiopancreatography (ERCP) were performed.

Results. Of 145 patients involved in this study, 127 (87.5%) were female and 18 (12.5%) were male; their mean age was 46.54 years. Nineteen of the patients were operated on after ERCP due to acute cholecystitis and 6 patients were operated on after ERCP due to choledocholithiasis. In 134 of the patients (92.4%), the operations were completed laparoscopically, while the process was converted to open surgery in 11 cases (7.6%). Male gender, chronic disease history, normal BMI level, increased thickness of the gallbladder wall, increased preoperative blood glucose level, leukocytosis, preoperative ERCP history, grade 3 or 4 (Blauer scoring system) adhesions determined during the operation, and multiple stone presence in the bladder were found to be statistically significant risk factors for conversion to open surgery.

Conclusions. Patients in the risk group should be informed by experienced laparoscopic surgeons about the potential conversion to open surgery and decision on such conversion should be made when necessary.

Key words: cholelithiasis, laparoscopic cholecystectomy, conversion

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Introduction

Laparoscopic cholecystectomy (LC) is superior to open cholecystectomy because of less postoperative pain, shorter hospitalization duration, shorter time for returning to daily activities, less surgical scarring, and better cosmetic results.[1] The reason for conversion to open surgery during LC is to prevent severe complications that may occur during the operation.[2] Conversion to open surgery should not be considered a complication, but as a procedure necessary to complete the operation safely. Knowing the risk factors for conversion to open surgery is important for informing the patient about this topic.

The aim of this study is to determine the risk factors leading to conversion from LC to open surgery.

Material and methods

One hundred and forty-five patients who underwent LC surgery in Malatya State Hospital (Malatya, Turkey) between January 2013 and May 2014 were involved in this study and their data was retrospectively examined. One hundred and forty-one patients were operated on due to choledocholithiasis, while 4 patients were operated on due to gallbladder polyp. The operations were performed by 3 exprienced surgeons in the American position with the classic 4 trocars method. Dissection was performed with a dissector in Calot's triangle. The patients were divided into 2 groups: LC patients and patients converted to open surgery. For the patients in both groups, the preoperative age, gender, body mass index (BMI), disease history, previous abdominal operations, and preoperative laboratory findings were recorded, as well as the fact if the abdominal ultrasonography (US) and endoscopic retrograde cholangiopancreatography (ERCP) were performed. The findings during the operation were also recorded. A >3 mm increase in the gallbladder wall in ultrasonography was considered increased wall thickness (acute cholecystitis). Fasting blood glucose (FBG), number of white blood cells (WBC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP) values were examined and recorded. White blood cells >10,000/mm^3, AST > 35 IU/L, ALT > 55 IU/L, GGT > 65 IU/L, ALP > 150 IU/L, and FBG > 105 mg/dL values were considered increased.

In all of the patients, the level of abdominal adhesion was evaluated in accordance with the Blauer scoring system (grade 0 – no adhesion; grade 1 – thin and narrow, and easy-to-separate adhesions; grade 2 – thick adhesions limited to a certain region; grade 3 – thick and widespread adhesion; grade 4 – thick and widespread adhesion, adhesion of viscera to the front and back abdominal wall).[3] Body mass index <20 was considered thin, 20–25 was considered normal, 25–30 – overweight, 30–35 – obese, and >35 – morbidly obese.

For statistical analyses, SPSS v. 18.0 software (SPSS Inc., Chicago, USA) was used. Evaluation of risk factors was performed using Pearson's χ^2 test. P-value <0.05 was considered statistically significant.

Results

Of the 145 patients involved in this study, 127 (87.5%) were male and 18 (22.5%) were female. Their mean age was 46.54 years (17–84). While the operation was accomplished laparoscopically in 134 (92.4%) patients, it was converted to open surgery in 11 (7.6%) cases. The reasons for conversion to open surgery are presented in Table 1. No complications were observed during or after the surgery.

It was observed that the conversion to open surgery was statistically significantly more frequent in male patients than in female patients ($p < 0.01$). Mean BMI of the patients was calculated to be 28.9. The risk of conversion to open surgery was statistically significantly higher in patients in the normal BMI category ($p = 0.01$) (Table 2). It was found to be statistically significantly higher in patients having chronic disease or increased gallbladder wall thickness in ultrasonography, and in those who underwent ERCP before the operation. The adhesion of the gallbladder to adjacent organs during the operation was found

Table 1. Reasons for conversion from laparoscopic cholecystectomy (LC) to open surgery

Reason	Number of patients
Adhesion	6 (54.5%)
Hemorrhage	2 (18.2%)
Cancer Mirizzi syndrome	1 (9.1%) 2 (18.2%)
Total	11

Table 2. Demographic factors

Parameter	LC group (n = 134 (92.4%))	Open surgery group (n = 11 (7.6%))	p-value
Advanced age >mean age (46.54 years) <mean age (46.54 years)	 61 (45.5%) 73 (54.5%)	 8 (73.0%) 3 (27.0%)	0.82
>65 years <65 years	15 (11.2%) 119 (88.8%)	1 (9.1%) 10 (90.9%)	0.83
Gender female male	 21 (90.3%) 13 (9.7%)	 6 (54.5%) 5 (45.5%)	0.005
BMI >mean BMI (28.91) <mean BMI (28.91)	 64 (47.7%) 70 (53.3%)	 3 (27.7%) 8 (73.7%)	0.19
BMI 20–25	16 (11.9%)	5 (45.5%)	0.01
BMI 25–30	55 (41%)	4 (36.3%)	
BMI >30	51 (38%)	2 (18.2%)	

LC – laparoscopic cholecystectomy; BMI – body mass index.

Table 3. History, physical examinations and intra-operative findings

Parameter	LC group (n = 134 (92.4%))	Open surgery group (n = 11 (7.6%))	p-value
Chronic disease	40 (29.9%)	7 (63.6%)	0.02
Previous abdominal surgery	30 (22.3%)	1 (9%)	0.3
ERCP history	5 (3.7%)	1 (9%)	0.03
Former cholecystitis attack	53 (39.5%)	7 (63.6%)	0.11
Increased wall thickness in USG	13 (9.7%)	6 (54.5%)	0.002
Multiple stone presence	88 (65.6%)	10 (90.9%)	0.03
Pre-operative adhesion grade 0 grade 1–2 grade 3–4	 59 (40.8%) 75 (51.7%) 11 (7.5%)	 0 (0%) 0 (0%) 11 (100%)	0.0001

LC – laparoscopic cholecystectomy; ERCP – endoscopic retrograde cholangiopancreatography.

Table 4. Laboratory values

Parameter	LC group (n = 134 (92.4%))	Open surgery group (n = 11 (7.6%))	p-value
WBC > 10,000/mm^3	17 (12.6%)	4 (36.6%)	0.03
AST > 35 IU/L	14 (10.4%)	1 (9%)	0.88
ALT > 55 IU/L	14 (10.4%)	0 (0%)	0.25
GGT > 65 IU/L	18 (13.4%)	1 (9%)	0.68
ALP > 150 IU/L	7 (5.2%)	0 (0%)	0.43
Glucose >105 mg/dL	40 (29.8%)	7 (63.3%)	0.02

LC – laparoscopic cholecystectomy; WBC – number of white blood cells; AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma-glutamyl transferase; ALP – alkaline phosphatase.

to be a risk factor for conversion to open surgery. According to the Blauer adhesion classification, there were grade 3 or 4 adhesions in all of the patients (11 cases) in case of whom operations were converted to open surgery ($p < 0.01$). In 75 patients having grade 1 and 2 adhesion and 59 patients having no adhesion (grade 0), the operation was accomplished laparoscopically. Among the reasons that may lead to adhesion, abdominal operation history was present in 31 patients. Conversion to open surgery was performed in only 1 of those patients (Table 3).

It was observed that the risk of conversion to open surgery was statistically significantly higher in patients having a preoperative blood glucose level higher than 105 mg/dL ($p = 0.02$). Of 17 patients having preoperative leukocytosis, 4 were converted to open surgery and that was statistically significant ($p < 0.03$) (Table 4).

Discussion

Laparoscopic cholecystectomy is presently the gold standard in surgical treatment of cholelithiasis and gallbladder diseases. But sometimes it may be inevitable to convert it to open surgery to safely end the procedure. Since the first LC in 1987, because of advancements in surgical experience and devices, the rate of conversion to open surgery has decreased gradually. In current publications, this rate is reported to be between 6.3% and 11.5%.[4,5] It was found to be 7.6% in our study.

Conversion to open surgery in LC is not a complication, but it may be a necessity to safely end the procedure. The real complications in LC are hemorrhage, perforation of the gallbladder, biliary leakage, biliary tract injury, and organ injury.[6] Conversion to open surgery may prevent these potential complications. In order to inform the patient before the operation and to continue the treatment process, it is important to determine the risk factors requiring the conversion to open surgery.

In much of the literature, it has been reported that advanced age, male gender, acute cholecystitis attack history, and accompanying chronic diseases are risk factors for conversion to open surgery.[6–11] In our study, we determined that male gender and accompanying chronic diseases may be risk factors for conversion to open surgery but advanced age (>65 years) and previous acute cholecystitis attack are not be risk factors. In patients operated on during acute cholecystitis attack, having increased bladder wall thickness and hydropic gallbladder, the conversion to open surgery increased statistically significantly ($p < 0.05$).

Salman et al. have determined that BMI >27 might be a risk factor for conversion to open surgery. They have stated that this risk may be caused by increased intraperitoneal fatty tissue. They believe that this increase in fatty tissue may make it difficult to control hemorrhage during the dissection.[11] But we determined that in the normal BMI group (20–25) frequency of conversion to open surgery was statistically significant ($p < 0.05$). High BMI was not found to be a risk factor for conversion to open surgery.

For patients with abdominal surgery history, the problem during LC is intraabdominal adhesions. Although intraabdominal adhesions generally depend on a surgical intervention and they are also seen in cases of peritonitis, endometriosis, pelvic inflammatory disease, long-lasting peritoneal dialysis, radiotherapy, and cancer.[12] In many studies, it has been stated that abdominal and epigastrium surgery might be a risk factor for conversion to open surgery.[13,14] However, there also are publications stating that it is not a risk factor.[15,16] In our study, we determined that prior abdominal surgery was not a risk factor but an adhesion makes dissection in Calot's triangle more difficult and this is a risk factor for conversion to open surgery ($p < 0.05$).

For patients having pancreatitis attack during the laparoscopic operation or obstruction in the biliary tract, operation after ERCP is recommended.[17] The ERCP has been reported to be a risk factor for conversion to open surgery due to the adhesions that may develop after sphincterotomy.[11] In our study, we determined that ERCP performed before the operation is a risk factor ($p < 0.05$).

Many authors have reported that preoperative increased WBC, FBG, AST, ALT, ALP, and GGT levels might be a risk factor for conversion to open surgery.[18,19] In our study, we determined that high FBG and WBC levels are the risk factors but high AST, ALT, ALP, and GGT levels are not.

Consequently, in our study, male gender, chronic disease history, normal (20–25) BMI level, increased gallbladder wall thickness, high preoperative blood glucose level and leukocytosis, ERCP history, grade 3 or 4 adhesions found during the operation, and the presence of multiple stones in the bladder were found to be statistically significant risk factors for conversion to open surgery ($p < 0.05$). Knowing these risk factors is important for planning the treatment phases and informing patients before the operation.

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Significance of apoptosis and autophagy of leukemic blasts for the outcomes of acute myeloid leukemia patients

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Abstract

Background. Cytostatic treatment induces apoptosis or other types of cell death like autophagy, necrosis, mitotic catastrophe, etc. Autophagy can play a role in the drug resistance of neoplastic cells, allowing the survival of blast cells under stressful conditions, such as the use of cytostatics. Studies on apoptosis and autophagy 12–24 h after the start of treatment have not been conducted until now.

Objectives. The study aimed to investigate the predictive and prognostic significance of autophagy and apoptosis in patients with acute myeloid leukemia (AML).

Material and methods. The study included 38 patients. Blood was collected before and 12–24 h after the start of treatment, since at that time point, the appropriate blast cell count was still available. Autophagy was measured with the expression of the ATG5, MAP1L3, LC3-I, and LC3-II proteins. The percentage of mononuclear cells in early and late apoptosis was evaluated with flow cytometry, using the annexin V and propidium iodide (PI) binding assay.

Results. The percentage of apoptotic blast cells before treatment was not associated with the response. However, in the remission group, the overall percentage of apoptotic cells measured 12–24 h after the start of treatment was higher than in non-remission patients, which was statistically significant. In neither group we found any difference in the level of autophagy before and 12–24 h after the start of treatment. Nevertheless, we observed an increasing tendency of the MAP1LC3 protein expression (not statistically significant) in the remission group 12–24 h after the start of treatment. Patients with a higher percentage of blast cells in apoptosis and with a higher expression of MAP1LC3 protein measured 12–24 h after the start of the therapy had longer overall survival (OS).

Conclusions. A higher percentage of apoptotic as well as autophagic blast cells measured 12–24 h after the start of the chemotherapy is an independent factor associated with better outcomes.

Key words: apoptosis, acute myeloid leukemia, autophagy

Introduction

The results of several studies have shown that chemotherapeutic drugs can induce non-apoptotic types of cancer cell death. These processes include autophagy, mitotic catastrophe and necrosis as well as ageing.[1–4]

Apoptosis is a programmed cell death, which under physiological conditions makes it possible to maintain tissue homeostasis, both in embryonic development and in adult life, by preserving a sufficient number of cells in the developing organism and by eliminating abnormal damaged cells. It is involved in the tissue regeneration process, but also in the organ involution process (thymus atrophy).[5–7] The induction of apoptosis can occur under the influence of intrinsic factors (derived from other cells) or extrinsic factors, such as ultraviolet radiation, ionizing radiation, high temperature, cytostatics, and free radicals.[8–10]

Autophagy, unlike other processes, does not always lead to cell death. It may be one of the mechanisms of cancer cell death, but on the other hand, it can be an adaptive process that allows the survival of tumor cells under stressful conditions, such as the use of cytostatics. In such situations, it can allow cells to adapt to stressors and is a survival strategy for them. There are few reports on the role of autophagy in the treatment of acute leukemia. It seems that autophagy is the most common mechanism of cancer cell proliferation through generating resistance to the chemotherapy. The results of the studies are ambiguous – some point to its cytoprotective role for leukemia cells, while others show its antitumor role. At present, we can observe constant attempts to investigate the effects of modifying autophagy in various types of cancers, including hematologic malignancies.[11–15]

The few reports on the role of apoptosis and autophagy in the treatment of patients with acute myeloid leukemia (AML) prompted us to investigate their role in the induction therapy.

This study investigated the percentage of early and late apoptotic cells. Autophagy was measured with the expression of the products of 2 important genes (*ATG5* and *MAP1LC3B*) – ATG5 and LC3B proteins.[16] The ATG5 protein is the part of the ATG12-ATG5-ATG16L complex, which is essential for the phagophore elongation. The oligomerization of this complex causes the lengthening of the phagophore. In the next stage, the phagophore closes, which leads to the formation of the autophagosome.

The autophagosome closes the part of the cytoplasm with components to be digested. During the autophagosome formation, the LC3 protein undergoes a lipid-mediated transformation of the cytosolic form (LC3-I form), thereby producing a lipid form (LC3-II), which is equivalent to the production of autophagosomes in the cell, and the expression of this protein reflects the number of produced autophagosomes.[17–20]

Thus, it was investigated whether cytostatic treatment activated the transcription of both genes as well as the post-translational transformation of the LC3B protein, required for the production of autophagosomes.

Objectives

The primary objective of the study was to investigate the predictive and prognostic significance of the autophagy and apoptosis of blast cells in patients with AML – before and 12–24 h after the start of the induction therapy. In this study, we attempted to determine the potential differences in the percentage of apoptotic cells and the expression of markers such as ATG5, MAP1LC3, LC3-IB, and LC3-IIB measured before (spontaneous apoptosis) and after the start of the treatment, depending on the response to the therapy. The study also investigated the correlation between the percentage of apoptotic cells, the level of expression of the *MAP1LC3* and *ATG5* genes before and after the start of the induction therapy, and overall survival (OS).

Material and methods

Patients

In total, this study enrolled 38 patients with AML (20 men and 18 women): 29 patients with de novo diagnosis, 2 patients with AML relapse, and 7 with AML secondary to myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD).

Depending on the remission status achieved after the induction therapy, 2 groups of patients were identified:

Group I: 20 patients (aged 18–67 years; median: 36 years) who achieved remission, including 18 patients with de novo diagnosis, 1 patient with a relapse (22 months after the completion of the treatment) and 1 with the blast crisis during MPD; the DAC (daunorubicin, cytosine arabinoside, cladribine) regimen was used in 8 patients and the DA (daunorubicin, cytosine arabinoside) regimen in 12 patients, without a reduction in the scheduled doses (Table 1 summarizes the DAC and DA regimens);

Group II: 18 patients (aged 35–77 years; median: 55.5 years) who did not obtain remission after the induction treatment, including 11 patients with de novo diagnosis, 6 patients with AML secondary to MDS or MPD and 1 patient with a relapse 3 years after the completion

Table 1. Protocols of the induction chemotherapy used in patients with acute myeloid leukemia (AML)

Age of patients [years]	Induction chemotherapy
<60	DA daunorubicin: 60 mg/m^2 iv. (day 1–3) cytarabine: 200 mg/m^2, continuous infusion (day 1–7) DAC daunorubicin: 60 mg/m^2 iv. (day 1–3) cytarabine: 200 mg/m^2, continuous infusion (day 1–7) cladribine 5 mg/m^2 (day 1–5)
>60	DA (Cancer and Leukemia Group B – CALGB) daunorubicin: 45 mg/m^2 iv. (day 1–3) cytarabine: 100 mg/m^2, continuous infusion (day 1–7)

D – daunorubicine; A – cytarabine; C – cladribine; iv. – intravenously.

of the maintenance treatment; the DA regimen was used in 17 patients and the DAC regimen in 1 patient. In 5 patients, it was necessary to reduce the dose of the drugs for various reasons: old age (n = 2), pregnancy (n = 1), venous thrombosis (n = 1), and prevoius use of low doses of cytosine arabinoside (20 mg/m^2 over 10 days) (n = 1).

In all patients, blood was collected before and 12–24 h after the start of the induction chemotherapy into 9-milliliter tubes to isolate mononuclear cells. The data is presented in Tables 1–3.

Measurement of early and late apoptosis

Simultaneous administration of propidium iodine (PI) and annexin makes it possible to distinguish between healthy cells and necrotic cells as well as early and late apoptotic cells. The percentage of mononuclear cells in early and late apoptosis and their viability was evaluated with flow cytometry before and 12–24 h after the start of the induction therapy with the use of the annexin V and PI (Becton Dickinson, Franklin Lakes, USA) binding assay. Half a million cells were incubated in 100 µL of the annexin V binding buffer (10 mM Hepes/NaOH, pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) with 5 µL of bound annexin V-FITC (fluorescein isothiocyanate) and 5 µL of PI at room temperature for 15 min. At the end of incubation 1 mL of the buffer was added and the cytometry analysis was performed (flow cytometer Partec PAS (Particle Analysing System), Partec GmbH, Münster, Germany). Next, the percentage of annexin V-binding cells corresponding to early apoptosis, not stained with PI, was measured. The annexin-binding and red-stained (PI) cells showed late apoptosis. The total percentage of apoptotic cells was expressed as the sum of early apoptosis and late apoptosis.

Molecular studies

Ribonucleic acid isolation

Total ribonucleic acid (RNA) was isolated from the mononuclear cells obtained from the peripheral blood of patients diagnosed with AML, using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A 10 × 10^6 cell pellet was

Table 2. Clinical characteristics of patients with remission after the start of the induction therapy

Clinical characteristics of patients with remission after the start of the induction therapy	n	M	min	max	25Q	75Q
AML de novo AML secondary to MPD Recurrence of AML	18 1 1			–		
Age [years]	20	36.0	18.0	67.0	26.0	56.5
Sex female male	 9 11			–		
FAB M0 M1 M2 M4 M5 M6	 0 4 9 4 3 0			–		
White blood cells [10^9/L]	20	47.4	4.7	225.6	19.4	134.1
Hemoglobin [g%]	20	9.60	6.40	14.10	8.65	10.70
Platelets [10^9/L]	20	44.5	17.0	292.0	30.0	89.0
Percentage of blasts in blood at diagnosis	16	85.0	7.0	96.0	37.0	89.5
Percentage of blasts in bone marrow at diagnosis	20	76.0	25.0	93.0	49.0	87.0
Unfavorable cytogenetic or molecular abnormalities	3			–		

AML – acute myeloid leukemia; FAB – French-American-British classification; M – median; MPD – myeloproliferative disease; n – number of patients; 25Q – upper quartile; 75Q – lower quartile.

Table 3. Clinical characteristics of patients without remission after the induction therapy

Clinical characteristics of patients without remission after the induction therapy	n	M	min	max	25Q	75Q
AML de novo AML secondary to MDS AML secondary to MPD Recurrence of AML	11 4 2 1			–		
Age [years]	18	55.5	35.0	77.0	46.0	64.0
Sex female male	 9 9			–		
FAB M0 M1 M2 M4 M5 M6 bilinear	 0 5 3 5 4 0 1			–		
White blood cells [10^9/L]	18	23.9	1.5	308.5	4.2	40.9
Hemoglobin [g%]	18	8.90	6.60	14.40	8.20	10.60
Platelets [10^9/L]	18	57.0	14.0	224.0	28.0	125.0
Percentage of blasts in blood at diagnosis	18	68.5	23.0	96.0	40.5	81.0
Percentage of blasts in bone marrow at diagnosis	17	26.0	1.0	88.0	16.0	67.0
Unfavorable cytogenetic or molecular abnormalities	3					

MDS – myelodysplastic syndrome.

lysed in 600 µL of the RLT buffer with the addition of 1 µL of 6-mercaptoethanol and homogenized by passaging several times through a syringe needle. After centrifugation (3 min, 13,400 rpm), the supernatant was transferred into new Eppendorf tubes. One volume of 70% ethanol was added to the supernatant and the sample was thoroughly mixed. The obtained solution was applied in 700-µL aliquots on a nucleic acid-binding silica column, mounted in 2-mL tubes, followed by rinsing with high ionic strength buffers supplied by the manufacturer, and rotating for 15 s, 10,000 rpm. The bound RNA was eluted into fresh tubes collecting RNase-free water with a volume of 30 µL (1 min, 10,000 rpm).

Reverse transcription

Complementary deoxyribonucleic acid (cDNA) was obtained on the matrix of the isolated RNA, using the High Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Foster City, USA) as recommended by the manufacturer. The reaction was performed at 37°C for 120 min in a DNA Engine® Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, USA). Reverse transcriptase was thermally inactivated by heating the samples at 85°C for 5 min. The obtained cDNA was stored at –20°C until polymerase chain reaction (PCR) was performed.

Real-time polymerase chain reaction

We assessed the expression of the *ATG5* and *MAP1LC3* genes with the use of real-time quantitative PCR (RQ-PCR). The primers and probes of Life Technologies (Carlsbad, USA), TaqMan® Real-Time PCR Assay, Hs00169468_m1 and Hs99999908_m1 were used for *MAP1LC3* and β-glucuronidase reference gene (*GUSB*), respectively. The reaction was performed in a Universal Master Mix (Life Technologies) on a 7500 Real-Time PCR System from Applied Biosystems. We used the following universal protocol: 45 cycles of 95°C for 15 s, 60°C for 1 min, with previous incubation at 50°C for 2 min, and 95°C for 10 min (the polymerase activation). Each assay was repeated 3 times. For each assay, the difference Ct was determined for the test and reference genes (ΔCt), and $2^{-\Delta Ct}$ was calculated. The value obtained was the measure of the expression of the test gene.

Western blot

Preparation of cell lysates

The cell lysis was performed at 4°C for 30 min after the suspension of the cell pellet in 60 µL of radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1mM of phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, USA). Then, the samples were centrifuged at 4°C for 10 min at 8000 × g and the supernatant was transferred into new tubes. After adding a sufficient quantity of the loading buffer (4 times concentrated), the samples were denatured by placing them in a boiling water bath for 5 min.

Electrophoresis of proteins in denaturing conditions (sodium dodecyl sulfate–polyacrylamide gel electrophoresis – SDS-PAGE)

After the denaturation in a boiling water bath, the protein samples were briefly centrifuged and then placed into 15% polyacrylamide gel wells. The quantity of lysate in the gel well corresponded to 40 µg of protein. The separation was held in the electrophoresis buffer, initially at 80 V and in the later phase at 110 V. The electrophoresis was conducted for approx. 2 h and was terminated when the loading buffer reached the lower gel layer.

Detection of the LC3-I and LC3-II proteins using western blot

Upon completion of gel electrophoresis, the gel protein was transferred to the nitrocellulose membrane by electroblocking. The transfer was carried out in the electrolytic buffer at 350 mA for 60 min. After the blocking step (1 h at room temperature) in a 5% solution of skimmed milk in TBST (tris-buffered saline, Tween 20) (Sigma-Aldrich), the membrane was incubated with mouse monoclonal antibody against human protein LC3B (MB-M186-3 anti-LC3; MBL International Corporation, Woburn, USA) in TBST with 0.5% skimmed milk. After overnight incubation at 4°C, the membrane was washed 4 times with TBST at room temperature and then incubated with secondary antibodies against Fc fragment of murine immunoglobulin G (IgG) conjugated with horseradish peroxidase (Dako A/S, Glostrup, Denmark), dissolved in TBST with 0.5% skimmed milk. After 4-fold membrane rinsing with TBST, the protein bands, bound by the antibody conjugated to the enzyme, were detected using the chemiluminescence assay. For this purpose, the West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, USA) reagent was used. This reagent contains luminol, which, under the influence of horseradish peroxidase, emits light. The solution of this reagent, prepared according to the manufacturer's instructions, was spot on the membrane and then the membrane was inserted into the plastic foil. After exposure to photographic film, the film was developed using the reagents Kodak® GBX developer and Kodak GBX fixer (Sigma-Aldrich).

The quantity of protein in individual polyacrylamide gel pathways was normalized using β-actin reference protein. For this purpose, the complexes of anti-LC3 Abs with secondary antibodies conjugated with the membrane were removed. Next, the membrane was incubated overnight with anti-β-actin murine Ab. After overnight incubation, the membrane was washed and then incubated with an antibody conjugated with horseradish

Table 4. Total percentage of apoptotic cells in the early and late apoptotic phases, determined after the induction therapy, depending on the remission status

Total percentage of apoptotic cells in the early and late apoptotic phases after induction therapy	Group of patients	M	min	max	25Q	75Q	p-value*
Percentage of apoptotic cells in the early and late apoptotic phases after the induction therapy	I (n = 18)	7.86	0.82	23.66	4.27	12.91	0.0078
Percentage of apoptotic cells in the early and late apoptotic phases after the induction therapy	II (n = 20)	26.3	0.8	62.2	6.7	47.4	

* Mann–Whitney U test; I – non-remission group of patients; II – remission group of patients.

peroxidase. The detection of chemiluminescence after spotting the substrate for horseradish peroxidase was similar to that of LC3. The LC3-II protein migrates faster than LC3-I in western blot.

Due to the differences in the migration rates of the LC3-I (18 kD) and LC3-II (16 kD) proteins, 2 separate bands were obtained in the western blot procedure.

Statistical methods

In each group, the median values (M), range (min–max), and lower and upper quartiles (25Q–75Q) of the continuous parameters were calculated. Statistical significance between the means for different groups was calculated with the non-parametrical Mann–Whitney U test, because the number of cases in the control group was small and the variances in the groups were not homogeneous (the homogeneity of variance was determined with Bartlett's test). Statistical significance between the means for dependent groups was calculated with the non-parametrical Wilcoxon parity test, because the number of cases in the control group was small and the variances in groups were not homogeneous (the homogeneity of variance was determined with Bartlett's test). Since the expected value in the cell was below 5, the Fisher test was used for discrete parameters to analyze the incidence of features in the study groups.

For the chosen pairs of parameters, a correlation analysis was performed and Pearson's correlation factor r was calculated. The p-value ≤0.05 was considered statistically significant.

Results

Evaluation of the total percentage of apoptotic cells (in the early and late apoptotic phase) determined 12–24 h after the start of the induction therapy, depending on the remission status

A statistically significant intergroup difference was found in the total percentage of apoptotic cells (in the early and late apoptotic phase) after the start of the induction therapy between the non-remission (n = 18) and remission (n = 20) groups (p = 0.0078). In the remission group, the overall

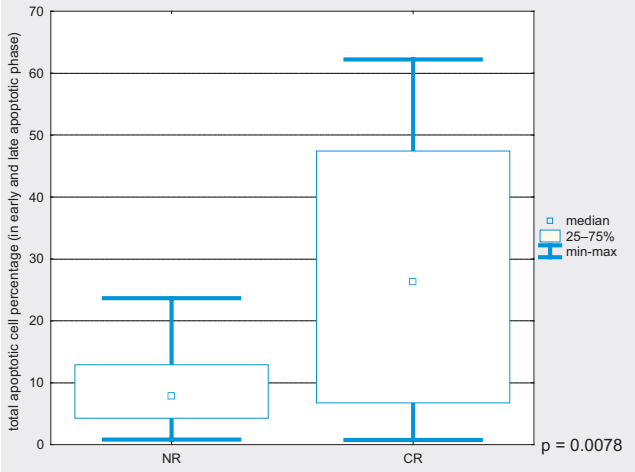

Fig. 1. Total percentage of apoptotic cells (in the early and late apoptotic phases) determined after the induction therapy, depending on the remission status

percentage of apoptotic cells after the start of the induction treatment was higher compared to non-remission patients. The data is presented in Table 4 and Fig. 1.

Comparison of the total percentage of apoptotic cells (in the early and late apoptotic phase) in patients with remission, before and after the start of the induction therapy

After the start of the induction therapy, a statistically significant increase in the total percentage of apoptotic cells (in the early and late apoptotic phase) was observed in the remission group compared to the values before treatment (p = 0.00059). The data is presented in Table 5 and Fig. 2.

Comparison of the MAP1LC3 protein expression in the remission group before and after the start of the induction therapy

In the remission group, the comparison of the MAP1LC3 protein expression before and after the start of the induction therapy demonstrated an increasing trend, although without statistical significance (p = 0.0930). The data is presented in Table 6 and Fig. 3.

Table 5. Percentage of apoptotic cells in the early and late apoptotic phases, determined in patients with remission, before and after the start of the induction therapy

Percentage of apoptotic cells in the early and late apoptotic phases, determined in patients with remission, before and after the start of the induction therapy	Group of patients	M	min	max	25Q	75Q	p-value*
Percentage of apoptotic cells in the early and late apoptotic phase before the induction therapy	I (n = 20)	11.9	0.6	55.6	2.9	20.1	0.00059
Percentage of apoptotic cells in the early and late apoptotic phase after the induction therapy	I (n = 20)	26.3	0.8	62.2	6.7	47.4	

* Wilcoxon test; II – remission group of patients.

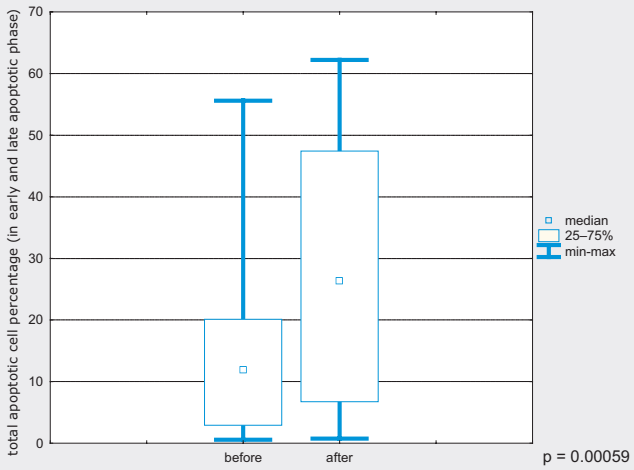

Fig. 2. Total apoptotic cell percentage (the early and late apoptotic phases) in the remission group before and after the start of the induction therapy

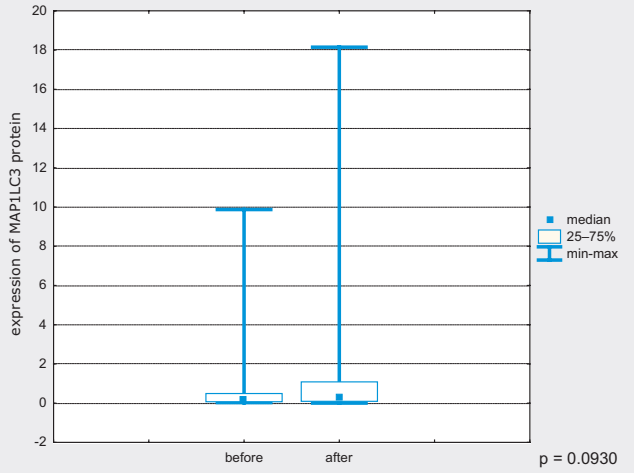

Fig. 3. Expression of the MAP1LC3 protein before and after the induction therapy in patients with remission

Results of the LC3-I and LC3-II protein expression assay performed with the use of the western blot procedure

In the remission group, cell pellets were tested in 16 of 20 patients, while in the non-remission group in 16 of 18 subjects. This was due to the inability to obtain a sufficient number of mononuclear cells in the precipitate in some patients (patients with leukopenia at diagnosis).

The differences in the speed of the migration of the LC3-I and LC3-II proteins resulted in 2 separate bands in the western blot procedure. LC3-II (16 kD) migrates faster than LC3-I (18 kD). The level of the LC3-II protein directly correlates with the quantity of autophagosomes. In the remission and non-remission groups, differences in the LC3-I and LC3-II expression before and after the start of the chemotherapy were not reported. The data is presented in Fig. 4.

Correlation between selected parameters and overall survival of patients

The longer OS was related to the percentage of cells in the late stages of apoptosis and to the total percentage of apoptotic cells (in early and late apoptosis) after the start of the induction therapy. The data is presented in Table 7.

Discussion

This study investigated whether the percentage of apoptotic cells as well as the expression of selected autophagic markers in mononuclear populations, before and after the start of the induction chemotherapy, may impact the therapeutic response in AML patients.

Table 6. Expression of the MAP1LC3 protein in patients with remission before and after the start of the induction therapy

Expression of the MAP1LC3 protein in patients with remission before and after the start of the induction therapy	Group of patients	M	min	max	25Q	75Q	p-value*
Expression of the LC3 protein before the induction chemotherapy	I (n = 20)	0.115	0.033	9.872	0.058	0.498	0.0930
Expression of the LC3 protein after the induction chemotherapy	I (n = 20)	0.54	0.00	18.15	0.10	1.19	

* Wilcoxon test; II – remission group of patients.

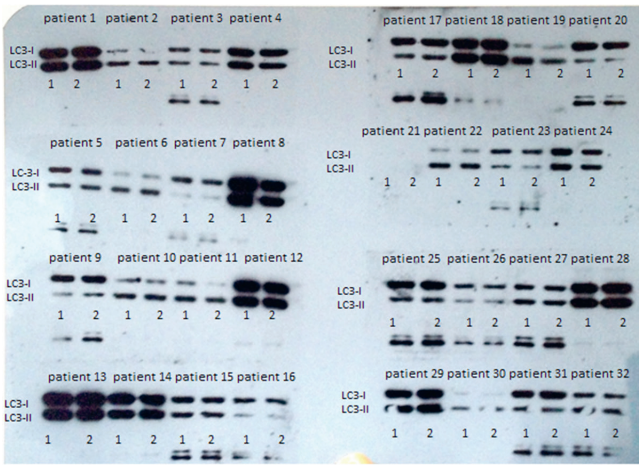

Fig. 4. Expression of the LC3-I and LC3-II proteins in patients diagnosed with acute myeloid leukemia (AML) determined with the western blot assay

Patients 1–16 – non-remission after the treatment; patients 18–32 – remission after the induction therapy; number: 1 – expression of the LC3-I and LC3-II proteins before the chemotherapy; 2 – expression of the LC3-I and LC3-II proteins after the chemotherapy.

Table 7. Correlation between overall survival (OS) and selected parameters

Parameter	OS
Difference in the percentage of cells in the late-stage apoptosis before and after the chemotherapy [%][1]	r = 0.36 p = 0.027
Difference in the total percentage of cells in early and late apoptosis before and after the chemotherapy [%][2]	r = 0.37 p = 0.024
Difference in the expression of the MAP1LC3 protein before and after the chemotherapy	r = 0.32 p = 0.054

[1] cell percentage in the late-stage apoptosis after the chemotherapy – percentage of cells in the late apoptotic phase before the chemotherapy/percentage of cells in the late apoptosis phase before the chemotherapy; [2] total percentage of cells in apoptosis after the chemotherapy – total percentage of cells in apoptosis before the chemotherapy/percentage of cells in the late apoptosis phase before the chemotherapy.

A small number of reports confirm the importance of apoptosis as a prognostic factor in the treatment of acute leukemia. A study by Smith et al. demonstrated that the percentage of blast cells in the spontaneous phase of apoptosis correlated with a good response following an intravenous (iv.) cytarabine administration.[21] The median proportion of blasts in the apoptosis phase in the blood of patients achieving complete remission (CR) after the treatment (after 24 h of culture) and in non-remission (NCR) subjects was 19.5% (range: 3.6–64%) and 4.2% (range: 1.8–7.0%), respectively. This intergroup difference was statistically significant (p = 0.0007).[21]

Other reports have also pointed at the importance of apoptosis as a prognostic factor in the treatment of acute leukemia. Its role has been confirmed through the measurement of apoptosis markers, such as the presence of Fas receptors and tumor necrosis factors (TNFs), the Smac/DIABLO expression levels, and the caspase-3 activity. It has been found that a loss of receptors for ligands like Fas or TNF before the induction therapy resulted in resistance to daunorubicin-induced apoptosis of the test Jurkat or U937 cell lines.[22] Other studies have demonstrated that a higher expression of Smac/DIABLO is an independent predictor of greater overall remission and longer OS ($p < 0.001$ and $p = 0.003$, respectively), while a reduced caspase-3 activity correlated with resistance to apoptosis.[23,24]

The results of our study did not show any statistically significant differences in the percentage of apoptotic cells before the induction therapy (cells in the spontaneous phase of apoptosis), depending on the achievement of remission after the treatment. There are few studies on the effects of cytostatic treatment on apoptosis, regarding its level before treatment and the remission status after therapy.[21–24] One study examined mononuclear cells isolated from the bone marrow of 31 patients diagnosed with de novo acute leukemia. The study demonstrated that the abovementioned cytostatics induced caspase-dependent apoptosis. Moreover, spontaneous apoptosis did not correlate with treatment-induced apoptosis, while induced apoptosis was significantly higher in patients who achieved CR after cytostatic treatment. Tests performed after the induction therapy showed that the percentage of cells in the apoptotic phase was significantly higher in those patients compared to the NCR group ($p = 0.0130$ and $p = 0.0078$, respectively).[25]

In our study, a significant increase in the percentage of apoptotic cells after the induction therapy in the remission group was crucial. However, the non-remission group patients did not obtain such a result.

There are few reports on the role of autophagy in the treatment of acute leukemia, and the results of the available studies are ambiguous. Some point to its cytoprotective role for leukemia cells, while others show its anticancer role. Modification of the autophagy process can also influence the proliferation of leukemia cells.

Bosnjak et al. demonstrated that cytarabine used in leukemia test cell lines inhibited the mammalian target of rapamycin (mTOR) kinase phosphorylation by inhibiting AKT kinase, and thus stimulated autophagy.[11] Adding autophagy inhibitors such as bafilomycin or chloroquine stimulated the apoptosis of leukemia cells.[11] This fact may indicate the cytoprotective role of autophagy in leukemia cells. In another paper, treatment with daunorubicin induced autophagy by stimulating ERK1/2 kinase.[12] The inhibition of this process by the use of the chloroquine inhibitor of the kinase and small interfering RNA (siRNA), which mute the expression of the *ATG5* and *ATG7* genes, reduced the viability of leukemia cells.[12] This may also indicate the cytoprotective role of autophagy in these cells. Palmeira dos Santos et al. showed that autophagy suppression may initially increase the antiproliferative effect of cytarabine on leukemia cells. After 24 h, however, this process may, quite to the contrary, promote the resistance of leukemia cells to treatment.[13]

One study evaluated the combination of daunorubicin and rapamycin in the therapy of patients with acute lymphoblastic leukemia (Ph+).[14] The synergistic effect of inhibiting the leukemia cell proliferation while using the above-mentioned drugs was associated with mTOR blockade, autophagy increase and cell cycle arrest in the G1 phase.[14] In mice implanted with myeloblasts, Willems et al. demonstrated that the inhibition of the mTORC1 and mTORC2 complex improved survival by reducing the tumor mass without generating visible toxicity effects.[15] The inhibitor stimulated apoptosis in leukemic cells without inducing that effect in normal immature CD34+ cells, and also intensively stimulated autophagy.[15]

In our studies, the expression of the ATG5 and MAP1LC3 proteins showed no intergroup difference, both before and after the treatment between the remission and non-remission groups. In the remission group, however, an upward trend in the expression of the MAP1LC3 protein after the start of the induction therapy is noteworthy ($p = 0.0930$).

Moreover, we did not observe any differences in the LC3-I and LC3-II protein expression, before and after the chemotherapy, between the remission and non-remission groups.

In the remission group, the observed that the increase in the MAP1LC3 expression after the start of the induction therapy was not associated with the change in the LC3-IB and LC3-IIB protein levels measured with western blot assay. It may, therefore, be suspected that the expression of the MAP1LC3 protein is further regulated by another factor, e.g., micro-ribonucleic acid (miRNA), which acts on the level of post-transcriptional regulation of gene expression. In the remission group, the increased expression of the MAP1LC3 protein after the start of the treatment may indicate higher susceptibility of mononuclear cells to enter a pathway of autophagy. Nevertheless, the negative factor acting at the level of post-transcriptional gene regulation eventually changes the course of this process.

Cytostatics used in the induction regimen potentially stimulate apoptosis, provided that an adequate supply of blasts to the chemotherapy is maintained. Blast cell sensitivity depends not only on cytogenetic prognosis, but also on the genesis of the disease (de novo vs secondary leukemia) and the intensity of the cytostatic treatment.

In our studies, longer OS was related to the percentage of cells in the late stages of apoptosis and to the total percentage of apoptotic cells (in early and late apoptosis) after the start of the induction therapy.

Conclusions

Our results indicate that patients with a good therapeutic response experience more intense cytostatic-induced apoptosis compared to non-remission subjects. Following the above, it is concluded that apoptosis disturbances are one of the causes of chemotherapy failure.

Based on the presented results, we can assume that apoptosis activity is a useful indicator of good prognosis in AML. At present, we need further studies to confirm the impact of autophagy on the therapeutic response of AML patients, to evaluate the possibility of modification of this process and to assess the effect of this change on patients' response.

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Life activity, disease acceptance and quality of life in patients treated with renal replacement therapy since childhood

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Abstract

Background. Advances in the treatment of chronic kidney disease (CKD) resulted in expanding therapy goals from simple prolongation of life to a return to normal social functioning and having an active and satisfactory life after reaching adulthood.

Objectives. The aim of the study was to evaluate life activity, disease acceptance (DA) and quality of life (QOL) in patients with end-stage renal disease (ESRD) treated with renal replacement therapy (RRT) since childhood.

Material and methods. We surveyed 117 patients aged ≥16 years on RRT since childhood. The control group included 25 healthy subjects. We used questionnaires that included a sociodemographic questionnaire (questions regarding education, work, family, and offspring), Acceptance of Illness Scale (AIS), Satisfaction With Life Scale, and Kidney Disease Quality of Life (KDQOL).

Results. A completed survey was returned by 45 respondents aged 27.16 ±6.78 years, among whom 82.2% had a transplanted kidney and 17.8% were on hemodialysis (HD). Higher education was reported by 18.18% of respondents, secondary and primary by 63.64% and 18.18%, respectively. Employment was reported by 46.67% of the respondents. A family was started by 35% of women and 4% of men. Good DA was found in 28.9% of the respondents. Satisfaction with life was lower in the study group compared to the control group. We found statistically significant correlations between the age when the kidney disease was diagnosed and satisfaction with life ($r = 0.33$), and between the time since the last change of RRT modality and emotional well-being ($r = 0.34$). The number of kidney transplantations correlated negatively with emotional component of QOL ($r = -0.66$) and emotional well-being ($r = -0.73$).

Conclusions. Patients treated with RRT were quite well adapted to their chronic disease but showed less ability to live independently. Young age at the diagnosis of kidney disease, loss of kidney transplant and living on social security benefit had a negative effect on their emotional well-being.

Key words: quality of life, dialysis, end stage renal disease, kidney transplant, childhood

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Introduction

Chronic diseases affect the functioning and mental status of patients. Patients with somatic disease experience many negative emotions and limitations caused by the disease, and the prolongation of life does not necessarily translate to an improvement in quality of life (QOL), which is considered a prognostic factor for patient morbidity and mortality.[1–3] Advances in the treatment of chronic kidney disease (CKD) resulted in expanding therapy goals from simple prolongation of life to a return to normal social functioning and having an active and satisfactory life after reaching adulthood. It is also known that patients treated with renal replacement therapy (RRT) since childhood have a feeling of dependence on other persons, isolation from their family and peers, and an awareness of worse life conditions compared to healthy people, which may lead to reduced QOL.[4–6]

The aim of the study was to evaluate life activity, disease acceptance (DA), satisfaction with life, and QOL in patients with end-stage renal disease (ESRD) treated with RRT since childhood.

Material and methods

We studied 117 patients aged ≥16 years who first received RRT due to ESRD as children in the Department of Pediatric Nephrology at the Medical University of Warsaw, Poland, in 1973–2007. Information on the disease course and RRT methods used during childhood were retrieved from medical records. Diagnosis of CKD was defined as decrease of glomerular filtration rate below 60 mL/min/1.73 m^2 or presence of permanent kidney damage (pathologic abnormalities or markers of damage in blood and urine tests, or imaging studies abnormalities).

The control group included 25 healthy adults (13 women and 12 men) at the mean age of 27 ±7.36 years, age- and gender-matched to the study group. Clinical data in the control group was collected using an interview.

The study was a cross-sectional survey performed using a set of questionnaires and scales:

1) a sociodemographic questionnaire that included information on further RRT after transition from pediatric to adult care, education, place of residence, professional status, and family situation; and

2) professional psychometric tools:

a) Acceptance of Illness Scale (AIS) by Felton et al. The studied subject evaluates whether negative disease consequences such as lack of self-reliance, feeling of dependence on others, reduced self-esteem, and disease-related limitations are present in his/her life. Overall score (8–40) corresponds to the degree of DA. Low disease acceptance was defined as score ≤23, moderate DA as score 24–36 and high DA as score ≥37.[7]

b) Satisfaction With Life Scale (SWLS) by Diener et al. Overall score ranges from 5 to 35. Scores 5–17 indicate low satisfaction with life, scores 18–23 indicate moderate satisfaction with life and scores 24–35 indicate high satisfaction with life.[7]

c) Kidney Disease Quality of Life – Short Form (KDQOL – SF™) by the RAND Group (University of Arizona, Tucson, USA) in Polish translation.[8] Kidney Disease Quality of Life is used to evaluate QOL in subjects receiving RRT and consists of 2 parts: a generic questionnaire (SF-36 form) to evaluate overall health related quality of life (HRQOL), and a specific questionnaire to evaluate QOL related to kidney disease. For each item, scores range from 0 to 100, where 0 indicates a negative answer and poor QOL, and 100 indicates good QOL.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the ethics committee at the Medical University of Warsaw, Poland. Informed consent was obtained from all individual participants included in the study.

Statistical analysis

Normal distribution of variables was examined using the Shapiro–Wilk and Lilliefors tests. Normally distributed data is shown as mean values ± standard deviations (SD), and non-normally distributed data is shown as medians and ranges. Significance of differences in the mean values between normally distributed independent or interrelated groups is evaluated using the Student's t-test for independent or paired samples, respectively. Non-normally distributed data was compared using the Mann–Whitney U test for independent sample or the Wilcoxon test for paired samples.

The Pearson's χ^2 test was used to compare frequencies between groups. Correlations between variables were evaluated using linear regression analysis. A value of $p < 0.05$ was considered statistically significant.

Statistical analyses were performed using the STATISTICA v. 9.0 software (StatSoft Inc., Tulsa, USA).

Results

Among 117 patients qualifying for the study (mean age 29.50 ±7.81 years), response was obtained from 71 patients (60.68%), including the consent for participation in the study and returned questionnaires from 45 respondents (20 women and 25 men) at the mean age of 27.16 ±6.78 years and information about patient death from the families of 26 patients. The most common causes of ESRD were congenital anomalies of the urinary tract (58%) and glomerulonephritides (31%).

Data was analyzed in 2 observation periods depending on the date of RRT initiation: group A with RRT initiated

in 1973–1992 and group B with RRT initiated in 1993–2007. Clinical characteristics of the study group are shown in Table 1. We found no significant differences between groups A and B in regard to the mean age at the time of the diagnosis of CKD and initiation of RRT. Men were significantly younger than women at the diagnosis of CKD (4.3 ±5.0 vs 7.7 ±4.8 years; $p < 0.05$) but gender had no effect on the age when RRT was initiated. The mean total duration of RRT was 15.6 ±6.6 years, including 4.2 ±2.9 years under pediatric care and 11.5 ±7.0 years under adult care. During childhood, peritoneal dialysis (PD) was used in 66.7% of the patients for the mean duration of 3.3 ±2.4 years and hemodialysis (HD) was used in 68.9% of the patients for the mean duration of 2.8 ±2.7 years. Kidney transplantation during childhood was performed in 73.3% of the patients. Overall, during RRT, kidney transplantation was performed in 97.8% of the patients, including 24% of the patients who received kidney transplant twice and 4.5% of the patients who received it 3 times. During adulthood, HD was used in 80% of the patients for the mean duration of 6.1 ±4.9 years and PD in 16% of the patients for the mean duration of 3.3 ±3.3 years. At the time of study, 82.2% of the respondents had a kidney transplant, including 73.9% in group A and 90.9% in group B, a non-significant difference. Hemodialysis was used in 17.8% of the respondents, including 26.1% in group A and 9.1% in group B.

Demographic and current life activity data is shown in Table 2. Urban place of residence was reported by 71% of the respondents, significantly more commonly women ($p < 0.05$). No significant differences in the level of education were seen in relation to gender and the period of RRT initiation. Among 30 patients who finished their education, 57% had no trained profession (finished primary school or general high school), significantly more commonly among those in whom RRT was initiated in 1973–1992. Lack of trained profession was observed more commonly in men (45.83%) than in women (30%), but

Table 1. Clinical characteristics of the study group

Parameters [years]	Total n = 45, mean ±SD (range)	Group A 1973–1992 n = 23, mean ±SD (range)	Group B 1993–2007 n = 22, mean ±SD (range)	♀ n = 20, mean ±SD (range)	♂ n = 25, mean ±SD (range)	p-value
Age	27.2 ±6.8 (16.3–45.1)	30.9 ±6.6 [1] (18.7–45.1)	23.2 ±4.4 [1] (16.3–29.5)	25.2 ±4.9 (16.3–32.4)	28.7 ±7.8 (18.3–45.1)	[1]$p < 0.0001$ A vs B
Age at CKD diagnosis	5.9 ±5.2 (0.0–15.6)	5.4 ±4.4 (0.4–14.3)	6.3 ±6.0 (0.0–15.6)	7.7 ±4.8[2] (0.6–15.3)	4.3 ±5.0[2] (0.0–15.3)	[2]$p < 0.05$ ♀ vs ♂
Age at start of RRT	11.5 ±4.4 (2.1–20.2)	10.4 ±4.5 (2.1–20.2)	12.6 ±4.1 (3.9–18.5)	11.5 ±4.0 (3.6–18.5)	11.6 ±4.9 (2.1–20.2)	NS
Total time of RRT	15.6 ±6.6 (3.0–30.8)	20.4 ±4.6[3] (15.9–30.8)	10.6 ±4.2[3] (3.0–16.2)	13.8 ±6.3 (3.0–25.8)	17.0 ±6.6 (4.5–30.8)	[3]$p < 0.0001$ A vs B

CKD – chronic kidney disease; RRT – renal replacement therapy.

Table 2. Demographic data of subjects from the study group

Parameters	Respondents n = 45 [%]	Group A n = 23 1973–1992 [%]	Group B n = 22 1993–2007 [%]	♀ n = 20 [%]	♂ n = 25 [%]	p-value
Place of residency						
1. rural area	28.89	34.78	22.73	15.00[1]	40.0[1]	[1,2]$p < 0.05$ ♀ vs ♂
2. urban area	71.11	65.22	77.27	85.00[2]	60.0[2]	
Education						
1. higher	18.18[4]	18.18	18.18	30.00	8.33	NS
2. secondary	63.64[4]	59.09	68.18	60.00	66.67	
3. primary	18.18[4]	22.73	13.64	10.00	25.00	
Trained profession						
1. yes	29.55	31.82	27.27	30.00	29.17	[3,4]$p < 0.05$ A vs B
2. no	38.64	54.55[3]	22.73[3]	30.00	45.83	
3. still learning	31.82	13.64[4]	50.00[4]	40.00	25.00	
Employment						
1. yes	46.67	47.83	45.45	50.00	44.00	NS
2. no	33.33	43.47	22.73	25.00	40.00	
3. no but still learning	20.00	8.70	31.82	25.00	16.00	
Own family	17.78	26.09	9.09	35.00[7]	4.00[7]	[7]$p < 0.05$ ♀ vs ♂
Children	9.89	8.70	9.09	15.00	4.00	NS

Table 3. Results of Satisfaction with Life Scale in study and control group

Examined groups	Total scores, mean ±SD	Satisfaction with life [%]		
		poor	average	good
Study group				
Total	19.56 ±5.95[1]	46.67[2]	22.22	31.11
Group A	18.48 ±5.32	56.52	26.09	17.39[4]
Group B	20.68 ±6.47	36.36	18.18	50.00[4]
♀	20.20 ±6.69[3]	40.00	20.00	40.00
♂	19.04 ±5.37	52.00	24.00	24.00
Tx	19.65 ±6.28	48.65	16.22	35.14
HD	19.13 ±4.39	37.50	50.00	12.50
Control group				
Total	22.12 ±5.47[1]	16.00[2]	40.00	44.00
♀	23.77 ±3.52[3]	–	53.85	46.15
♂	20.33 ±6.72	33.33	25.00	41.67
p-value				

[1]p = 0.08: study group vs control group; [2]p < 0.05: study group vs control group; [3]p = 0.09: ♀ study group vs ♀ control group; [4]p < 0.05: A vs B; SD – standard deviation; HD – hemodialysis; Tx – kidney transplantation.

the difference was not statistically significant. Employment was reported by 46.7% of the respondents at the mean age of 29.96 ±6.9 years (range 20.1–45.1 years). Among those who did not work, 37.5% of the respondents at the mean age of 18.92 ±2.23 years were still studying.

Among 37 patients after kidney transplantation, 51.4% reported employment, 24.3% were still studying and 24.3% neither worked nor studied. Among 8 patients treated with HD, 2 (25%) were professionally active and 6 (75%) did not work. The unemployment rate was significantly higher ($p < 0.01$) among patients treated with HD compared to those living with a transplanted kidney.

Among 45 respondents, 30 (66.7%) received social security benefits due to incapacity to work, significantly more commonly ($p < 0.01$) those in whom RRT was initiated in 1973–1992 vs 1993–2007 but with no difference in relation to gender. Social security benefits were received by 56.7% of the respondents after kidney transplant compared to 87.5% of the respondent treated with HD, a non-significant difference.

Among the respondents, women were significantly more likely ($p < 0.05$) to start a family. Four respondents (9.9%) reported having children, including 3 women and 1 man. Eighty-eight percent of men and 58.82% of women still lived with their parents. Among the respondents aged ≥25 years, 34.5% lived independently or with their families, including 63.6% of women and 16.7% men. No significant differences in the mean age at the time of the diagnosis of CKD, age at the time of RRT initiation, and duration of dialysis therapy before adulthood were found between the respondents living independently or still with their parents.

Disease acceptance, satisfaction with life and quality of life

Among the respondents, 28.9% reported high DA, and 26.7% of the respondents reported low DA, with no significant differences in relation to gender, period of RRT initiation and the current RRT modality. Patients living in rural areas reported significantly lower ($p < 0.05$) DA compared to those living in urban areas (AIS score 26.23 ±6.52 vs 31.10 ±7.81, respectively). The lowest scored item was the statement "I will never be self-reliant to the degree I would like to be" (mean score 3.07 ±1.69).

A comparison of SWLS scores in the study and control groups is found in Table 3. Low satisfaction with life was reported significantly more frequently ($p < 0.05$) by the respondents in the study group compared to the control group. Good satisfaction with life was reported significantly more frequently ($p < 0.05$) by patients with RRT initiated in 1993–2007 compared to those in whom RRT was initiated in 1973–1992. We showed a positive correlation between the age at the time of the diagnosis of CKD and current satisfaction with life ($r = 0.33$, $p < 0.05$) (Fig. 1). Patients living in urban areas reported significantly better ($p < 0.05$) satisfaction with life (mean score 20.66 ±6.31) compared to those who lived in rural areas (mean score 16.85 ±3.95). Significantly lower ($p < 0.05$) satisfaction with life and DA was reported by those who received social security benefits due to incapacity to work compared to those who did not (25.3 ±5.3 vs 17.96 ±5.15). No significant differences in SWLS and AIS scores were found in relation to education, marital status, professional status, and the presence of concomitant diseases. A positive correlation was found between AIS and SWLS scores ($r = 0.56$, $p < 0.0001$) (Fig. 2).

Results for specific items of the KDQOL questionnaire in patients with ESRD are illustrated on a graph (Fig. 3). Lowest values were obtained for general health perception and work status. Patient satisfaction was assessed low

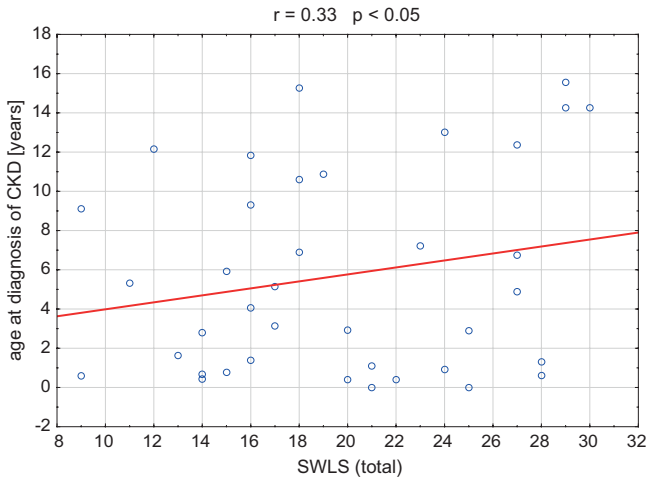

Fig. 1. Correlation between age of the diagnosis of chronic kidney disease (CKD) and results of Satisfaction with Life Scale (SWLS)

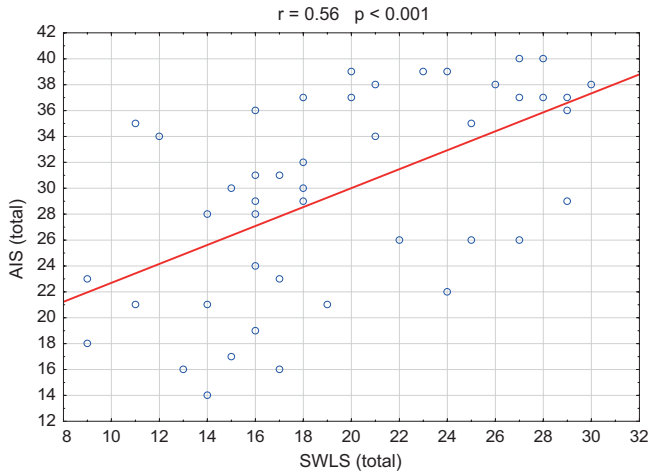

Fig. 2. Correlation between results of Acceptance of Illness Scale (AIS) and Satisfaction with Life Scale (SWLS)

in the group of patients who started RRT between 1973 and 1992, but the difference in comparison to group B was not statistically significant. The KDQOL scores of patients in whom RRT was initiated in 1993–2007 were significantly higher ($p < 0.05$) compared to those of patients who started RRT in 1973–1992 in regard to general health perception (59.82 ±18.05 vs 44.57 ±18.40) and dialysis staff encouragement (83.33 ±17.13 vs 63.75 ±15.84). No significant differences in physical and emotional components of QOL were found between groups A and B and between women and men. The KDQOL questionnaire results in patients living in rural areas indicated significantly higher ($p < 0.05$) disease-related burden and lower emotional well-being compared to patients living in urban areas. The KDQOL questionnaire results were not related to receiving social security benefits. We found that the physical component of QOL was significantly worse ($p < 0.05$) among the respondents who reported primary education compared to those who reported secondary or vocational education.

We found negative correlations between the duration of treatment with HD and the physical functioning component of KDQOL, and between the number of kidney transplantation and the emotional component of QOL ($r = -0.66$, $p < 0.05$) and emotional well-being ($r = -0.73$, $p < 0.05$). The time since the last change of RRT modality correlated positively with emotional well-being in our study group ($r = 0.34$, $p < 0.05$). No correlations were found

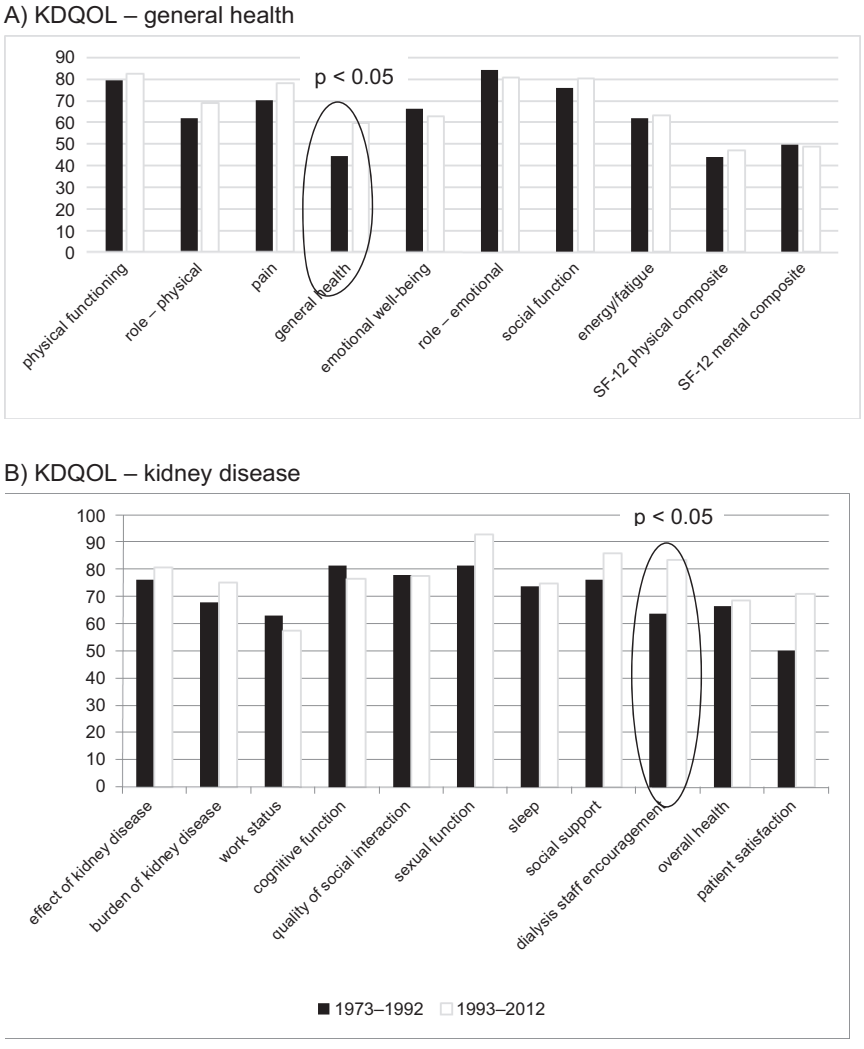

Fig. 3. Results of Kidney Disease Quality of Life (KDQOL) questionnaire – comparison of patients entering RRT between 1973–1992 and 1993–2012: A) KDQOL – general health; B) KDQOL – kidney disease

between KDQOL questionnaire results and the current age of the respondents, the age at the time of RRT initiation, overall duration of living with a transplanted kidney, and the number of RRT modality changes.

Discussion

Life activity

Based on our findings, patients treated with RRT since childhood seem quite well adapted to their chronic disease but showed less ability to live independently compared to the general population, as few of them were professionally active and started their own families, while many continued to live as adults with their parents.

The educational profile of our patients treated with RRT since childhood was similar to that reported for the general Polish population (higher education reported by 18.2% of our patients compared to 16.5% in the general Polish population of working age; secondary education reported by 63.6% and 58.8%, respectively).[9] These results in our study group are similar to those reported by European patients, with 11–18% of them reporting higher education and about 1/3 reporting vocational education in the study evaluating the level of education among young Europeans treated with RRT since childhood.[10] However, a major problem identified in our study group was the unemployment rate (53.3%), which was about 10% higher compared to the general Polish population of working age.[9] Similarly, Groothoff et al. found the unemployment rate among patients treated with RRT since childhood to be twice as high compared to the general population.[6] In contrast, full- or part-time employment was reported by 70–84% of the patients treated with RRT since childhood in the USA,[11,12] and 67.4% of such patients in the Netherlands.[6] It has also been reported that the proportion of professionally active subjects among those treated with RRT since childhood continues to grow with each decade.[5,10,11] In a German study, the proportion of professionally active subjects among patients in whom RRT was initiated in 1970–1984 was 39% compared to 62% among those with RRT initiated in 1985–1994.[13] In our group of patients treated with RRT since childhood, we did not find a significant difference in the proportion of professionally active subjects depending on the decade of initiation of RRT (group A 1973–1992 vs group B 1993–2007). This likely resulted from a 50% proportion of patients continuing their studies among those in whom RRT was initiated in 1993–2007. In the future they will hopefully increase the rate of professionally active.

Compared to the general population, our patients were much less likely to start their own families. According to the national census data for Poland, 47.89% of the population aged 15–44 years is married (51.97% of women and 57.89% of men),[9] compared to 17.8% in our study group (35% of women and 4% of men). Similarly, in a 1992 European study that included patients aged 21–35 years treated with RRT since childhood, only 13.5% were married and 8% had children.[13] In an American study by Bartosh et al. who evaluated patients after kidney transplantation in childhood, 48% started their own families and 28% had children.[12] Similarly to our study group, other authors also reported that men with ESRD had more difficulties with finding a life partner compared to women,[6,14] which may be related to social beliefs (men are perceived as those who should provide primary financial support for the family) and physical factors.

Our patients often (76%) continued to live with their parents after reaching adulthood, which is consistent with the results reported in other studies on the population treated with RRT since childhood. In a Dutch study, 34% of patients aged 29.3 ±5.4 years continued to live with their parents.[6] This may be related to financial factors (high unemployment rate, low social security benefits, difficulties with obtaining loans) and social characteristics (low level of independence, lack of social support).

Disease acceptance

Compared to the general Polish adult population of dialyzed patients, we found better AIS scores in our study group (25.32 ±6.03 vs 29.7 ±7.7),[8] which might have been affected by an earlier disease onset in our respondents and a large proportion of subjects after kidney transplantation, which significantly reduces disease-related burden. Better DA following kidney transplantation compared to dialyzed patients was reported previously.[15] In another Polish study, lower AIS scores were reported in both patients treated with HD and those treated with PD (24.53 ±1.86 and 24.24 ±2.20, respectively).[16] The lowest scored AIS item in the study group was the statement "I will never be self-reliant to the degree I would like to be" which correlated positively with the emotional component of QOL in the KDQOL questionnaire ($r = 0.47$, $p < 0.01$) and was scored much lower compared to the reference population of adult dialyzed patients (3.81 ±1.51). This result highlights the burden of disease-related limitations, which prevent patients to participate in life as actively as they would like to, and attests to the importance of perceived independence in the assessment of QOL by young people treated with RRT.

Satisfaction with life and quality of life

In our study group, we found a trend ($p = 0.08$) towards lower satisfaction with life among patients treated with RRT compared to the control group. In addition, low satisfaction with life was reported significantly more frequently compared to the control group. The mean SWLS score was similar to that reported by Juczyński in adult dialyzed patients (19.51 ±4.65).[7] Lower satisfaction with

life compared to healthy peers was also shown by Rosenkranz et al.[5]

When comparing KDQOL scores in our study group with the results reported in a group of adult patients treated with HD in the European DOPPS study,[17] physical and emotional components of QOL were rated about 10 points higher by men in our study group, while women rated the physical component also about 10 points higher but their rating of the emotional component was similar to the abovementioned European study. However, these findings might have been affected by the type of RRT modality used, as shown in other studies.[15,18–20] In our study group, the total duration of dialysis therapy, including HD and PD, and the duration of living with a kidney transplant had no effect on the overall rating of QOL, satisfaction with life and DA. Other studies on the effect of RRT duration and modality on QOL in patients with ESRD yielded inconsistent results. British and Italian studies showed a negative effect of the duration of dialysis therapy on QOL.[21,22] In the study by Rosenkranz et al., no association was found between either total duration of RRT or duration of treatment with specific RRT modalities and satisfaction with life, but the latter was found to be positively affected by the time since successful kidney transplantation.[5]

The lowest values among domains of KDQOL questionnaire were obtained for general health perception and the group of patients who started RRT between 1973 and 1992 had statistically significant lower results. In another Polish study evaluating HRQOL of 69 adult patients with ESRD (44 patients on HD, mean age 49 years, and 25 on PD, mean age 42 years) before and 12 months after kidney transplantation, mean result of KDQOL domain "general health" was 34 points in patients on HD and 32 points in patients on PD which is lower in comparison to our study group. After kidney transplantation KDQOL mean scores in domain "general health" were 40 points (regardless previous RRT method) – similar as in group A in our study group, but lower than in group B.[23] Similar result in our group A may be connected with comparable total time of RRT, but the younger age of patients in our group B and shorter time of RRT, may contribute in less comorbidities, which probably influences better assessment of "general health" in KDQOL questionnaire.

Patient satisfaction and dialysis staff encouragement were assessed significantly lower in the group of patients who started RRT between 1973 and 1992 in comparison to group B (1993–2007). The results achieved by patients from group B were similar to the group of patients on chronic HD or after kidney transplantation in another Polish study.[23] The difference between group A and B may be a result of improvement in patient support, introduction of educational programs directed to patients with chronic renal failure or shorter time of waiting for kidney transplantation. Currently, goals of treatment in patients with CKD include not only prolongation of life and prevention of complications but also a return to normal peer environment and active, satisfactory life.

We found that the physical component of QOL was significantly worse among the patients who reported primary education compared to those who reported secondary or vocational education. Declining of perceived HRQOL with decreasing health status was reported previously.[24,25]

In our study, significantly worse DA, satisfaction with life and disease-related burden and lower emotional well-being in KDQOL questionnaire were found among patients living in rural areas compared to those living in urban areas. Lower chronic DA reported by patients living in rural areas was shown by Niedzielski et al.[26] Worse DA and adaptation to the burden of chronic disease in patients living in rural areas may be the result of more difficult access to health and social services, support groups and educational programs in comparison to patients living in urban areas. Rural areas have also a higher level of unemployment and poverty, which directly results in a less convenient life and worse emotional well-being. Zagozdzon at al. showed that rural residence was a strong determinant of health-related QOL of Polish females.[27] They showed that rural residence was positively associated with mental health but negatively with the physical HRQOL.[27] Significantly worse ($p < 0.05$) satisfaction with life and DA was found in our study among patients who received social security benefits due to incapacity to work compared to those who did not. In contrast, no association was seen between employment and QOL. In other studies, unemployment was found to be associated with poorer QOL in patients treated with RRT.[6,17,28]

In our study group, longer time since the last change of RRT modality correlated positively with emotional well-being. Similarly higher number of kidney transplantations (experience of the loss of transplanted organ function) was associated with significantly worse emotional component of QOL and emotional well-being. Such observation is similar to the results described by Griva et al., who showed that the fear of losing transplanted organ function is associated with a significantly worse QOL among patients after kidney transplantation.[29]

In summary, our findings may offer guidance for pediatric nephrologists, who should pay due attention to the appropriate social development and independence of children with ESRD, and inform parents about the importance of their children's appropriate social development and interactions with peers. Results of this study indicate a serious need to create therapeutic teams that would include a psychologist and a social worker.

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The effect of high fructose corn syrup on the plasma insulin and leptin concentration, body weight gain and fat accumulation in rat

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Abstract

Background. Studies on the effects of high fructose corn syrup (HFCS) on the metabolism are scarce and their results are inconsistent.

Objectives. The aim of this research was to examine in an animal model the effect of replacing sucrose with HFCS-55 on the levels of glucose, insulin and leptin, and on the consumption of feed, body weight gain and fat storage.

Material and methods. The experiment was carried out on 30 Wistar male rats aged 5 months, fed 3 different diets, containing whole grains (group I), 10% sucrose (group II) and 10% HFCS (group III).

Results. It was found that the amount of daily energy intake was similar for all the groups of animals. There was no difference in fasting glucose and insulin level and homeostatic model assessment for insulin resistance (HOMA-IR) index. The higher leptin level was determined in blood plasma of the animal fed a feed with sucrose (group 2) compared to group 1 and group 3 (360 ng/mL vs 263 and 230 ng/mL, respectively). Despite the similar amounts of consumed energy, the animals fed with modified feeds achieved higher weight gain and the effect of HFCS-55 was similar to the effect of sucrose.

Conclusions. The obtained results indicate similar metabolic effects of HFCS-55 and sucrose in feed, at the level of 11% dietary energy value, on the energy intake, body weight gain and periorgan adipose tissue accumulation in rats. The results suggest that accusations against HFCS as the major dietary contributor to overweight and obesity are unfounded, and the total elimination of HFCS from the diet seems to be unnecessary. The modified feeds (containing both sucrose and HFCS) produced greater absolute weight gain and weight gain per kilojoule consumed compared to standard feeds. This may indicate not just a basic thermodynamic consequence of consuming more energy, but a change in the metabolic efficiency when consuming a diet with simple sugars and refined carbohydrates.

Key words: insulin, leptin, body weight, HFCS, fat tissue

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Introduction

The widespread use of high fructose corn syrup (HFCS) in the food industry has dynamically increased its consumption (by over 1,000% between 1970 and 1990), but studies on the health effects of HFCS have begun relatively recently.[1,2] For many years, HFCS has been considered a replacement for sucrose, having comparable metabolic effects. However, the increased number of obese people observed since the introduction of HFCS, for example in the USA, suggests a positive correlation between body weight gain and HFCS consumption.[3,4] Currently, this dietary component is being attributed a significant role in the development of obesity, which is classified as an extended metabolic disease that increases the risk of type 2 diabetes, hypertension, lipid disorders, cardiovascular disease, gout, kidney stones, and certain cancers.[5,6]

Statistics suggest that regular consumption of products containing HFCS increases appetite and promotes obesity and lipid disorders.[7] However, in population studies, other factors that also affect weight gain and nutrient metabolism, not just those related to food and HFCS intake, have not been assessed at the individual level. In addition, in many review papers the metabolic effect of HFCS is treated as an equivalent to that of pure fructose, which is clearly a mistake. Despite its name, the most widely used HFCS-55 form contains 55% fructose, 42% glucose and 3% other sugars, which is quite similar to the composition of sucrose (50% fructose and 50% glucose). Moreover, most studies have been conducted with the use of HFCS solutions drunk by animals, although HFCS is also present in solid foods. Animal model studies on the metabolic effects of HFCS are scarce and their results are divergent; however, many of them have confirmed adverse metabolic effects of HFCS,[8] due to possible alternations in energy homeostasis resulting from changes in leptin and insulin concentrations. However, not all studies confirm the adverse impact of HFCS on humans and experimental animals.[9–11] Organizations such as the American Medical Association (AMA) and the American Dietetic Association (ADA) have even issued a statement confirming metabolic equivalence of HFCS and sucrose.[12,13]

The aim of this research was to examine the effect of replacing sucrose with HFCS-55 (55% fructose and 42% glucose) in an animal model on the levels of insulin and leptin, and also on the consumption of feed, body weight gain and fat storage.

Material and methods

Material

The experiment, after approval of the Local Ethics Commission for Animal Experiments in Szczecin (approval No. 1/2012), was carried out in the vivarium of the Department of Human Nutritional Physiology, West Pomeranian University of Technology, Szczecin, Poland, on 30 Wistar male rats aged 5 months, of initial body weight 398 ±32.6 g. Rats were obtained from the animal husbandry of Chair and Department of Toxicology, Poznan University of Medical Sciences.

Methods

Following a week-long conditioning on the standard rat laboratory chow in the vivarium environment (temperature 21–22°C, humidity 55–60%, 12 h/12 h light/dark cycle), the animals were sorted into 3 equinumerous groups (n = 10) of equal body weight, housed in individual cages and fed ad libitum on pelleted feeds composed of the same components, besides those differentiating, produced by the Feeds and Concentrates Plant in Kcynia, Poland, after having implemented the procedure 5.14.5. "Cleaning of machines and devices". Group I was fed standard feed (Labofeed H), while group II received modified feed 1 and group III – modified feed 2. In the modified feed 1, 83.5% of wheat was substituted with wheat flour and 50% of corn grain was substituted with sucrose (in relation to standard feed). In modified feed 2 sucrose was substituted with high fructose corn syrup-55 (HFCS-55 – 55% content of fructose, in powder) in relation to modified feed 1. The selection of HFCS-55 resulted from the fact that it contains more fructose (than HFCS-42), which exerts adverse metabolic effects. HFCS-55 is added not only to beverages but also to solid foods.

Sucrose or HFCS accounted for 11.6% of the energy value of the diet. The percentage of the remaining components was unchanged (Table 1). Changes of feed components

Table 1. Component composition of feeds used in the experiment [g/100 g]

Component	Basic feed	Modified feed 1	Modified feed 2
Wheat	36.4	6	6
Corn grain	20	10	10
Wheat bran	20	20	20
Dry whey	3	3	3
Fodder salt[1]	0.3	0.3	0.3
Soya-bean grain	17	17	17
Fodder chalk[2]	1.5	1.5	1.5
Phosphate 2-CA[3]	0.8	0.8	0.8
Vitamin-mineral premix[4]	1	1	1
Wheat flour	–	30.4	30.4
Sucrose	–	10	–
High fructose corn syrup-55 (in powder)	–	–	10

[1] – mainly NaCl; [2] – mainly $CaCO_3$; [3] – $CaHPO_4$; [4] – vitamin-mineral composition used in animals feeds content per kg: IU: A 1500000, vitamin D_3, 100000; mg: vitamin E 8000; vitamin K 300, vitamin B_1 1200, vitamin B_2 1200, vitamin B_6 1000, vitamin B_{12} 8, Se 100, Fe 16000, Mn 4500, Zn 6000, Cu 1300, I 100, Co 200

were designed to reflect the changes taking place today in the composition of diets, which contains simple sugars and refined carbohydrates. For drinking, animals were provided pure, settled tap water.

Analyses

The prepared diets were subjected to chemical analysis[14] to determine the contents of total nitrogen with Kjeldahl's method, on Kjeltec 2100 apparatus (Foss, Hilleroed, Denmark), converted to quantity of protein, crude fat with Soxhlet's method, on Soxtec 1046 apparatus (Foss, Hilleroed, Denmark), dry matter (using a gravimetric method), ash (with a gravimetric method), and fiber with an ANKOM 220 apparatus (Ancom Technologies, New York, USA). The content of digested carbohydrates was derived from the difference between dry matter and the remaining solid components. The metabolic energy was calculated using commonly applied energy equivalents: protein – 4.0 kcal/g (16,76 kJ/g), fat – 9.0 kcal/g (37.71 kJ/g) and digested carbohydrates – 4.0 kcal/g (16.76 kJ/g) (Table 2).

The experiment lasted for 7 weeks, during which the amount of feed consumed by the animals was recorded daily, whereas once a week the animals were weighed. The amount of feed consumed was calculated from the difference between the weight of the feed given to the feeder and the mass of feed, which was left in the feeder, and the one that fell to the bottom frame. Upon completion of the experiment, the animals were fasted overnight (12 h) and anesthetized with an intramuscular injection (10 mg/kg b.w.) of Ketanest (Pfizer Ireland Pharmaceuticals, Cork, Ireland). Blood was sampled from the heart to tubes with anticoagulant and centrifuged at 2,000 g for 10 min at 4°C (MPW 350-R; MPW Medical Instruments, Warszawa, Poland). Plasma samples were stored at 4°C and assayed within 24 h.

Intraperitoneal and retroperitoneal fat was dissected out immediately after sacrificing the rats and weighed. Blood plasma obtained after clot centrifugation was assayed for the concentration of glucose with colorimetric method (biotest kit ref. No. 11503 BioSystems, Barcelona, Spain) on the Metertech spectrophotometer (Metertech, Taipei, Taiwan), insulin and leptin with enzyme-linked immunosorbent assay (ELISA) kit (Rat ELISA kit Demeditec Diagnostics, Kiel, Germany, insulin ref. No. DE2048; leptin ref. No. DEE006), according to the manufacturer's instructions. Assays were performed using EnVision apparatus (PerkinElmer Inc., Waltham, USA). To quantify insulin resistance and beta-cell function, a homeostatic model assessment for insulin resistance (HOMA-IR) was used, where: HOMA-IR = fasting glucose [mmol/L] × fasting insulin [μU/L]/22.5.[15]

Statistics

Biochemical data is shown as mean (Me) and standard deviation (SD). The resulting data was tested for normality of distribution (Shapiro–Wilk test) and processed statistically with STATISTICA software package v. 9 (StatSoft Inc., Tulsa, USA), using the post hoc Duncan test at the significance level $\alpha = 0.05$.

Results

The analysis of the results revealed that rats with a sucrose-containing diet consumed statistically significantly less food, although the amount of daily energy intake was similar for all the groups of animals (Table 3).

There was no difference in fasting glucose and fasting insulin level and HOMA-IR index between the groups (Table 3). There was, however, a marked difference in the level of leptin determined in fasting blood. A higher leptin level was determined in blood plasma of the animal fed the modified feed with sucrose compared to the other groups of animals.

Table 2. Chemical composition of feeds used in the experiment

Component	Basic feed	Modified feed 1	Modified feed 2
Total protein [%]	23.1 ±0.58^a	22.1 ±0.85^a	22.7 ±0.99^a
Crude fat [%]	2.76 ±0.07^a	2.90 ±0.14^a	2.82 ±0.12^a
Carbohydrates [%]			
total	60.1 ±0.48^a	61.9 ±0.51^a	60.1 ±0.62^a
fiber	4.48 ±0.13^b	4.03 ±0.15^a	4.06 ±0.09^a
digested	55.6 ±0.52^a	57.8 ±0.42^b	56.9 ±0.61^b
Total ash [%]	6.03 ±0.14^b	5.77 ±0.27^a	5.52 ±0.10^a
Dry matter [%]	92.0 ±0.12^a	92.6 ±0.19^a	92.0 ±0.11^a
Metabolizable energy			
[kcal·g^{-1}]	3.40 ±0.03 ^{a}	3.45 ±0.05^b	3.44 ±0.02^b
[kJ·g^{-1}]	14.20 ±0.20 ^{a}	14.42 ±0.12^b	14.38 ±0.10^b

$^{a, b}$ – means that denoted different letters in the same line are statistically different, $p \leq 0.05$.

Table 3. Effect of diet type on feed and energy intake, plasma glucose, insulin and leptin concentration and HOMA-IR index in rats, ±SD, n = 30

Trait	Group I	Group II	Group III
Feed intake [g/day]	17.7 ±1.03^a	17.0 ±1.22^a	17.8 ±0.91^a
Feed intake [g/100 g body weight/day]	3.90 ±0.11^b	3.77 ±0.09^a	3.89 ±0.11^b
Energy intake [kJ/day]	251 ±14.7^a	245 ±17.5^a	255 ±13.0^a
Energy intake [kJ/100 g body weight/day]	55.4 ±2.15^a	54.4 ±1.93^a	55.6 ±1.62^a
Glucose [mmol/L]	7.12 ±1.05^a	7.48 ±1.65^a	7.11 ±0.88^a
Insulin [pmol/L]	42.0 ±11.6^a	47.3 ±18.6^a	44.0 ±16.3^a
HOMA-IR	1.94 ±0.57^a	2.10 ±0.62^a	1.89 ±0.60^a
Leptin [ng/mL]	263 ±84.2^a	360 ±61.1^b	230 ±59.7^a

$^{a, b}$ – means denoted different letters in the same line are statistically different, $p \leq 0.05$; HOMA-IR – homeostatic model assessment.

Table 4. Effect of diet type on body weight gain and amount and localization of fatty tissue in rats, ±SD, n = 30

Trait	Group I	Group II	Group III
Initial body weight [g]	398 ±36.9[a]	399 ±31.9[a]	397 ±30.2[a]
Final body weight [g]	447 ±42.1[a]	457 ±37.0[a]	460 ±31.5[a]
Body weight gain [g]	48.4 ±9.6[a]	57.2 ±13.4[b]	63.1 ±11.1[b]
Body weight gain [g/100 g feed]	5.54 ±0.89[a]	6.82 ±1.47[b]	7.24 ±1.26[b]
Body weight gain [g/1000 kJ]	3.90 ±0.63[a]	4.73 ±1.02[b]	5.04 ±0.87[b]
Intraperitoneal fat [g]	3.57 ±0.99[a]	3.58 ±0.72[a]	3.34 ±0.70[a]
Intraperitoneal fat [g/100 g b.w.]	0.779 ±0.190[a]	0.787 ±0.118[a]	0.724 ±0.074[a]
Intraperitoneal fat [g/100 g feed]	0.409 ±0.105[a]	0.427 ±0.069[a]	0.383 ±0.074[a]
Intraperitoneal fat [g/1000 kJ]	0.288 ±0.074[a]	0.296 ±0.048[a]	0.267 ±0.051[a]
Retroperitoneal fat [g]	3.18 ±1.31[a]	3.08 ±0.99[a]	3.13 ±0.88[a]
Retroperitoneal fat [g/100 g b.w.]	0.685 ±0.211[a]	0.678 ±0.179[a]	0.675 ±0.165[a]
Retroperitoneal fat [g/100 g feed]	0.362 ±0.135[a]	0.366 ±0.094[a]	0.357 ±0.091[a]
Retroperitoneal fat [g/1000 kJ]	0.255 ±0.090[a]	0.254 ±0.065[a]	0.249 ±0.063[a]
Sum of intra- and retroperitoneal fat [g]	6.75 ±1.02[a]	6.66 ±0.82[a]	6.47 ±0.76[a]
Sum of intra- and retroperitoneal fat [g/100 g b.w.]	1.46 ±0.40[a]	1.46 ±0.29[a]	1.40 ±0.24[a]
Sum of intra- and retroperitoneal fat [g/100 g feed]	0.770 ±0.226[a]	0.793 ±0.155[a]	0.741 ±0.141[a]
Sum of intra- and retroperitoneal fat [g/1000 kJ]	0.542 ±0.159[a]	0.550 ±0.107[a]	0.515 ±0.098[a]

[a, b] – means denoted different letters in the same line are statistically different, $p \leq 0.05$; b.w. – body weight; SD – standard deviation.

Despite the similar amounts of consumed energy, the animals fed with modified feeds achieved higher weight gain, both in absolute terms and per 100 g of consumed food and per unit of consumed energy, and the effect of HFCS-55 was similar to sucrose (Table 4).

When analyzing the obtained results, we observed no significant effect of replacing sucrose with HFCS on the amount of intraperitoneal and retroperitoneal adipose tissue (Table 4). The amount of periorgan adipose tissue was similar in all groups of animals.

Discussion

It was found that changes in the diet composition influenced the feed intake per body weight. Rats fed a sucrose-containing diet consumed statistically significantly less food, although the daily energy intake was similar for each animal group. Similar energy consumption at lower feed intake may result from higher energy value of modified feeds. Lower consumption of feed containing sucrose compared to standard feed may have been caused by its higher energy value and its better digestibility due to the lower fiber content. DiMeglio and Mattes[16] showed that the consumption of sugars in solid foods, similarly to our experiment, results in compensatory leveling of the energy intake through the modification of the amount of consumed food. However, when carbohydrates are given in fluids, this regulation is less precise and the administered fluids increase the energy intake and body weight gain. The lower intake of feed by animals from group II may have also resulted from the increased level of leptin, which reduces food intake by stimulating the satiety center.

One of the arguments against the use of HFCS is its potential ability to affect insulin and leptin levels, as fructose, unlike glucose, does not stimulate the secretion of insulin and leptin, and may increase the intake of food. In this study, HFCS reduced fasting leptin level, which in the long-term may have been the cause of increased food intake by animals from group III compared to group II. There were no differences in insulin levels between the groups.

In a study by Monsivais et al.,[17] solutions of HFCS-55 and sucrose exerted similar effects on insulin levels, satiety, and food intake in rats. Similar results were also reported by Akhavan and Anderson[18] in men, and Melanson et al.[9] in women. Soenen and Westerterp–Plantenga[19] observed no effect of HFCS consumed in soft drinks on either satiety, energy intake or body weight in men and women. They observed similar changes in the concentrations of ghrelin, insulin and glucose resulting from the consumption of HFCS- and sucrose-containing drinks.

HFCS-55 administered in solid foods or fluids, in the amounts equivalent to 10–15% of dietary energy value, did not differ significantly from sucrose in terms of the effect on either levels of hormones regulating food intake, the sensation of satiety or energy intake. However, in the aforementioned studies,[18,19] the insulin and leptin levels were determined several times a day, directly after the consumption of drinks or foods containing HFCS-55 or sucrose. In our study, insulin and leptin levels were determined in fasting blood samples, so their changes should have resulted from long-term physiological processes and may affect long-term food intake. Despite the lower leptin levels in rats fed with a HFCS-containing mixture, their energy intake was not higher than in group II.

Despite the similar energy intake, the animals fed with modified feeds achieved greater weight gain, both absolute and relative (per 100 g of food consumed and per energy unit consumed), the effect of HFCS-55 being in this respect similar to sucrose. Insignificant effects of HFCS on body weight gain were also observed during the 8–10-week experiments by Akar et al.[20] in male rats and by Light et al.[21] in female rats. Similarly to our experiment, the female rats which consumed HFCS-55 had higher body weight gain compared to the control group but comparable

to the animals receiving sucrose.[21] Similar results were also obtained by Figlewicz et al.[22] Detailed studies on the effect of HFCS on body weight gain was performed by Bocarsly et al.,[23] who, in contrast, observed a significantly higher weight gain in animals receiving HFCS compared not only to controls, but also to sucrose-receiving rats.

However, the form of administration of sugars was different than in our study, i.e., in aqueous solution, which may modify the rate of absorption of monosaccharides, increase the glycemic effect, enhance energy overconsumption, and lead to fatty tissue accumulation. It is interesting that the modified feeds (both sucrose- and HFCS-containing one) produced not just greater weight gain than the standard feed, but also greater weight gain per energy unit consumed. This may indicate not just a basic thermodynamic consequence of consuming more calories, but a change in the metabolic efficiency when consuming a diet with simple sugars and refined carbohydrates.

Therefore, the observed higher weight gain in animals fed with mixtures containing simple sugars and refined carbohydrates were not associated with intra-abdominal fat accumulation. They may have resulted from higher absorption of sodium and water in the digestive tract, enhanced by glucose present in food,[24] and from higher synthesis of glycogen (which binds water) or from fat accumulation in regions other than the examined visceral area.

In an experiment by Bocarsly et al.,[23] male rats receiving 8% HFCS solution accumulated much higher amounts of fat around the urinary tract and intra-abdominal fat, but not perivisceral fat, compared to rats receiving 10% sucrose solution. Marini et al.[25] observed that 10% HFCS solution had a similar effect on the accumulation of adipose tissue to the analogous solution of sucrose; a significant effect of HFCS in this regard was observed at a 20% concentration. Bravo et al.,[26] who administered 8%, 18% and 30% solutions of HFCS and sucrose to people, observed no difference in their effects on body weight, total body fat as well as intramuscular and hepatic fat between 8% and 18% sugars solutions. Consumption of 30% solutions of both sugars increased the body weight but not fat content, and there was no difference between HFCS and sucrose.

Conclusions

In conclusion, the obtained results indicate similar metabolic effects of HFCS-55 and sucrose in feed, at the level of 11% dietary energy value, on the energy intake, body weight gain and periorgan adipose tissue accumulation in rats. The results suggest that accusations against HFCS as the major dietary contributor to overweight and obesity are unfounded, and the total elimination of HFCS from the diet seems to be unnecessary. The modified feeds (both sucrose- and HFCS-containing) produced greater, compared to the standard feeds, absolute weight gain and weight gain per kilojoule consumed. This may indicate not just a basic thermodynamic consequence of consuming more energy, but a change in the metabolic efficiency when consuming a diet with simple sugars and refined carbohydrates.

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Design and characteristics of new experimental chlorhexidine dental gels with anti-staining properties

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Abstract

Background. Chlorhexidine-based products are often used in medicine and dentistry as dental hygiene and therapeutic products, especially by patients with various oral tissue diseases. However, these products have disadvantages, such as low stability, as well as discoloration of the teeth and dental reconstruction materials.

Objectives. The aim of this study was to create and evaluate experimental chlorhexidine (CHX) gels with anti-staining properties and to compare them with 3 commercially available products.

Material and methods. For this study, 4 new formulations containing 1% CHX and different anti-staining agents were developed. The properties of these gels were compared with 3 commercial CHX-based dental products. The pH, viscosity, disintegration in water, and anti-staining properties were evaluated.

Results. The pH level of the 4 new CHX gels ranged from 5.92 to 6.33. The viscosity of the experimental gels was higher (85.7÷217.7 Pa·s) than the commercial ones (11.6÷72.7 Pa·s). Among the experimental formulations with 1% CHX, the formulation with 5% polyvinylpyrrolidone (PVP) and 0.2% citric acid and the formulation with 1% citric acid were the most stable in terms of pH and viscosity. The disintegration times of the experimental gels were longer (50–70 min) as compared with the commercial products (approx. 20 min). These 2 CHX gels caused less color change of glass ionomer cements in black tea solution.

Conclusions. To conclude, 2 new experimental dental gels based on 1% CHX, one with 1% citric acid and the second with 5% PVP and 0.2% citric acid, had the most favorable physicochemical properties. Further research is needed to evaluate their therapeutic potential in the treatment of diseases of the oral cavity.

Key words: viscosity, disintegration time, gel, chlorhexidine rinse, anti-staining properties

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Chlorhexidine (CHX) was developed in the 1940s by Imperial Chemical Industries (ICI, Macclesfield, UK) and marketed since 1954 as a general disinfectant.[1] It is a bisbiguanide antiseptic, active against both Gram-positive and Gram-negative organisms, including aerobes and anaerobes, yeast and fungi. Its mechanism of action leads to the rupture of the bacterial cell wall and precipitation of the cytoplasmic contents. Higher concentrations of CHX result in better efficacy, but also increase its side effects, such as staining of the teeth and restorations.[1–3] Najafi et al. reported similar effectiveness of both 0.2% and 0.12% digluconate CHX mouth rinses in the reduction of plaque index and gingival index. They also found that 0.2% CHX was more effective in terms of the gingival bleeding index but caused much more teeth staining than 0.12% CHX.[4]

Chlorhexidine has superior antiplaque activity due to its ability to adsorb and bind to soft and hard tissues. When CHX is used after brushing, an interval of at least 30 min should be kept between tooth brushing and rinsing with this chemical because of possible inactivation between various positively charged dentifrice detergents and the cationic CHX.[5–7] This property of CHX has still not been clearly verified, but it was described for the first time in the 1970s.[5]

Chlorhexidine salts are available in various formulations for dental applications (mouth rinses, gels and toothpastes).[4–9] Some studies have reported that CHX might be also released from methacrylic resins and experimental glass ionomer cements.[9–11] Mucoadhesive dosage forms, including gels and films, have been extensively developed for the treatment of oral diseases. They are frequently used in local therapy of periodontal inflammations. Although one of the limitations of gel formulations is their inability to deliver a quantified dose of the drug to the site, gels have some advantages over other formulations, such as ease of preparation and administration, relatively faster release of the incorporated drug, as well as higher biocompatibility and mucoadhesivity.[12]

However, an important issue is the instability of the gels. Several studies have reported on the instability of CHX gels, based on measurements of pH changes and viscosity over a given period of time.[13,14]

One of the disadvantages of CHX is staining following long-term use. It is commonly accepted that prolonged CHX use can change the color of the teeth and dental restorative materials (composites and cements). Different combinations have been introduced to reduce the brown pigmentation and other side effects caused by CHX. Various products, such as peroxoborate, polyvinylpyrrolidone (PVP), sodium metabisulfite, and ascorbic acid, are added to CHX.[15–19] Natural teeth have been used in in vitro tests, but the color of natural dentition depends on many factors and the typical deviation is very high.[20,21] To reduce the influence of individual factors, some authors have used dental restorative materials or hydroxyapatite disks for the evaluation of color change.[22–24]

Due to its various advantages, CHX is a common agent in the treatment of different oral diseases. However, there are still some unfavorable effects related to its use. The development of new formulations with improved composition could reduce the side effects of CHX. The purpose of this study was to develop new experimental mucoadhesive gels in vitro with 1% CHX and to compare them with 3 commercially available gels.

Material and methods

Preparation of the experimental gels

For this study, 4 new formulations containing 1% CHX with different anti-staining agents were prepared. The raw materials used for the preparation of these experimental gels are summarized in Table 1. All the materials were used without undergoing any purification process. A dark glass bottle (60 mL) was filled with 30 g of distilled water, and all the ingredients were then added. Toward the end of the process, 1.5 g of hydroxyethyl cellulose (Natrosol 250, Ashland Specialty Chemical Co., Convington, USA) was added as a gelling agent, together with 2.5 g of CHX. The samples were mixed using a magnetic stirrer (Sunlab SU1200, Mannheim, Germany) until a colorless solution was obtained. After preparation, all gels were stored at room temperature (23°C) in a dark place. The properties of the experimental gels were compared with 3 commercial gels: Curasept 1%, Curasept 0.5% (both from Curaden International AG Healthcare S.p.A., Saronno VA, Kriens, Switzerland) and Dentosan 0.5% (Recordati S.p.A, Milan, Italy) (Table 2).

Table 1. Raw materials used to prepare the gels

Formulation	Composition
Experimental gel No. 1–4	CHX digluconate 1% (Medichem, Germany)
	hydroxyethyl cellulose Natrosol 250 (Aqualon, USA)
	Aroma 56041 Mint (Lipo Technologies, USA)
	sodium hydroxide (Brenntag, Germany)
	Tween 80 (Acumedia Manufacturers, Inc., USA)
	glycerol (Brenntag)
Experimental gel No. 1	polyvinylpyrrolidone 5% (Plastodone K29/32 ISP, Ashland, USA)
	citric acid monohydrate 0.2% (Brenntag)
Experimental gel No. 2	malic acid 0.5% (Brenntag)
Experimental gel No. 3	citric acid monohydrate 1% (Brenntag)
Experimental gel No. 4	potassium oxalate monohydrate 1% 60425 (Sigma-Aldrich, USA)
	citric acid monohydrate 0.22% (Brenntag)

CHX – chlorhexidine.

Table 2. Commercial chlorhexidine gels used in the study

Formulation	Composition
Curasept 1% (Curaden International AG)	water, propylene glycol, hydroxyl ethyl cellulose, PVP/VA copolymer, PEG 40, hydrogenated castor oil, CHX digluconate, sodium acetate, aroma, acetic acid, sodium metabisulfite, ascorbic acid
Curasept ADS 350 0.5% (Curaden International AG)	propylene glycol, glycerol, xylitol, hydroxyl ethyl cellulose CHX digluconate, ascorbic acid, PEG 40, hydrogenated castor oil, sodium metabisulfite, aroma, methylparaben
Dentosan 0.5% (Recordati S.p.A)	propylene glycol, sorbitol, hydroxyl ethyl cellulose glycerin, CHX digluconate, xylitol, PEG 40, hydrogenated castor oil, menthol, aroma, methylparaben, citric acid

PEG – polyethylene glycol; CHX – chlorhexidine.

Stability of the pH of the gels

For the evaluation of the stability of the gels, quantification of pH values was chosen. The pH was measured initially after the preparation of the gels, and then after 1, 2 and 4 months of storage at room temperature using a Voltcraft PHT-01 ATC pH meter (CEI Conrad International Ltd., Hong Kong, China).

Stability of the viscosity of the gels

Viscosity tests were performed according to the standard procedure using a Haake rheometer (Thermo Fisher Scientific, Waltham, USA). Each experimental gel (0.5 g) was placed on the plate of the rheometer. The upper plate of the machine was pulled down and the instrument was run at a rotary speed of 1 rpm.

Gels and their disintegration in water

The purpose of this experiment was to observe the disintegration of the experimental gels in water. For these tests, the gel dissolution protocol found in the U.S. Pharmacopeia was followed, according to the description of the "paddle over disk" method.[18] The disk assembly for holding the gels was made of acrylic resins. A distance of 2 ±2 mm was maintained between the paddle and the surface of the disk assembly. The test container was filled with distilled water and an acrylic plate covered with 1 g of CHX gel was affixed to the bottom. The temperature was maintained at 32 ±0.5°C. To simulate the saliva flow inside the mouth, the paddle rotation speed was set at 300 rpm.

Anti-staining properties of the gels

To measure the anti-staining properties of the gels, 24 glass ionomer disks (3 for each experiment and 3 as controls) were prepared (Kavitan LC glass ionomer cement, color A3; SpofaDental AS, Jičín, Czech Republic). The diameter of each disk was 20 mm, and the thickness was 3 mm. The cement was prepared by mixing 1.2 g of powder and 0.5 g of liquid with a spatula to obtain the required paste consistency. The material was then placed inside a metal form and covered with polyethylene (PE) foil on both sides. The samples were cured with a Demi Ultra curing unit (Kerr Dental, Bioggio, Switzerland) for 20 s for each side. After curing, the samples were removed from the metal form, and after 24 h of storage in a dark place, the initial color of the disks was measured using an eXact^{TM} colorimeter (X-Rite, Grand Rapids, USA) in normal standard light to obtain values for the lightness of the color (L), its position between red and green (a) and its position between yellow and blue (b). In the next step, the samples were covered with CHX gels using a plastic spatula. All the disks were then put in a black tea solution for 1 week, since CHX gel can change the color of teeth or oral mucosa in the presence of tea or coffee as a result of the Maillard reaction.[25] Lipton tea (Unilever Food Solutions, London, UK) was prepared by placing 1 tea bag in 200 mL of boiling water for 5 min. The tea solution was then poured in 10 plastic cups (about 20 mL each). Tweezers were used to put the disks into the tea solution. Three disks without CHX gel were also immersed in the black tea solution as a control group.

After 1 week of storage at room temperature, the samples were removed from the tea solution and cleaned with a brush under a stream of water. The color was measured for the second time with the colorimeter to obtain values L1, a1 and b1. Color changes were calculated as ΔE, according to the equation:

$$\Delta E = \sqrt{(L1 - L)^2 + (a1 - a)^2 + (b1 - b)^2}$$

Statistical analysis

The results were expressed as the mean value of 3 measurements for each sample. The data was analyzed using STATISTICA v. 12 software (StatSoft Inc., Tulsa, USA). The significance of the difference between the mean values of different groups and the control group was assessed using Student's t-test, with p-values ≤0.05 or ≤0.005 taken as statistically significant.

Results

Stability of the pH of the gels

The results of the measurements of pH stability are presented in Fig. 1. The initial pH levels of experimental gels 3 and 4 were found to be 6.21 and 6.31, respectively,

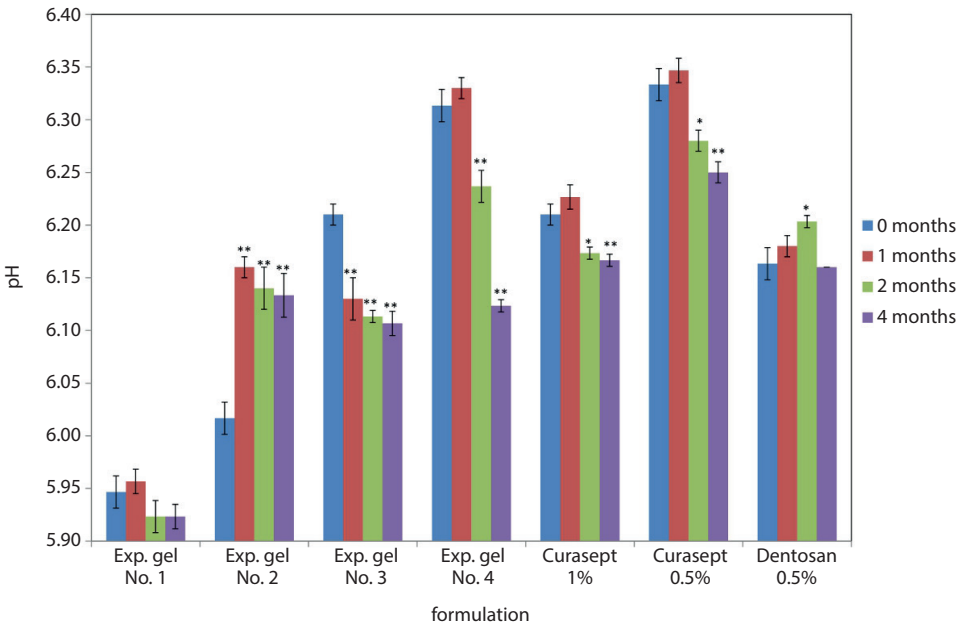

Fig. 1. The stability over time of the pH levels of the gels measured after the preparation of the gels and 1, 2 and 4 months later; changes in pH level with p-values ≤0.05 or ≤0.005 were considered statistically significant

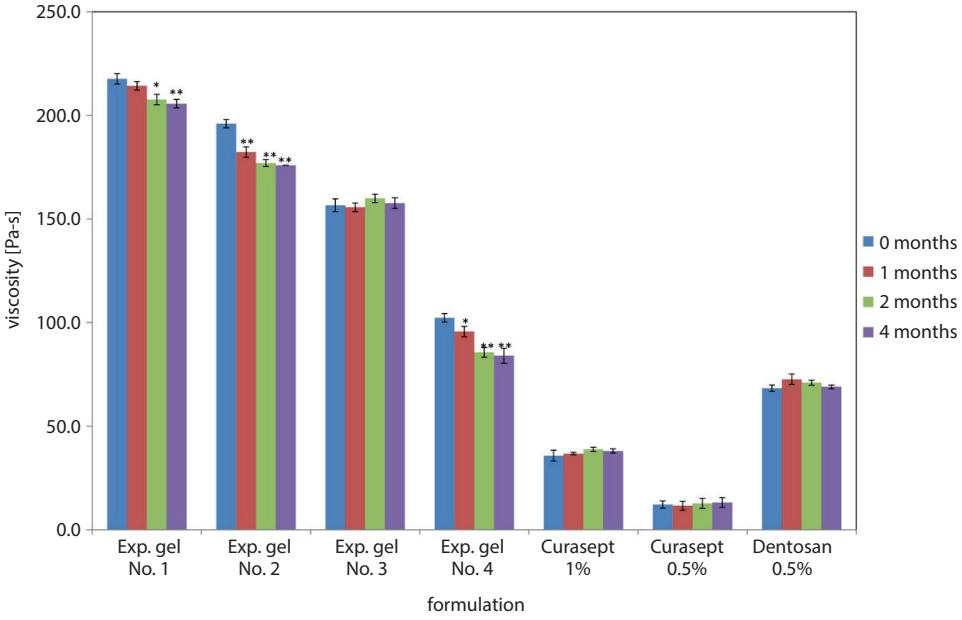

Fig. 2. The stability over time of the viscosity of the gels measured after the preparation of the gels and 1, 2 and 4 months later; changes in viscosity with p-values ≤0.05 or ≤0.005 were considered statistically significant

which were comparable to the initial pH levels of the commercial products (6.16–6.33). Experimental gels 1 and 2 had lower initial pH levels (5.95 and 6.02, respectively). After being stored for 1, 2 and 4 months, the pH values of both the experimental and the commercial gels changed. The maximum change in pH was observed in experimental gel 4 (3% of the initial value). The most stable gel in terms of pH was experimental gel 1, in which a pH change of only 0.4% was noted.

Stability of the viscosity of the gels

The results of measurements of the stability of the viscosity of the gels are presented in Fig. 2. Experimental gels had a higher initial viscosity (102.3–217.7 Pa·s) than the commercial products (12.2–68.3 Pa·s). Gels 1 and 3 had stable viscosity, as did the commercial products. After 2 months of storage, the viscosity of gels 2 and 4 had decreased by 10% and 16%, respectively.

Gels and their disintegration in water

The results of the measurements of the disintegration time of the gels in water are summarized in Fig. 3. The commercial gels had a shorter disintegration time (19.3–22.7 min) than the experimental gels used in this experiment (49.3–71.3 min).

Anti-staining properties of the gels

Changes in the color of the glass ionomer cement after 1 week of contact with black tea solution and the CHX gels

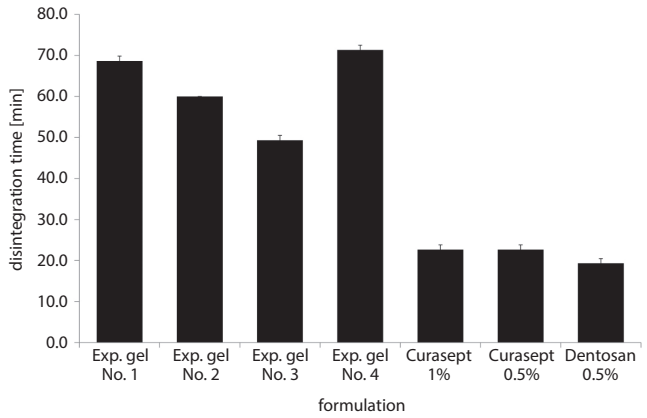

Fig. 3. Gel stability in water: a model of disintegration

are presented in Fig. 4. Black tea alone had a major influence on the color of the glass ionomer cement. In the control group, ΔE was 9.2 after 1 week. In the disks that were covered with commercial gels with a lower concentration of CHX (0.5%), the color change was lower (for Curasept 0.5% ΔE was 2.9%, and for Dentosan 0.5 it was 5.6%). Treatment with Curasept with 1% CHX resulted in the highest ΔE value: 11.87%. The same result was obtained for experimental gel 4. However, the disks covered with experimental gels 1–3 showed less color change. For the experimental gel 3, ΔE was found to be 4.0%.

Discussion

Some of the raw materials used to improve the anti-staining properties of CHX gels are very strong anionic substances, such as sulfide or disulfide. However, CHX gel is not stable in the presence of anionic substances.[1,2,25–27] Aqueous solutions of CHX are most stable within the pH range from 5 to 8. Above pH 8.0, CHX base is precipitated and under more acidic conditions, a gradual degradation of the gel and reduction of its antibacterial activity can be observed.[27]

Our study revealed that after the addition of anionic substances, pH of the CHX gels changes over a period of time, and they start to release an unpleasant smell. For consumer acceptance, manufacturers of commercially available products containing sulfite ions need to add other substances to prevent these undesirable consequences.

In the literature, it is hypothesized that the extrinsic tooth staining associated with CHX and metal salts occurs due to the formation of metal sulfides. Chlorhexidine denatures proteins in the acquired pellicle by splitting disulfide bridges. This leads to the production of reactive sulfhydryl groups, which can react with iron or tin ions to produce pigmented products.[28] The use of substances with reductive properties, like ascorbic or citric acids, as protection against the browning reaction was described by Ozdemir.[25] Another method to obtain this effect is to use agents that can form a complex with metal ions. However, we found that during storage, not all gels incorporated with such agents are stable. For example, after 1 month of storage, gels with ascorbic acid and isoascorbic acid become yellow. Li et al. tested gels with anti-staining additives and observed that they were capable of reducing the side effect of staining of CHX staining.[29] In our study, the best anti-staining properties (the smallest color change) were demonstrated for gels with PVP and citric acid. These compounds were able to protect glass ionomer disks from color changes resulting from a 1-week immersion in black tea solution.

The polymer used in gel preparation is an important water-soluble excipient and also serves in controlled oral drug-delivery systems. It provides thickening properties and contributes to pH stability, water retention and adhesion power. A similar effect of prolonged CHX release has also been observed in the presence of acidic polymers carrying carboxylic groups, such as polyacrylic acid or alginate. This may suggest that the formation of a complex of these substances with CHX may be responsible for CHX retention in the gel, which may be used to control its release.[26] Gels made by Fini et al. with a higher concentration

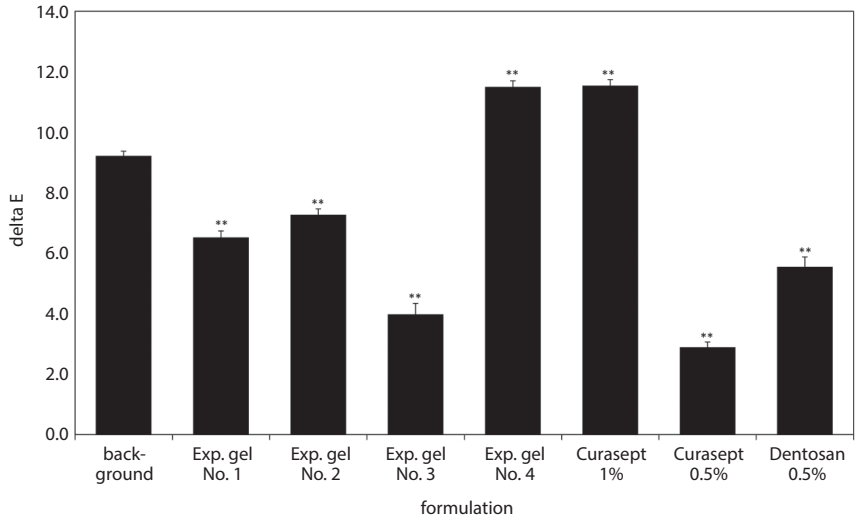

Fig. 4. Color changes in glass ionomer cement covered with CHX gels after 7 days of storage in black tea solution. Differences in the results for different gels and the controls (glass ionomer cement without gel but with black tea solution) with p-values ≤0.005 were considered statistically significant

of the gelling agent Lutrol (15–25%) had a higher viscosity than commercial CHX products and other materials prepared during their study.[26] For example, a gel with 25% Lutrol had a higher viscosity (7413 cps.) than one with 20% Lutrol (827 cps.) at 37°C.

In our study, the experimental gels had also a higher viscosity than the commercial products. The adhesion of experimental gels to the disk during the disintegration tests was better than the performance of commercial gels: disintegration times ranged between 50 and 70 min for the experimental gels, compared to the complete disintegration of the commercial products after approx. 20–25 min.

Conclusions

To conclude, 2 new experimental gels, based on 1% CHX, one with 1% citric acid and the other with 5% polyvinylpyrrolidone and 0.2% citric acid, showed the best physicochemical properties among the gels tested in our study. Further research is still needed to evaluate their therapeutic potential.

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Relationship between the dietary glycemic load of the adult Polish population and socio-demographic and lifestyle factors – results of the WOBASZ II study

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Conflict of interest
None declared

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Abstract

Background. Cardiovascular disease (CVD) occurs more often among people with a low sociodemographic status, so it is worth knowing if any sociodemographic factor also has an impact on diet quality, defined by glycemic load (GL).

Objectives. Assessment of the relationship between the sociodemographic and lifestyle factors, health status self-assessment and dietary GL of the adult Polish population based on WOBASZ II study results.

Material and methods. The study included a representative group of the Polish population aged ≥20 years (2,554 men and 3,136 women). A 24-hour dietary recall was collected to assess the dietary intake. The total GL was calculated by summing the GL values of the consumed food.

Results. The average GL/1,000 kcal was significantly higher in women's than in men's diets (74.0 ±15.9 vs 71.2 ±15.7). Dietary GL/1,000 kcal increased with age (men: aged <35 – 70.3 and aged ≥65 – 73.9, women: 73.5 and 76.5, respectively). The lowest dietary GL/1,000 kcal was found among people living in large population centers. Dietary GL/1,000 kcal decreased with education level (men with primary, secondary and higher education: 73.4, 69.5 and 68.9, respectively, and women: 76.7, 73.4 and 70.9, respectively). Dietary GL decreased as an income increased. The highest GL/1,000 kcal was observed in the diets of participants who performed less physical activity. The highest GL/1,000 kcal was observed in the participants who defined their health status as very poor/poor and the lowest among those who defined their health status as good/very good.

Conclusions. Nutritional education about the proper selection of products that are sources of carbohydrates in the diet should be addressed mainly to people with low sociodemographic status, such as: people in the older age group, living in small population centers, and with lower levels of education and lower income. It should also be directed to people with a lower level of physical activity. Greater awareness of the choices of carbohydrate products is recommended to improve diet quality in these groups of people.

Key words: carbohydrates, socio-demographic factors, glycemic load, WOBASZ II

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Introduction

The European Society of Cardiology has emphasized that low socioeconomic status as well as other psychosocial factors (e.g., lack of social support, stress at work and in family life, depression, anxiety) increase the risk of developing cardiovascular disease (CVD) and contribute to a worse prognosis of CVD treatment.[1] Kollia et al., based on a 10-year follow-up observation, proved that there is a consistent inverse relation between socioeconomic status and the incidence of CVD.[2] In a study conducted by Kozakiewicz et al. in a random Polish population, increase in socioeconomic status was related to a decreased proportion of high CVD risk subjects in the overall study population and in participants aged 30–39 years.[3]

Taking into account the fact that an unhealthy diet is also one of the risk factors for the development of CVD, it is worth knowing what sociodemographic factors have an impact on diet quality. Kwaśniewska et al., based on results obtained in the Multi-centre National Population Health Examination Survey in Poland (Project WOBASZ), observed that sociodemographic factors associated with fruit and vegetable consumption included: gender, age, place of residence, and health status self-assessment.[4] Other authors showed that factors such as gender, age and level of education had a significant impact on the nutritional value of the diets of people residing in Świętokrzyskie voivodeship.[5]

One of many values that can be used to describe diet quality is glycemic load (GL). It is calculated based on the glycemic index (GI) of food and the available carbohydrate content in the portion of the consumed food. Glycemic load provides an indication of glucose available for energy or storage following a carbohydrate-containing meal. It classifies food as having low (≤10), medium (>10 – <20) or high GL (≥20).[6] The results of many studies have shown that high dietary GL plays an important role in increasing the risk of CVD development.[7,8]

The mechanism of impact of high dietary GL on the human body can be diverse and can lead to several disorders which have been observed by, among others, Murakami et al.[9] Their cross-sectional study conducted among 1,354 Japanese women aged 20–78 years showed that dietary GL was independently negatively correlated with high-density lipoprotein (HDL)-cholesterol, and positively correlated with fasting triacylglycerol and glucose level in blood.[9] Other authors have observed that the intake of high GI/GL foods may increase the risk of type 2 diabetes and lead to oxidative stress.[10,11]

Taking into account the arguments that dietary GL may be related to the risk of CVD development and that CVD occurs more often among people with lower sociodemographic status, it seems important to known which sociodemographic factors also have an influence on dietary GL. This is also relevant in view of the fact that there are no Polish studies concerning this topic.

The aim of the study was to assess the relationship between the sociodemographic and lifestyle factors, health status self-assessment and dietary GL in adult Polish population based on the WOBASZ II study results.

Material and methods

The study group consisted of a representative group of the Polish adult population aged ≥20 years who participated in the project WOBASZ II conducted in the years 2013–2014. The assumption and methods of WOBASZ II, including the method of sample drawing, have been previously described by Drygas et al.[12]

This study was conducted among 5,690 participants (2,554 men and 3,136 women), who completed the 24-hour dietary recalls, based on which the dietary GL was computed. Moreover, the study participants were asked if they were on a diet (none, reducing diet, low fat/low cholesterol/antidiabetic, other, e.g., anti-allergic) during the study.

The sociodemographic factors analyzed in this study included: age (which was categorized as: <35 years old, 35–49 years old, 50–64 years old, ≥65 years old), size of the population center (small, medium, large) inhabited, level of education (primary, secondary, higher), net monthly income per member of family (<1,000 PLN, 1,000–2,000 PLN, 2,000–3,000 PLN, >3,000 PLN), marital status (married, others: single, widow/widower, divorced, separated), smoking status (current smoker, former smoker, never-smoker), and level of physical activity during leisure time (low, moderate, high). Physical activity was assessed at a low level when there was no physical activity such as jogging, cycling, swimming, or gardening for at least 30 min a day or only occasional activity (once a month, several times a month, several times a year); middle level – physical activity such as jogging, cycling, swimming, or gardening for at least 30 min a day, every second or third day or once a week; high level – physical activity as given above every day or almost every day. Moreover, the study participants were asked for their health status self-assessment (very good and good, moderate, poor and very poor).

The nutritional value of the daily food rations (DFRs) was assessed with the use of Food Composition Tables.[13] The GL values were computed based on the digestible carbohydrate content in the portion of the individual foods and the GI values of these foods, using the following formula:

$$GL = [\text{digestible carbohydrate content in the portion of food [g]} \times GI]/100.$$

The total GL of each diet was calculated by summing the GL values of the foods consumed. There is no Polish database with the GI values of foods, so they were taken from international tables wherein GI values were determined experimentally for different food products and dishes.[14–16] However, the GL values for typical Polish dishes which do not exist in international tables were computed based on their

Table 1. Comparison of the mean dietary glycemic load, energy and macronutrient intake between the men and women studied

Dietary compound	Men		Women		p-value
	X ±SD	95% CI	X ±SD	95% CI	
Glycemic load/day	163.6 ±72.2	160.8–166.4	124.1 ±54.2	122.2–126.0	<0.0001
Glycemic load/1,000 kcal	71.2 ±15.7	70.5–71.8	74.0 ±15.9	73.5–74.6	<0.0001
Energy [kcal/day]	2,317 ±935	2,281–2,354	1,678 ±639	1,656–1,701	<0.0001
Carbohydrates [g/day]	287.5 ±119.1	282.9–292.1	224.5 ±90.6	221.3–227.7	<0.0001
Carbohydrates [% energy]	47.1 ±9.2	46.8–47.5	49.8 ±9.4	49.5–50.1	<0.0001
Saccharose [g/day]	57.3 ±46.6	55.5–59.1	51.0 ±38.5	49.6–52.3	<0.0001
Starch [g/day]	169.4 ±73.6	166.6–172.3	119.0 ±52.5	117.2–120.9	<0.0001
Dietary fiber [g/day]	20.9 ±9.1	20.6–21.3	17.5 ±7.8	17.3–17.8	<0.0001
Protein [g/day]	86.0 ±36.9	84.6–87.5	61.4 ±24.5	60.6–62.3	<0.0001
Protein [% energy]	15.4 ±3.8	15.2–15.5	15.1 ±4.0	14.9–15.2	<0.0001
Fats [g/day]	97.0 ±49.3	95.1–98.9	66.2 ±32.3	65.1–67.4	<0.0001
Fats [% energy]	37.5 ±8.4	37.2–37.8	35.1 ±8.5	34.8–35.4	<0.0001

X ±SD – average ± standard deviation; 95% CI – 95% confidence interval.

recipes described in the Food Composition Tables.[13] In order to assess the sources of GL of all foods and dishes, taking into account their nutritional value, they were classified into the following groups: dairy, refined and unrefined grains, sweets, vegetables, fruits, dried fruits, nuts and cocoa, potatoes and potato products, soups, juices, sweetened beverages, mixed dishes, alcoholic beverages, and others. Dietary GL strongly depends on energy intake and the amount of carbohydrates in the diet,[17] therefore, for the analysis of values of dietary GL in this study, they were calculated for energy intake (GL/1,000 kcal), as it was done by other authors.[9]

Statistical analysis

The obtained results were summarized by average (X) and standard deviation (SD). Dietary GL was adjusted for energy intake (GL/1,000 kcal). The differences in the nutritional values of the diets between 2 groups were compared using Mann–Whitney U test. The differences in the GL values of the diets between more than 2 groups were compared with the Kruskal–Wallis test. The results obtained were considered to be statistically significant at $p < 0.05$. Statistical analysis was performed using SAS software v. 9.2 (SAS Institute, Cary, USA).

Results

Table 1 shows a comparison of GL, energy and macronutrient intake of diets between men and women. The average GL in men's diets was significantly higher than in women's diets (163.6 ±72.2 vs 124.1 ±54.2); however, GL/1,000 kcal was significantly higher in women's diets than in men's diets (74.0 ±15.9 vs 71.2 ±15.7). Energy intake as well as the intake of all macronutrients was significantly higher in the DFRs of men than of women. The percentage of energy from proteins and fats was significantly higher in the diets of men than in the diets of women, while the percentage of energy from carbohydrates was significantly lower for men's diets than for women's diets.

The contribution of food groups in the dietary GL of men and women participating in the study is summarized in Table 2. The main sources of GL in the DFRs of men as well as in the DFRs of women were refined grain products (44.0% and 35.3%, respectively) and sweets (20.2% and 24.9%, respectively).

Table 3 shows the relationship between dietary GL (GL/1,000 kcal) and sociodemographic factors

Table 2. The contribution of food groups in the glycemic load of the diets of men and women participating in the study

Food group	Men		Women	
	GL (X ±SD)	%	GL (X ±SD)	%
Dairy	1.7 ±3.2	1.0	1.8 ±2.6	1.4
Refined grains	71.9 ±48.9	44.0	43.9 ±32.8	35.3
Unrefined grains	11.1 ±22.9	6.8	9.5 ±16.3	7.7
Sweets	33.1 ±34.7	20.2	30.9 ±31.4	24.9
Vegetables	3.7 ±5.5	2.3	3.5 ±4.8	2.8
Fruits	8.2 ±13.0	5.0	9.5 ±12.1	7.6
Dry fruits	0.2 ±2.2	0.1	0.3 ±3.0	0.3
Nuts and cocoa	0.2 ±1.6	0.2	0.2 ±1.2	0.1
Potatoes and potato products	5.0 ±16.7	3.0	3.6 ±11.7	2.9
Soups	6.7 ±11.1	4.1	6.1 ±9.3	4.9
Juices	1.8 ±6.0	1.1	1.7 ±5.2	1.4
Sweetened beverages	3.8 ±12.8	2.3	1.9 ±7.2	1.5
Mixed dishes	8.9 ±21.5	5.5	6.9 ±17.3	5.6
Alcoholic beverages	1.0 ±3.9	0.6	0.1 ±0.9	0.1
Other	7.0 ±10.4	4.3	5.0 ±9.0	4.0
Total	163.6 ±72.2	100	124.1 ±54.2	100

GL – glycemic load; X ±SD – average ± standard deviation.

Table 3. Relationship between dietary glycemic load (GL/1,000 kcal) and sociodemographic factors in the study population by sex

Factor		Men		p-value	Women		p-value
		X ±SD	95% CI		X ±SD	95% CI	
Age	<35 years old (n = 602^{M}/662^{W})	70.3 ±15.5	69.0–71.5	0.0016	73.5 ±15.7	72.3–74.7	<0.0001
	35–49 years old (n = 692^{M}/825^{W})	70.6 ±15.7	69.5–71.8		73.4 ±16.5	72.3–74.5	
	50–64 years old (n = 792^{M}/1,011^{W})	70.6 ±15.7	69.5–71.7		73.3 ±15.3	72.3–74.2	
	≥65 years old (n = 468^{M}/637^{W})	73.9 ±15.6	72.5–75.3		76.5 ±16.1	75.3–77.8	
Size of the inhabited population center	small (n = 864^{M}/1,139^{W})	73.3 ±15.0	72.3–74.3	<0.0001	76.4 ±15.6	75.5–77.3	<0.0001
	medium (n = 784^{M}/961^{W})	70.9 ±15.1	69.8–71.9		74.0 ±15.9	73.0–75.0	
	large (n = 906^{M}/1,036^{W})	69.3 ±16.6	68.2–70.4		71.4 ±16.0	70.4–72.4	
Level of education	primary (n = 1,135^{M}/1,136^{W})	73.4 ±15.6	72.5–74.3	<0.0001	76.7 ±16.0	75.8–77.7	<0.0001
	secondary (n = 938^{M}/1,200^{W})	69.5 ±15.9	68.5–70.6		73.4 ±15.5	72.6–74.3	
	higher (n = 478^{M}/796^{W})	68.9 ±14.9	67.5–70.2		70.9 ±15.9	69.8–72.0	
Net month income per member of family	<1,000 PLN (n = 908^{M}/1,274^{W})	73.3 ±15.0	72.4–74.3	<0.0001	75.9 ±15.5	75.0–76.7	<0.0001
	1,000–2,000 PLN (n = 861^{M}/1,056^{W})	70.5 ±15.9	69.4–71.6		73.6 ±15.6	72.7–74.6	
	2,000–3,000 PLN (n = 253^{M}/233^{W})	68.3 ±15.4	66.4–70.3		68.3 ±16.3	66.1–70.4	
	>3,000 PLN (n = 122^{M}/72^{W})	66.0 ±17.8	62.8–69.2		68.0 ±17.3	63.9–72.1	
Marital status	married (n = 1,792^{M}/2,003^{W})	71.0 ±15.9	70.3–71.8	0.5428	73.7 ±16.0	73.0–74.4	0.0744
	other* (n = 761^{M}/1,133^{W})	71.4 ±15.3	70.3–72.5		74.6 ±15.8	73.7–75.5	

X ±SD – average ± standard deviation; 95% CI – 95% confidence interval; ^{M} – men; ^{W} – women; * – single, widow/widower, divorced, separated.

Table 4. Relationship between dietary glycemic load (GL/1,000 kcal) and sociodemographic factors in the overall study population

Factor		Overall		p-value
		X ±SD	95% CI	
Age	<35 years old (n = 1,264)	72.0 ±15.7	71.1–72.8	<0.0001
	35–49 years old (n = 1,517)	72.1 ±16.2	71.3–73.0	
	50–64 years old (n = 1,803)	72.1 ±15.5	71.4–72.8	
	≥65 years old (n = 1,105)	75.4 ±16.0	74.5–76.4	
Size of the inhabited population center	small (n = 2,003)	75.1 ±15.4	74.4–75.7	<0.0001
	medium (n = 1,745)	72.6 ±15.6	71.9–73.3	
	large (n = 1,942)	70.4 ±16.3	69.7–71.1	
Level of education	primary (n = 2,271)	75.1 ±15.9	74.4–75.7	<0.0001
	secondary (n = 2,138)	71.7 ±15.8	71.1–72.4	
	higher (n = 1,274)	70.1 ±15.5	69.3–71.0	
Net month income per member of family	<1,000 PLN (n = 2,182)	74.8 ±15.3	74.2–75.5	<0.0001
	1,000–2,000 PLN (n = 1,917)	72.2 ±15.8	71.5–72.9	
	2,000–3,000 PLN (n = 486)	68.3 ±15.8	66.9–69.7	
	>3,000 PLN (n = 194)	66.7 ±17.6	64.3–69.2	
Marital status	married (n = 3,795)	72.4 ±16.0	71.9–72.9	0.0299
	other* (n = 1,894)	73.3 ±15.7	72.6–74.0	

X ± SD – average ± standard deviation; 95% CI – 95% confidence interval; * – single, widow/widower, divorced, separated.

in the study population by sex. Dietary GL/1,000 kcal increased significantly with the age of participants: among men aged <35 years, it was on average 70.3 ±15.5 and among men aged ≥65 years it was 73.9 ±15.6, while among women it was on average 73.5 ±15.7 and 76.5 ±16.1, respectively. Significant differences in dietary GL/1,000 kcal were also observed depending on the size of the population center inhabited – the lowest was found in the DFRs of participants living in large population centers. The level of education also determined the GL value of the diets significantly: dietary GL/1,000 kcal of the diets decreased with increasing level of education, both in men and women. Moreover, dietary GL/1,000 kcal decreased significantly as the income increased. Marital

Table 5. Relationship between dietary glycemic load (GL/1,000 kcal) and selected lifestyle factors in the study population by sex

Lifestyle determinant		Men	p-value	Women	p-value
		X ±SD		X ±SD	
Level of physical activity	low (n = 1,203^{M}/1,402^{W})	71.9 ±15.8	0.0435	74.8 ±16.0	0.0088
	moderate (n = 642^{M}/831^{W})	70.3 ±14.7		72.8 ±15.8	
	high (n = 700^{M}/892^{W})	70.7 ±16.4		73.8 ±16.0	
Smoking status	current smoker (n = 738^{M}/585^{W})	72.3 ±16.0	0.0525	74.8 ±16.2	<0.0001
	former smoker (n = 857^{M}/590^{W})	71.2 ±15.8		71.2 ±15.9	
	never smoker (n = 954^{M}/1,960^{W})	70.4 ±15.3		74.6 ±15.8	

X ±SD – average ± standard deviation; ^{M} – men; ^{W} – women.

Table 6. Comparison of dietary glycemic load (GL/1,000 kcal) in the study population depending on the health status self-assessment

Health status self-assessment	Men	p-value	Women	p-value
	X ±SD		X ±SD	
Very good and good (n = 1,733^{M}/2,019^{W})	70.8 ±15.8	0.0426	73.4 ±15.8	0.0191
Moderate (n = 662^{M}/916^{W})	71.6 ±14.9		75.2 ±16.3	
Poor and very poor (n = 101^{M}/148^{W})	74.7 ±17.6		75.3 ±15.1	

X ± SD – average ± standard deviation; ^{M} – men; ^{W} – women.

Table 7. Comparison of dietary glycemic load (GL/1,000 kcal) in the study population depending on the type of declared diet

Type of diet	Men	p-value	Women	p-value
	X ±SD		X ±SD	
None (n = 2,311^{M}/2,779^{W})	71.4 ±15.4	0.0739	74.2 ±15.6	0.0509
Reducing diet (n = 22^{M}/41^{W})	69.5 ±19.5		65.6 ±23.9	
Low-fat/low-cholesterol/antidiabetic (n = 182^{M}/251^{W})	69.6 ±17.0		73.6 ±17.1	
Other, e.g., anti-allergic (n = 39^{M}/65^{W})	64.9 ±19.2		71.1 ±18.1	

X ±SD – average ± standard deviation; ^{M} – men; ^{W} – women.

status of the study group was not related to dietary GL/1,000 kcal. Table 4 shows the relationship between dietary GL/1,000 kcal and sociodemographic factors in the overall study population. The relationships observed in the overall study population were similar to those observed among men and women separately, aside from the marital status – dietary GL/1,000 kcal observed in the diets of married people was significantly lower than among others.

The relationship between GL/1,000 kcal and selected lifestyle factors in the study population by sex is presented in Table 5. The highest GL/1,000 kcal was observed in the diets of participants who declared low physical activity. Smoking status did not affect the GL/1,000 kcal in the diets of men. In contrast, among women, the lowest GL was observed in the DFRs of former smokers.

The lowest GL/1,000 kcal was observed in the diets of those participants who defined their health status as very good and good compared to those who defined their health status as moderate, poor or very poor (Table 6). The dietary GL/1,000 kcal was not significantly determined by the type of diet declared by the respondents (Table 7).

Discussion

Dietary GL is a value resulting from the amount of carbohydrates in food and their GIs. The average GLs were different between men's and women's diets; however, in both groups the main source of GL included refined grains followed by sweets. Products in these groups have high GI and are rich in carbohydrates and therefore their consumption has a dominant influence on the total GL. The contribution of other food groups in dietary GL was below 10%. The contribution of all grain products in total GL in the women's diets was 43%, which was similar to the results obtained among women participating in the Australian Longitudinal Assessment of Ageing in Women, where the contribution of grain products in total GL was 40.9%.[18] However, in the diets of Australian women sweets were the source of 13.6% of GL while in the Polish women it was 24.9%. It is also worth noting that in the DFRs of Australian women, fruits have almost 2-fold higher contribution to total GL compared to the DFRs of Polish women (14.2% vs 7.6%), while the contribution of vegetables was similar and amounted to 2.8%. Taking into account

the abovementioned differences, it can be assumed that the diets of women from the study population contained less fruits and more sweets than the diets of Australian women.[18] The contribution of all grain products in total GL in the studied men's diet was 50.8%, which was more than in the diets of Swedish men, where these products were the source of 36.7% of total GL.[19] On the other hand, in the DFRs of the men studied, potatoes and potato products were the source of 3.0% of GL, while in the DFRs of Swedish men it was 12.7%. However, a similar contribution of sweets (20.2% vs 22.6%, respectively) and fruits (5.0% vs 5.2%) in the diets of men from both groups was observed.[19] The GL in the diets of Polish women was higher compared to Australians (124.1 vs 115.0 per 1,000 kcal), while the GL in the diets of Polish men was lower compared to Swedes (163.6 vs 211.0 per 1,000 kcal).[18,19] Differences between food contribution in dietary GL among the abovementioned countries show that various dietary habits have an influence on dietary GL.

As was mentioned before, dietary GL is associated with energy intake. To minimize the influence of very low and very high energy diets on the dietary GL in this study, dietary GL was calculated per 1,000 kcal. Such calculation made it possible to compare the presented results with other authors, who also adjusted their results to the energy value of the diet.[10,20–24]

In this study, the relationships between sociodemographic factors such as age, size of the population center inhabited, level of education and net monthly income per member of family, and the value of dietary GL/1,000 kcal were found. We have observed that dietary GL/1,000 kcal increased with age, which was also observed by other authors.[10,20,21] However, in the Krishnan et al. study conducted in US black women, energy-adjusted GL was negatively associated with age.[22] We have also observed that dietary GL/1,000 kcal was lower among people residing in large population centers compared to those from small population centers. The same significant relationship was found for total GL. There are few studies which have examined such relationships; however, Hlaing et al. found a significantly lower total GL among people from suburban in comparison to urban areas in Myanmar.[23]

Another factor that was related to dietary GL was the level of education. Higher level of education among the Polish population was related to lower GL/1,000 kcal, and also to lower total GL. Villegas et al. observed a similar relationship in middle-aged Chinese women. Among people from the 5th quintile of energy-adjusted GL, there were significantly fewer people with college education (6.3%) and significantly more people with no education (36.5%) in comparison to the 1st quintile (19.8% and 7.4%, respectively).[10] Similar results were obtained by Sahyoun et al. in the Health, Aging and Body Composition Study conducted among 70–79-year-old people from Pittsburgh and Memphis, USA.[24] Levitan et al. also found that among middle-aged and older Swedish men from the 4th quartile of energy-adjusted GL, there were significantly fewer people with university education (13.5%) and significantly more people with less than high school education (73.8%) compared to the 1st quartile (20.9% and 63.1%, respectively).[21]

In the present study, an inverse relationship between the net monthly income per family member and dietary GL/1,000 kcal was observed. Villegas et al. also found that higher income was more often found among middle-aged Chinese women whose diets had low GL (energy-adjusted).[10] These 3 factors (place of residence, level of education and income) may be connected to each other because the level of education of people living in rural areas is often lower and people with lower level of education often have lower income. Such relationships were observed in the WOBASZ study conducted between 2003 and 2005.[25] In small population centers, 75.4% of men had elementary education, 19.9% had secondary education and 4.7% had university education, while in large population centers these percentages were 47.7%, 37.8% and 14.5%, respectively. Moreover, in small population centers, 83.0% of men reported that their net monthly income per member of family was <700 PLN, 15.4% reported 700–1500 PLN and 1.6% reported more than 1500 PLN. Similar observations were noted in the group of women.[25]

What is interesting is that marital status in the study population did not have any impact on dietary GL/1,000 kcal. Levitan et al. showed that marital status was related to dietary GL in the group of Swedish men.[21] In the 4th quartile of energy-adjusted GL, compared to the others, the percentage of married men was the lowest and the percentage of single men was the highest. However, the highest percentage of divorced men was observed in the 1st quartile of dietary GL. On the other hand, there was a similar percentage of widowed men in every quartile of dietary GL.[21]

Besides the abovementioned sociodemographic factors, we also included 2 lifestyle determinants in our analyses: level of physical activity and smoking status. The 1st one was to a large extent related to the GL/1,000 kcal of the diets in the study population. Sahyoun et al. in the Health, Aging and Body Composition Study did not observe significant differences in physical activity between participants from different quintiles of energy-adjusted dietary GL.[24] However, in postmenopausal Spanish women from the PREDIMED study, the highest level of physical activity (185.0 METs-h/w) was noted among the participants from the 1st tertile of energy-adjusted GL compared to the 2nd (162.8 METs-h/w) and 3rd (171.8 METs-h/w) tertiles.[20] On the other hand, Krishnan et al. found the highest percentage of US black women with vigorous physical activity in the 5th quintile of energy-adjusted GL in comparison to other quintiles.[22] In middle-aged and older Swedish men, the highest level of physical activity (58.6 min/day) was observed in participants from the 4th quartile of energy-adjusted GL in comparison to other quartiles (1st – 53.9 min/day, 2nd – 56.7 min/day, 3rd – 57.6 min/day).[21]

Smoking status was associated with dietary GL/1,000 kcal only in the diets of women – the lowest GL was found in the DFRs of former smokers. However, the results of other studies differ between each other. Krishnan et al. observed the highest rate of cigarette use in US black women from the 1st quintile of energy-adjusted GL compared to the other groups.[22] Among Swedish men, the lowest percentage of never-smokers (31.4%) was found in the 1st quartile of energy-adjusted GL compared to the 2nd, 3rd and 4th quartile (37.7%, 40.1% and 40%, respectively). Taking into account former and current smokers, an inverse relationship was found.[21] On the other hand, Castro-Quezada et al. did not observe significant differences in the percentage of current smokers, former smokers and never-smokers among Spanish women between tertiles of energy-adjusted dietary GL.[20]

Based on the study results, it was also shown that the health status self-assessment was related to the value of dietary GL/1,000 kcal, in contrast to the type of declared diet. People who assessed their health status as good and very good had significantly lower dietary GL than other people. Such relationships have not been analyzed by other authors. Probably the health status self-assessed as poor or very poor may be caused, among others, by unhealthy lifestyle, including unhealthy diet. However, such analyses were not assessed in this study.

Conclusions

Nutritional education about the proper selection of products that are sources of carbohydrates in the diet should be addressed mainly to people with low sociodemographic status, such as people in the older age group, living in small population centers, with lower levels of education and lower income, and also to people with lower levels of physical activity. Greater awareness of the choices of carbohydrate products is recommended to improve the quality of diet in these groups of people.

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The prevalence of mucosa-associated diffusely adherent *Escherichia coli* in children with inflammatory bowel disease

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Abstract

Background. The relationship of diffusely adherent *Escherichia coli* (DAEC) with pediatric inflammatory bowel disease (IBD) has not been previously studied. Diffusely adherent *E. coli* are a common cause of long-lasting childhood diarrhea and we postulated that they may induce inflammation of the intestinal mucosa, contributing to the development of IBD in susceptible children.

Objectives. The aim of the study was to investigate the relationship between DAEC and pediatric IBD, including Crohn's disease (CD) and ulcerative colitis (UC). Diffusely adherent *E. coli* isolates were also assessed regarding their pathogenicity.

Material and methods. Diffusely adherent *E. coli* were screened among 130 *E. coli* strains isolated from intestinal biopsy specimens from 26 children with IBD using polymerase chain reaction (PCR) with primers specific to the pathotype and adherence assays to HEp-2 cells. Diffusely adherent *E. coli* were further analyzed for their ability to adhere to and invade polarized Caco-2 cells. The immunomodulatory effect of DAEC on the secretion of tumor necrosis factor α (TNF-α) by human monocyte-derived macrophages (MDM) was assessed using an immunoenzymatic assay.

Results. Diffusely adherent *E. coli* were recovered from 18 (69.2%) of the 26 intestinal biopsy specimens from both CD and UC patients. Most DAEC isolates carried AfaE3 adhesin, adhered to and were internalized by Caco-2 cells, and induced secretion of elevated levels of TNF-α.

Conclusions. The study demonstrated the internalization of DAEC by intestinal epithelial cells and their ability to induce secretion of increased level of TNF-α in a Caco-2/macrophage compartmentalized culture. This indicated that the pathovar should be considered a pathobiont inducing inflammation of the intestinal mucosa in pediatric patients with IBD.

Key words: childhood inflammatory bowel disease, diffusely adherent *E. coli*, AfaE3 adhesin

Inflammatory bowel disease (IBD) is a chronic inflammation of the digestive system. The principal types of IBD include Crohn's disease (CD) and ulcerative colitis (UC). The currently prevailing view is that genetic background combined with intestinal dysbiosis and a related aberrant immune response contribute to IBD pathogenesis.[1–3]

Escherichia coli (*E. coli*) has been implicated in the etiopathogenesis of the ileal form of CD in adults, and many independent studies have demonstrated an increased number of *E. coli* bacteria in the intestinal mucus layer of patients with CD in comparison with healthy individuals.[4,5] Investigating the composition of mucosa-associated microflora in colonoscopic biopsy specimens from children with newly diagnosed CD, Conte et al. found *E. coli* bacteria in 74% of the samples examined.[6]

The relationship between diffusely adherent *E. coli* (DAEC) and IBD has not been studied extensively, although Darfeuille-Michaud et al. reported the isolation of *E. coli* strains hybridizing with the *daaC* probe specific to DAEC from adult patients with CD.[4] Prorok-Hamon et al. found that mucosa-associated *afaC*-positive *E. coli* strains were more common in CD and colorectal cancer than in UC and the controls.[7] Diffusely adherent *E. coli* causes urinary tract infections in humans and diarrhea in children aged from 18 months to 5 years, but is also isolated from healthy children and adults.[8,9] It has been suggested that carrying DAEC may predispose to chronic inflammation of the intestinal mucosa and the development of CD.[9]

Diffusely adherent *E. coli* are divided into 2 main classes: Afa/Dr-positive (Afa/Dr$^+$) strains that express *afa*, *dra* and *daa* operons encoding Afa/Dr adhesins, and Afa/Dr-negative (Afa/Dr$^-$) strains. The subclass of Afa/Dr$^-$ diarrhea-associated DAEC strains expressing AIDA-I adhesin represent a subgroup of atypical enteropathogenic *E. coli* (EPEC).[8] The Afa/Dr$^+$ group is further divided into 2 subgroups: Afa/Dr$_{DAF/CEACAM}$ DAEC possessing AfaE3, Dr and F1845 adhesins binding to hDAF, CEACAM-1, CEA, and CEACAM6 cell receptors; and Afa/Dr$_{DAF}$ DAEC presenting AfaE1 and Dr-II adhesins that bind to hDAF but not to CEACAMs.[9] The adherence of Afa/Dr strains to differentiated, polarized epithelial cells through membrane-bound receptors such as hDAF, CEACAMs and CEA induces structural and functional lesions in the epithelial barrier that generate an increase in paracellular permeability, transepithelial migration of polymorphonuclear leukocytes and the production of pro-inflammatory cytokines, i.e., interleukin (IL)-8, tumor necrosis factor α (TNF-α) and IL-1β.[10–12]

In the present study, we searched for DAEC among 130 *E. coli* isolates obtained from intestinal biopsy specimens from 26 children with IBD. Diffusely adherent *E. coli* isolates were assessed with regard to their pathogenicity, i.e., their ability to adhere to and invade polarized epithelial intestinal cells, the type of adhesins involved in their adherence to intestinal mucosa and their ability to induce TNF-α from monocyte-derived macrophages (MDM) co-cultured with Caco-2 cells infected with DAEC.

Material and methods

Biopsy specimens

Biopsy specimens were obtained from 26 consecutive children and adolescents (mean age 11.1 years, ranging from 7 to 18 years) with IBD (17 patients with CD and 9 patients with UC) diagnosed at the Department and Clinic of Pediatrics and Gastroenterology of Wroclaw Medical University (Poland). Biopsies from inflamed intestinal mucosa were collected from each patient for routine histopathologic examination and punch biopsies from the terminal ileum or colon were obtained for culture. The Ethics Committee of Wroclaw Medical University approved the study. The specimen for culture was washed in saline and immediately dispersed onto MacConkey (MCA) agar to isolate *E. coli* strains. Five lactose-positive colonies were isolated from every biopsy and defined as *E. coli* using standard biochemical testing. In total, 130 *E. coli* strains were screened for DAEC. Two *E. coli* reference strains, i.e., C1845 DAEC[13] and non-pathogenic *E. coli* K-12, were included in the study as positive and negative controls, respectively.

Polymerase chain reaction assay

The genes screened with polymerase chain reaction (PCR) assay included the *afaC* gene present in the *afa–3*, *afa–7* and *afa–8* operons of the Afa/Dr adhesins, the *afaBC* gene of the conserved region of the *afa* operons and the genes encoding *afaE1*, *afaE2* and *afaE3* adhesins.[14] In addition, the *daaE* gene encoding the adhesion of the C1854 strain was investigated. *Escherichia coli* isolates were assigned to one of the 4 phylogenetic groups (A, B1, B2, and D) using a multiplex PCR-based method.[15]

Cell cultures

The human epithelial HEp-2 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with heat-inactivated 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution (penicillin 100 U, streptomycin 100 µg/mL, amphotericin B 25 µg/mL). HEp-2 cells were seeded at a density of 4×10^5 cells per well on 24-well plates with round glass coverslips (1 cm in diameter) in each well, and cultured for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Caco-2 cells (ATCC HTB-37™; ATCC, Manassas, USA) were maintained in a minimal essential medium (MEM) with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics (penicillin 100 U, streptomycin 100 µg/mL). Caco-2 cells were seeded at a density of 5×10^4 cells per well and cultured for 10 days to differentiate. Twenty-four hours before the assays, the cells were washed 3 times with phosphate-buffered saline (PBS; pH 7.2), and MEM with 2% FBS and D-mannose (1%, w/v) without antibiotics was added.

In the co-culture experiments, Caco-2 cells were seeded at a density of 2×10^5 cells onto polyethylene terephthalate (PET) insert membranes (0.3 cm^2, 0.4 μm pore diameter) and cultured for 10 days post-confluence. The integrity of the Caco-2 cell monolayers was checked by measuring transepithelial electrical resistance (TEER) using a Millicell ERS-2 ohm meter (Merck Millipore, Darmstadt, Germany). Caco-2 cell monolayers with TEER ≥ 500 Ω × cm^2 were used for experiments. Human monocytic cell line THP-1 (ATCC TIB-202™) was routinely cultured in RPMI-1640 medium with 10% FBS. THP-1 cells were differentiated into monocyte-derived macrophages (MDM) with 200 nM phorbol-12-myristate 13 acetate (PMA) in 24-well plates. After 2 days, the PMA-supplemented medium was removed, the cells were washed twice, supplemented with 2% FBS and allowed to rest in fresh PMA-free culture medium for 3 days to acquire the phenotypic characteristics of macrophages.

Bacterial adhesion and invasion assays

Qualitative mannose-resistant adherence assays to HEp–2 cells and quantitative mannose-resistant adherence and invasion assays to Caco-2 cells were carried out as described by Cravioto et al.[16] Overnight *E. coli* cultures at 37°C in Luria broth (LB) were used to infect epithelial cells at a multiplicity of infection (MOI) of 50 bacteria per cell. In the adherence assay to HEp-2 cells, after 3 h of incubation with *E. coli* isolates, infected epithelial cells were washed to remove unbound bacteria, fixed with 70% methanol and stained with Giemsa stain. To assess the pattern of adherence, glass slides were examined under light microscopy (BX50; Olympus Corp., Tokyo, Japan). To quantify the total number of cell-associated bacteria (surface-adherent and internalized) in the adherence assay to Caco-2 cells, after 3 h of incubation, cells infected with *E. coli* strains were washed and lysed with 1% Triton X-100. The collected lysates were serially diluted in PBS and plated on MCA for assessment of bacterial colony-forming units (cfu). The invasion of *E. coli* was performed the same way as the adherence assay, but after 3 h of incubation and washing away unbound bacteria, a culture medium containing gentamycin (100 μg/mL) was added for an additional 1 h to kill off adherent bacteria. Then the cells were washed and lysed with Triton X-100 as described above. Invasion indices ≥0.1% were considered significant. Each assay was repeated at least 3 times.

Co-culture of Caco-2 and THP-1 cells

A co-culture model was used to analyze the immunomodulatory effect of DAEC on the secretion of TNF-α. Caco-2 cells grown on inserts were infected apically with *E. coli* at the MOI = 10 for 3 h. After incubation, the Caco-2 cell monolayers were washed and a culture medium containing 100 μg/mL of gentamycin was added to both apical and basal compartments for 1 h to kill adherent bacteria. After that, a culture medium containing 20 μg/mL of gentamycin to avoid bacterial overgrowth was added to the apical compartments of the inserts with Caco-2 cell monolayers, which were transferred to 24-well plates with MDM cultures. After 16 h of incubation, the culture medium from the basal compartment was harvested for quantification of TNF-α using a Quantikine® ELISA Human TNF-α Immunoassay (R&D Systems Europe, Ltd., Abingdon, UK).

Statistical analysis

Pearson's χ^2 test and Student's t-test p-values ≤0.05 were considered statistically significant, and the Pearson correlation coefficient (r) was used to analyze the results.

Results

Adherence pattern

Qualitative mannose-resistant adherence to HEp-2 cells was the first criterion to evaluate the diffuse adherence (DA) pattern of *E. coli* isolated from the biopsy specimens. Diffusely adherent *E. coli* were recovered from 18 (69.2%) of the 26 biopsies. There was no difference in the distribution of DAEC among CD and UC patients: they were isolated from 12 (70.6%) out of 17 children with CD and 6 (66.7%) out of 9 children with UC (p = 0.8).

Distribution of Afa/Dr DAEC genes among *E. coli* isolates

Escherichia coli were screened for DAEC-specific genes, i.e., *afaC* and *afaBC*. The *afaBC*-positive and *afaC*-positive (Afa/Dr^+) strains were isolated from 14 (53.8%) of the 26 patients with IBD. The *afaBC*-negative but *afaC*-positive DAEC isolates (Afa/Dr^-) were obtained from 3 (17.6%) patients with CD and 1 patient with UC (11.1%). Screening for AfaE subtypes among the Afa/Dr^+ DAEC indicated that they all carried only 2 different subtypes of AfaE adhesins, i.e., AfaE2 and AfaE3, which were associated with 2 (14.3%) and 12 (85.7%) isolates, respectively (p = 0.0002). The AfaE3 adhesin was carried by 8 (88.9%) and 4 (80%) Afa/Dr^+ from CD and UC, respectively (p = 0.7; Table 1). None of the examined *E. coli* carried the *daaE* gene, except for the reference C1845 strain.

Phylogroup distribution

Ten (55.5%) and 8 (44.4%) of the 18 DAEC belonged to the B2 and D phylogroups, respectively. There was no statistically significant difference between CD and UC isolates in the prevalence of the B2 and D phylogroups (p = 0.4).

Quantitative adherence and invasion assays to Caco-2 cells

The mean adherence levels of DAEC isolated from the UC and CD patients were 2.8 ±0.7 × 10^6 cfu/mL and 4.5 ±1.3 × 10^6 cfu/mL, respectively (p = 0.08), whereas the mean invasion indices for DAEC from UC and CD were 1.9 ±0.2% and 0.8 ±0.1%, respectively (p = 0.054). The non-pathogenic *E. coli* $K12_{C600}$ strain was not internalized by differentiated Caco-2 cells (Table 1; Fig. 1). There was a negative correlation between the adherence and invasion levels among the DAEC from UC (r = –0.66), but a positive correlation among those from CD (r = 0.67).

Immunomodulatory effect of *E. coli* on monocyte-derived macrophages co-cultured with Caco-2 cells

To determine effect on TNF-α secretion by MDM a co-culture of the epithelial cells and macrophage-like cells

Table 1. Characteristics of the 14 Afa/Dr$^+$ and 4 Afa/Dr$^-$ diffusely adherent *Escherichia coli* (DAEC) from children with inflammatory bowel disease (IBD)

Escherichia coli strain/group	Adherence [×10^6 cfu/well][a]	Invasion index [%]	Afa/Dr genes			Phylogroups
			afaC	*afaBC*	*afaE2/3*	
Afa/Dr$^+$						
UC38	6.0 ±1.8	0.8 ±0.1	+	+	3	D
UC28	1.7 ±0.5	2.7 ±0.1	+	+	3	B2
UC30	3.8 ±0.3	3.7 ±0.3	+	+	3	B2
UC20	1.9 ±0.3	0.4 ±0.06	+	+	2	D
UC36	3.8 ±1.5	0.9 ±0.3	+	+	3	D
CD21	3.0 ±1.3	0.05 ±0.01	+	+	3	B2
CD16	2.1 ±0.7	1.0 ±0.2	+	+	3	B2
CD45	9.9 ±3.6	2.8 ±0.06	+	+	3	B2
CD18	4.1 ±2.0	1.2 ±0.1	+	+	3	D
CD11	1.2 ±0.3	0.08 ±0.01	+	+	2	B2
CD15	6.0 ±1.3	0.5 ±0.04	+	+	3	D
CD33	6.3 ±1.1	0.07 ±0.01	+	+	3	B2
CD3	6.1 ±4.0	1.6 ±0.3	+	+	3	B2
CD13	1.3 ±0.03	0.3 ±0.1	+	+	3	B2
Afa/Dr$^-$						
UC46	1.6 ±0.3	2.8 ±0.1	+	–	–	B2
CD39	7.0 ±2.3	0.5 ±0.1	+	–	–	D
CD43	3.8 ±1.7	0.4 ±0.03	+	–	–	D
CD27	4.5 ±1.3	0.8 ±0.04	+	–	–	D
Reference						
C1845	2.8 ±1.9	0.76 ±0.25	+	+	*daaE*	B2
E. coli K12	0.2 ±0.01	0.0 ±0.00	–	–	–	A

cfu – colony-forming units; CD – Crohn's disease; UC – ulcerative colitis; [a] – quantitative adherence assay to Caco-2 cells. The values of adherence and invasion indices are presented as means ± standard deviation (SD).

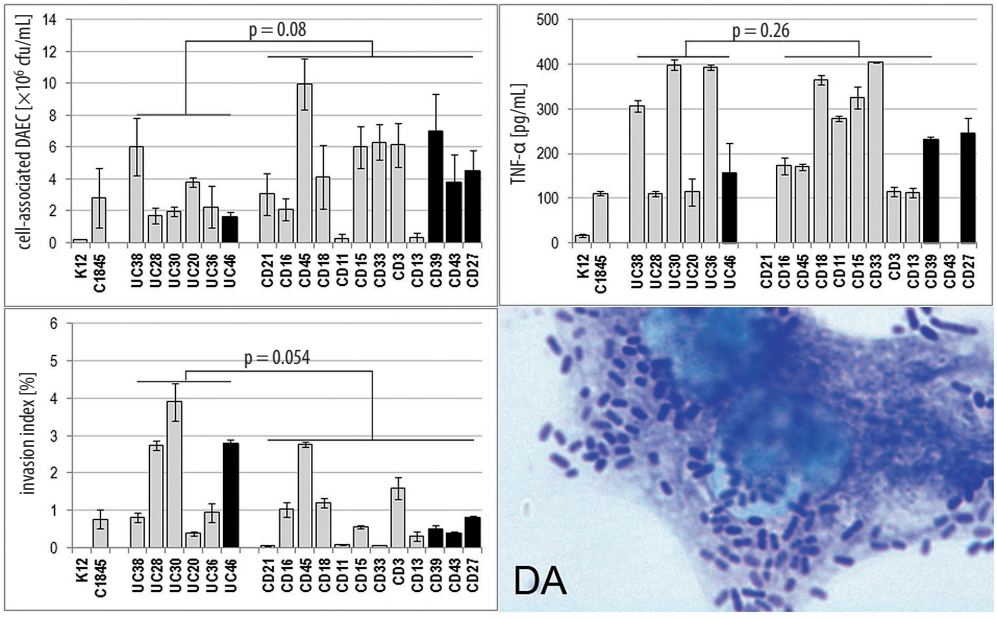

Fig. 1. The adherence levels (cfu × 10^6/mL), internalization indexes (%) of diffusely adherent *Escherichia coli* (DAEC) to Caco-2 cells and the concentration of TNF-α analyzed in Caco-2/HMDM compartmentalized culture. Grey bars represent Afa/Dr$^+$ DAEC whereas black bars represent Afa/Dr$^-$ DAEC. Data represents the means ± standard deviation (SD) of at least 3 experiments, each performed in triplicate. Giemsa stain, magnification ×100

DA – diffuse adherence pattern.

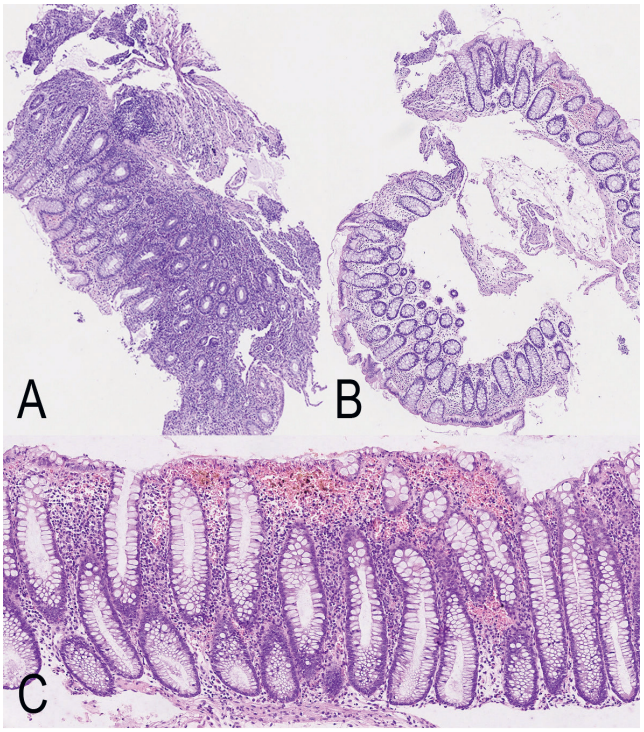

Fig. 2. For routine histopathologic examination biopsy specimens were fixed with buffered formaldehyde, paraffin–embedded and stained with hematoxylin and eosin (H&E)

Table 2. Histopathologic findings and comparison of diffusely adherent *E. coli* (DAEC) characteristics in acute and chronic pediatric inflammatory bowel disease (IBD)

Biopsies findings	Number of biopsies (%) Inflammation	
	acute (n = 9)	chronic (n = 9)
Epithelial damage	8 (88.9)*	0
Architectural changes	3 (33.3)	2 (33.3)
Mononuclear cells in lamina propria	9 (100)	7 (77.8)
Polymorphonuclear cells in lamina propria	9 (100)	5 (55.5)
Neutrophils in epithelium	7 (77.8)*	0
Erosion or ulceration	8 (88.9)*	0
Crohn's disease (n = 12)[a]	5 (41.7)	7 (58.3)
Ulcerative colitis (n = 6)[a]	4 (66.7)	1 (33.3)
Mean level of TNF-α [pg/mL]	199.7 ±22.6	233.7 ±18.7
Mean level of adherence [$\times 10^6$ cfu per well]	3.9 ±1.2	3.9 ±1.4
Mean invasion index [%]	1.6 ±0.2	0.9 ±0.2
AfaE2 (n = 7)[b]	0	2 (22.2)
AfaE3 (n = 7)[b]	7 (100)	5 (71.4)

[a] – number of DAEC-positive samples; [b] – number of Afa/Dr$^+$ strains carrying AfaE2 or AfaE3 adhesins; cfu – colony-forming units; * statistically significant difference ($p \leq 0.01$).

was performed. Infection of Caco-2/MDM compartmentalized culture with Afa/Dr$^+$ and Afa/Dr$^-$ DAEC isolates induced the secretion of elevated levels of the cytokine in comparison with the control level (Caco-2 cells co-cultured with MDM without bacteria; Fig. 1). The mean levels of TNF-α secreted by MDM after infecting Caco-2 cells with DAEC from CD and UC were comparable: 247 ±22 pg/mL and 202 ±11 pg/mL, respectively ($p = 0.26$). In general, all but 2 (88.9%) DAEC isolates induced secretion of TNF-α at levels similar to or greater than the reference C1845 strain. The level of TNF-α released after infection of Caco-2 cells with the *E. coli* K-12_{C600} strain was negligible. These results indicated that the infection of Caco-2 cells co-cultured with MDM with Afa/Dr DAEC induced the secretion of TNF-α independently of strain origin (UC vs CD), the level of adherence and invasion, and the affiliation to Afa/Dr$^+$ or Afa/Dr$^-$ DAEC. There was no correlation between the adherence or invasion levels and TNF-α secreted by DAEC from UC and CD ($r = 0.1$ and $r = 0.2$, respectively).

Histopathology

The histopathologic findings in the biopsy specimens were graded according to the scoring system proposed by Geboes et al., in which grade 0 corresponds to normal mucosa; grade 1 indicates inflammation in the lamina propria with slight invasion of plasma cells and/or lymphocytes and granulocytes; grade 2 denotes moderate or severe inflammation with destructive crypt abscesses, abundant lymphocytes and granulocytes in the lamina propria; and grade 3 means severe inflammation in the lamina propria, partial granular atrophy, crypt dilation, and microgranulomas.[17] Acute inflammation included cryptitis, crypt abscesses and neutrophils in the lamina propria and in the epithelium, whereas chronic inflammation was assumed when chronic inflammatory infiltrate consisting of mononuclear cells was present in the lamina propria but there was no crypt destruction or epithelial ulceration (Table 2; Fig. 2). Acute and chronic inflammation were present in 9 (50%) of the 14 Afa/Dr$^+$ and 4 Afa/Dr$^-$ DAEC-positive biopsies. There was no correlation between the isolation of Afa/Dr$^+$ DAEC or Afa/Dr$^-$ DAEC and acute or chronic inflammatory lesions.

Discussion

Adherent-invasive *E. coli* (AIEC) is implicated in the etiopathogenesis of ileal CD in adults[5]; however, the role of AIEC in the pathogenesis of childhood CD is not as clear as among adult patients. According to a study published by Conte et al., AIEC-like isolates of an aggregative adherence pattern were identified in children with CD as well as in non-IBD controls.[6] *Escherichia coli* colonizes infants in the first months of life and is present in the intestines of humans throughout life. However, diet and the composition of intestinal microbiota affect the quantitative ratios

of various *E. coli* pathovars, leading to the predominance of one over another. Martinez-Medina et al. demonstrated that a high fat and high sugar Western diet led to dysbiosis in mice, with a particular increase in the *E. coli* population.[18] Microbiotas are different in children and adults, and pathogenic *E. coli* (i.e. DAEC) are common in children's intestinal microflora.[19] Mansan-Almeida et al. showed that DAEC isolated from children were remarkably different from DAEC isolated from adults in terms of the diversity of adhesins and virulence factors.[19] Moreover, they found that DAEC from children had a greater ability to colonize the gastrointestinal tract.

Bacterial adhesins are known virulence factors that permit pathogens to colonize the intestinal mucosa. Adherent-invasive *E. coli* is characterized by mannose-sensitive adherence to epithelial cells through type 1 fimbriae. Most studies examining *E. coli* from patients with IBD have focused on *E. coli* isolates adhering through mannose-sensitive adhesins, which are common among pathogenic and non-pathogenic *E. coli* strains. In the present study we preselected *E. coli* isolates using an adherence assay to HEp-2 cells in the presence of mannose to eliminate the influence of type 1 fimbriae on the adherence of *E. coli*.

The results of the study indicated that DAEC were isolated from 18 (69.2%) of the 26 children. Screening for AfaE subtypes indicated that AfaE3-positive strains represented 85.7% of the Afa/Dr$^+$ isolates. According to other studies, AfaE3-positive DAEC were isolated at a much lower frequency. Zhang et al. isolated *afaE3*-carrying *E. coli* from 12% of 787 isolates from urinary tract infections and diarrhea cases.[20] Mansan-Almeida et al. detected *afaE3*-positive DAEC in 2% and 1.7% of children with diarrhea and healthy controls, respectively, and in 7.4% and 6.7% of adults with diarrhea and controls, respectively.[19] In a study of human diarrheal isolates, Le Bougenec et al. detected DAEC carrying *afaE1*, *afaE3* and *afaE5* subtypes with similar frequency (21.4%).[14] Those investigators suggested that there might be an association between the subtype of AfaE adhesins and the physiological site of infection caused by *afa*-positive strains. In their study they found that the AfaE8 subtype is predominant in sepsis patients but absent from diarrhea-associated strains. Similarly, Zhang et al. demonstrated that the AfaE5 subtype occurred 3 times as often among Afa/Dr$^+$ DAEC causing urinary tract infections than in fecal strains.[20] The predominance of the AfaE3 subtype among Afa/Dr$^+$ strains demonstrated in the present study raises the question if the result simply reflects frequent colonization of the gastrointestinal tract of children in our region with AfaE3-positive DAEC, or if this subtype of adhesins is actually connected with childhood IBD. Hence, further studies are necessary.

Receptors for the AfaE3 adhesin, like DAF and CEACAM6, are exposed on the apical membrane of intestinal epithelial cells, providing a docking site for colonization of the intestinal mucosa by Afa/Dr$^+$ DAEC. Interestingly, both of these molecules are upregulated in patients with CD, so they may promote enhanced adherence of DAEC. In addition, CEACAM engagement triggers endocytosis of bacteria into epithelial cells.[21] Internalization of microorganisms through interaction with CEACAMs prevents exfoliation of the epithelium, favoring persistent infection.[22] Indeed, in young children, diarrhea caused by Afa/Dr+ DAEC can become persistent.[9] Furthermore, it has been demonstrated that infecting cultured epithelial Caco-2 cells with Afa/Dr$^+$ DAEC increased the expression of major histocompatibility complex (MHC) class I-related MICA, a molecule that is expressed to a greater extent on the colonic epithelium of patients with CD.[23] In patients with UC, there is no difference in the expression level of the CEACAM6 molecule compared to patients with CD, although, like patients with CD, DAF expression is enhanced on colonic epithelial cells of patients with UC in relation to the severity of the mucosal inflammation.[24,25] Thus, upregulated expression of DAF, CEACAM6 and MICA may predispose pediatric patients with IBD to colonization by Afa/Dr$^+$ DAEC.

Acute IBD is characterized by increased expression of pro-inflammatory cytokines, e.g. TNF-α, which can modulate DAF expression, favoring the adherence of Afa/Dr$^+$ DAEC to the intestinal mucosa.[26] Both CD and UC exhibit elevated levels of TNF-α.[27,28] In our study, we investigated whether the adherence and/or invasion of DAEC to epithelial cells induces secretion of TNF-α by MDM co-cultured with epithelial cells. The results indicated that all but 1 Afa/Dr$^+$ and all but 1 Afa/Dr$^-$ DAEC induced secretion of elevated levels of TNF-α independently of the strain origin (CD vs UC) or IBD activity (acute vs chronic). This indicates that Afa/Dr DAEC colonizing intestinal epithelium can promote their own adherence and invasiveness via induction of TNF-α release, which in turn modulates the expression of cellular receptors for these pathogens.

Conclusions

In summary, the results of the present study indicated that although DAEC was the most prevalent pathotype among the *E. coli* associated with biopsy specimens from pediatric patients with IBD, Afa/Dr$^+$ strains were not specifically associated with CD or UC. Moreover, the study revealed that Afa/Dr$^+$ DAEC from children with IBD most commonly presented the AfaE3 subtype of adhesins. The study demonstrated the internalization of Afa/Dr isolates by intestinal epithelial cells and their ability to induce increased secretion of TNF-α from monocytes/macrophages co-cultured with epithelial cells, which indicated that DAEC should be considered one of the pathobionts responsible for inducing inflammation of the intestinal mucosa in childhood IBD.

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The Polish Prevalence of Infection in Intensive Care (PPIC): A one-day point prevalence multicenter study

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Abstract

Background. Infections in critically ill patients are the main reasons for a lack of therapeutic success and increased mortality in intensive care units (ICUs). There have been many analyses of the incidence of infections in ICUs; however, no large studies of this kind have been conducted either in Poland or in Eastern and Central Europe.

Objectives. The aim of the research was to undertake a one-day study of the prevalence of infections in ICUs in Warszawa and the Mazovian region of Poland.

Material and methods. A prospective questionnaire survey analysis — a one-day prevalence study of infections — was carried out on June 25, 2014, in 28 ICUs in Poland.

Results. Among 205 ICU patients (193 adults and 12 children), 134 infections were found in 101 patients (99/193 adults (51.30%) and 2/12 children (16.70%)), and bacterial colonization in 19/205 (9.3%) patients. In 66.42% of the cases, more than 1 site of infection was diagnosed. On the day of the study, 75.40% of the diagnosed infections had positive microbiological results. The most frequent were respiratory tract infections (53.73%), wound infections (18.65%) and bloodstream infections (14.92%). Most of the infections (64.10%) were caused by Gram-negative bacteria (GN), followed by Gram-positive bacteria (GP; 31.80%) and fungi (4.10%). The most frequently reported GN microorganisms were Enterobacteriaceae (44.7%). Methicillin-resistant *Staphylococcus aureus* (MRSA) infections were found in 8.80% of the patients. Antibiotics were administered to 75.60% of the adult patients, in 69.20% as targeted treatment. Mechanical ventilation, central vein catheterization and urinary bladder catheterization were used in 67.80%, 85.85% and 94.63% of the patients, respectively.

Conclusions. On the day of the study, more than half of the patients had infections, mostly from GN bacteria. Respiratory tract infections were the main type found. In about 2/3 of the patients, antibiotics were administered, mainly as targeted therapy.

Key words: intensive care unit, hospital infection, one-day prevalence study

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Introduction

Infections in critically ill patients are the main reasons for a lack of therapeutic success and increased mortality in intensive care units (ICUs) all over the world. Unfortunately, the incidence of infections is still high.[1–3] Many epidemiological studies have analyzed this problem, mainly in the populations of Western Europe, North America and developing countries.[4–8] Among all these studies, one-day prevalence studies are favored because they can be carried out quickly and easily in different medical centers. The prototype for this type of analysis was the European Prevalence of Infection in Intensive Care (EPIC) Study.[4] There have been no large studies of this type focused on ICU patients either in Poland or in Eastern and Central Europe. The objective of our research was a one-day study of the prevalence of infections in ICUs in Warszawa and the Mazovian region of Poland (about 7 million citizens).

Material and methods

Study population and data collection

Our study was a questionnaire-based survey analysis of the epidemiological status of critically ill patients who were hospitalized in ICUs on Tuesday, June 25, 2014. The study covered 205 patients in 28 ICUs in Poland. The study protocol was approved by the institutional Bioethics Committee and performed in accordance with the Declaration of Helsinki. We asked 15 questions about infections and pathogens "occurring in the ICU", and about all other aspects of therapy important to our analysis. The questionnaire was sent to 33 ICUs in 500- to 1000-bed university and municipal hospitals, as well as to smaller district hospitals (up to 250 beds). Two large pediatric hospitals (PD) were also included in our study. Of the 33 ICUs that received the questionnaire, 85% completed the questionnaires and were included in the study. The patient characteristics are shown in Table 1.

Table 1. Patient characteristics

Total number of patients		205
Gender	male, n (%)	120 (58.5)
	female, n (%)	85 (41.5)
Type of medical problem	general, n (%)	58 (28.3)
	surgical, n (%)	147 (71.7)
Age	>18 years of age, n (%)	193 (94.1)
	<18 years of age, n (%)	12 (5.9)
ICU admission source: other departments of the same hospital*, n (%)		143 (69.8)
Emergency departments* or other hospital, n (%)		62 (30.2)

Data is presented as number of patients and percentage value;
* data from departments for adult patients only.

We performed global epidemiological analyses in 28 ICUs (205 patients) as well as detailed analyses in Warszawa (WA – 11 hospitals), large Mazovian provincial hospitals (MPH – 5 hospitals) and district hospitals (DH – 12 hospitals). Additionally, we compared some results of our study to the European Prevalence of Infection in Intensive Care (EPIC) and EPIC II studies.[4,5]

Definitions and diagnostic methods

Infections were diagnosed based on the criteria of the Centres for Disease Control and Prevention's National Healthcare Safety Network (CDC/NHSN) and the European Centre for Disease Control (ECDC).[9] All materials submitted for microbiological analysis were sampled and assessed qualitatively and quantitatively according to accepted standards. The susceptibility of microorganisms was determined in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[10] Colonization was defined when microorganisms were found at a normally sterile site on the patient, without clinical or laboratory signs of infection. Patients were considered surgical if emergency surgery was performed immediately before admission or if elective surgery was performed within 1 month before admission. All other patients – e.g. with respiratory, cardiac or renal insufficiency – were considered medical. The incidence of infections was calculated as the number of patients with infections per 100 hospitalized patients.

Statistical analysis

The statistical analyses were performed using STATISTICA software v. 10 (StatSoft Inc., Tulsa, USA). Descriptive statistics were computed for all study variables. Discrete variables are expressed as counts (percentages) and mean ± standard deviation (SD). The data was analyzed using the χ^2 test or χ^2 test with Yates's correction, as appropriate. P-value <0.05 was considered statistically significant.

Results

There were 238 intensive care beds for adults and 18 for children in the analyzed ICUs on the day of our study (WA – 135, MPH – 49, DH – 54). Among them, 193/238 intensive care beds (81.1%) were occupied (WA – 90.4%, MPH – 73.5%, DH – 64.8%, PD – 66.7%). Of the adult patients, 143/193 (74.1%) were admitted to ICUs from other departments of the same hospital (WA – 84.4%, MPH – 47.2%, DH – 65.7%), whereas 50/193 (25.9%) were admitted from the emergency department or from other hospitals (WA – 16.4%, MPH – 50%, HD – 28.6%). We do not have such data for 12 of the pediatric patients.

Among the 205 patients hospitalized in an ICU, 134 infections were found. In total, 101 patients (49.26%) – 99/193 adults (51.30%; WA – 53.30%, MPH – 47.20%, DH – 51.40%) and 2/12 pediatric patients (16.70%) – were considered infected. Of these, 101/134 infections (75.40%) had positive microbiological results, whereas 33/134 (24.63%) infections were diagnosed without positive microbiological results on the day of the study. Bacterial colonization was diagnosed in 18/193 of the adult cases (9.3%; WA – 10.7%, MPH – 5.6%, DH – 8.6%). No bacterial colonization was diagnosed in the pediatric patients. Of the adult patients, 146/193 (75.6%) received antimicrobial treatment. Among these patients, in 36.3% of the cases, 1 antimicrobial agent was administered, whereas 2 agents were administered in 45.9%, 3 in 15.8%, and 4 in 2.1%. In 69.2% of the patients, there was targeted therapy, whereas in 22.6% and in 8.2%, both empirical and prophylactic treatments were provided.

The prevalence of infections and primary sites of infections in the different types of hospital are shown in Table 2.

More than 1 site of infection was diagnosed in 89/134 cases (66.42%; WA – 35.20%, MPH – 38.80%, DH – 17.10%). The incidence of infections in ICUs for adults was 99/193 patients (51.30%), mainly due to lung infections 70/193 (36.70%). Respiratory tract infections were the most frequently found type of infections in adults (53.03%), followed by bloodstream (15.50%), urinary tract (12.88%), abdominal (11.36%), and wound infections (7.57%).

The microorganisms responsible for infections in different types of hospitals are shown in Fig. 1. Among the isolated microorganisms (n = 170), the most common Gram-negative (GN) bacteria (n = 109) were *Klebsiella pneumoniae* (32.9%), *Escherichia coli* (20.0%), *Acinetobacter baumannii* (5.3%), *Pseudomonas aeruginosa* (4.1%), and other microorganisms (1.8%). Gram-positive (GP) bacteria

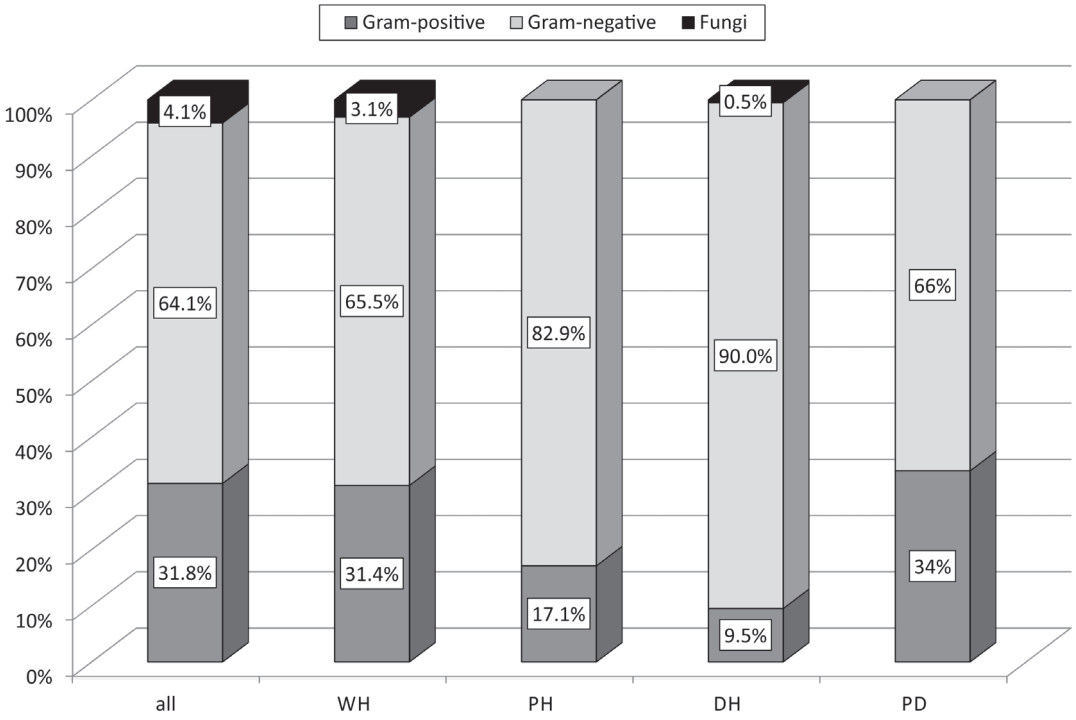

Fig. 1. The structure of isolated pathogens inanalyzed hospitals

WH – Warsaw hospitals; PH – Mazovian provincial hospitals; DH – district hospitals; PD – pediatric departments.

Table 2. Prevalence of infections in different types of hospitals

Variables	WH	MPH	DH	PD	Total
Total number of patients, n	122	36	35	12	205
Patients with no infections, n (%)	94 (48.7)			10 (83.3)	104 (50.7)
Patients with infections, n (%)	99 (51.3)			2 (16.7)	101 (49.3)
Total number of infections, n (%)	82 (67.2)	20 (55.6)	30 (85.7)	2 (16.7)	134 (65.4)
Lung infections, n (%)	43 (35.2)	9 (25.0)	18 (51.4)	2 (16.7)	72 (35.1)
Bloodstream infections, n (%)	11 (9.0)	7 (19.4)	2 (5.7)	0 (0.0)	20 (9.8)
Urinary tract infections, n (%)	12 (9.8)	4 (11.1)	1 (2.9)	0 (0.0)	17 (8.3)
Abdominal infections, n (%)	6 (4.9)	0 (0.0)	9 (25.7)	0 (0.0)	15 (7.3)
Wound infections n, (%)	10 (8.2)	0 (0.0)	0 (0.0)	0 (0.0)	10 (4.9)

Data is presented as number of patients and percentage value. WH – Warsaw hospitals; MPH – Mazovian provincial hospitals; DH – district hospitals; PD – pediatric departments.

Table 3. Device utilization ratios in different types of hospitals

Variable		WH	MPH	DH	PD
Total number of patients		122	36	35	12
Mechanical ventilation	total	87 (71.3%)	23 (63.9%)	20 (57.1%)	9 (75.0%)
	via endotracheal intubation	66 (75.9%)	20 (87.0%)	17 (85.0%)	9 (100.0%)
	via tracheostomy	21 (24.1%)	3 (13.0%)	3 (15.0%)	0 (0.0%)
Central vein catheter		116 (95.1%)	23 (63.9%)	29 (82.9%)	8 (66.7%)
Urinary catheter		120 (98.4%)	36 (100.0%)	33 (94.3%)	5 (41.7%)

Data is presented as number of patients and percentage value. WH – Warsaw hospitals; MPH – Mazovian provincial hospitals; DH – district hospitals; PD – pediatric departments.

(n = 54) were Methicillin-resistant *Staphylococcus epidermidis* (MRSE; 10.6%), Methicillin-resistant *Staphylococcus aureus* (MRSA; 10.0%), Methicillin-susceptible *Staphylococcus epidermidis* (MSSE; 10.6%), and *Enterococcus* spp. (5.9%). The fungi found (n = 7) were non-albicans *Candida* (100%). The most frequently isolated pathogens responsible for pneumonia were GN bacteria (47/70, 67.1%), mainly species of Enterobacteriaceae (21/47, 44.7%) and non-fermenting GN strains such as *A. baumannii* (9/47, 19.1%) and *P. aeruginosa* (7/47, 14.9%). The GN pathogens (5/47, 10.6%) (*K. pneumoniae,* n = 3; *E. coli,* n = 1; *Enterobacter cloacae,* n = 1) had extended-spectrum beta-lactamase (ESBL) mechanisms of bacterial resistance. Among the GP species (23/70, 32.9%), *S. aureus* was the most frequently isolated (12/23, 54.0%), specifically MRSA (8/12, 70.0%).

The majority of bloodstream infections (11/20, 55%) were caused by GN bacteria (mainly Enterobacteriaceae), while 45% of the bloodstream infections were due to GP bacteria species, mainly Staphylococci (n = 6) and *Enterococci* (n = 3). Two cultures of vancomycin-resistant *Enterococcus faecium* were isolated.

The main pathogens responsible for urinary tract infections (UTI) were *E. coli* (12/17, 71.0%), *Enterococcus faecalis* (3/17, 18.0%) and other pathogens (2/17, 11.0%). Methicillin-resistant *Staphylococcus aureus* infections were diagnosed in 17/193 patients (8.8%). All *A. baumannii* strains found in this study were multi-drug resistant (MDR).

Non-albicans Candida infections were diagnosed in 7/193 patients (3.6%; *Candida glabrata* (n = 3), *Candida kruzei* (n = 3), *Candida tropicalis* (n = 1)). Only 1 case of *Clostridium difficile* infection was diagnosed in this study.

Mechanical ventilation, central venous catheters and urinary catheters are considered risk factors for infections in critically ill patients. These factors are shown in Table 3.

Discussion

One-day point prevalence studies of infections in ICUs have been performed mainly in the USA, North European countries and Australia.[4,5,11] Several such studies, carried out in ICUs or in all hospital departments, have focused on community-acquired infections (CAIs) and hospital-acquired infections (HAIs) or on HAIs only, although some of these studies also included analyses of the antimicrobials used.[12–15] A one-day point prevalence study (PPS) and incidence study in Poland evaluated the epidemiology of infections in Polish long-term care facilities.[16]

The first Polish one-day PPS on infections in ICUs was performed in 59 ICUs in 1994.[17] The most important differences in comparison to this study include the predomination of GP bacteria in the earlier study, which were isolated in 61.8% of the patients (of these, 67.0% were MRSA species). In our study, the percentage of GN bacteria was nearly double in comparison with the findings of the earlier Polish survey (35.2%); Candida infections were also twice more prevalent (2.6%).[17]

Because Poland lacks a history of one-day infection PPS performed in ICUs, we could compare our data only to the one-year prevalence study of Polish ICUs, which analyzed 1,043 critically ill patients with sepsis.[18] In this earlier study, patients with GN bacterial sepsis were less frequent than in our study (48.0% vs 64.1%; *P. aeruginosa* 14.2% vs 4.1%; *A. baumannii* 15.3% vs 5.3%). The main site of infection found in the previous study was the abdominal cavity (47.0%), which is a contrast to the present study, where respiratory tract infections were predominant.[18] In this regard, our findings were similar to the Sepsis Occurrence in Acutely Ill Patients (SOAP) study; other one-day PPSs also determined that the respiratory tract was the most common site of infection (68.0%, 58.5%, 28.0%, and 20.0% according to Vincent et al.,[2] Toufen et al.,[13] Marioka et al.,[14] and Esen et al.,[15] respectively).[2,13–15] However, in other studies (mainly analyzing HAIs), bloodstream infections were predominant.[12,19]

We analyzed data from 28 Polish ICUs, whereas the EPIC study analyzed data from 1,417 European ICUs; the EPIC II study analyzed data from 1,265 ICUs in North and South America, Western Europe, Asia, Oceania, Australia, and Africa.[4,5] The incidence of infections in our study was comparable both to EPIC (44.80%) and EPIC II (51.40%). The incidence of lung infections in our study was also similar to EPIC II (36.30% vs 32.60%). Nevertheless, in our study, respiratory tract infections constituted 53.03% of all

Table 4. Comparative analysis of point prevalence infection studies

Variable	PPIC (adults)	EPIC[4]	EPIC II[5]	p-value
Number of hospitalized patients	193	10,038	13,796	–
Number of patients with infections	99	4,501	7,087	–
Total number of infections/total number of hospitalized patients	132/193 (68.39%)	4,501/10,038 (44.8%)	7,087/13,796 (51.4%)	p = 0.0000 p* = 0.0000
Respiratory tract infections including pneumonia	70/193 (36.27%)	967/10038 (9.63%)	4,503/13,796 (32.64%)	p = 0.0000 p* = 0.983
	70/132 (53.03%)	967/4501 (21.48%)	4,503/7,087 (63.5%)	p = 0.0000 p* = 0.2858
Bloodstream infections	20/193 (10.36%)	247/10,038 (2.46%)	1,071/13,796 (7.76%)	p = 0.0000 p* = 0.1811
	20/132 (15.15%)	247/4,501 (5.49%)	1,071/7,087 (15.1%)	p = 0.0000 p* = 0.9900
Urinary tract infections	17/193 (8.81%)	363/10,038 (3.62%)	1,011/13,796 (7.33%)	p = 0.0002 p* = 0.4339
	17/132 (12.88%)	363/4,501 (8.06%)	1,011/7,087 (14%)	p = 0.0470 p* = 0.6515
Patients receiving antibiotics	146/193 (75.6%)	6,250/10,038 (62.3%)	9,084/13,796 (71%)	p = 0.0001 p* = 0.0043

p – PPIC vs EPIC; p* – PPIC vs EPIC II; PPIC – Polish Prevalence of Infection in Intensive Care; EPIC – European Prevalence of Infection in Intensive Care.

infections, in comparison to 63.50% in EPIC II. The incidence of blood infections in our study was also similar to the EPIC II study (10.40% vs 7.80%), as was the incidence of UTIs (8.80% vs 7.30%). We did not find any difference in the incidences of respiratory tract infections and bloodstream infections when we compared our study to the EPIC study. The incidence of UTIs in our study was higher than in the EPIC study (8.80% vs 3.62%).[4,5] A comparative analysis of these 3 PPSs is shown in Table 4.

Data from the European Centre for Disease Control and Prevention point prevalence survey showed that the prevalence of HAIs in pediatric ICUs was 15.5%.[19] That was supported by our observations; nevertheless, our study showed lung infections as the most common, while in the ECDC point prevalence survey, bloodstream infections were the most common type of infection (45.0%).[19] The EPIC and EPIC II studies reported the incidence of infections caused by GN bacteria as 32.0% and 62.0%, respectively.[4,5] Only the EPIC II microbiological results were consistent with our findings, in which GN bacterial infections were predominant. The main GN pathogens in our study were members of the Enterobacteriaceae family. This is similar to other studies, such as those from Brazil (33.8%) and Japan (27.6%).[13,14]

A high number of infections caused by *A. baumannii,* as well as resistance to many groups of antibiotics among GN bacteria, was noted in studies by Weiner et al.[20] and Harris et al.[21] We did not observe this phenomenon in our study. The relatively small number of *A. baumannii* infections in our observations may result from sample size; Weiner et al. analyzed information from 4,515 hospitals. Moreover, our data was collected more than 3 years ago, when the number of such infections was lower in comparison to the present day. In addition, the number of infections caused by *A. baumannii* may be a picture of the epidemiological situation only on the particular day the study was performed.

The frequency of MRSA infections was lower in our study in comparison to the results of one-day multicenter PPS from Turkey (18.20%) and Brazil (16.90%) as well as the EPIC study (20.00%), and was similar to the frequency observed in EPIC II (10.20%).[4,5,13,15] We found no infections caused by colistin-resistant *A. baumannii*, although the literature includes data on *A. baumannii* resistance to colistin amounting to 2.95%.[22] The low percentage of infections caused by GN pathogens that produce ESBL in our study was similar to the observations of Coque et al.[23] The rate of *C. difficile* infections (only 1 isolated pathogen) was lower in our study than in Bartlett's work.[24] This may result from the methodology of our study. We decided to analyze only *C. difficile* infections confirmed with microbiological tests. Moreover, according to data of Kübler et al.,[18] metronidazole is administered to septic patients in Polish ICUs very often because about half of the infections in the critically ill originate in the abdominal cavity. Metronidazole is effective against *C. difficile*, so it may be the reason such infections were not noted in this one-day study.

In summary, the most effective way to control infection problems in ICUs is to strictly follow antiseptic rules (hand hygiene, the use of alcohol-based hand rub solution and HAI monitoring) and to assess compliance with protocols related to these infections that are being implemented in Polish hospitals.[6,25,26]

Our study had several limitations. First, the analysis could have been affected by the respondents' level

of carefulness in completing the questionnaires. Second, our study included multiple hospitals, but the ICUs were situated within 1 geographic area, and the epidemiology of infections in the rest of Poland may not be the same. Third, it is quite noticeable that the data on pediatric patients is rather small: there were 12 patients, and only 2 of them had infections. This is not a representative sample, and all the comparisons involving this group should be considered very cautiously. Fourth, preventive methods could have influenced the rate and epidemiology of HAIs, and this factor was not analyzed. Fifth, we did not precisely analyze the origin of infections, so some could be community-acquired and some hospital-acquired. Also, the use of medical devices may promote infections in hospitalized patients. However, because of the methodology of the study and the nature of the data received, it was not possible to perform analyses that would take these factors into account. Sixth, we did not analyze survival because we completed our observations within 24 h. Finally, some limitations resulted from the methodology of the study; nevertheless, the small number of published studies with the same methodology indicates that there is limited research in this field and shows that our analysis is very important for this part of Europe.

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Complications in cardiac surgery: An analysis of factors contributing to sternal dehiscence in patients who underwent surgery between 2010 and 2014 and a comparison with the 1990–2009 cohort

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Abstract

Background. Sternal dehiscence is a serious postoperative complication of cardiac surgery observed in 0.2–5% of procedures performed by median sternotomy.

Objectives. Assessment of factors, including the method of sternum closure, which may affect the incidence of this complication.

Material and methods. A total of 5,152 consecutive patients undergoing surgery with median sternotomy access in the Cardiac Surgery Department of the Pomeranian Medical University between 2010 and 2014 were included in the study. The analysis centered on cases of sternal dehiscence, which occurred in 45 patients (0.9%).

Results. Factors such as age ($p < 0.05$), body mass ($p < 0.005$) and coronary artery bypass surgery (CABG) ($p < 0.005$) were found to be significant risk factors. Diabetes and chronic obstructive pulmonary disease (COPD) also had an impact on an increased risk of sternal dehiscence ($p < 0.006$ and $p < 0.015$). However, the differences were only significant in the whole study group. Apart from CABG, the type of operation did not affect the incidence of dehiscence. Logistic regression analysis found independent risk factors for the development of sternal dehiscence: body mass index (BMI) (odds ratio (OR): 2.1; $p < 0.019$), diabetes (OR: 2.4; $p < 0.004$), COPD (OR: 2.7; $p < 0.016$), and redo procedure (OR: 3.0; $p < 0.014$). There were no significant differences in postoperative mortality between these groups – 6.7% in the group with sternal dehiscence and 3.9% in the group without dehiscence.

Conclusions. Introducing a more durable sternum stabilization method with 8+ loops helped to improve conditions for bone union and reduced the risk of dehiscence. Therefore, we suggest that centers which still use 6-loop sternal closure should consider shifting to a stronger technique.

Key words: risk factors, cardiac surgery, median sternotomy

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Introduction

Sternal dehiscence after a cardiac surgery procedure is still a very serious complication observed in 0.06–12.50% of patients and associated with an increase in perioperative mortality.[1–4] In a previous publication we described 298 cases (2.1%) of sternal dehiscence after 14,117 procedures performed between 1990 and 2009.[5] The previous analysis was performed due to modification of the postoperative sternum closure technique, which happened at the beginning of 2010. Instead of 6 single wire loops we started to use a closure with 8–12 single loops, depending on the quality of osseous tissue of the sternum, but mostly depending on the body weight of the patient with a simple assumption of 1 loop for every 10 kg of body weight and no fewer than 8 loops per patient.

After 5 years from the introduction of the new sternal closure technique, we decided to examine whether it had an impact on the incidence of postoperative sternum dehiscence. Naturally we are aware of the fact that the results of both papers are not comparable due to the retrospective nature of the studies they describe. The scope of the procedures, demographic characteristics of the assessed group, environmental factors and methods of treatment have changed. The patients are older (therefore diabetes occurs more often) and have a higher body mass index (BMI), which means they are more susceptible to sternal dehiscence. Although we have a specific standard of preventive antibiotic therapy, the drug resistance of some bacteria has reached almost 100%. The previously predominating Gram-positive bacteria were replaced by Gram-positive and opportunistic strains that were not a risk factor in the past.[6] However, there are some positive changes.

One of the new techniques that increase the effectiveness of sternum closure is the application of cement, similar to the one used in dentistry.[7] Yet it seems that the most significant breakthrough has been made in the techniques employed for dealing with complications. The introduction of vacuum-assisted closure (VAC), a still undervalued modality in our opinion, seems to be a very important element that shapes the treatment of surgical wound healing complications such as sternal dehiscence.[6]

The study aimed to analyze factors which may have an influence on the incidence of sternal dehiscence in patients undergoing surgery in our center during the period of 5 years from the introduction of a new sternal stabilization method.

We compared groups with sternal dehiscence and without this condition by performing an analysis of population factors, concomitant diseases and perioperative data. The study was based on data retrieved from the computerized Departmental Database that contains data gathered from our institution since 1990.

The statistical analysis was performed using STATISTICA v. 10 (StatSoft, Inc., Tulsa, USA). The χ^2 test, t-test, Mann-Whitney U test, logistic regression analysis, and discriminant analysis were used in the study. The results have been presented as percentage and mean values ± standard deviation (SD). The threshold for statistical significance was $p < 0.05$.

Material and methods

The retrospective study included consecutive patients who underwent surgery with sternotomy access in the period from 1/01/2010 to 31/12/2014. Sternal dehiscence requiring surgical intervention was found in 45 (0.87%) patients out of 5,152 operations with median sternotomy access.

Fifty-four patients were not included, as they had valve replacement procedures (in 1 case also a graft of ascending aorta prosthesis) with minimal access by partial upper sternotomy and the operative technique precluded full sternal dehiscence.

The technique of primary sternum closure was identical to that used in the years 1990–2009 apart from increasing the number of loops to 8+. As to secondary closures, if the dehiscence was mainly due to loosening of wire loops, we tried tightening them by pulling and twisting the wire. If a full dehiscence occurred, the standard, Robitsek or Martinez closure technique was used. In a few cases we additionally used bone cement to stabilize the closure.

The introduction of VAC as a method of choice whenever sternal wound infection was suspected was a significant improvement. Fluid collection or purulent discharge from the wound, skin dehiscence, a fistula, or a suspicion of hematoma in the wound were indications for partial or complete opening of the wound and applying VAC therapy. During the period of the study, we used systems of several companies, predominantly Hartmann (Paul Hartmann AG, Heidenheim, Germany), KCI (Kinetic Concepts, Inc., San Antonio, USA) and Smith&Nephew (Smith & Nephew, Inc., London, UK). In VAC therapy, the wound is drained using a controlled level of negative pressure. This eliminates the necessity of frequent dressing changes (a few times a day when using a conventional approach). Vacuum assisted closure dressings are changed every 3 to 4 days with a simultaneous lavage or debridement of the wound and culture. Two subsequent negative results of culture were indications for closing the wound. Due to a controlled level of negative pressure, the VAC dressing causes a local vasodilation, increases the temperature of neighboring tissues and is conducive to the migration of leucocytes as well as removing wound discharge. By restricting contact with the infected wound secretion (the VAC foam is covered with a self-adhesive, microporous membrane) the risk of environmental contamination and transfer of bacteria to the staff or other patients is reduced. The use of VAC therapy obviously entails an increase in treatment cost. Nevertheless, a comparison of all expenses involved in treatment with VAC and conventional therapy shows that the former is much more cost-efficient.[6]

The entire study group was composed of 5,152 patients aged 17–90 years (mean 64.7 ±9.28). There were 3,646 men (age: 63.7 ±9.04 years) and 1,506 women (age: 67.1 ±9.56 years). The mean BMI was 28.7 ±4.44 in the whole study group, 28.0 ±4.07 in men and 28.6 ±5.23 in women. In spite of a very similar result, the differences were statistically significant. There were no statistically significant sex-specific differences in normal body weight and obesity. The majority of underweight patients were men, and as far as overweight, and severe, morbid and super obesity were concerned, the majority were women.

Due to the rising average age of patients undergoing surgery, there were more patients with diabetes and chronic obstructive pulmonary disease (COPD) in comparison to the previous study period. Diabetes was reported in 1,649 (32.0%) patients, significantly more often in women than in men. The COPD was reported in 327 (6.3%) patients, without any sex-specific differences. The most prevalent disease was hypertension; 3,528 (68.4%) of the patients were treated for this condition, women again forming the majority. The same trend was observed for coexisting chronic renal failure, which was diagnosed in 409 (7.9%) patients. In contrast, myocardial infarction was found significantly more often in men. Overall there were 2,188 (42.4%) patients with this diagnosis. Furthermore, we also analyzed the impact of an additional factor defined as movement limitations. Such condition may occur as a consequence of many diseases such as: stroke with significant paresis, advanced degenerative changes in facet joints and lower limb joints, multiple sclerosis, morbid and super obesity, amputation in the area of lower limbs, as well as elephantiasis and edema of lower limbs in the course of post-thrombotic syndrome. Movement limitations may heighten the risk of sternal dehiscence due to increased stress on the shoulder girdle during recovery (when getting up from bed and walking). Movement limitations were found in 369 (7.2%) of the patients undergoing surgery, women representing a significant majority.

Another indicator of the functional status of the heart that we investigated was ejection fraction (EF). Mean EF value was around 50% in the entire group; a significantly higher result was reported in women.

Mean cardiopulmonary bypass (CPB) duration, which may be treated as an approximate indicator of the severity of surgery, was 65.2 min in the entire group, significantly longer in women.

The mean value of the perioperative mortality risk index Euroscore Logistic (ESL) was 6.8%. Women had a significantly higher mortality risk (9.3% vs 6.4%). This was reflected in actual perioperative mortality, exactly half the predicted value (3.9%), but also with a significant majority of deaths in female patients (5.5% vs 3.3%). The data is presented in Table 1.

In the reported interval, most of the operations (4,701) were conducted in CPB. The most frequently performed procedures were: coronary artery bypass grafts (CABG – 2,964), procedures for heart valves (590), aortic aneurysms (287), and combinations of CABG with 2 other types of procedures (822). Among procedures performed without cardiopulmonary bypass (CPB) (451), off-pump coronary artery bypass surgery (OPCAB) formed the largest group (424). The annual number of patients who had surgery with median sternotomy access was between 997 and 1,093 (mean 1,030 ±38.7) in the reported period. Table 2 summarizes the types of operations performed. Due to a large number of combinations, we only included the main types of procedures.

Out of 4,222 CABG operations, 3,798 (90.0%) were performed using the standard approach in cardiopulmonary bypass, whereas 454 (10.0%) were off-pump procedures. The dominating model of operation was a single bypass graft from the left internal thoracic artery (LITA) to the branch of the left anterior descending artery (LAD) and a bypass graft from the saphenous vein, usually sequential, to other coronary vessels. Apart from LITA, the right internal thoracic artery (RITA) and radial artery from the non-dominant hand, i.e. mostly left, was used.

Using at least one thoracic artery as a bypass conduit to the LAD belongs to the so-called golden standard. We use it in our center whenever it is possible and indicated. We avoid using ITAs in exceptional situations, especially when: 1. the patient undergoes emergency surgery, is being resuscitated, or presents with low output syndrome and harvesting the ITA could delay revascularization and pose a risk of hypoperfusion in the site of anastomosis due to contraction of the ITA under the influence of high doses of catecholamines often used in such situations; 2. if a difference in blood pressure of more than 30 mm Hg to the detriment of the planned side of ITA harvesting was detected or stenosis of the ITA was diagnosed angiographically; 3. when LAD is not included in planned revascularization; 4. during reoperation, when one ITA was previously used or damaged and the other one is not suitable for harvesting; 5. when the harvested ITA cannot be grafted (atherosclerotic lesions, dissection or other type of damage inflicted during harvesting) or when an area of the patient's chest including the ITA was previously injured or irradiated.

Of the 3,798 patients included in the CABG group, a single ITA (SITA) was used in 3,247 patients. The ITA was harvested but was unsuitable for grafting in 32 patients and replaced by a venous bypass graft. This subset of patients was included in the SITA group. To this group we also added 1 patient from outside the CABG group whose ITA was harvested but could not be grafted because the LAD had severe atherosclerotic lesions and was too narrow.

Both ITAs were used in 329 patients. Five patients, who had 2 arteries harvested but only 1 (4) or none (1) suitable for grafting, were added to this group. For reasons mentioned above, no bypass grafts with ITAs were performed in 148 patients. They had 1–4 venous bypass grafts and in 2 cases also a graft from the radial artery.

Table 1. Pre- and perioperative data of whole patient group treated by sternotomy in years 2010–2014

Patient data		Number of patients	%	p-value
Sex	men women	3,646 1,506	70.7 29.3	–
M/F ratio		2.4:1	–	–
Age		64.7 ±9.28	–	–
Age	men women	63.7 ±9.29 67.1 ±9.69	– –	p < 0.001
Body mass index (BMI)		28.7 ±4.44	–	–
BMI	men women	28.0 ±4.07 28.6 ±5.23	–	p < 0.001
Underweight	men women	60 41	2.8 1.7	p < 0.02
Normal weight	men women	632 279	17.6 19.0	p = 0.32
Overweight	men women	1,718 569	38.8 47.9	p < 0.001
Obesity	men women	1,014 421	28.3 28.7	p = 0.93
Severe obesity	men women	203 165	5.7 11.3	p < 0.001
Morbid and super obesity	men women	18 32	0.5 2.2	p < 0.001
Diabetes mellitus (DM)		1,649	32.0	–
DM	men women	1,110 539	30.4 35.8	p < 0.0001
COPD		327	6.3	–
COPD	men women	225 102	6.2 6.8	p = 0.42
Hypertension (HTN)		3,528	68.5	–
HTN	men women	2,431 1,097	66.7 72.8	p < 0.0001
History of chronic renal failure (CRF)		403	7.8	–
CRF	men women	260 143	7.1 9.5	p = 0.005
History of myocardial infarction (MI)		2,188	42.4	–
MI	men women	1,657 531	45.4 35.3	p < 0.0001
Movement limitations (ML)		369	7.2	–
ML	men women	241 128	6.6 8.5	p < 0.02
Ejection fraction (EF)		49.2 ±11.44	–	
EF	men women	48.0 ±11.47 52.1 ±10.86	– –	p < 0.001
Cardiopulmonary bypass time (CPB)		65.2 ±30.11	–	–
CPB time	men women	64.5 ±29.60 67.1 ±31.18	– –	p < 0.009
EuroScore Logistic (ESL)		6.8 ±8.80		
ESL	men women	5.9 ±8.15 8.9 ±9.92	– –	p < 0.001
Early mortality		203	3.9	–
Death	men women	120 83	3.3 5.5	p < 0.0002

COPD – chronic obstructive pulmonary disease; NYHA – New York Heart Association; CCS – Canadian Cardiovascular Society.

Table 2. Type of procedures performed by sternotomy on patients operated on in years 2010–2014

Type of procedure	Isolated procedures	Procedures combined with CABG	In total	%
Aortic aneurysm repair*	287	85	372	7.2
Aortic valve and mitral valve procedures	64	45	109	2.1
Aortic valve procedures	304	354	658	12.8
Mitral valve procedures	198	317**	515	10.0
Isolated tricuspid valve procedures***	24	21	45	1.0
Other procedures in CPB	26	12	38	0.7
CABG	2,964	–****	2,964	57.5
OPCAB	424	–	424	8.2
Other off-pump procedures	27	–	27	0.5
In total	4,318	834	5,152	100.0

CPB – cardiopulmonary bypass; CABG – coronary artery bypass grafts; OPCAB – off pump coronary artery bypass; * Valve procedures were omitted if they were performed together with aortic aneurysm repair; ** 1 patient from the isolated procedures group was included in the CABG group, because his left ITA was harvested (but not grafted); *** Tricuspid valve procedures were omitted if they were performed together with aortic or mitral valve procedures; **** 833 CABG procedures were not included here, as they were performed with other operations listed above.

Among 424 patients who had off-pump surgery, one ITA was used in 234 (55.2%) patients. In 3 patients, however, the ITA was not suitable for grafting. Both ITAs were harvested in 184 patients (43.4%), although in one case a damaged ITA was not used. Six (1.4%) patients received a planned graft from the saphenous vein. The data is presented in Table 3.

Sternal dehiscence was found in 45 (0.9%) of the patients undergoing surgery by median sternotomy. The onset of dehiscence was calculated as the difference between the date of the first surgery and the date of the first sternal reconstruction procedure. Enlarging the wound opening and/or applying VAC dressing were not counted as reconstruction procedures. Early dehiscence up to 30 days from surgery developed in 38 (84.4%) patients, delayed dehiscence in seven (15.6%) patients. Six patients had previously undergone a revision of the operative wound (one of the patients twice) due to bleeding (4 patients) and/or thrombus that pressed on the right atrium (3 patients).

The partial reconstructions, performed 45 times in 33 patients, mostly entailed pulling and tightening the loose wire loops due to perceived mobility of the separated sternum and, in some cases, adding wire loops (1–2) to the lower part of the sternum body. If a patient developed full dehiscence, complete sternal osteosynthesis was needed, and this was performed 25 times in 22 patients. In total, 70 different types of reconstruction were conducted.

A partial reconstruction only was required in 23 patients. A single reconstruction was sufficient in 19 patients, whereas 2 were needed in 4 patients (27 procedures, in total). Complete sternal osteosynthesis was required in 22 patients. In 17 patients, the Robicsek closure (10 patients), the standard technique (5 patients), the Martinez closure (1 patient), and the standard technique with Kryptonite reinforcement (1 patient) were used. In 12 patients there was a single procedure, whereas 5 complete reconstructions were followed by 11 partial procedures.

In 4 patients, first reconstructions were partial, followed by the Robicsek closure (2 patients), the standard technique (1 patient) and the standard technique with Kryptonite reinforcement (1 patient). In total, 6 partial reconstructions were performed in this group. One patient had 1 partial and 4 complete procedures of sternum closure (2 with the standard method, 1 with the Robicsek closure and 1 with steel plates used to stabilize the chest due to destruction of the sternum).

Furthermore, 29 patients required treatment with VAC therapy. Two hundred and thirty-seven VAC kits were used in the early dehiscence group, 47 in the late dehiscence group (284 in total). The data is presented in Table 4.

Concomitant bacterial wound infection was diagnosed in 26 of 45 (57.8%) dehiscence cases. In the rest

Table 3. Type of revascularisation procedures performed on patients operated on in years 2010–2014

Category	CABG	OPCAB	In total	p-value
Number of used (harvested) single ITAs	3,280 (86.4%)	234 (55.2%)	3,514 (83.2%)	$p < 0.00001$
Number of used (harvested) bilateral ITAs	334 (8.8%)	184 (43.4%)	518 (12.3%)	$p < 0.00001$
Number of revascularizations without using an ITA	184 (4.8%)	6 (1.4%)	190 (4.5%)	$p < 0.01$
Total number of patients undergoing surgery	3,798 (100.0%)	424 (100.0%)	4,222 (100.0%)	–
Mean number of bypass grafts per patient	3.02 ±0.95	2.27 ±0.68	2.95 ±0.95	$p < 0.001$
Mean number of arterial grafts per patient	1.19 ±0.52	1.75 ±0.78	1.24 ±0.58	$p < 0.001$

CABG – coronary artery bypass grafts; OPCAB – off pump coronary artery bypass; ITA – internal thoracic artery.

Table 4. Primary procedures and methods of treatment patients after early and late dehiscence of sternum

Type of procedure	Early dehiscence	Late dehiscence
Number of patients with sternal dehiscence	38 84.4%	7 15.6%
Type of primary procedure: Aortic aneurysm and/or heart valve procedure + CABG CABG OPCAB Aortic aneurysm/heart valve procedure without CABG	 12 23 2 1	 – 7 – –
Time between surgery and sternal dehiscence [days]	5–30 17.5 ±7.1	36–125 66.6 ±9.9
Postoperative re-explorations before sternal dehiscence	4	2 (3)
Procedures due to partial dehiscence or instability of the sternum.	40	5
Full sternal closures	22	3
Number of patients who received VAC treatment	27	2
Number of VAC kits used	2–29 9.63 ±6.98	7–17 12 ±7.01

CPB – cardiopulmonary bypass; CABG – coronary artery bypass grafts; OPCAB – off pump coronary artery bypass; VAC – vacuum-assisted closure.

of the patients, cultures from the sternal wound were negative. Lysogenic conversion of 3–4 bacteria in the wound was observed in isolated cases, conversion of 2 bacteria was observed in 10 patients. One type of bacteria dominated in the remaining 14 cases. Half of the cultured strains were Gram-positive bacteria (21), the other half (20) were Gram-negative. Details for the bacterial strains are shown in Table 5.

Table 5. List bacterial strains cultured from the wound of sternum in patients after dehiscence

Bacteria	Gram stain	Number of cases
Enterococcus faecalis	G+	6
Enterococcus faecium	G+	5
Staphylococcus epidermidis	G+	5
Staphylococcus aureus	G+	4
Peptoniphilus asaccharolyticus	G+	1
Enterobacter cloacae	G–	5
Pseudomonas aeruginosa	G–	4
Escherichia coli	G–	2
Klebsiella pneumoniae	G–	2
Morganella morganii	G–	2
Serratia marcescens	G–	2
Heamophilus influenzae	G–	1
Proteus mirabilis	G–	1
Stenotrophomonas maltophilia	G–	1
In total	G+/–	41

Results

Sternal dehiscence occurred about 1.7 times more often in men than in women (M:F = 4:1 in the dehiscence group; 2.4:1 in patients without dehiscence), but the differences were not statistically significant. Yet, it should be remembered that both groups differed in terms of age. Patients in the dehiscence group were significantly older, although when adjusted for sex it may be noted that the differences were significant only in men, while the mean age of women was similar in both groups. Obesity dominated in the dehiscence group, whereas overweight was prevalent among patients without this complication. This translated into significant differences in mean BMI values between both groups – 30.2 ±4.51 vs 28.2 ±4.45. The disparities remained significant when the groups were analyzed by sex. Diabetes was the only condition among concomitant and previous diseases found significantly more often in patients with sternal dehiscence. Differences were also statistically significant with regard to COPD, but after incorporating sex differences they were only significant in men. No statistically detectable differences were found for hypertension, renal failure, previous myocardial infarction, poor mobility, NYHA (New York Heart Association) class IV, or stage IV of CCS (Canadian Cardiovascular Society). The mean EF values and CPB duration also did not differ significantly between the 2 groups. We found, however, that the risk of dehiscence doubled in patients who required a redo procedure due to severe hemodynamic instability, ischemia, bleeding, or compression of the heart by thrombus (5.2% vs 13.3%, $p < 0.035$).

There were also no statistically significant differences in predicted (using the EuroScore Logistic index) and actual early mortality rate. In the sternal dehiscence group, actual mortality was nearly the same as the predicted rate. In the group without dehiscence, actual mortality was about half the predicted mortality. The data is presented in Table 6.

Unilateral or bilateral ITA use during a procedure increased the risk of dehiscence up to 1.1% and 0.96%, respectively. In the remaining subgroups, the risk of dehiscence was markedly lower and did not exceed 0.5%. The results are presented in Tables 7 and 8.

The only accompanying procedure that was never performed by sternotomy as an independent procedure was RF ablation to treat abnormal conduction pathways in the atria. In total, 230 (4.5%) such procedures were performed, including 93 ablation procedures combined with isolated CABG in CPB and 4 OPCAB procedures. Only one (0.4%) patient from the sternal dehiscence group had a previous accompanying RFA procedure.

In order to analyze the effect of individual risk factors more accurately, we performed logistic regression analysis (LRA) of 16 risk factors: sex, age, BMI, EF, ESL, movement limitations, chronic renal failure, diabetes, COPD, CABG, use of 1 or 2 ITAs, CPB time, NYHA class IV,

Table 6. Differences in pre- and perioperative data between patients with and without dehiscence after sternotomy

Results		Patients with dehiscence	Patients without dehiscence	p-value
Number of cases		45 (0.9%)	5,107 (99.1%)	–
Sex	men women	36 (80.0%) 9 (20.0%)	3,610 (70.7%) 1,497 (39.3%)	p = 0.17
M/F ratio		4:1	2.4:1	–
Age		66.8 ±6.54	64.7 ±9.56	p < 0.05
Age	men women	63.3 ±8.9 66.8 ±7.7	59.0 ±10.0 61.5 ±7.8	p < 0.0004 p < 0.0002
Body mass index (BMI)		30.2 ±4.51	28.2 ±4.45	p < 0.005
BMI	men women	29.9 ±4.96 31.6 ±1.13	28.0 ±4.06 28.6 ±5.25	p < 0.04 p < 0.001
Diabetes mellitus (DM)		23 (51.1%)	1,626 (31.8%)	p < 0.006
DM	men women	15 (41.7%) 8 (88.9%)	1,095 (30.3%) 531 (35.5%)	p = 0.14 p < 0.003
COPD		7 (5.6%)	320 (6.3%)	p < 0.015
COPD	men women	6 (16.7%) 1 (11.1%)	225 (6.2%) 102 (6.8%)	p < 0.015 p = 0.88
Hypertension (HTN)		36 (80.0%)	3,492 (68.4%)	p = 0.09
HTN	men women	27 (77.2%) 9 (100.0%)	2,404 (66.6%) 1,088 (72.7%)	p = 0.29 p = 0.14
History of chronic renal failure (CRF)		7 (15.6%)	396 (7.7%)	p = 0.052
CRF	men women	5 (13.9%) 2 (22.2%)	255 (7.1%) 141 (9.4%)	p = 0.11 p = 0.19
History of myocardial infarction (MI)		21 (46.7%)	2,167 (42.4%)	p = 0.57
MI	men women	18 (50.0%) 3 (33.3%)	1,639 (45.4%) 528 (35.3%)	p = 0.58 p = 0.9
Movement limitations (ML)		4 (8.9%)	365 (7.1%)	p = 0.65
ML	men women	4 (11.1%) 0 (0.0%)	237 (6.6%) 128 (8.5%)	p = 0.57 p = 0.75
Ejection fraction – EF [%]		46.2 ±12.42	49.2 ±11.43	p = 0.11
EF [%]	men women	45.8 ±13.10 47.8 ±9.72	48.1 ±11.45 52.1 ±10.87	p = 0.31 p = 0.22
NYHA Class IV		3 (6.67%)	300 (5.87%)	p = 0.92
NYHA IV	men women	3 (8.33%) 0 (0.0%)	176 (4.87%) 124 (8.28%)	p = 0.92
CCS Stage IV		12 (26.7%)	903 (17.7%)	p = 0.17
CCS IV	men women	8 (22.2%) 4 (44.4%)	644 (17.8%) 259 (17.3%)	p = 0.64 p = 0.09
Cardiopulmonary bypass time [min]		65.9 ±29.09	65.2 ±30.10	p = 0.87
CPB time [min]	men women	67.9 ±29.23 58.7 ±29.02	64.4 ±29.60 67.1 ±31.20	p = 0.5 p = 0.41
EuroScore Logistic [%]		6.3 ±6.55	6.8 ±8.82	p = 0.65
ESL [%]	men women	6.4 ±6.85 6.2 ±5.56	5.9 ±8.16 8.9 ±9.94	p = 0.71 p = 0.19
Re-exploration after primary procedure (RE-EX)		6 (13.3%)	264 (5.17%)	p = 0.035
RE-EX procedures	men women	6 (16.7%) 0 (0.0%)	209 (5.79%) 55 (3.67%)	p = 0.035
Early mortality		3 (6.7%)	200 (3.9%)	p = 0.57
Death	men women	2 (5.5%) 1 (11.1%)	118 (3.3%) 82 (5.5%)	p = 0.31 p = 1.0

COPD – chronic obstructive pulmonary disease; NYHA – New York Heart Association; CCS – Canadian Cardiovascular Society.

Table 7. Frequency of dehiscence of sternum depending on isolated coronary or valve procedures and combined operation

Type of procedure		Sternal dehiscence	%	p-value
Aortic aneurysm repair*	isolated with CABG	1 1	0.3 1.2	p = 0.94
Aortic and mitral valve procedures	isolated with CABG	0 1	0.0 2.2	p = 0.86
Aortic valve procedures	isolated with CABG	0 4	0.0 1.1	p = 0.17
Mitral valve procedures	isolated with CABG	1 5	0.5 1.6	p = 0.49
Other procedures	isolated with CABG	0 0	0.0 0.0	–
CABG		30	1.0	p = 0.42
OPCAB		2	0.5	
In total		45	0.9	5,152

* Valve procedures performed together with aortic aneurysm repair were treated as aneurysm repair procedures.

Table 8. Frequency of dehiscence of sternum depending on using ITAs grafts

Category		Sternal dehiscence	Without dehiscence	Dehiscence rate	p-value
1.	Surgery with 1 (harvested) ITA	38	3,476	1.09	$p < 0.005^{1,4}$
2.	Surgery with 2 (harvested) ITAs	5	513	0.96	$p < 0.03^{2,4}$
3.	Other procedures without using (harvesting) an ITA	2	1,120	0.18	$p < 0.005^{3}$
	In total	45	5,107	0.87	–

ITA – Internal Thoracic Artery; [1]1. vs 3; [2]2. vs 3; [3]1.+2. vs 3; [4]1. vs 2., p = 0.81.

stage IV of CCS, and reexploration after primary procedure. The LRA showed that only BMI (p < 0.015; OR = 1.08; 95% CI = 1.02–1.15), diabetes (p < 0.007; OR = 2.34; 95% CI = 1.27–4.31), COPD (p < 0.019; OR = 2.68; 95% CI = 1.18–6.11), and re-exploration (p < 0.012; OR = 3.12; 95% CI = 1.29–7.5) were independent factors which significantly affected the incidence of sternal dehiscence. Results were confirmed with discriminant analysis. The data is presented in Table 9.

Table 9. Results of logistic regression analysis and discriminant analysis

Independent risk factor	Odds ratio (OR)	–95% CI	+95% CI	p-value
Logistic regression analysis				
BMI	2.1	1.13	3.82	p < 0.019
Diabetes	2.4	1.33	4.45	p < 0.004
COPD	2.7	1.2	6.24	p < 0.016
Re-exploration	3.0	1.24	7.21	p < 0.014
Discriminant analysis				
BMI	–	–	–	p < 0.013
Diabetes	–	–	–	p < 0.002
COPD	–	–	–	p < 0.01
Re-exploration	–	–	–	p < 0.009

BMI – body mass index; COPD – chronic obstructive pulmonary disease.

Discussion

Sternal dehiscence is a surgical complication which has been reported since 1957, when Julian presented his idea of opening the chest through median sternotomy,[7] soon accepted as the best method of access to the anterior mediastinum. Unfortunately, mortality reached 50% when concomitant mediastinitis presented. The introduction of a new method of close wound treatment in 1963, which used irrigation drainage with an antibiotic-containing solution, helped to reduce mortality to 20% in the most severe cases.[8] The first studies on the causes of sternal dehiscence appeared at the end of the 1960s due to the rapid increase in the number of CABG procedures and the postoperative complications that had been reported. More than 20 different causes that could directly or indirectly affect the incidence of sternal dehiscence have been identified in the literature. It has been established that this complication develops more frequently in men due to the musculature of the chest, but also in women with large breasts and patients with diabetes, patients who are overweight, patients who regularly take steroids, patients receiving immunosuppressive treatment, patients who smoke tobacco, and patients with COPD. Advanced age was also identified as another factor increasing the risk of sternal dehiscence.[2,9,10]

The technique of proper incision has been stressed as an important perioperative factor since the beginnings of median sternotomy as a surgical procedure.[11,12] A serious deviation from the symmetry axis of the incision may often result in complicated healing. Mistakes in sternal closure may have a similar effect.[4,12] Moreover, such factors as time of the operation and exposition of the mediastinum, use of hypothermia, cardiopulmonary bypass, increased blood transfusions, reoperations due to bleeding, and most importantly – using 1, and especially 2 internal thoracic arteries as conduits during surgery were indicated as having an impact, although bilateral ITA use is sometimes challenged in the literature.[13–17] Postoperative factors that may increase the risk of sternal dehiscence include chest compression during resuscitation, intensive cough among tobacco smokers and patients with COPD, but also intake of specific medicines.[18,19] In addition, other lung complications that result in strong cough, prolonged intubation and mechanical ventilation may increase the risk of sternal dehiscence. The most serious complication that

often accompanies sternal dehiscence is an infection of the wound, soft tissues, sternum and mediastinum.[14–16,20]

Prevention of sternal dehiscence is mostly accomplished by eliminating all controllable factors, for example, discontinuation of smoking at least 1 month before surgery and reduction of body mass. Using proper techniques of chest opening and closure is also of vital importance.[1,21–29] Reoperation due to bleeding or other causes is also a risk factor confirmed in clinical trials.[26]

Infection of the operative wound and sternal dehiscence are 2 closely associated complications, although it is rarely possible to determine which one causes the other. Therefore, appropriate aseptic and antiseptic procedures can have a major impact on the incidence of these complications.[20,21] In patients with diabetes, it is crucial to maintain glycemic control with insulin infusions in the early postoperative period and appropriately titrated doses in later phases of treatment.[10,29] Early weaning from mechanical ventilation and rehabilitation from the moment of awakening after surgery contribute to reducing the rate of respiratory complications and consequently to decreasing sternal dehiscence rates.[30]

In 2009, we conducted a similar analysis that covered patients undergoing surgery between 1990 and 2009. We observed at that time that sternal dehiscence was much more frequent among men. Furthermore, patients with sternal dehiscence were significantly older and had a higher BMI. Diabetes and COPD were significantly more frequent in the dehiscence group.[5] Similar results were obtained in the current study. In comparison to the previous study period, the patients are older and have a higher BMI. The incidence of diabetes and COPD, factors associated with age, has also increased significantly. Moreover, we monitored the impact of other factors that had not been analyzed before, such as concomitant hypertension, renal failure, previous myocardial infarction, and movement limitations, but we did not find any influence on the incidence of the observed cases of sternal dehiscence. In contrast to the previous study, although men dominated in the dehiscence group, the differences attributed to sex lost their statistical significance.

The largest differences pertain to the association between using internal thoracic arteries and postoperative sternal dehiscence. The results of both studies show that harvesting thoracic arteries significantly increased the risk of postoperative dehiscence. However, using our strengthened method of sternum closure reduced that risk by half (2.1% vs 1.1%). In the previous study, use of both thoracic arteries increased the risk of dehiscence to 4.6%. In the current study using 1 or 2 thoracic arteries did not have any influence on the risk (1.1% vs 1.0%). The risk of dehiscence after operations without revascularization of the myocardium fell 14 times in comparison to the previous study – from 1.4% to 0.1%.

The discriminant analysis of the influence of risk factors on sternal dehiscence performed during previous studies demonstrated the significance of 3 independent risk factors: age, sex and BMI.[5]

In the present study, an LRA was performed. Diabetes, COPD, BMI and re-exploration were identified as independent risk factors. These results were confirmed by DA.

Certainly, it may be argued that the current and previous study group are too different from each other to allow a comparison. However, 2 opposing processes need to be taken into consideration. On one hand, there are the growing risks due to ongoing trends in modern societies such as population ageing, an increasing rate of lifestyle diseases such as diabetes and obesity, and an intensification of unfavorable changes in the surrounding environment – a rising number of antibacteria-resistant bacteria. On the other hand, the operative techniques and methods of postoperative patient management are improving, aseptic and antiseptic techniques in the operative field have been enhanced, and the conditions of hospital stay have been ameliorated. All of the above processes took place gradually, in contrast to the modification of sternal closure, which happened at the beginning of the 5-year study period. Though the results may be referred to 1 specific cardiac surgery center, we believe that some general suggestions may be inferred.

Conclusions

Introducing a more durable sternum stabilization method with 8+ loops helped to improve conditions for bone union and reduced the risk of dehiscence. Therefore, we suggest that centers which still use 6-loop sternal closure should consider shifting to a stronger technique.

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Patient awareness, perception and attitude to contrast-enhanced CT examination: Implications for communication and compliance with patients' preferences

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Abstract

Background. Despite the high volume of contrast-enhanced computed tomography (CECT) examinations, there is limited awareness about its risks among patients and little is known about the influence of patient information sheets.

Objectives. The objective of this study was to assess patients' awareness and perception of risks related to CECT examination and how they are influenced by an information sheet.

Material and methods. A total of 263 adult patients scheduled for a CECT examination completed a questionnaire. The first page evaluated patients' characteristics, their fear and awareness about examination-related risks, and source of information. Page 2 contained the Zung self-rating anxiety scale. After reading the information sheet, patients completed page 3 that surveyed how their awareness and fear had changed.

Results. Nearly half of the patients underestimated the risk of secondary malignancy (n = 121, 46%), or the risk of renal impairment (n = 110, 42%). The vast majority (n = 227, 86%) stated that they were not instructed to maintain fluid intake up to 1 h before the procedure. After reading the information sheet, patients generally corrected their knowledge, but 195 (74%) reported experiencing greater fear ($p < 0.0001$). Fear was more pronounced in younger female patients who had not undergone CT previously. Patients feared the result more than examination-related risks. Most patients (n = 204, 78%) would feel uncomfortable before receiving the examination result.

Conclusions. Most patients do not assess risks related to CECT examination correctly. Although the information sheet improves patients' understanding of CECT-related risks, it lacks empathically delivered reassurance and increases their fear. Fast communication of examination results would make patients feel more comfortable.

Key words: multidetector computed tomography, radiation exposure, patient education, test anxiety scale, patient preference

Introduction

Over the last decade, the utilization of cross-sectional imaging by computed tomography (CT) has been increasing due to better availability, broader range of examinations, new treatments in oncology, and also medico-legal considerations.[1,2] Concerns about radiation exposure to the population[3] resulted in numerous technical innovations in the CT industry aiming at reducing the radiation dose, while maintaining adequate diagnostic performance.[4,5]

However, little effort has been devoted to the assessment of patients' awareness, perception and attitude to radiation exposure during CT examination and its associated risks.[3,6] Scarce data showed that not only patients but also medical professionals have limited insight into the amount of radiation delivered during diagnostic procedures and their debated effect on human health.[3,7,8] Communicating the potential hazards associated with radiation exposure and contrast material administration, in addition to the uncertainty of the examination result, contribute to patients' fear.[3,9]

In this study, we assessed patients' evaluation of risks related to contrast-enhanced CT examination and the impact that informing the patient has on their awareness and fear of the examination.

Methods

This questionnaire study was performed in accordance with the Helsinki Declaration and approved by the local Institutional Review Board. Written informed consent was obtained from all participants.

The study was performed in a single tertiary university hospital from May to June 2017. A total of 315 adult patients consented to complete a questionnaire while waiting for a scheduled contrast-enhanced CT examination of the abdomen and pelvis ± thorax. Patients with a physical or visual impairment preventing them from reading or completing the questionnaire were not addressed. The English translation of the questionnaire is in Appendix A.

Apart from questions related to patients' characteristics, we surveyed their awareness about radiation exposure and risks, and their fear of the CT examination. Patients also indicated sources of their information and particular pieces of information they had obtained. Page 2 contained the Zung self-rating anxiety scale.[10] Patients then read the information sheet about the CT examination, including the presence of X-rays and their potential to induce cancer in a small fraction of cases. The risks of contrast material administration (adverse reaction, renal impairment) were explained. The last page of the questionnaire surveyed the way in which the awareness of radiation exposure, risks of CT examination and patients' fear changed. Final questions related to the communication of the examination result.

Finally, a radiologist (LL) reviewed the examination report and marked those with adverse result (disease progression or a major complication).

Statistical evaluation was performed with SPSS v. 19 (IMB Corp., Armonk, USA) using Kendall's tau-b bivariate correlation (τ), χ^2 test and Mann–Whitney U test as appropriate. A p-value below 0.05 was considered significant.

Results

From 315 questionnaires, 263 (83%) were returned completed. Most patients (n = 239, 91%) had previously undergone at least 1 CT examination. Patient characteristics are listed in Table 1.

Table 1. Patients' characteristics

Patients, n	263
Age [years]	62 ±12
Gender (males)	99 (38%)
Malignant condition	237 (90%)
Education	
basic education	45 (17%)
high school	118 (45%)
higher education	24 (9%)
university	76 (29%)
Number of previous CT examinations	
0	24 (9%)
1–2	67 (25%)
3–4	74 (28%)
≥5	98 (37%)
Type of CT examination	
abdomen + pelvis	81 (31%)
thorax + abdomen + pelvis	182 (69%)
Radiation exposure	
dose-length product (mGy·cm)	801 ±254

In risk assessment, 25 (10%) patients underestimated radiation exposure, 121 (46%) underestimated the risk of developing a secondary tumor and 110 (42%) underestimated the risk of renal impairment (Fig. 1). After reading the information, the patients generally corrected their evaluation of the risks of carcinogenesis and renal impairment but were more likely to overestimate radiation exposure. Males (τ = 0.16, p = 0.010) and older (τ = −0.11, p = 0.029)

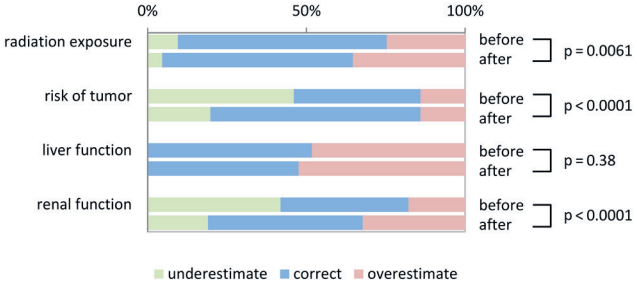

Fig. 1. Assessment of CT-related risks by patients undergoing CT examination before and after being given information about the examination

Table 2. Patients' responses according to age, gender, education, and number of previous CTs expressed as Kendall's tau

Variable	Age years		Gender 1 = female, 2 = male		Education 1 = primary school, 4 = university		Previous CTs number	
	tau	p-value	tau	p-value	tau	p-value	tau	p-value
Before reading the information sheet (1 = underestimate, 2 = correct, 3 = overestimate)								
radiation exposure	−0.120	0.012	−0.147	0.011	0.069	0.212	−0.036	0.481
tumor development	−0.108	0.029	0.156	0.010	0.042	0.464	−0.207	0.000
liver impairment	−0.011	0.830	0.047	0.445	0.021	0.717	−0.076	0.167
renal impairment	−0.001	0.988	0.040	0.499	0.038	0.505	−0.114	0.033
Improvement of knowledge after reading the information sheet (improved = 1, unchanged = 2, worse = 3)								
radiation exposure	−0.050	0.328	−0.016	0.794	−0.054	0.362	−0.013	0.818
tumor development	−0.028	0.581	0.025	0.692	−0.047	0.428	−0.078	0.160
liver impairment	0.108	0.040	−0.074	0.246	−0.121	0.051	0.057	0.325
renal impairment	0.021	0.681	0.047	0.451	0.161	0.007	−0.209	0.000
Fear (1 = no fear, 2 = little fear, 3 = great fear)								
fear from CT examination	−0.168	0.001	0.158	0.010	0.043	0.463	−0.063	0.247
fear from intravenous contrast material	−0.089	0.079	0.111	0.071	0.044	0.452	−0.047	0.389
fear from the examination result	−0.049	0.314	0.184	0.002	−0.048	0.403	−0.131	0.014
Fear change after reading the information sheet (markedly decreased = 1, no change = 3, markedly increased = 5)								
change in fear from the examination	−0.096	0.048	−0.235	0.000	0.143	0.012	0.036	0.496

patients and those who had had previous experience with CT examination ($\tau = -0.21$, $p < 0.0001$) tended to underestimate the risks of radiation exposure more (Table 2). Patients who had previously undergone CT examinations underestimated the possible impact of contrast material on renal function more ($\tau = -0.11$, $p = 0.033$). Reading the information sheet confused 6% to 18% of patients, depending on the question. The vast majority of patients (n = 227, 86%) stated that they were not instructed to maintain fluid intake up to 1 h before the procedure.

Sources of patients' information are shown in Table 3. The most frequent source of information was the referring physician (67% patients).

Patients feared the result of the examination more than the injection of contrast material or the examination itself ($p < 0.0001$, Fig. 2). Fear of the CT examination was more pronounced in younger ($\tau = -0.22$, $p = 0.0003$) and female patients ($\tau = -0.17$, $p = 0.0009$). Patients who had previously undergone CT examinations feared the result of the examination less ($\tau = -0.17$, $p < 0.006$). After reading the information sheet, 195 (74%) patients reported experiencing greater fear of the examination ($p < 0.0001$, Fig. 2), in particular females ($\tau = -0.24$, $p < 0.0001$), younger patients ($\tau = -0.096$, $p = 0.0048$) and those with a higher level of education ($\tau = 0.143$, $p = 0.012$). Especially younger patients reported increased fear of the radiation exposure and contrast material administration ($\tau = -0.22$, $p = 0.0003$; $\tau = -0.14$, $p = 0.025$, respectively). The majority

Table 3. Sources of patients' information and particular pieces of information they had obtained

Information	Yes	No
No food at least 5 h prior to CT	251 (95%)	12 (5%)
Can drink up to 1 h prior to CT	36 (14%)	227 (86%)
How CT examination proceeds	87 (33%)	176 (67%)
Contrast agent	144 (55%)	119 (45%)
Radiation burden	50 (19%)	213 (81%)
Alternative to CT examination	51 (19%)	212 (81%)
Information source	**yes**	**no**
Referring physician	176 (67%)	87 (33%)
Previous examinations	144 (55%)	119 (45%)
Internet	21 (8%)	242 (92%)
Friends, relatives, other patients	12 (5%)	251 (95%)
Radiology staff	18 (7%)	245 (93%)

Table 4. Patients' responses and preferences regarding the examination result

Question	Response			
I will know the result within	3 days	week	month	>month
	164 (62%)	76 (29%)	16 (6%)	6 (2%)
I will find out the result	by phone	by post	by e-mail	by visiting the physician
	79 (30%)	0 (0%)	0 (0%)	184 (70%)
I wish to know the result within	1 h	1 day	3 days	1 week
	61 (23%)	88 (33%)	30 (11%)	84 (32%)
Before I know the result, I will be	comfortable	mildly uncomfortable	severely uncomfortable	
	58 (22%)	154 (59%)	50 (19%)	

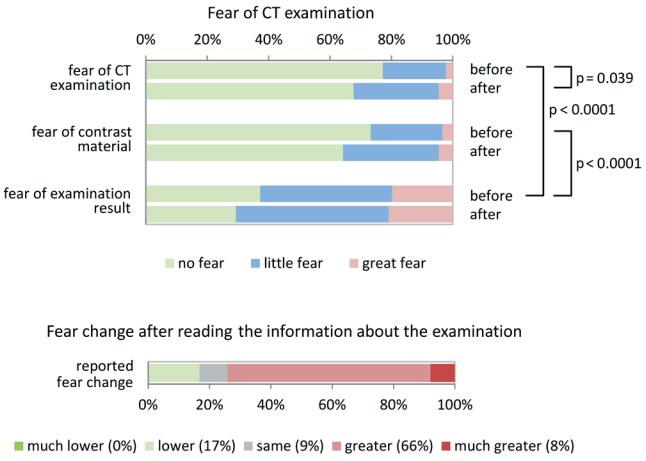

Fig. 2. Fear of CT examination before and after reading the information sheet

of patients (79%) reported that they had learned new information or had refined their knowledge. None of the patients refused to undergo the examination based on the information presented in the questionnaire.

Mean anxiety score rated by the Zung self-rating anxiety scale was 34 points (interquartile range, 7 points), which is within the normal range (20–44 points). There was no correlation between the score and fear of the CT examination, contrast material administration or the result of the examination. Likewise, we found no correlation with fear reported after reading the information, or with the actual result of the examination (25% patients had progression of the disease or a major complication).

Two-thirds of patients stated that they would receive the examination results from the referring physician within 3 days. Most patients admitted that they would be uncomfortable before they receive the result. One-quarter of the patients would like to know the result within 1 h (Table 4).

Discussion

The first part of this study showed that roughly half of the patients do not evaluate radiation exposure as well as other risks related to CT examination correctly. Patients fear the result of the examination more than the potential risks associated with the examination. Supplying this information results in a better appreciation of the risks, but also increases fear, especially in younger women with a higher level of education.

In this study, patients had better awareness of the radiation exposure with 66% being correct, compared to a study in emergency patients of a younger age conducted by Bauman et al. with 45%[6] or even less in other studies.[3,11,12] On the other hand, 46% patients were not aware of the impact of radiation on human health. The risks were underestimated, especially by older male patients with previous CT examinations. Similarly to a study conducted by Singh et al., most participants were not educated about radiation exposure by their referring physician in any way.[12] Our study showed that patients read and tried to comprehend information presented to them by the medical staff in print, which resulted in improved assessment of examination-related risks.

As shown in patients' response to questions about their fear, they believe correctly that the result of the examination has a larger influence on their health compared to risks related to it. The risk of having an adverse result of the CT examination (25% in our sample) is greater than the risk of a severe adverse reaction to non-ionic contrast material (0.04%) or the risk of developing a secondary malignancy decades later (0.05%).[11,13,14] After reading the information, patients generally experience greater fear irrespective of their general anxiety level measured by the Zung self-rating anxiety scale.[10] This effect is pronounced especially in younger women who had little experience with CT and therefore may require more counseling.

The information about the examination is primarily delivered by the referring physician (67% of patients in this study), who has a bond of trust and who can provide the patient with an information letter.[17,18] In a study conducted by Caoili et al., 47% of the respondents were educated about CT by their physicians, and 70% of them regarded this input as important.[3] Although nearly all patients knew that they should abstain from food for 5 h, only 14% were aware that soft drinks are allowed up to 1 h before the examination. This recommendation, which may decrease the risk of contrast-induced nephropathy, should also be pointed out to the physicians.[14]

Although information sheets may be distributed among patients (by the referring physicians) with the effect of improving their understanding of CT-related risks, they may lack cognitive reassurance delivered with empathy that would decrease their fear.[15] We believe that when communicating radiation concerns, medical professionals should inform the patients about its presence and reassure them that subtle health risks may be dismissed in the view of a much greater benefit of exposing an important finding that may guide their further treatment.[9,16]

Two-thirds of patients will receive examination results within 3 days, mostly when visiting the physician. In our experience, composing a report with a concise conclusion in a timely manner facilitates patient workflow in a clinical department and reduces the number of phone queries that interrupt work. Unfortunately, under normal circumstances, our department is unable to cope with ¼ of respondents requesting the report within 1 h.[19] However, rapid communication of the examination result would make patients feel more comfortable and might be regarded as an extra service.

This study has the following limitations. It was performed in patients from a single center undergoing 1 particular type of CT examination. Selection of patients may have been affected by convenience sampling bias by not including those who would not cope with the questionnaire. Because of the paucity of similar studies, we were unable to comment on trends in patients' awareness, perception and attitude to contrast-enhanced CT examinations.

Conclusions

More than half of the patients do not evaluate radiation exposure and other risks related to CT examination correctly. Although information sheet improves patients' understanding of CT-related risks, it may lack empathetically delivered reassurance and increase fear especially in younger women with higher level of education and without previous experience with CT. Patients are primarily educated by their referring physicians who frequently fail to communicate important points. Patients generally fear the result of the examination more than potential risks associated with the examination. Most patients would be uncomfortable before they receive the examination result.

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Appendix A: Questionnaire

FIRST PAGE

1. **Gender**	Female	Male		
2. **Education**	Basic school	High school	Higher education	University

3. **How many previous CT examinations have you undergone?**	0	1	2	3	4	more

4. **What is the radiation exposure associated with CT examination?**	None	Low (eg. chest x-ray, flight)	Moderate (eg. annual radiation background)	High (eg. radiotherapy)

What impact can your CT examination have on your health?					
5a.	**Tumor development**	No risk	Low risk	Moderate risk	High risk
5b.	**Liver impairment**	No risk	Low risk	Moderate risk	High risk
5c.	**Renal impairment**	No risk	Low risk	Moderate risk	High risk

6.	**From CT examination, I have**	No fear	Little fear	Great fear
7.	**From intravenous contrast material, I have**	No fear	Little fear	Great fear
8.	**From the examination result, I have**	No fear	Little fear	Great fear

What information about the CT examination were you given by the referring physician?		
9a.	**I should abstain from food at least 5 hours prior to the examination**	
9b.	**I can drink up to one hour prior to the CT examination**	
9c.	**How CT examination proceeds**	
9d.	**Contrast material administration**	
9e.	**Radiation burden**	
9f.	**Whether there is an alternative to the CT examination**	

I have information about the CT examination from		
10a.	**the referring physician**	
10b.	**previous examinations**	
10c.	**the internet**	
10d.	**friends, relatives, other patients**	
10e.	**the radiology staff**	

AFTER READING INFORMATION FOR PATIENTS

1. **What is the radiation exposure associated with CT examination?**	None	Low (eg. chest X-ray, flight)	Moderate (eg. annual radiation background)	High (eg. radiotherapy)

What impact can your CT examination have on your health?				
2a. **Tumor development**	No risk	Low risk	Moderate risk	High risk
2b. **Liver impairment**	No risk	Low risk	Moderate risk	High risk
2c. **Renal impairment**	No risk	Low risk	Moderate risk	High risk

3a. **From CT examination, I have**	No fear	Little fear	Great fear
3b. **From intravenous contrast material, I have**	No fear	Little fear	Great fear
3c. **From the examination result, I have**	No fear	Little fear	Great fear

After reading information about the examination	
4a. **I learned completely new information**	
4b. **I updated my knowledge about the examination**	
4c. **I learned nothing new**	
4d. **I am still decided to undergo the examination**	

5. **After reading information about the examination, my fear from the examination**	markedly decreased	somewhat decreased	did not change	somewhat increased	markedly increased

6a. **I will receive the examination result within**	three days	one week	a month	more than a month
6b. **I will receive the examination result**	by phone	by post	by e-mail	by visiting the physician
6c. **I wish to know at least a preliminary result within**	one hour	one day	three days	one week

7. **Before I receive the examination result, I will be**	comfortable	mildly uncomfortable	severely uncomfortable

Effect of fenofibrate on serum nitric oxide levels in patients with hypertriglyceridemia

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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. Fenofibrate, a peroxisome proliferator-activated receptor-α (PPARα) agonist, is used to treat patients with hypercholesterolemia and hypertriglyceridemia in order to reduce the risk of development of the atherosclerotic cardiovascular disease. However, it exerts pleiotropic effects beyond correcting atherogenic dyslipidemia to treat hypercholesterolemia.

Objectives. The aim of this study was to investigate the potential effects of fenofibrate on endothelial function by analyzing the serum nitric oxide (NO) levels in patients with hypertriglyceridemia.

Material and methods. Lipid profiles and serum NO levels were assessed in 56 healthy adults aged 29 to 84 years, before and after 12 weeks of fenofibrate (250 mg/d; n = 30) or placebo (n = 26). Appropriate dietary suggestions for hypertriglyceridemia were made for all patients. This study was randomized, double-blind and placebo-controlled in design.

Results. Total cholesterol, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and triglyceride levels significantly decreased; high-density lipoprotein (HDL) and NO levels significantly increased after 12 weeks of fenofibrate therapy. We observed a statistically significant correlation between the increase in serum NO levels and decrease in serum triglyceride levels ($r = -0.42$, $p = 0.02$) in the fenofibrate group.

Conclusions. The positive effect of short-term fenofibrate treatments on vascular endothelial functions in patients with hypertriglyceridemia has been demonstrated by increasing the serum NO levels. Agents such as fenofibrate targeting PPARα-associated signaling pathways show promise as an alternative treatment of vascular dysfunction related to advanced age and hyperlipidemia.

Key words: fibrate, hypertriglyceridemia, nitric oxide

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Introduction

The endothelium plays a major role in not only regulation of the function but also the protection of the normal structure of vascular tissue mainly by the production of nitric oxide (NO). Endothelium-derived NO is a strong endogenous vasodilator, acting through the activation of the soluble guanylate cyclase resulting in cyclic guanosine monophosphate (cGMP) production.[1–3] Nitric oxide takes a role in regulating vascular tone; on the other hand, some important steps, such as smooth muscle proliferation in vascular tissue, platelet accumulation and endothelial cell-leukocyte interaction in the development of atherosclerosis and thrombosis, are also inhibited by NO.[4–9] The presence of cardiovascular risk factors may lead to impaired endothelial integrity as a result of decreased levels of NO released from endothelium.[10] Hypercholesterolemia, an important cause of atherosclerosis formation and progression, also results in decreased vascular NO activity.[11,12]

In animal studies, the inhibition of NO synthesis in hypercholesterolemia gives rise to the progression of development of atherosclerosis, while the increase in NO levels slows down or even regresses it.[13–16] The mechanism underlying the impairment of endothelial NO syntheses by hypercholesterolemia has remained unclear; however, it is essential to define new treatment modalities for improving vascular endothelial function by increasing vascular NO activity in patients with hyperlipidemia.

Fenofibrate, a peroxisome proliferator-activated receptor (PPAR)-α agonist, exerts several pleiotropic effects beyond treating atherogenic dyslipidemia by lowering plasma triacylglycerides and consequently modifying the morphology of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particles. Fatty acids and derivatives activate the PPAR nuclear receptors. In experimental studies, it has been demonstrated that PPAR-α receptor activation plays an important role in all stages of the development of atherosclerosis such as vascular inflammation, suggesting that the antiatherogenic actions of PPAR-α occur at the level of the vascular wall.[17] Fenofibrate improves endothelium-dependent dilation (EDD) in the microcirculation (i.e., small mesenteric arteries) in old rats, and increases brachial artery flow-mediated dilatation (FMD) in patients with type 2 diabetes mellitus.[18,19] Impairment of the NO synthase (NOS) pathway contributes to the inhibition of EDD.[20] Fenofibrate induces an increase in endothelial NOS expression in humans, as well as in cultured endothelial cells and the aorta of rats.[21–23] However, plasma levels of malondialdehyde and nitrate in humans were not changed by therapy with fenofibrate.[24]

The objective of this study was to investigate the effect of fenofibrate therapy on serum NO level in hypertriglyceridemia.

Material and methods

Subjects and study design

The Ethics Committee of the Ankara University Faculty of Medicine approved the study and all procedures were carried out according to the Declaration of Helsinki. All participants gave written informed consent. The study was funded by the Association of Turkish Clinical Vascular Biology.

This study was randomized, prospective and placebo-controlled in design. Sixty-eight hypertriglyceridemic patients who were admitted to our outpatient clinic in the Cardiology Department of the Ankara University School of Medicine between February 2014 and February 2015 were evaluated to enroll in the study. A total of 8 patients, of whom 3 patients declined to participate the study and 5 patients did not meet the inclusion criteria, were excluded from the study. The study population consisted of 60 patients who were allocated to the 2 arms (Fig. 1). The exclusion criteria included known vascular disease, stable angina pectoris, smoking, serum creatinine >1.5 mg/dL, abnormal liver or muscle enzymes, alcohol consumption, or use of antioxidant or lipid-regulating therapy. The past medical history of all participants was examined in detail and 12-lead electrocardiography, and a physical examination with measurement of liver enzymes, renal function test, fasting lipid profile, and glucose were performed. Sixty patients were randomly assigned to receive either oral treatment with 250-mg micronized fenofibrate (n = 30) or placebo capsules (n = 30) once daily for 12 weeks. A computer-generated table of random numbers (1:1) was used for accurate randomization. Four patients in the placebo group dropped out of treatment on their own decision during the study after randomization, so 26 patients reached the final analyses in the placebo group. Both investigators and patients were blinded to the treatment. A physician was assigned to count the pills to assess treatment compliance. Minor adverse effects that did not require drug withdrawal, such as abdominal pain in 2 patients, dyspepsia in 1 patient, and dizziness in 1 patient, were observed in the treatment group during the follow-up period. In our study, appropriate dietary suggestions for hypertriglyceridemia were made for all patients with no objective follow-up of participants' compliance with diet.

Laboratory assays

Venous blood samples were taken in the morning after overnight fasting to study the biochemical parameters at the beginning and at the end of both treatment periods.

It is recommended that patients take fenofibrate treatment with their evening meal. Patients were reviewed every 2 weeks to assess compliance with the treatment, adverse effects, body weight, and blood pressure. Plasma liver and muscle enzymes were monitored at 6-week intervals.

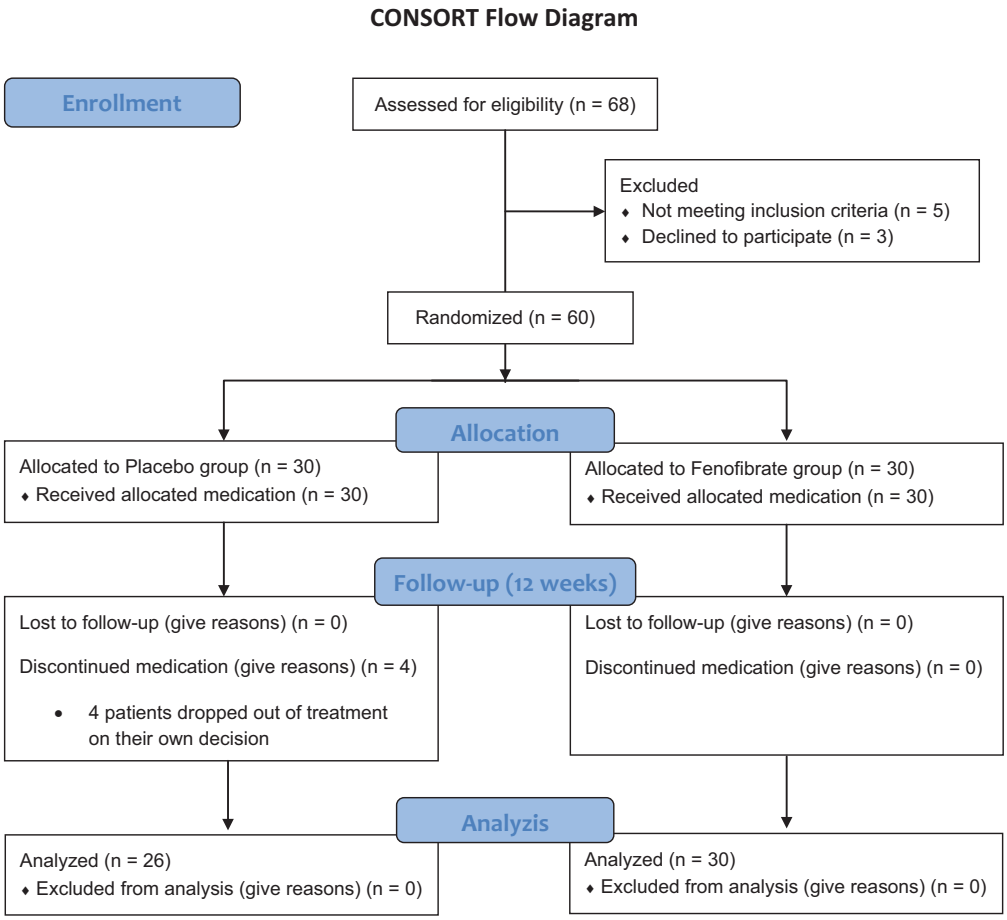

Fig. 1. Consolidated Standards of Reporting Trials (CONSORT) flow diagram of patients enrolled in the study

Fasting triglyceride, HDL cholesterol and total cholesterol were examined using enzymatic kits (Roche Diagnostics, Risch-Rotkreuz, Switzerland), and LDL-cholesterol was calculated using the Friedewald formula. Serum creatinine and CK were measured using Roche Diagnostics reagents and a Hitachi 917 analyzer (Hitachi Ltd., Tokyo, Japan).

For the vast majority of convenient methods, it is impossible to detect NO because of its volatile and transient character. However, as a result of NO oxidizing to nitrite (NO2–) and nitrate (NO3–), we used these anions' concentrations as a quantitative measurement of NO production. We performed the spectrophotometric measurement of NO2– by performing the Griess reaction after the conversion of NO3– to NO2–. Nitric oxide can be determined with this assay, which is achieved by nitrate reductase activity converting nitrate to nitrite. After the reaction, the nitrite is detected with colorimetric methods as an azo dye product of the Griess reaction. Nitric oxide concentration is indirectly measured by identifying both nitrite and nitrate levels in the specimen.

Statistical analysis

We analyzed the data with the Statistical Package for the Social Sciences (SPSS) v. 20.0 for Windows (IBM Corp., Armonk, USA). Categorical variables were summarized as percentages; continuous variables are presented as mean ± standard deviation (SD) or median with interquartile range according to normal distribution. The data was analyzed with the Shapiro–Wilk test for normal distribution. Groups were compared with χ^2 tests with Yates's correction or Fisher's exact test for categorical variables and the independent sample t-test or Mann–Whitney U test according to the distribution of data for continuous variables. Pre- and post-treatment parameters were compared using paired sample t-test for normally distributed variables, and the Wilcoxon signed rank test was used for non-normally distributed variables. Changes in serum NO levels and the changes in blood lipid parameters were distributed normally, therefore the Pearson test was used to analyze correlations. A value of $p < 0.05$ was considered statistically significant.

Results

A comparison of patients with placebo (n = 26; 10 male and 16 female; mean age 53.5 ±15.1 years) and those with fenofibrate therapy (n = 30; 8 male and 22 female; mean age 55.7 ±7 years) regarding baseline characteristics and laboratory findings is demonstrated in Table 1. No significant differences were detected between the 2 groups in terms of sex, age, the presence of hypertension, diabetes mellitus, hypothyroidism, chronic obstructive pulmonary

Table 1. Baseline characteristics of the study and control group

Variable	Placebo (n = 26)	Fenofibrate (n = 30)	p-value
Age, years	53.5 ±15.1	55.7 ±7	0.19
Male gender, n (%)	10 (38.5)	8 (26.7)	0.5
Hypertension, n (%)	18 (69.2)	26 (86.7)	0.21
Diabetes mellitus, n (%)	6 (23.1)	10 (33.3)	0.58
Hypothyroidism, n (%)	2 (7.7)	–	0.21
COPD, n (%)	1 (3.8)	3 (10)	0.6
ACE-I or ARB use, n (%)	16 (61.5)	24 (80)	0.22
β-blocker use, n (%)	6 (23.1)	6 (20)	1
Diuretics use, n (%)	2 (7.7)	2 (6.7)	1
Calcium channel blockers use, n (%)	–	2 (6.7)	0.49
Total cholesterol [mg/dL]	217.1 ±28.4	238.3 ±52.8	0.09
LDL cholesterol [mg/dL]	127.5 ±22.5	141.2 ±30	0.08
HDL cholesterol [mg/dL]	39.9 ±8	40.6 ±10.7	0.81
VLDL [mg/dL]	52 (36–55)	68.7 (46–77)	0.07
Triglycerides [mg/dL]	330 ±25.4	380.9 ±139.4	0.12
NO [μM]	26.4 ±11.9	30.9 ±12.4	0.17
Creatinine [mg/dL]	0.74 (0.65–0.92)	0.83 (0.68–0.98)	0.5
Creatinine kinase [u/L]	128 (79–250)	85.5 (62.75–160.75)	0.39
Adverse effect, n (%)	–	4 (13.3)	0.12

COPD – chronic obstructive pumonary disease; VLDL – very low-density lipoprotein; LDL – low-density lipoprotein; HDL – high-density lipoprotein; NO – nitric oxide.

disease (COPD), cardiovascular drug use, blood lipid panel, and NO level.

Biochemical measurements recorded at baseline and after 12 weeks of fenofibrate or placebo are listed in Table 2. Baseline triglyceride levels decreased significantly after 12 weeks in the placebo group ($p < 0.001$); however, there was no significant change in other types of blood lipid parameters and NO level. Total cholesterol, LDL, very low-density lipoprotein (VLDL) and triglyceride levels significantly decreased; HDL and NO levels significantly increased after 12 weeks of fenofibrate therapy. Treatment with fenofibrate resulted in a significant increase in serum NO levels compared to the placebo group ($p < 0.001$ vs $p = 0.06$).

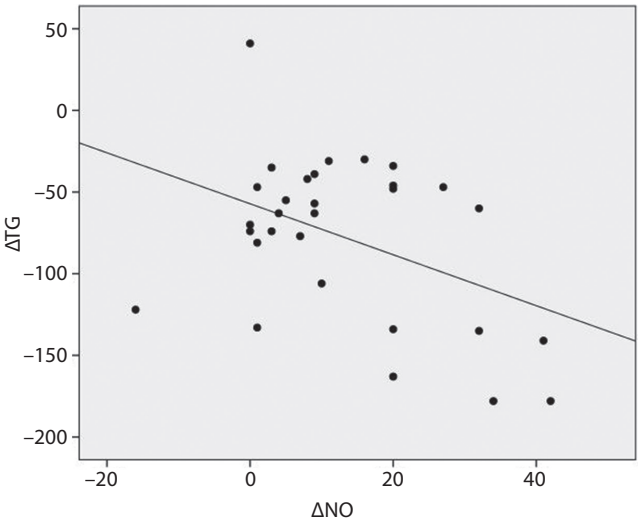

Fig. 2. Statistically significant correlation between the increase in serum NO levels and decrease in serum triglyceride levels ($r = -0.42$, $p = 0.02$). (Δ NO: change in serum NO levels; Δ TG: change in serum triglyceride levels)

Table 3 compares the magnitude of both groups' various parameter changes during the study. This comparison revealed that the increase in HDL and creatinine, and the decrease in creatinine kinase, are similar in the placebo and fenofibrate groups, but that the decreases in total cholesterol, LDL, VLDL and triglycerides, and the increase in NO, are significantly higher in the fenofibrate group.

We observed a statistically significant correlation between the increase in serum NO levels and decrease in serum triglyceride levels ($r = -0.42$, $p = 0.02$; Fig. 2), with a non-significant correlation for other changes in the lipid variables (HDL: $r = 0.04$, $p = 0.84$; VLDL: $r = 0.12$, $p = 0.55$; total cholesterol: $r = 0.35$, $p = 0.07$; and LDL $r = 0.03$, $p = 0.87$; Table 4) in the fenofibrate group.

Discussion

In our study, it has been demonstrated that fenofibrate significantly decreased total cholesterol, LDL cholesterol, VLDL cholesterol and triglycerides, and increased HDL

Table 2. Biochemical variables at baseline and 12 weeks for placebo and fenofibrate groups

Variables	Placebo group			Fenofibrate group		
	baseline	12th week	p-value	baseline	12th week	p-value
Total cholesterol [mg/dL]	217.1 ±28.4	213 ±36.3	0.3	238.3 ±52.8	186.7 ±36.6	p < 0.001
LDL cholesterol [mg/dL]	127.5 ±22.5	123.2 ±22.9	0.07	141.2 ±30	106.8 ±33.5	p < 0.001
HDL cholesterol [mg/dL]	39.9 ±8	41.9 ±7.8	0.15	40.6 ±10.7	43.5 ±8.6	0.001
VLDL [mg/dL]	52 (36–55)	35 (30–54)	0.89	68.7 (46–77)	38 (31–56)	0.001
Triglycerides [mg/dL]	330 ±25.4	280.4 ±38.9	p < 0.001	380.9 ±139.4	303.5 ±120	p < 0.001
Nitric oxide [μM]	26.4 ±11.9	27.1 ±12.4	0.06	30.9 ±12.4	43.9 ±15.8	p < 0.001

VLDL – very low-density lipoprotein; LDL – low-density lipoprotein; HDL – high-density lipoprotein.

Table 3. Comparison of the magnitude of the changes from baseline to 12th week follow-up evaluation in placebo and fenofibrate groups

Variables	Change in placebo group after 12 weeks (n = 26)	Change in fenofibrate group after 12 weeks (n = 30)	p-value
Total cholesterol [mg/dL]	–14.5 ±50.1	–51.6 ±45.1	0.02
LDL cholesterol [mg/dL]	–4.3 ±10.9	–34.3 ±30.3	p < 0.001
HDL cholesterol [mg/dL]	2 ±6.2	2.9 ±4.3	0.53
VLDL [mg/dL]	–0.5 ±13.1	–33.9 ±47.2	0.01
Triglycerides [mg/dL]	–49.6 ±26.5	–77.4 ±50.6	0.01
Creatinine [mg/dL]	0.03 ±0.14	0.03 ±0.09	0.87
Creatinine kinase [u/L]	–16.7 ±7.4	–10.3 ±18.4	0.44
Nitric oxide [μM]	0.77 ±1.9	12.9 ±13.7	p < 0.001

VLDL – very low-density lipoprotein; LDL – low-density lipoprotein; HDL – high-density lipoprotein.

Table 4. Correlation between changes in serum NO levels and changes in blood lipid parameters in the fenofibrate group

Variables	Δ NO level	
	r	p-value
Δ Triglycerides	–0.42	0.02
Δ VLDL	0.12	0.55
Δ HDL	0.04	0.84
Δ LDL	0.03	0.87
Δ Total cholesterol	0.35	0.07

r – correlation coefficient; VLDL – very low-density lipoprotein; LDL – low-density lipoprotein; HDL – high-density lipoprotein; NO – nitric oxide.

in patients with hypertriglyceridemia. Besides being a hypolipidemic drug, there are some important molecular and metabolic pleiotropic effects of fenofibrate on the vascular endothelium. Previous studies have demonstrated the effect of fibrates on vasomotor function as a pleiotropic effect on vascular endothelium. It has been shown that 12 weeks of fenofibrate therapy improves FMD in patients with type 2 diabetes mellitus after oral fat loading.[25] On the other hand, 3 weeks of gemfibrozil therapy did not show similar beneficial effects in healthy volunteers.[26] When compared with a placebo, bezafibrate therapy increases coronary artery dilation after exercise in patients with coronary artery disease; however, FMD was not changed significantly by either gemfibrozil usage alone or combination therapy with niacin.[27,28] Endothelium-dependent dilation in patients with hyperlipidemia and type 2 diabetes mellitus as well as in healthy middle-aged and older normolipidemic individuals were improved by therapy with fenofibrate.[19,21,24,29–32]

Although the results were controversial, it was observed in most of the studies that fenofibrate therapy significantly improves FMD. It is plausible that the hypolipidemic action of fenofibrate may account for this pleiotropic effect. From this hypothesis, it can be proposed that there was a significant correlation between the alteration in lipid levels and amelioration in FMD. On the other hand, it was also reported that improvements in EDD with fenofibrate was independent of reductions in lipids.[21,32] In these patients, improvements in EDD were provided by the increased activity of endothelial NOS, which plays a major role in ensuring NO release from vascular endothelial cells in healthy humans.[21] In another clinical trial, it was also shown that treatment with fenofibrate in patients with metabolic syndrome improved the endothelial protective effects of HDL by demonstrating the advanced ability of HDL to induce the expression of endothelial NOS on human umbilical vein endothelial cells.[33] Furthermore, PPAR-α agonists induced production of endothelial NOS in cell cultures, which leads to decreased oxidative stress, with increased bioactivity of NO.[22,34] Naturally occurring PPAR agonists can impede the inducible NOS enzyme pathway. Thus, the production of eicosanoid breakdown products as a result of inflammation may assist in its eventual resolution by activation of the PPAR pathway. In addition to these anti-inflammatory effects, PPAR-α ligands may improve endothelial NOS activity.[35] The activation of PPAR-α by fenofibrate in mice not only improved endothelial vasodilatation but also prevented myocardial ischemic injury, so treatment with fenofibrate ameliorated post-ischemic contractile dysfunction and decreased myocardial infarct size by an antioxidant mechanism that enhanced the bioavailability of NO.[36] Similarly, cardioprotection in a rat model of diabetic and acute myocardial infarction is also generated by fenofibrate + metformin combination therapy and fenofibrate therapy alone via decreased inducible NOS (iNOS) activity and increased NO bioavailability.[37] Additionally, fenofibrate therapy remained significantly associated with increased NO production and decreased serum asymmetric dimethylarginine level, leading to amelioration of inflammation and oxidation in rats.[38] However, the plasma levels of malondialdehyde and nitrate in humans were not changed by therapy with fenofibrate.[24]

Most of the studies showed that fenofibrate therapy significantly improved FMD, whereas serum NO levels were not measured in participants, so it had not been investigated whether there was a correlation between improved FMD and change in serum NO levels. In the literature, there was only 1 study which showed that fenofibrate therapy did not alter plasma levels of malondialdehyde and nitrate in humans, despite a significant improvement in FMD.[24] Thus, it is claimed that fenofibrate may not have clinically important antioxidant effects. Here, we demonstrate for the first time that compared with placebo, fenofibrate significantly increases the serum NO levels in patients with hypertriglyceridemia. These results indicate that fenofibrate targeting the PPARα-related signaling pathways shows promise for the treatment of vascular dysfunction associated with dyslipidemic status including hypertriglyceridemia, and the prevention of cardiovascular disease.

On limitation is this result is not supported by any clinical test such as FMD, etc. demonstrating the relationship between improved endothelial function and increased serum NO levels as a pleiotropic effect of fenofibrate. Additionally, the small patient population is another limitation of this study.

In conclusion, fenofibrate significantly changes lipoprotein levels by decreasing total cholesterol, LDL cholesterol, VLDL cholesterol and triglycerides, and increasing HDL-C. It also significantly increases the serum NO levels in patients with hypertriglyceridemia as a pleiotropic effect.

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Tick-borne pathogens *Bartonella* spp., *Borrelia burgdorferi* sensu lato, *Coxiella burnetii* and *Rickettsia* spp. may trigger endocarditis

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Abstract

Background. Infections caused by tick-borne pathogens such as *Bartonella* spp., *Borrelia burgdorferi* s.l., *Coxiella burnetii*, and *Rickettsia* spp. are capable of causing serious lesions of the mitral and aortic valves, leading to a need for valve replacement.

Objectives. The aim of the study was to determine whether such cases are sporadic or frequent. An additional goal was to establish effective diagnostic methods to detect these infections.

Material and methods. The study involved 148 patients undergoing valve replacement. Blood samples were drawn for serological testing. Samples of the removed mitral and aortic valves were tested with polymerase chain reaction and immunohistochemical staining.

Results. Specific antibodies to *Bartonella* spp. were detected in 47 patients (31.7%) and in 1 of the healthy controls (1%) ($p < 0.05$). Antibodies to *B. burgdorferi* spirochetes were found in 18 of the patients (12.2%) and in 6 blood donors from the control group (5.8%) ($p < 0.1$). Antibodies to *Rickettsia* spp. were detected in 12 (8.1%) and to *C. burnetii* phase I and II antigens in the serum of 1 patient. All the participants in the control group were seronegative to *C. burnetii* and *Rickettsia* spp. antigens. Polymerase chain reaction (PCR) tests for detection of *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* and *Rickettsia* spp. DNA in the valve samples were all negative. Inflammation foci with mononuclear lymphoid cells in the aortic and mitral valves were seen in sections stained with hematoxiline and eozine. In sections dyed using the indirect immunofluorescence method with hyperimmune sera, *Bartonella* spp. and *Rickettsia* spp. were found.

Conclusions. The results obtained indicate that laboratory diagnostics for patients with heart disorders should be expanded to include tests detecting tick-borne zoonoses such as bartonelloses, Lyme borreliosis, rickettsioses and Q fever.

Key words: endocarditis, tick-borne diseases, heart valve

There is increasing evidence that infectious diseases transmissible from animals to humans may trigger various heart disorders. Very often fastidious, nonculturable pathogens, such as *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, *Bartonella* spp. and *Rickettsia* spp. are responsible.[1,2] This is the reason infections with these bacteria are very frequently undiagnosed. The symptoms of endocarditis and valvular heart disease may indicate the bacterial etiology of the disease, and detection of significant titers of specific antibodies allows the origin of the disease to be identified.[2–4]

The aim of the present study was to establish whether tick-borne infections can contribute to serious heart disorders, resulting in a need for heart valve transplantation, and to determine whether diagnoses of *Bartonella* spp., *B. burgdorferi*, *C. burnetii* or *Rickettsia* spp. infections should be considered in every case of infectious endocarditis with negative blood cultures.

Material and methods

The patients

A group consisting of 148 patients undergoing valve replacement due to clinically recognized infective endocarditis were treated in the Cardiac Surgery and Transplantology, Department of the National Institute of Cardiology in Warszawa, Poland. The age range of the patients was 25–86 years, with the great majority from 66 to 86 years old. The group included 58 inhabitants of small villages (39.2%) and 90 residents of big cities (60.8%). The patients qualified for surgery did not have any records of previous antibiotic treatment.

Sera from 101 blood donors (47 women and 54 men) aged 31–65 years old (median age 48.5 years) were included as a control group in the serologic part of the study. The controls in the molecular and histological part of the study were samples of the mitral valves, aortal valves and myocardia from 70 individuals qualified as organ donors for transplantation after their deaths in accidents. These tissues were included after undergoing a number of serological, molecular, and bacteriological examinations. Infections with aerobic and anaerobic bacteria, viral or fungal infections were excluded by cultivating the blood and tissues.

The study was approved by the Institutional Review Board (IRB) at the National Institute of Public Heath – National Institute of Hygiene, Warszawa, Poland (approval No. 1/2012, issued on June 28, 2012).

Material

Blood samples were drawn for serological testing. Samples of the removed mitral and aortic valves were collected. Tissue samples were frozen at –75°C until tested.

Histology

Double imprint preparations were prepared from all the tissue samples. The slides were air-dried. Half of the slides were stained with hematoxylin and eosine and the other half were stained using the indirect immunofluorescence method.

Hematoxylin and eosin staining

The slides were fixed with acetone, washed with water for 5 min, then stained with Meyer's hematoxylin and 1% eosin and washed again with water. Next, the slides were dehydrated in ethanol (96–100%), cleared with xylene and embedded in Canadian balsam.

Immunohistochemical staining

The slides were fixed in a 1:1 mixture of methanol and acetone for 1 min at room temperature. Next, the slides were covered with human hyperimmune sera against *Bartonella* spp. (diluted 1:1600 in PBS), *B. burgdorferi* (1:400 dilution), *C. burnetii* (1:1024) or *Rickettsia* spp. (1:80) and incubated in a wet chamber for 20 min at 37°C.

The slides were washed 3 times in PBS with gentle agitation and then stained with diluted (1:80) goat anti-human fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich, St. Louis, USA) and incubated in a wet chamber for 20 min at 37°C. The slides were washed 3 times in PBS with gentle agitation and embedded in glycerol, then immediately examined under a fluorescence microscope (Nikon, Tokyo, Japan) at 40×10 magnification.

Polymerase chain reactions

Each tissue sample was cut and 30 mg was homogenized. DNA was extracted with a QIAamp Tissue kit (QIAGEN Gmbh, Hilden, Germany) according to the manufacturer's instructions. To ascertain whether the size of the valve samples was sufficient, DNA was extracted and amplified from 300 mg of 5 tissue samples.

The following primers were used to detect DNA of:

- *Bartonella* spp.: BhCS.781 and BhCS1137n primers for citrate synthase gene fragments;
- *Borrelia burgdorferi* sensu lato: L2 and P1 primers for 16 S r RNA and OA149 and OA319 specific primers for the *OspA* gene fragments characteristic of all *Borrelia burgdorferi* s. l. genospecies;
- *Coxiella burnetii*: isIIIIf and isIIIIr for the *htpAB* gene fragment (3,5,6,7,8) (Table 1).

The 50 µL reaction mixtures contained 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% gelatin, 200 µM dNTPs, 50 pmol of each primer and 1.5 U Taq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, USA). An aliquot of 5 µL of extracted DNA template was added to each reaction mixture. Each polymerase chain reaction (PCR)

Table 1. Primers and nucleotide sequences used in the study

Pathogen	Primers	Gene fragment (size bp)	Nucleotide sequences	References
Bartonella spp.	Bh.CS.781p BhCS.1137n	Citrate synthase gene (400)	5′-CCTATGGCTATTATGCTTGC 5′-AATGCAAAAGAACAGTAAACA	5
Borrelia burgdorferi sensu lato	L2 P1	16S rRNA (600)	5′-GGTCAAGACTGACGCTGAGT 5′-TCGCTTTGTACAGGCCATTG	6
	OA149 OA319	OspA gene	5′-TTATGAAAAAATATTTATTGGGAAT 5′-CTTTAAGCTAAGCTTGTCTACTGT	7
Coxiella burnetii	isIIIIf isIIIIr	htpAB	5′-CGCTCTCGTTTATGCGAGC 5′-CCAACAACACCTCCTTATTC	3
Rickettsia spp.	RpCS. 409D RpCS. 1258n	Citrate synthase gene (850)	5′-CCTATGGCTATTATGCTTGC 5′ATTGCAAAAAGTACAGTGAACA	8

test included negative (water) and positive controls containing the DNA of *B. afzelii*, *B. garini*, *B. henselae*, *C. burnetii* Henzerling strain and *Rickettsia conori* H24 strain, all from the collection of the National Institute of Public Heath – National Institute of Hygiene. For *B. burgdorferi* and *Rickettsia* spp. the cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 1 min denaturation at 95°C, annealing for 1 min at 55°C, elongation for 1 min at 72°C and final elongation for 7 min at 95°C. For *Bartonella* spp. the cycling conditions were as follows: 10 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C and finally 10 min at 72°C. For *C. burnetii* the cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 57°C, 1 min at 72°C, and finally 7 min at 95°C. Polymerase chain reactions were performed in a Mastercycler EP Gradient PCR/thermal cycler (Eppendorf AG, Hamburg, Germany).

Serology

We looked for serum antibodies to *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Bartonella* spp. and *Coxiella burnetii* in the patients' sera. To detect IgG *Rickettsia* spp. antibodies, micro immunofluorescence (MIF) – the reference method for a serological diagnosis of rickettsioses – was used. Inactivated *R. typhi* and *R. rickettsii* antigens were used (Rickettsia IFA IgG, Focus Diagnostics, Diasorin Molecular LLC, Cypress, USA). Control sera were delivered by the manufacturer. The screening was performed at a titer of 1:16. All serum samples negative in this titer were regarded as negative to both groups of rickettsiae. According to Unité des Rickettsies (Marseilles, France) an IgG titer ≥64 is considered indicative of infection by these specific *Rickettsia* species.[3]

Levels of *Bartonella* spp. IgG antibodies were determined by indirect immunofluorescence assay (Bartonella IFA IgG; Focus Diagnostics). Vero cells infected with *B. henselae* or *B. quintana* strains were used as the antigens. A titer of IgG antibodies ≥64 was considered positive. Positive and negative controls were delivered by the manufacturer.

B. burgdorferi IgM and IgG antibodies were tested with ELISA kits (EIA Borrelia 14kDa+OspC IgM and EIA Borrelia IgG+VisE, DRG-Medtek, Marburg, Germany). Positive results were confirmed by the western blot method (Euroline Borrelia-RN-AT IgM, Euroline Borrelia-RN-AT IgG; EUROIMMUN, Lübeck, Germany). Results were interpreted as seropositive according to the criteria of the German Society of Hygiene and Microbiology.[9]

The statistical analysis (χ^2 test) of the serologic test results was done using Statistica software (StatSoft Inc., Tulsa, USA). P-values <0.05 were considered statistically significant.

Results

The patients

The blood cultures of all 148 patients admitted to the clinic for valve replacement with symptoms of infective endocarditis were negative. During and after surgery; however, all the patients received 1 g of cefazolinum as perioperative antibiotic therapy, sometimes up to 1–2 days, to prevent the infection from spreading. Five patients were additionally treated with gentamicin/amoxycilline + clavulonic acid/ceftriaxone due to high temperatures (Table 2).

Serology

All the patients were examined serologically for the presence of specific antibodies to *Bartonella* spp., *B. burgdorferi*, *C. burnetii* and *Rickettsia* spp. Among the inhabitants of big cities, 42 (67%) were found to be seropositive for these tick-borne pathogens, as were 21 of the residents of villages (33%). Specific antibodies were found in the sera of 63 patients (42.6%). The seropositive group consisted of 47 patients (31.7%) with specific *Bartonella* spp. antibodies, 18 patients (12.2%) with *B. burgdorferi* s.l. antibodies, 12 patients (8.1%) with antibodies to *Rickettsia* spp., and 1 patient (0.7%) with specific *Coxiella burnetti* antibodies. The following ranges of the serum antibody titers were detected: *Bartonella* spp. 64 – 2048, *Rickettsia* spp. 64 – 128,

Table 2. Patients treated with antibiotics before surgery

Patient's number/initial	Additional antibiotics[a]	Duration	Presence of antibodies to:
31/P.B.	amoxicillin+ clavulanic acid (Taromentin)	7 days	negative
45/J.S.J.	cefazolin (Tarfazolin, Biofazolin)	4 days	negative
58/N.W.	ceftriaxone (Biotraxone) cefazolin (Biofazolin)	1 day 1 day	*B. burgdorferi*
P77/O.S.	ceftriaxone (Biotraxone) cefazolin (Biofazolin)	7 days 1 day	*Bartonella* spp.
P78/Pa.	gentamicin cefazolin (Biofazolin)	1 day 1 day	*B. burgdorferi Bartonella* spp.

[a]All the patients were given 1-day treatment with 1 g cefazolinum before or during surgery.

Table 3. Patients with recognized Lyme borreliosis (positive results of a 2-step serological test for *Borrelia burgdorferi*)

Ordinal No.	Patient's number/ initials	Detected fractions[a]
1	P2	p17, p19, p25, p30, p31, p41, VlsE
2	P.kr	p100, p39, p18, VlsE
3	K.kr	p100, p25, VlsE
4	H.Gen.	p18, VlsE
5	P.B.	p18, VlsE
6	P.A	p100, p58. p39, p18, VlsE
7	M.C.	p100, p58. p39, p18, VlsE
8	K.A.	p100, p58, p41, VlsE
9	P58	p58, VlsE
10	P59	p58, p18, VlsE
11	P61	p100, p58, p39, p18, VlsE
12	P65	p100, p18, VlsE
13	P69	p100, p41, p18, VlsE
14	P78	p58, p18, VlsE
15	P109	p100, p25, p18, VlsE
16	P110	p100, p18, VlsE
17	P117	p18, VlsE
18	P122	p58, p39, VlsE
19	P126	p18, VlsE
20	P132	p18, VlsE
21	P141	p18, VlsE

[a] specificity confirmed by immunoblotting: The presence of 2 fractions from the following: p83/100, p58, p43, p41 int., p39, OspC, DbpA (p17/18), VlsE indicates an IgG positive result.

C. burnetii phase I 32 and phase II 256. The specificity for *B. burgdorferi* was confirmed by immunoblotting (Table 3). The presence of antibodies to *B. burgdorferi* p18 and VlsE antigens is characteristic of chronic infections. According to the Modified Duke Criteria for the Diagnosis of Infective Endocarditis, 63 patients (42.6%) fulfill the criteria for infective endocarditis: A characteristic echocardiogram picture (the major clinical criterion) and the presence of significant levels of specific antibodies to *Bartonella* spp., *B. burgdorferi* s.l., *Rickettsia* spp. or *C. burnetii* (a minor criterion).[10,11]

In the study group, co-infections of *Bartonella* spp. with *B. burgdorferi* s.l. were detected in 8 patients; with *Rickettsia* spp. in 3 patients; and with *C. burnetii* in 1 patient. Also, 1 patient presented specific antibodies to the antigens of *Bartonella* spp., *B. burgdorferi* s.l. and *Rickettsia* spp., indicating co-infection with 3 pathogens.

In the control group (the blood donors), specific IgG antibodies to *B. henselae* antigen were detected in a titer of 128 in 1 person (1%; p-value <0.05). Specific IgG antibodies to *B. burgdorferi* sl. were detected in the sera from 6 blood donors (5.8%; p-value <0.1). All the control sera were seronegative to *C. burnetii* and *Rickettsia* spp. antigens.

Histopathology

Macroscopic evaluation of the valve samples obtained from the patients showed vegetation and other degenerative changes, mostly fibrosis and calcification. Light microscopic examination of hematoxylin & eosin stained valve preparations showed diffusely infiltrating mononuclear inflammatory cells. The infiltrates were composed predominately of mononuclear cells, including lymphocytes and plasma cells (Fig. 1A). In some patients mononuclear lymphoid cells were sparsely accompanied by polymorphonuclear leukocytes. Occasionally, small aggregates of lymphocytes and macrophages were found. Additionally, we observed numerous fibrocytes forming a cellular net with mononuclear inflammatory cells (Fig. 1B).

Immunofluorescent staining with specific *Bartonella* spp., *B. burgdorferi*, *Rickettsia* spp. and *C. burnetii* immune sera showed positive reactions to *Bartonella* spp. and *Rickettsia* spp. cells (Fig. 2A,B).

Structural valvular changes were not observed in the tissues from organ donors group.

Apparent discrepancies between serology and immunohistochemistry

Among the 63 patients seropositive to *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* or *Rickettsia* spp., the presence of bacterial cells was also found in some of the tested tissue sections. *Bartonella* spp. bacterial cells were detected in 9 valve sections removed from 47 seropositive patients; *Rickettsia* spp. bacteria were seen in 5 tissue samples

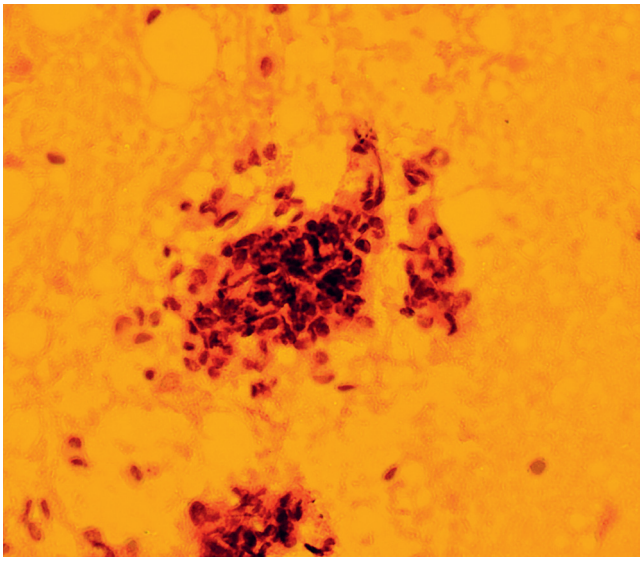

Fig. 1A. Sections of valves stained with hematoxylin & eosin (H&E) (×400): A. accumulation of mononuclear lymphoid cells in valve's section from patient seropositive to *Bartonella* spp. (serum titer 1:128)

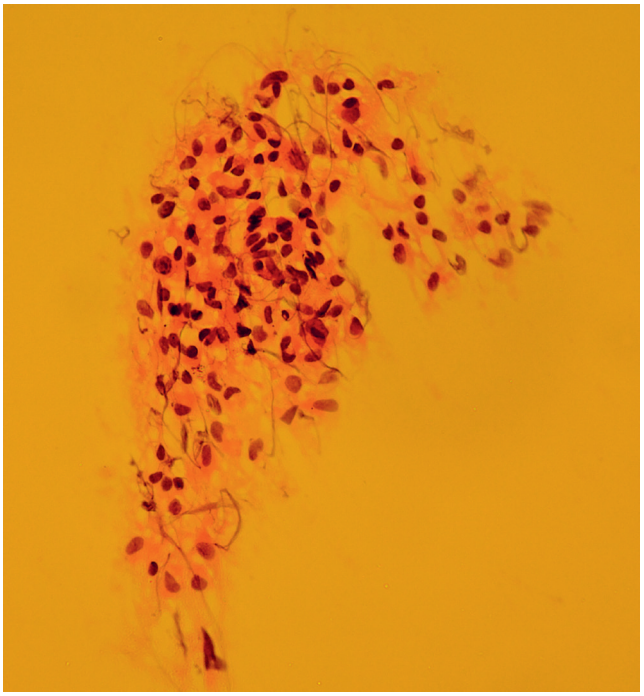

Fig. 1B. Sections of valves stained with hematoxylin & eosin (H&E) (×400): B. mononuclear inflamatory cells surrounded by fibrocytes in valve from patient seropositive to *Bartonella* spp. (serum titer 1:128)

from 12 seropositive patients; and *B. burgdorferi* spirochetes were observed in 1 out of 18 seropositive patients. *C. burnetii* bacteria were not found in any tissue section. These results indicate the different sensitivity of the tests employed rather than discrepancy.

Polymerase chain reactions

All tests performed to detect *Bartonella* spp./*B. burgdorferi* s.l./*C. burnetti*/*Rickettsia* spp. DNA were negative.

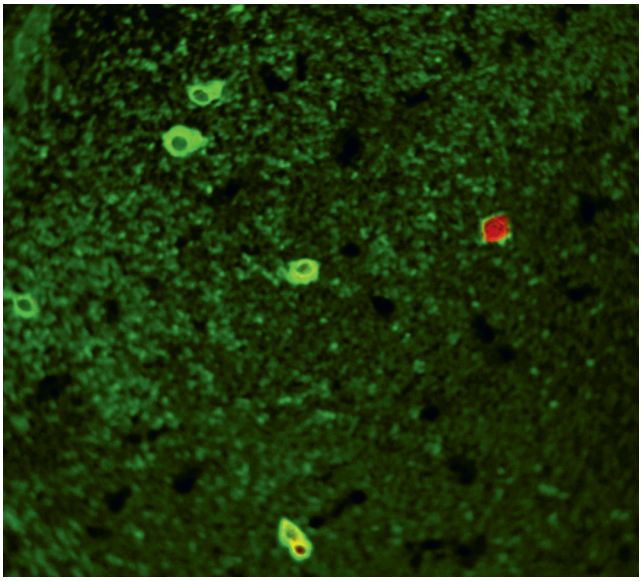

Fig. 2A. Sections of valve from patients with antibodies to *Bartonella* spp. (serum titer 1:128) and *Rickettsia* spp. antigents (titer 1:64), dyed with indirect immunofluorescence (×400): A. immunofluorescence of mononuclear cells with *Bartonella* spp. hyper immune serum

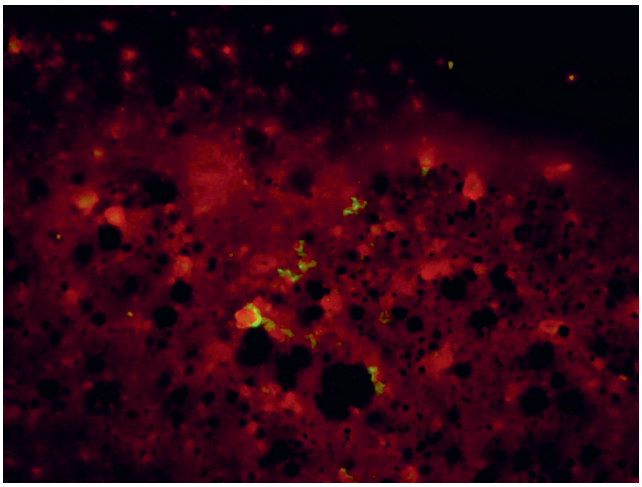

Fig. 2B. Sections of valve from patients with antibodies to *Bartonella* spp. (serum titer 1:128) and *Rickettsia* spp. antigents (titer 1:64), dyed with indirect immunofluorescence (×400): B. immunofluorescence with *Rickettsia* spp. hyper immune serum

Discussion

The aim of our study was to establish whether infections caused by tick-borne pathogens such as *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* and *Rickettsia* spp. are capable of causing serious lesions of the mitral and aortic valves, leading to a need for valve replacement. One goal was to determine whether such cases are sporadic or frequent; an additional goal was to establish effective diagnostic methods to detect these infections.

The results obtained indicate that tick-transmitted pathogens (*B. burgdorferi* s.l., *Bartonella* spp., *Rickettsia* spp., *C. burnetii*) play a significant role in the development

of endocarditis, causing irreparable damage requiring valve replacement.

Out of 148 patients referred for valve replacement, 63 (42.6%) showed the presence of specific antibodies to *Bartonella* spp., *B. burgdorferi* spirochetes, *Rickettsia* spp. and *C. burnetii*. It is surprising that 67% of the patients infected with these tick-borne pathogens were residents of big cities, and only 33% live in the countryside. This can be explained as a consequence of lifestyle changes. Recently, society favors active recreation, various forms of tourism, extreme sports and exotic excursions. This sometimes has negative effects due to more frequent contact with new pathogens, creating new high-risk groups contracting tick-borne zoonoses. Wild animals (birds, mammals) can be reservoirs of the etiologic agents of these diseases. Many of these infections appear seasonally, during the active periods of various species of arachnids, which are very often the vector of various microorganisms. In our climatic zone ticks transmit infections from the spring to the autumn.

In Poland, Q fever endocarditis (caused by *Coxiella burnetii*) has never been diagnosed,[12] possibly because the *Coxiella burnetii* strains isolated in Poland represent phenotypes that correlate with acute disease; they are different from the strains that have appeared in France.[4,13] The "gold standard" for the diagnosis of an infection is the isolation of the etiologic agent in a culture. In the case of tick-borne diseases, such as rickettsioses, bartonelloses, Q fever and Lyme borreliosis, the causative microorganisms are rarely cultured. In addition, negative results may develop from sampling errors due to the bacteria being present in the tissues in low concentrations.[14–16]

The delay in the recognition of tick-borne diseases may be related in part to the fact that many cases appear to be asymptomatic or subclinical. When symptoms are present, the typical presentation of an undifferentiated febrile illness (including fever, headache, malaise, myalgias, and arthralgias) may be difficult to distinguish since these diseases have similar epidemiological distribution and are transmitted by the same ticks.[17–19] Moreover, infectious cardiac diseases may be para-infectious phenomena, e.g., inflammation, rather than the result of direct action by viable microorganisms.

Efforts to detect bacterial nucleic acids are also limited by many other factors. Some authors have reported that studies using single biopsies produce consistently lower estimates for intracellular pathogens than studies that examine multiple biopsies.[15,16,20,21] Molecular techniques such as PCR improve the likelihood of identifying an infectious agent, but should be used judiciously and in conjunction with traditional diagnostics such as culture, serology and histology. Knowing the limitations of PCR, it is important to take appropriate samples, selecting those that are most likely to have a significant bacterial DNA load.[18,22,23]

PCR testing is of relatively low sensitivity after antibiotic therapy. Patients with infective endocarditis due to *Bartonella* spp. are usually treated with aminoglycosides for more than 14 days or with doxycycline for 6 weeks in combination with gentamicin for 14 days.[19,24] Differences in the sensitivity of PCR in tissue specimens may be explained by the use of antibiotics prior to sampling, which may reduce the amount of bacterial DNA.

In addition, the results may vary depending on the type of tissue sample – e.g. vegetation or valve tissue. This implies that the type of tissue biopsied may be critical, with vegetation more likely than heart valves to yield a positive result. This may not be surprising, as vegetation is believed to contain dense clumps of bacteria (and hence high bacterial DNA loads) within a matrix of fibrin, platelets and erythrocyte debris.[15,25]

The principles of diagnosis and recognition of infective endocarditis caused by tick-borne pathogens have not been clearly established. In 2012, new criteria for recognizing Q fever endocarditis were proposed.[26] Currently, there is a need to establish criteria for *Bartonella* and *B. burgdorferi* endocarditis. Some suggestions concerning *Bartonella* were submitted by Raoult et al. in 2015; however, these need further analysis.[4,19,24]

Recently, it has been shown that many tick-borne zoonotic diseases contribute to valve and myocardium damage. It has been observed in the course of Lyme borreliosis, bartonelloses, rickettsioses and Q fever. These diseases are recognized as emerging or re-emerging infections and serious public health problems. If the role of tick-borne infections in the development of serious heart malfunctions is suspected, it should be obligatory to perform diagnostic serological tests, especially in cases of negative blood cultures. Generally, serological tests are fast and not as invasive as blood cultures. Performing a serological examination makes it possible to confirm cases of infective endocarditis and myocarditis and to apply antibiotic treatment before irreversible damage is done.

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Neonatal brain and body imaging in the MR-compatible incubator

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Abstract

Background. The use of a specialized MR-compatible incubator (INC) is very poorly described in the literature and only with regard to brain imaging.

Objectives. To present our own experience with brain and body magnetic resonance imaging (MRI) in the INC in a large cohort of neonates.

Material and methods. A total of 555 examinations were performed in 530 newborns with the use of a 1.5T system and Nomag IC 1.5 incubator, equipped with head and body coils.

Results. More than half of neonates (54%) were prematurely born at 22 + 6–36 + 6 gestational weeks. They were examined from the first to 153 days of life (median: 18.5, mean: 37.7) with body weights 600–5000 g (mean: 3051 g), 23% of less than 2500 g. The proportion of brain MRIs to other body regions was 533:85 = 86%:14%. In 36.6% of cases, MRI showed more abnormalities than ultrasound (USG), in a further 21.8%, MRI diagnosis was completely different, in 4.7%, a pathology described on a USG was ruled out on MRI. The superiority of MRI over USG was 63.1%.

Conclusions. MR-compatible incubator significantly increased the availability of MRI to newborns, especially to premature and unstable newborns. The integration of body coils into the INC increased the spectrum of examinations and made possible the scanning not only of the brain but also the body. Dedicated neonatal coils improved image quality and allowed more accurate diagnosis than the previously used adult coils. Immobilization of the babies in the INC by means of Velcro belts and head fixation inserts is better than in adult coils. The closed space of the INC isolates newborns to a greater extent from the negative influence of noise in the MR environment.

Key words: body imaging, brain imaging, magnetic resonance imaging, MR-compatible incubator, neonates

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Introduction

Since the time when we published our first experience with magnetic resonance imaging (MRI) in neonates with the use of a specialist incubator compatible with an MRI system (INC),[1] 5 more papers including the keywords "MRI", "neonates" and "MR compatible incubator" have been added to PubMed and 2 of these are ours.[2,3] Although the first publication concerning INC dates back to 2003,[4] there are only 22 articles devoted to this subject in PubMed now (including 3 of ours) and almost all of them are focused on safety issues and brain imaging.[4–7] Our INC, equipped with a head coil and a body coil, allows more than neuroimaging, and we have noticed the growing role of neonatal body MRI in everyday clinical practice. Our first reports concerned the first 27 and 47 newborns examined, respectively.[1,3] Now we present our experience with brain and body MRI after having performed 555 of the procedures at the Institute of Mother and Child in Warszawa, Poland.

Material and methods

Patients

Over 4 years (2013–2017), we performed 555 MRI examinations (in 287 boys and 268 girls) in 530 neonates (275 boys, 255 girls), always after ultrasound (USG), performed by a radiologist. Institutional Bioethics Committee approval was acquired even though this is a retrospective study (decision No. 26/2017). In each case, written informed consent for clinical MRI was obtained from the patients' parents or legal guardians. In urgent cases, in the absence of parents/guardians, consent to the study was signed by a commission of 3 doctors. The examinations were always performed in the presence of an anesthesiologist.

Equipment

All neonates were examined using a GE Signa HDxT 1.5 T system (GE Healthcare, Chicago, USA) and MR-compatible incubator Nomag IC 1.5 by Lammers Medical Technology Co. (Lübeck, Germany), with temperature and humidity adjustment, equipped with non-magnetic gas cylinders with a capacity of 5 L of oxygen at a maximum pressure of 200 bar, with a non-magnetic gas cylinder pressure reducing valve with flow adjustment in the range of 0–5 L/min, respirator with a non-magnetic air pressure reducing valve, pulse oximeter measuring heart rate within the range of 20–300 bpm and partial oxygen pressure within the range of 1–100%. From the point of view of imaging, the most important equipment were 3 MRI coils: An 8-channel, phased-array neonatal head coil and a 12-channel phased-array coil for the body, consisting of an 8-channel coil integrated in the INC bed and a separate 4-channel surface coil. The neonates were immobilized with Velcro belts and head fixation inserts. Separate neonatal noise attenuators were not used as the dual-wall structure of the INC lowers the noise level around the patient by approx. 12–15 dB and the head fixation inserts mentioned above provide further protection.

Anesthesia

To induce anesthesia, atropine at a dose of 0.01 mg/kg of body weight and thiopental at a dose of 4–7 mg/kg were used in all children. Four patients from the study group required respiratory support with a ventilator integrated with the INC, the others kept their own breath. In the freely breathing group, passive oxygen therapy was used in the INC. Vital signs, heart rate and oxygen saturation were monitored using a wireless oximeter adapted to work in a magnetic field. The medical gases came from non-magnetic cylinders. The babies did not require a catecholamine supply. During the study, due to a lack of infusion pumps adapted for operation in a magnetic field, the supply of intravenous fluids was stopped. The time without infusion did not exceed 30 min.

Image analysis

Assessment of the quality of the MR examinations was performed on the basis of a visual inspection. The images were assessed in agreement by 2 radiologists (MBF, 26 years of experience with MRI, 21 years of experience with neonatal MRI, and one of the remaining authors) in each case and final diagnoses were reached by consensus. The superiority of MRI over USG was defined as the MR visualization of elements and features that were not visualized by USG.

Results

Characteristics of patients

Most of the neonates were prematurely born: in 286/530 cases (54%), they were born at a gestational age (GA) of 22 + 6–36 + 6 weeks. For the whole study group, the mean age at birth was 32.5 gestational weeks (GW) (range 22–41 GW). Neonates were examined from the 1st day of postnatal life to 153 days (mean: on the 37.7th day of life), depending on clinical status and suspected pathology. Seventeen neonates (3.2%) were scanned on the 1st day of postnatal life and 124 neonates (23.4%) within the 1st week. On the day of the MRI, the body weight of the newborns ranged between 600 g and 5000 g, with a mean of 3051 g (median 3040 g). At the moment of the examination, 109 neonates (19.6%) had body weight of less than 2500 g, 16 (2.9%) of less than 1500 g and 3 (0.5%) of less than 1000 g.

Characteristics of examinations

Five-hundred eighteen examinations (518/555 = 93.5%) were performed under pharmacological sedation, 37 (6.5%) in physiological sleep. There were no anesthetic complications in our material.

In the analyzed material there were 533 brain examinations, 5 studies of orbits, 21 of the vertebral column, spinal canal and cord, 18 of the head and neck, 6 of the thorax, 33 of the abdomen and pelvis, and 2 of joints. In 31 neonates we scanned 2 body regions, in 3 babies 3 regions and in 2 babies 4 regions, at one time. Therefore, 555 procedures totaled 598 examined regions of the body. Twenty-four babies had an initial examination and a follow-up study and 1 was scanned 3 times. The proportion of brain MRIs to the scans of other body regions was 533 : 85, i.e., 86% vs 14%.

The quality of MR examinations was found satisfactory in all but 4 cases (99.3%). In 1 of these examinations, the neonate was thought to be in natural sleep but started crying, but the quality was sufficient to confirm the continuity of the spinal cord which was questioned by the parents. In 2 cases anesthesia was very difficult and the studies were stopped, but the completed sequences revealed no evident brain pathology. In the 4th case, MRI of the brachial plexi was requested and it was impossible to assess the particular nerve roots forming the plexi due to insufficient spatial resolution.

Result of magnetic resonance imaging versus ultrasonography

In 203 cases (203/555 = 36.6%) the MRI showed more abnormalities than the USG and in a further 121 cases (121/555 = 21.8%) the diagnosis after MRI was completely different than after USG. In 26 cases (26/555 = 4.7%) the pathology described on the USG was ruled out on MRI. The superiority of MRI over USG in the visualization of pathological lesions and normal structures was found in 63.1% of cases in the collected material in neuroimaging and body imaging.

Discussion

General remarks

Ultrasonography is and will remain the first-line imaging method in neonates. However, it has its well-known limitations and additionally, some parts of the brain, e.g., the sella, and of the body, e.g., the chest, do not lend themselves to USG examination and are most appropriately examined using MRI. Computed tomography (CT) is another option but according to the ALARA rule (as low as reasonably achievable), the radiologist should establish a diagnosis with the use of a radiation dose that is as low as possible and no dose of ionizing radiation is the best solution. Moreover, the tissue resolution of CT is much lower than that of MRI so the routine use of CT in the youngest group of patients is not justified. Therefore, MRI, by definition, has higher diagnostic usefulness, not only in this age group.

The added value of MR-compatible incubator

Some national societies have published recommendations on the use of MRI in neonates, preterm in particular.[8,9] Safety issues such as transport of the neonates to MRI units, maintaining the temperature and monitoring vital functions have limited performing MRI so far, especially in premature infants. The equipment of the MR-compatible incubator, making it possible to control the temperature and humidity and to maintain and monitor vital parameters, ensures safe conditions for the babies. Therefore, even preterm and unstable neonates can be scanned safely now. Skeptics argue that the advantages of INC are unclear: it does not obviate the need for anesthesia or sedation and it even obscures the anesthesiologist's view of the patient; neonatal coils are available without the INC; transport can be safely accomplished in a standard neonatal incubator after placing the patient onto the MRI table with appropriate hearing protection; intravenous fluids can be administered with the pumps kept outside of the MRI chamber and tubes running through a hole in the wall or with MR-compatible pumps. However, before the era of INC, neonatologists would not entrust their most preterm and unstable neonates to radiologists, at least because of the impossibility to maintain proper temperature, and also because the whole procedure is complex. Introduction of INC to the clinical practice allowed examination of even the smallest neonates with extremely low body weights – our smallest neonate, weighing 600 g, is still the smallest neonate examined to date, as shown in the available literature,[1] although followed very closely by a 620-gram baby reported in the newest paper devoted to this subject that was published last year.[10] In our material, as many as more than half of the examined neonates were preterm (54%) and almost 1/4 (23%) weighed less than 2500 g – equivalent of low birth weight (LBW) – on the day of MRI. Seventeen neonates (3.2%) were scanned on the 1st day of postnatal life and 124 (23.4%) within the 1st week. Before introduction of INC, MRI was performed on such newborns only in exceptional situations.

Our material constitutes the largest cohort of neonates examined in INC to date. Cho et al. reported 154 newborns over the course of more than 4 years.[10] Earlier reports are based on much smaller groups of neonates (from 13 to 129).[6,7] Our group of 383 neonates with 400 MR examinations performed was collected in 3 years (2013–2016), which reflects the need for these studies in specialized tertiary referral centers.

Anesthesia

In the vast majority of MRI examinations in INC – 93.5% – pharmacological sedation was used. In 6.5% of cases, after feeding 30 min before scanning, we achieved physiological

sleep peaceful enough to be able to perform the procedure during it. Despite comfortable conditions inside the INC (optimal temperature and humidity, insulation from noise), in most newborns physiological motion artifacts occur during sleep hindering the precise evaluation of the images. In children studied in natural sleep, attempts have been made to use pacifiers to calm them down, but motion artifacts associated with suction made us abandon this method. Another argument against placing the just-fed neonate in an INC chamber is the risk of regurgitation and aspiration of food into the airways. Premature neonates are subject to this risk to the greatest extent. During scanning, newborns must be in a horizontal position, the upper body cannot be lifted to prevent aspiration. One should also keep in mind that an anesthesiologist does not have visual supervision of a baby in INC. His or her assessment is based on the recordings of the monitoring equipment, which gives results with a delay of tens of seconds, which may have impact on the effect of rescue operations in case of aspiration of food into the airways. Taking into account the above considerations, the authors believe that only neonates in good condition may be subjected to MRI in physiological sleep: neonates with no history of episodes of aspiration pneumonia and those in whom MRI is carried out as part of planned prospective control programs, where the inevitable motion artefacts do not have significant impact on the precision of image evaluation. It should also be remembered that conversion of the procedures towards anesthesia is possible only after 6 h after feeding in fed infants in case of failure to obtain physiological sleep allowing the execution of MRI.

At our center, the vast majority of children undergoing MRI in INC are premature neonates with congenital malformations, CNS hemorrhages or after severe perinatal hypoxia. In these babies, the results of MRI have great therapeutic and prognostic implications, which is why this procedure – in order to obtain optimal image quality – is performed under general anesthesia although the use of sedation is discussed in the literature and some authors stress that the sedative drugs may have a negative influence on the immature brain of extremely premature babies.[11–13] However, sedation is used worldwide and, e.g., Cho et al. report that the oral sedative agent chloral hydrate was used in their babies when possible, and in other cases midazolam or ketamine hydrochloric acid were administered intravenously.[10]

There were no anesthetic complications in the examined newborns in our material.

Brain magnetic resonance imaging

The study of the brain is now an important challenge in neonatology and the development of imaging techniques and – above all – of MRI offers great opportunities for assessment and analysis of brain morphology in vivo. At the same time, a lot of questions are being posed about the place of MRI as a tool for assessment and prognosis of the development of newborns, both those born at term and those extremely premature. In the USA, at least since 2004, it is recommended to perform MRI at term equivalent in preterm babies due to its prognostic value and to perform MRI if any neurological symptoms are present, as diffusion-weighted sequence (DWI) in particular is capable of reliable assessment of CNS injury.[14] The date, 2004, coincides with the time of the introduction of incubator into clinical practice.[4]

The brain can be injured at any stage of its development, and the damage may be congenital (developmental abnormality) or acquired (e.g., hypoxic-ischemic or hemorrhagic). There is no doubt that transfontanelle USG is and will remain the primary diagnostic method – non-invasive, readily available and cheap. But it has limitations, and at present MRI is the most accurate method of brain imaging. Thanks to visibility of all the structures, including basal ganglia, internal capsule, brainstem and cerebellum, and the possibility to measure the volume of these structures (e.g., cerebellum), MRI provides additional information about which drug treatment or rehabilitation can be implemented and facilitates the prognosis for the babies' further development.

Neurologists emphasize that each stage of brain development is very important, but the formation and selection of synapses and myelination seem to be the most important for the brain. This type of information can only be obtained using MRI, but images must be of very high quality and newborns subjected to MRI may be in various clinical conditions: from stable and apparently healthy to unstable.

Among neonates born at term, the study is performed most often in the first days of life and mainly in 2 clinical situations: birth defects and after mild to severe perinatal hypoxia, a few days after therapeutic hypothermia. Sometimes these newborns require respiratory support (from nCPAP to endotracheal intubation and mechanical ventilation) and inotropic agents (catechol amines).

In the group of premature neonates, so far MRI has been performed only in exceptional situations in the first days of postnatal life, when their clinical condition is very unstable, because at that time USG is the primary diagnostic tool. Magnetic resonance imaging is performed more often between 36 and 40 weeks of postconceptional age as it brings additional information in relation to USG that may help predict prognosis for the neonates' further development.[15]

Our experience shows that MRI in INC is feasible in preterm newborns even on the first days of postnatal life (Fig. 1). This observation is in agreement with those of other authors who stated that the interval between birth and MRI was significantly shorter after the introduction of INC, i.e., 54.3 ±2.6 days vs 70.5 ±4.4 days without INC.[10] Our results show even earlier availability of MRI in INC as compared to that of Cho et al., with a median of 18.5 days and mean of 37.7. When performed at term equivalent,

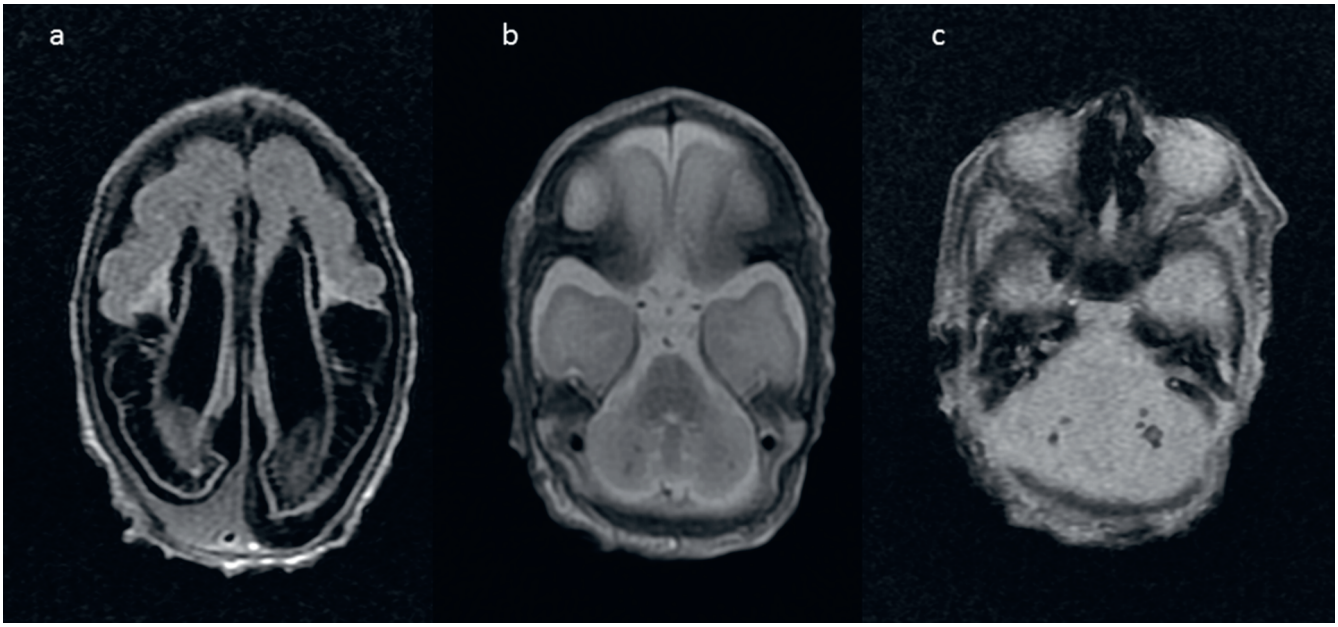

Fig. 1. Male preterm neonate born at 26 GW, examined at 29 GW. (a) FLAIR shows relatively small cavities in the frontal lobes and extensive encephalomalacia in the posterior parts of the brain. Pericerebral hematoma on the right. Axial FSE/T2-weighted (b) and GRE/T2*-weighted (c) images show bilateral cerebellar hemorrhages

MRI allows assessing brain injury in neonates with known pathology and reveal unexpected findings in neurologically and otherwise healthy ones. One example is a baby born at 27 GW and examined at 38, before discharge, who turned out to have diffuse polymicrogyria involving almost the entire left cerebral hemisphere. Abnormal gyration is extremely difficult to be revealed sonographically. Magnetic resonance imaging is necessary in these cases and such a finding carries significant implications for the future. The dedicated neonatal coils in the INC provide better signal-to-noise ratio and smaller fields of view and these factors allow better visualization of small details.[5,6,10] The percentage of cerebellar injury detected with MRI is incomparably greater than that diagnosed with USG. As in case of neuronal migration abnormalities, the damage to the cerebellum is often difficult to visualize and evaluate on USG and, in these cases, MRI is also a method of choice. Although mastoid fontanelle USG enables a better view of the posterior fossa, MRI shows more abnormalities and enables assessment of the real state of the cerebellum and brain stem in which the incidence of defects and damage turned out to be greater than expected.[16,17] In a recent paper by Steggerda et al., USG allowed to detect abnormalities in the posterior fossa in 41% of infants (in the vast majority these abnormalities were only seen on mastoid fontanelle views) while MRI revealed them in 66%.[18] In our material, in 71 cases (71/533 = 13.3% of brain scans) the sonographic picture of the cerebellum was normal while MRI revealed cerebellar abnormalities (cases with hemorrhage only in pericerebellar space that was not visualized by USG were not counted) (Fig. 2A,B). Also, in the case of known pathology mentioned at the beginning of this paragraph, MRI can show its consequences which are occult to USG. Examples are pre-Wallerian (and later Wallerian) degeneration or crossed cerebellar diaschisis, which carry significant clinical consequences.[19,20] (Fig. 3A,B).

Body imaging in MR-compatible incubator

In these very few papers that have been published so far (very few despite the fact that INC was introduced to clinical practice at least 15 years ago), only neuroimaging was described. Only Blüml et al. mentioned 2 cardiac and 2 pelvic MR examinations in their paper, but it is mainly a technical and safety-concerning publication. By the way, Blüml et al. found that the signal-to-noise ratio (SNR) of images acquired with INC had been 2.3 times greater than the SNR of images acquired with standard "adult" equipment at MRI of age-matched babies.[6] These authors used the same incubator and the same MRI system as in our case. O'Regan et al., using another vendor's 1.5 T scanner and the same INC, achieved a 3-fold increase of SNR in brain imaging.[5] Our experience shows that neurological indications are still the most frequent in neonatal MRI (in our study, brain scans constituted the majority of cases – 86%) but neurological indications include also spinal ones, and imaging of the vertebral column, spinal canal and cord is now possible and scheduled. Our material contains 21 cases of such examinations with diagnoses of myelomeningocele, diastematomyelia, tethered cord, or exclusion of suspected pathology (Fig. 4A,B). Although USG of the spinal canal and cord is a reliable method in newborns, and in patients with normal findings no further imaging is necessary in most cases, (neuro) surgeons usually request MRI before surgical intervention in the case of spinal malformations. The examination

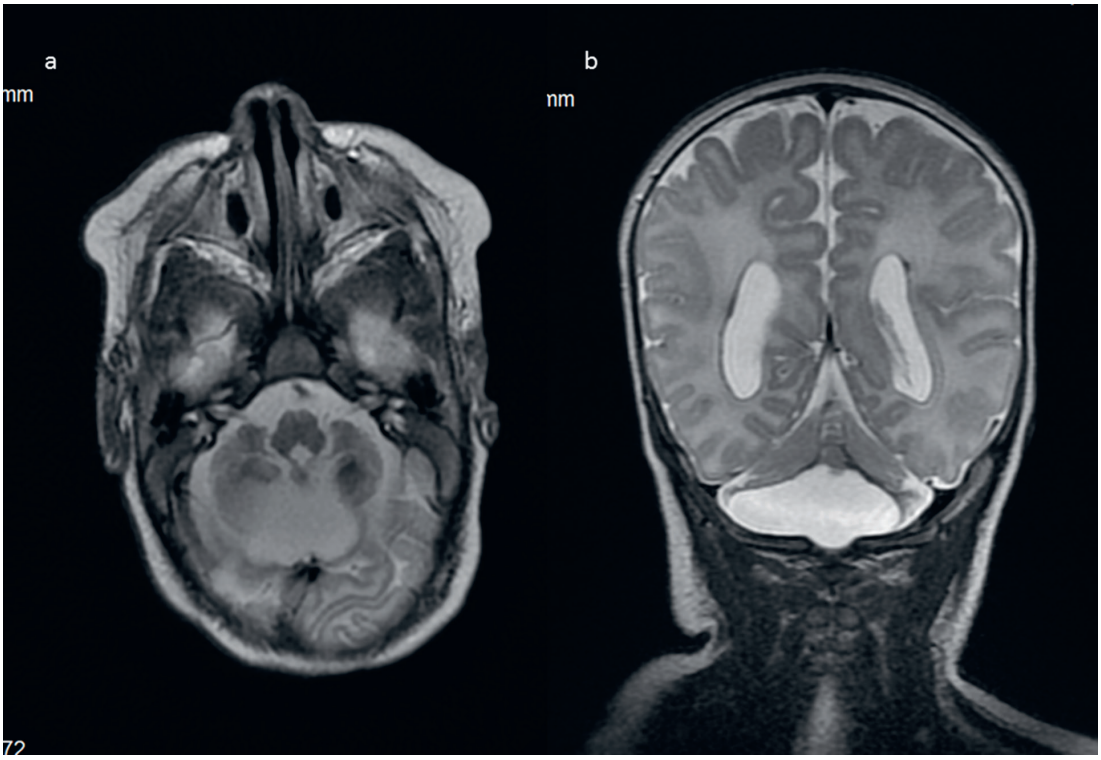

Fig. 2. Female second twin born at GA of 25 weeks, examined at 42. FSE/T2-weighted images in axial (a) and coronal projection (b). Posthemorrhagic destruction of both cerebellar hemispheres with hypointense deposits of hemosiderin and hyperintense cystic space between and below the hemispheres

is also performed for medical-legal reasons, e.g., when spinal cord injury is suspected due to traumatic birth. In our material, in 1 case this was the reason to perform spinal MRI when the depressed parents of a sick baby accused obstetricians of having injured the spinal cord secondarily to traction forces during delivery. The examination showed a normal spinal cord without evidence of any lesions.

What was shown in our study is that MR body imaging has started to play a growing role in neonatal care thanks to the development of body coils. This equipment consists of an 8-channel coil integrated in INC and a 4-channel surface coil as mentioned above. They enable scanning of not only the spine but also the neck, thorax, abdomen, and pelvis.

What we find significant is that neonatologists, surgeons and oncologists have started to refer neonates to non-CNS MRI thanks to the fact that we have body coils and to the safety of MRI in INC. There is no doubt that USG still is and will be the first line imaging modality, but whenever it is insufficient, MRI in INC is the most valuable and safest non-invasive tool that we have to answer clinical questions which cannot be answered by USG, and it is of utmost importance in neonates that must be operated on. In our material, we dealt with a female neonate whose mother did not see the obstetrician during pregnancy and did not receive prenatal USG. The baby was diagnosed with persistent cloaca after birth and surgical repair was scheduled, but MRI turned out to be necessary to confirm a sonographic diagnosis of bilateral renal agenesis and agenesis of the bladder before the decision to give up treatment.

We have already described a case in which the association of abdominal cyst with the biliary ducts was excluded.[3] In another baby, this relationship was found on MRI and MR-cholangiography, and a common bile duct cyst was diagnosed implying referral of the newborn to another tertiary reference center that deals with biliary malformations. There is also a case of gallbladder duplication in our material.

In the case of lymphangioma, its extent frequently cannot be assessed by means of USG, e.g., if it penetrates to the retropharyngeal space or to the mediastinum, as it was in one of our babies. Magnetic resonance examinations of the chest belong to the least frequent in the analyzed material, which is quite obvious knowing the limited (at least so far) value of this method in this part of the human body. In another case, we easily visualized the lesion, its smooth borders and fluid contents, but the question whether this was a bronchogenic cyst or a cystic teratoma remained unsolved.

In another case, the results of USG were equivocal, indicating a complex ovarian cyst or presacral teratoma. Different surgical approaches required unequivocal diagnosis and MRI gave it, clearly showing sacrococcygeal teratoma type IV according to Altman.

Inborn tumors are a challenge for all involved specialists, especially when unclear at presentation. Magnetic resonance imaging was also performed at our center after discrepant orthopedic and sonographic evaluations suggesting a congenital anomaly of the left hip and unspecified mass, respectively. Magnetic resonance imaging revealed a giant tumor of the left buttock and thigh extending to the pelvis, inseparable from the rectum and vagina, with enlarged lymph nodes in both groins, around the iliac vessels and the aorta up to the level of the diaphragm. After

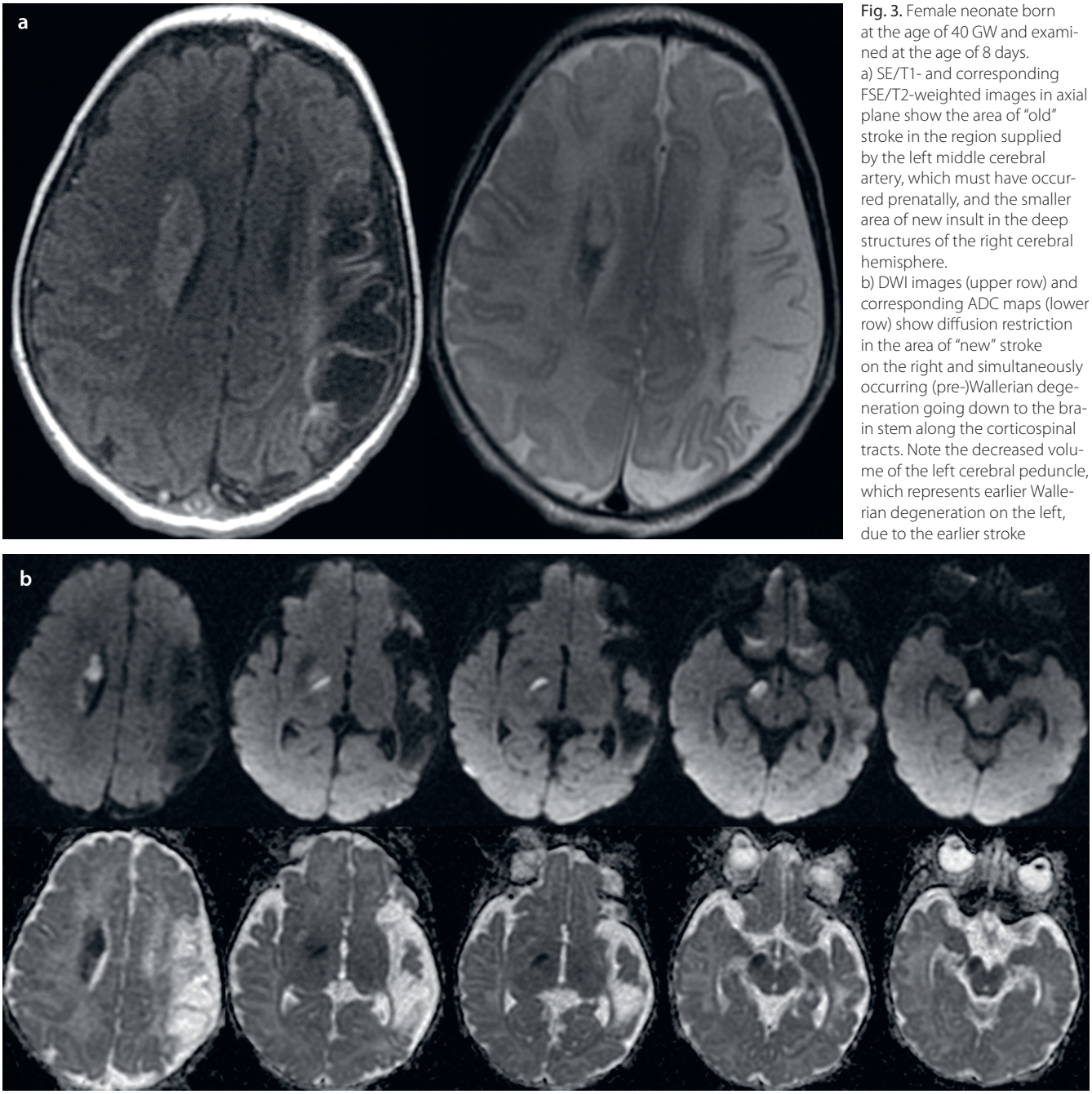

Fig. 3. Female neonate born at the age of 40 GW and examined at the age of 8 days. a) SE/T1- and corresponding FSE/T2-weighted images in axial plane show the area of "old" stroke in the region supplied by the left middle cerebral artery, which must have occurred prenatally, and the smaller area of new insult in the deep structures of the right cerebral hemisphere. b) DWI images (upper row) and corresponding ADC maps (lower row) show diffusion restriction in the area of "new" stroke on the right and simultaneously occurring (pre-)Wallerian degeneration going down to the brain stem along the corticospinal tracts. Note the decreased volume of the left cerebral peduncle, which represents earlier Wallerian degeneration on the left, due to the earlier stroke

biopsy and numerous pathologic consultations, a diagnosis of infantile myofibroma was established.

As stated before, literature concerning neonatal MRI in INC is very scant and there are no descriptions of body imaging. In this aspect, our observations are the first in world literature. In our material, MRI body scans constituted 14% and, to the best of our knowledge, this is the first report on body scanning in INC in a large cohort of neonates. Our results show that body imaging in INC is feasible and more and more frequently required, not only to establish diagnosis but also to follow up the patient after surgery. One example is a follow-up examination after resection of huge facial hemangiopericytoma with no residual disease. In our material, we have 2 cases of serial follow-up studies in INC in preterm neonates who were examined due to unclear sonographic appearance after surgery for sacrococcygeal teratoma, with a diagnosis of pelvic abscess in 1 case (Fig. 5) and pelvic hematoma in the other, and subsequently – the regression of lesions. Residual or recurrent tumors were thus ruled out.

Final remarks

Altogether, in 36.6% of our material, MRI showed more abnormalities than USG and in a further 21.8%, MRI changed the diagnosis. In 4.7% of cases, pathological lesions noted on USG were ruled out on MRI. The superiority of MRI over USG was 63.1% in the collected material in neuroimaging

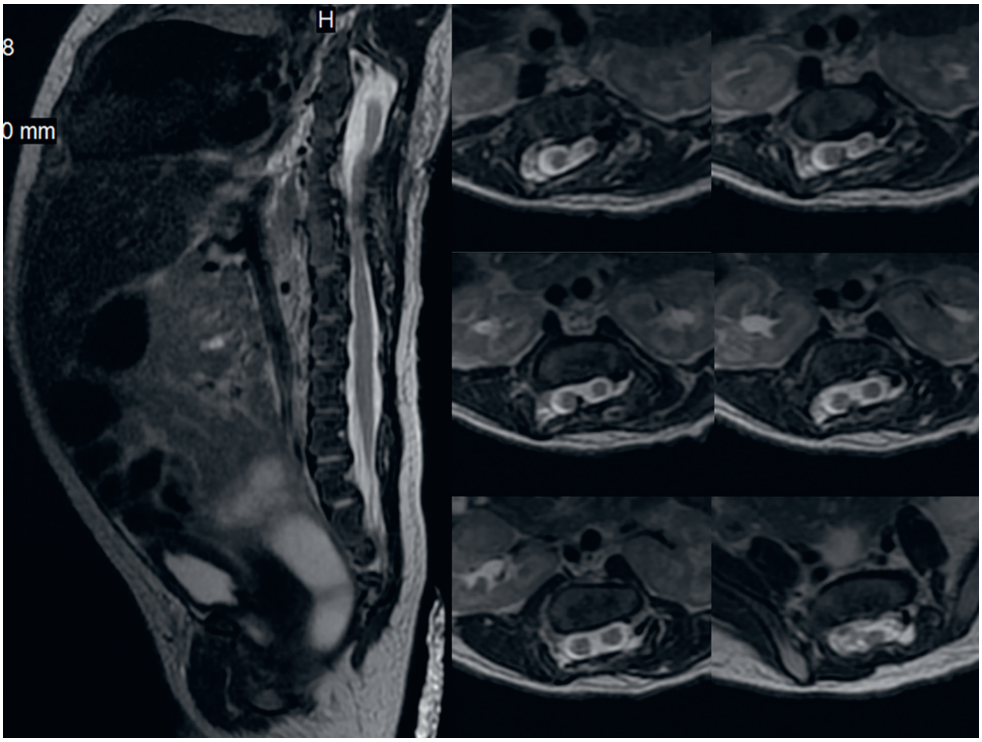

Fig. 4. Female term neonate examined at the age of 7 days. FSE/T2-weighted images in sagittal A) and axial B) projection. Vertebral anomalies, tethered cord A) and diastematomyelia B) are observed

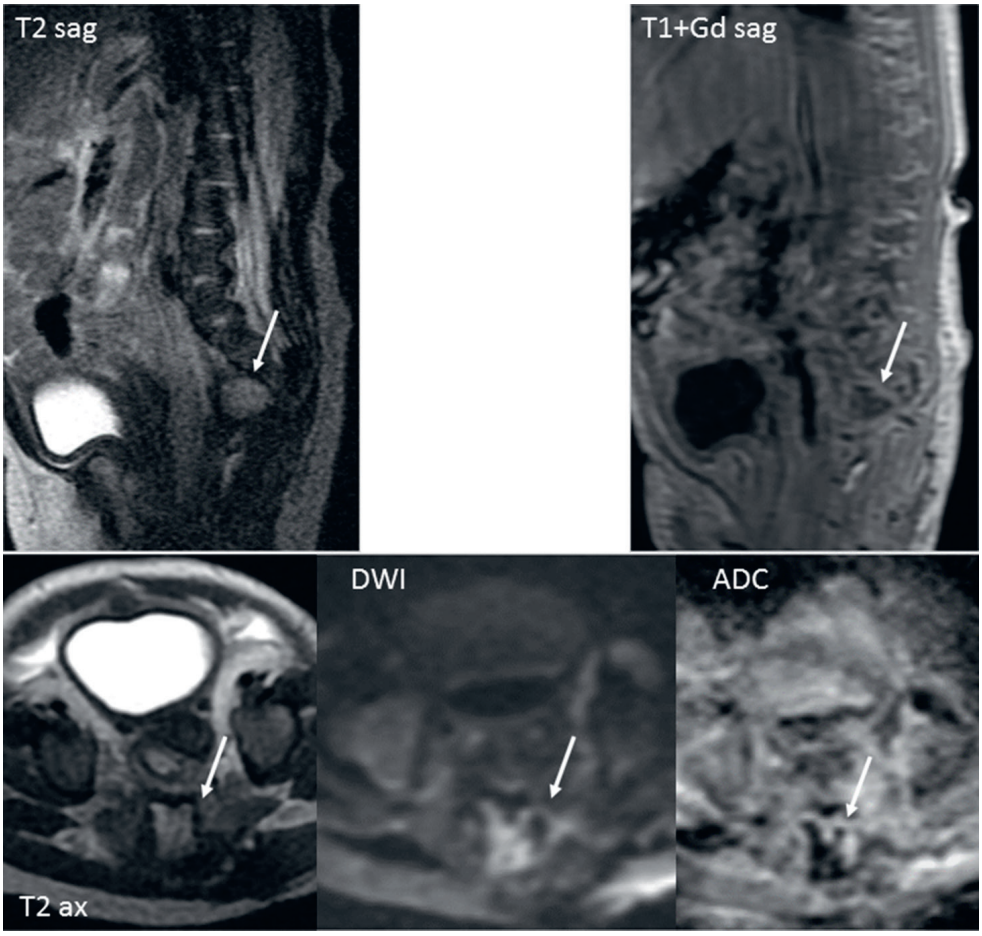

Fig. 5. Follow-up study still in the neonatal period after surgery for sacrococcygeal teratoma due to inconclusive sonographic findings. A lesion with enhancing wall, filled with thick fluid showing diffusion restriction was typical of abscess (arrows)

and body imaging. Obviously, picking up more abnormalities (i.e., higher sensitivity) with MRI does not necessarily equate to a change of the care that the patient receives at the moment. A good example is the abovementioned case of diffuse polymicrogyria detected at the asymptomatic moment of the baby's life. However, even if such a finding

causes increased parental anxiety, it is crucial for the prognosis and spares future examination when seizures appear. So the significance of MRI findings is not only related to the change of current therapeutic procedures, but also to the outcome and implications for the child's future life. This increased sensitivity of MRI makes possible progress in medical knowledge as well, as in the case of the very little-described issue of pre-Wallerian degeneration in the corticospinal tracts and in the corpus callosum after brain insult.[20] This increased sensitivity of MRI; however, is not always equivalent to finding clinically meaningful information. The classic example of this is diffuse excessive high signal intensity (DEHSI) of the white matter seen on neonatal MRI and invisible on USG, which is a highly qualitative finding and does not appear to have clear clinical significance.[21] In our material we did not count DEHSI as an element of MRI superiority over USG.

Although we have been trying for years to convince clinicians that prenatal MRI can be the basis of treatment after birth, it still happens that they wish to have postnatal confirmation of radiological prenatal findings, which is relatively easily available with INC. In our study, we have shown that in most such cases, there was no added value of postnatal MRI as compared to prenatal. However, MRI in INC allowed a visualization of small details that could not have been noticed on prenatal MRI or required contrast medium administration to be noticed.[2]

Limitations of MR-compatible incubator

The size of an extracorporeal lesion is one of the limitations of the use of INC. Besides the presented material, we had 2 newborns who did not fit into the space provided in the head coil: one with suboccipital encephalocele of large dimensions and another one with a tongue tumor which turned out to be part of blue rubber bleb nevus syndrome. The coil could not be closed around the neonate's heads and we had to give up INC. Another limitation is spatial resolution of the body coil. For instance, brachial plexus injury can be visualized if edema is seen within it. However, in the absence of edema, it is practically impossible to follow the particular nerve roots leaving the spinal canal in the cervical and thoracic region and forming the plexus. The lesions in the extremities limit the use of INC – in the absence of small local coils we had to give up INC in a newborn with congenital rhabdomyosarcoma of the forearm. There is still room for technical progress, but progress has already been made by introducing body coils to the INC.

Conclusions

In conclusion, the MR-compatible incubator constitutes a significant advancement in neonatal diagnostics. It has significantly increased the availability of MRI to newborns, especially those who are premature or otherwise unstable. Integration of body coils into INC has increased the spectrum of possible examinations and made possible scanning not only of the brain but also of the body. Dedicated neonatal coils have improved image quality and allowed more accurate diagnosis than the adult coils used previously. The immobilization of the neonate in INC with the use of Velcro belts and head fixation inserts are better than in adult coils. The closed space of INC isolates newborns to a much greater extent than before from the negative influence of noise in the MR environment.

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Vitamin D receptor (*VDR*) TaqI polymorphism, vitamin D and bone mineral density in patients with inflammatory bowel diseases

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Conflict of interest
The Author(s) represents that the conflict of interest applies to the conflict-of-interest statement: IKK received travel grants from Alvogen, Abbvie and Astellas; PE received lecture fees from Abbvie Poland and travel grants from Astellas, Abbvie Poland and Ferring; AD received travel grants from Alvogen, Abbvie and Astellas and lecture fees from Abbvie Poland and Alvogen; LLS received travel grants from Alvogen, Astellas and Abbvie Poland; AST received travel grant from Abbvie Poland; KSE received travel grants from Alvogen. MKR, MSZ, MS, SzH, MM and RD have nothing to disclose.

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Abstract

Background. A common feature in the etiology of inflammatory bowel disease (IBD) and osteoporosis is a complex genetic background. Moreover, it has been shown that some of the susceptibility loci overlap for both diseases. One of the genes that may be involved in the pathogenesis of IBD as well as decreased bone mass is the vitamin D receptor (*VDR*) gene.

Objectives. The aim of this study was to investigate the association of the TaqI polymorphism (rs731236, c.1056T >C) in the *VDR* gene with serum vitamin D concentration and bone mineral density (BMD) in patients with IBD.

Material and methods. A total of 172 IBD patients (85 with Crohn's disease (CD) and 87 with ulcerative colitis (UC)) and 39 healthy controls were enrolled in the study. Polymorphism was determined with polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Bone mineral density was measured at the lumbar spine (L2–L4) and the femoral neck (FN) using dual-energy x-ray absorptiometry (DEXA). Serum concentrations of 25-hydroxyvitamin D were determined using electrochemiluminescence binding assay (ECLIA).

Results. Our studies revealed that serum vitamin D concentration in IBD patients was not lowered in comparison with healthy controls. Patients with CD presented more advanced osteopenia and osteoporosis. Individuals with UC carrying the TaqI tt genotype of *VDR* gene showed significantly higher FN BMD than carriers of TT and Tt genotypes ($p = 0.02$). Moreover, tt genotype was present with higher frequency in UC patients than in controls and CD patients (23% vs 7.7% and 16.5%, respectively).

Conclusions. The tt genotype may have a protective effect on BMD in UC patients.

Key words: vitamin D, bone mineral density, inflammatory bowel diseases, vitamin D receptor gene polymorphism

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Introduction

Inflammatory bowel disease (IBD) is an idiopathic disease caused by uncontrolled mucosal immunity of the gastrointestinal tract, but its pathogenesis is not fully understood.[1] It has been suggested that it originates from a combination of environmental (intestinal microbiota), immunological (impaired regulation of T-helper lymphocytes, Th) and complex genetic factors.[2,3] Inflammatory bowel disease does not concern only the gastrointestinal system and should be treated as a systemic disease with a number of manifestations outside the digestive tract, among which osteopenia and osteoporosis are the most common.[4] Even 22–77% of patients with IBD suffer from osteopenia and 17–41% of suffer from osteoporosis.[5–7] Lean body structure, age over 65, low-protein and low-calcium diet, cigarette smoking, alcohol drinking, vitamin D deficiency, the use of some pharmaceutical drugs, and compound genetic factors are osteoporosis risk factors. Osteoporosis susceptibility genes encode proteins that control bone formation and the maintenance of the normal bone tissue structure. They belong to different families of biological factors such as cytokines, growth factors, matrix components, and calciotropic hormone receptors.[8,9] To date, almost 200 susceptibility loci have been identified for IBD. Some of them are characteristic for Crohn's disease (CD) or ulcerative colitis (UC) and some of them overlap. Moreover, susceptibility loci may be also common for IBD and osteoporosis.[10,11] One of such genes is the vitamin D receptor (*VDR*) gene, encoding the vitamin D ($1,25(OH)_2D_3$) receptor belonging to a family of nuclear receptors and acting as a ligand-activated transcriptional factor. Activation of the *VDR* through direct interaction with $1,25(OH)_2D_3$ prompts the rapid binding of the receptor to regulatory regions of target genes, where it causes directed changes in transcription. This receptor plays a central role in the biological actions of vitamin D; moreover, *VDR* regulates the expression of numerous genes involved in calcium/phosphate homeostasis, cellular proliferation and differentiation, and immune response.[12] Vitamin D is a prohormone that is metabolized to its active form, 1,25-dihydroxycalciferol (calcitriol), having both calcemic and non-calcemic (pleiotropic) effects. The most important calcemic effects include inducing calcium and phosphate absorption in the digestive system, increasing renal calcium reabsorption and inducing bone turnover.[9]

According to the latest data, patients with IBD suffer from vitamin D deficiency.[13] Decreased exposition to sunlight,[14] inappropriate diet, inflammatory changes in the bowel mucosa, and consequences of the digestive tract resection are considered the main reason for this problem.[15–17] This leads to osteomalacia (defective bone mineralization with the maintenance of the normal bone mass) and osteoporosis (a decrease of the properly mineralized bone mass). Moreover, vitamin D may have an important effect on the course of the disease through modulation of the inflammation mechanisms.

It has been shown that polymorphisms in the *VDR* gene are related with changes in bone mineral density (BMD). Furthermore, earlier reports showed that the *VDR* alterations might increase the risk of IBD in Caucasians and Asians.[18,19] The mechanism of this phenomenon is difficult to explain. The 3' region of the *VDR* gene is involved in the regulation of gene expression, which affects the stability of mRNA. In turn, it may lead to a decreased concentration of mRNA resulting in decreased vitamin D levels and a reduction in its inhibitory effects on interleukin 12 (IL-12). Changes in the immune system result in increased Th1-dependent reaction, which may explain the susceptibility to CD. What is also significant is the linkage disequilibrium (LD); the *VDR* gene polymorphisms may be in strong linkage with another, unknown sequence variant, which has a causative effect.[20] Considering the above premises, we decided to deal with this issue. The aim of this study was to analyze the association between the TaqI polymorphism in the *VDR* gene and serum vitamin D concentration as well as BMD in patients with inflammatory bowel disease.

Materials and methods

Patients and clinical data

The study group included 172 IBD patients from the Clinic of Gastroenterology, Human Nutrition and Internal Diseases of the Poznan University of Medical Sciences (Poland), comprising of 85 CD and 87 UC patients. The control group consisted of 39 healthy volunteers without IBD or any bone disorders. Peripheral blood and clinical data were collected from all study subjects. The clinical examination included measurements of BMD of the lumbar spine (L2–L4 levels) and of the femoral neck (FN). The data on weight, height and age at densitometry was also collected. Patients included into the study were not taking any vitamin D supplements. Densitometry measurements were conducted with dual energy X-ray absorptiometry (DEXA) using the DPX-Plus device (Lunar, GE Healthcare, Chicago, USA). Serum concentrations of 25-hydroxyvitamin D (25(OH)D), as markers of vitamin D sufficiency in the organism, were determined using an electro-chemiluminescence binding assay and a Cobas e601 analyzer (Roche, Basel, Switzerland). The functional sensitivity of these assays is 4.01 ng/mL (coefficient of variation: 18.5%). All patients gave their written consent to genetic testing scheduled within this study. The study was approved by the local Ethical Committee of the Poznan University of Medical Sciences (approval No. 92/09).

DNA extraction and *VDR* polymorphism analysis

DNA was isolated from peripheral blood leukocytes using guanidine isothiocyanate and phenol-chloroform

as described elsewhere.[21] DNA fragments including the polymorphic sites of the *VDR* gene were amplified using polymerase chain reaction (PCR). Each amplification reaction contained 100 ng of genomic DNA, 0.25 mM dNTP, 7.5 pmol of each primer, and 0.5 unit of the Taq polymerase (Sigma Aldrich, Saint Louis, USA) in 20 µL total volume. The reaction was conducted in 35 cycles of the following steps: initial denaturation at 94°C for 4 min; denaturation at 94°C for 40 s; primer annealing at 64°C for 40 s; elongation at 72°C for 100 s; and final incubation at 72°C for 180 s. PCR primers: forward CAG AGC ATG GAC AGG GAG CAA and reverse GCA ACT CCT CAT GGC TGA GGT CTC were used.[22] The amplification products were subsequently hydrolyzed using TaqI restriction enzyme (New England Biolabs, Ipswich, USA) at 65°C for 2 h. Digestion products were separated in an agarose gel (1.5%) with ethidium bromide. Alleles of TaqI polymorphism (allele T(c.1056C): 494+251 bp and allele t (c.1056T): 293+251+201 bp) were identified in comparison to the control samples determined with Sanger sequencing.

Statistical analysis

We conducted an analysis of the distribution of genotype concordance with the Hardy–Weinberg equilibrium. First, the normality of the distribution and the homogeneity of variable variances were conducted in the studied groups using the Shapiro–Wilk t test and Levene's test, respectively. In the event of non-concordance with 2 or at least 1 condition, the non-parametric Kruskal–Wallis H test was used to compare the groups. In the event of a statistically significant heterogeneity between the groups, multiple comparisons were conducted using the Dunn's test in order to evaluate an association between qualitative variables (the 3 study groups vs groups carrying different *VDR* genotypes). All analyses were conducted using STATISTICA v. 10.0 software (StatSoft Inc., Tulsa, USA) and the calculator on the http://ihg.gsf.de/cgi-bin/hw/hwa1.pl website. P-values below 0.05 were considered as indicative of a statistical significance.

Results

The basic characteristics of the study subjects have been summarized in Table 1. The comparison of investigated groups has shown differences in BMI, which occurred significantly reduced in IBD patients than in controls. Moreover, CD patients presented lower BMI than UC patients. We observed decreased BMD of lumbar spine and FN in patients with CD and UC. Individuals with CD presented more advanced osteopenia and osteoporosis than patients with UC. However, we did not find statistically significant differences in vitamin D concentration between studied groups (Table 1). The genotypes distribution in examined groups was concordant with the Hardy–Weinberg equilibrium.

Table 1. Basic characteristics and clinical parameters of the study subjects.

Parameter mean	CD patients n = 85	UC patients n = 87	Controls n = 39	p-value
Age [years]	35.24 (SD 12.12)	39.40 (SD 14.43)	30.74 (SD 8.6)	$^{c}p = 0.040$
Weight [kg]	63.20 (SD 13.86)	68.63 (SD 14.92)	73.62 (SD 13.65)	$^{b}p < 0.001$
Height [cm]	171.39 (SD 10.30)	170.80 (SD 9.74)	172.69 (SD 9.35)	$p = 0.70$
BMI [kg/m^2]	21.36 (SD 3.62)	23.43 (SD 4.32)	24.57 (SD 3.45)	$^{a}p < 0.001$ $^{b}p < 0.001$
L2–L4 BMD [g/cm^2]	1.12 (SD 0.18)	1.16 (SD 0.14)	1.22 (SD 0.08)	$^{b}p < 0.001$ $^{c}p = 0.01$
L2–L4 T-score	–0.82 (SD 1.45)	–0.46 (SD 1.15)	0.09 (SD 0.70)	$^{b}p < 0.001$ $^{c}p = 0.007$
L2–L4 Z-score	–0.41 (SD 1.32)	–0.18 (SD 1.19)	0.09 (SD 0.66)	$^{b}p = 0.03$
FN BMD [g/cm^2]	0.95 (SD 0.18)	0.98 (SD 0.15)	1.07 (SD 0.16)	$^{b}p < 0.001$ $^{c}p = 0.003$
FN T-score	–0.65 (SD 1.28)	–0.31 (SD 1.14)	0.41 (SD 1.03)	$^{b}p < 0.001$ $^{c}p = 0.001$
FN Z-score	–0.27 (SD 1.08)	0.06 (SD 1.04)	0.38 (SD 0.99)	$^{b}p = 0.002$
25-OHD [ng/mL]	21.43 (SD 12.32)	22.06 (SD 9.27)	21.56 (SD 9.11)	$p = 0.77$

BMD – bone mineral density; CD – Crohn's disease; FN – femoral neck; ns – non-significant; SD – standard deviation; UC – ulcerative colitis. All results are presented as means with standard deviations (SD); ^{a}CD vs UC; ^{b}CD vs controls; ^{c}UC vs controls.

We observed that tt genotype was present with higher frequency in UC patients than in controls and CD patients (23% vs 7.7% and 16.5%, respectively). Comparing the whole group of IBD patients with controls we pointed out that tt genotype was almost 3 times more frequent than TT+Tt genotypes (OR = 2.96), but this observation was borderline significant (p = 0.07). The same analysis performed in UC group revealed that in these patients tt homozygotes occurred even more frequently (OR = 3.58, 95% CI = 1.00–12.87, p-value = 0.04) (Table 2).

In the next step, we carried out the analysis of bone mass parameters and serum vitamin D levels in correlation with TaqI genotypes of *VDR* gene. Patients with CD did not significantly differ in lumbar spine (L2–L4) and FN BMD, T-score, Z-score, nor in 25(OH)D serum concentration (p > 0.05 for all comparisons); however, we have noticed slightly higher bone mass parameters values in tt genotype carriers, while in TT homozygotes they were the most decreased. Considering UC patients, lumbar spine values of BMD, T-score and Z-score as well as FN, have emerged higher in tt homozygotes even more clearly; the differences between FN BMD values in different genotypes carriers were statistically significant (overall p = 0.02, post hoc p: [tt] vs [Tt] = 0.02) (Table 3). In controls, we made the same observations, although in post hoc tests they remained borderline significant or insignificant.

Table 2. Alleles and genotypes frequency in *VDR* TaqI (rs731236, c.1056T>C) loci

Group	Genotype frequencies (%)	Allele frequencies (%)			
	TT	Tt	tt	T	t
IBD (all patients), n = 172	59 (34.3)	79 (45.9)	34 (19.8)	197 (57.3)	147 (42.7)
UC patients, n = 87	31 (35.6)	36 (41.4)	20 (23.0)	98 (56.3)	76 (43.7)
CD patients, n = 85	28 (32.9)	43 (50.6)	14 (16.5)	99 (58.2)	71 (41.8)
Controls, n = 39	13 (33.3)	23 (59.0)	3 (7.7)	49 (62.8)	29 (37.2)
Group	**Comparisons of allelic and genotypic frequencies between groups under study**				
	[tt] vs [TT+Tt]	[TT] vs [Tt+tt]	[tt] vs [TT]	[T] vs [t]	[t] vs [T]
IBD vs controls OR, 95% CI p-value	OR = 2.96 (0.86–10.18) p = 0.07	OR = 1.04 (0.50–2.18) p = 0.91	OR = 2.45 (0.66–9.39) p = 0.17	OR = 0.79 (0.48–1.32) p = 0.37	OR = 1.26 (0.76–2.09)
UC vs controls OR, 95% CI p-value	**OR = 3.58 (1.00–12.87) p = 0.04**	OR = 1.11 (0.50–2.46) p = 0.80	OR = 2.80 (0.71–11.06) p = 0.13	OR = 0.76 (0.44–1.32) p = 0.33	OR = 1.31 (0.76–2.27)
CD vs controls OR, 95% CI p-value	OR = 2.37 (0.64–8.77) p = 0.19	OR = 0.98 (0.44–2.20) p = 0.97	OR = 2.17 (0.53–8.87) p = 0.28	OR = 0.83 (0.48–1.43) p = 0.49	OR = 1.21 (0.70–2.10)
CD vs UC OR, 95% CI p-value	OR = 0.66 (0.31–1.41) p = 0.28	OR = 0.89 (0.47–1.67) p = 0.71	OR = 0.78 (0.33–1.82) p = 0.56	OR = 1.08 (0.71–1.66) p = 0.72	OR = 0.93 (0.60–1.42)

In bold were marked statistically significant ($p < 0.05$) and borderline results. CI – confidence intervals; OR – odds ratio.

Discussion

In this study, we analyzed *VDR* gene TaqI polymorphic variants and their relation to BMD parameters and serum vitamin D levels in a particular group of patients with IBD. The *VDR* gene was one of the first genes studied with regard to its possible role in the development of osteoporosis.[23–25] Vitamin D binds to a specific steroid receptor that has a transcription factor activity. The formation of vitamin D steroid receptor complex results in the activation or silencing of target gene expression. This leads to synthesis regulation of proteins that actively participate in bone metabolism and calcium homeostasis.[26] Alterations in the *VDR* gene may result in alterations of the structure, function and/or activity of the VDR protein, i.e., affect the transcription factor participating in the signal transduction from vitamin D to genes that are under its control. Studies on *VDR* gene polymorphisms showed divergent results in the analyzed groups of patients and populations.[27] A meta-analysis carried out by Xue et al. in 2013 describes 9 studies conducted in the years 1995–2011. What follows is that ff genotype of the FokI polymorphism in *VDR* gene is associated with a significant risk of UC in Asians, while the TaqI polymorphism (tt genotype) was associated with an increased risk of CD in Europeans as well as in Asians.[18] In turn, Wang et al. did not report any correlation between ApaI, BsmI and FokI polymorphisms and IBD. Wang et al. paid particular attention to differences in the genetic profiles of studied patients regarding ethnic differences between them. They have demonstrated no significant differences in the distribution of allele frequencies between the examined groups of IBD and controls, either any relationship between *VDR* polymorphisms or the occurrence of the disease.[20] In turn, other studies carried out in the European populations (UK, Germany) revealed the association of the TaqI polymorphism tt genotype with the occurrence of IBD. The authors found that tt genotype was more frequent in patients with CD with numerous fistulas and stenoses.[28] Interestingly, the latest meta-analysis conducted by Zhang et al. involved 17 studies on patients with postmenopausal osteoporosis and showed no significant relationship between *VDR* TaqI polymorphism and osteoporosis susceptibility in Caucasians and the overall populations as well.[29] An earlier report by Sikorska et al. is in concordance with Zhang's findings, although the Polish scientists pointed out the relation of *VDR* alteration with osteoporotic fractures susceptibility.[24] Another paper concerning Polish patients with UC has shown no differences in the distribution of *VDR* TaqI polymorphism in comparison to healthy control subjects.[30]

In our study, we observed the protective effect of the *VDR* gene TaqI t (c.1057T) allele on BMD in IBD patients and controls. Particularly, UC patients with tt genotypes had a significantly higher FN bone mass. Our observations are contrary to those reported by Morrison et al., who showed that tt homozygotes had a lower bone mass.[25] In the study by Noble et al., no association between TaqI *VDR* polymorphisms and bone mass was shown, and the only independent factor associated with osteoporosis was low BMI.[28] In our study, we did not show significant differences

Table 3. Analysis of the TaqI polymorphism genotypes with respect to BMD, T-score, Z-score, and 25(OH)D concentration in the study groups

Parameter mean (SD)	Crohn's disease			
	TT n = 28	Tt n = 43	tt n = 14	p-value
L2–L4 BMD [g/cm^2]	1.11 (0.19)	1.12 (0.18)	1.15 (0.19)	p = 0.98
L2–L4 T-score	−0.87 (1.45)	−0.85 (1.44)	−0.61 (1.61)	p = 0.97
L2–L4 Z-score	−0.44 (1.41)	−0.44 (1.26)	−0.23 (1.40)	p = 0.90
FN BMD [g/cm^2]	0.94 (0.15)	0.95 (0.19)	0.96 (0.19)	p = 0.99
FN T-score	−0.71 (1.00)	−0.66 (1.36)	−0.52 (1.58)	p = 0.98
FN Z-score	−0.30 (0.82)	−0.28 (1.14)	−0.17 (1.41)	p = 0.92
25 OHD [ng/mL]	20.04 (12.44)	21.95 (11.47)	22.62 ±15.07	p = 0.62
Parameter	Ulcerative colitis			
	TT n = 31	Tt n = 36	tt n = 20	p-value
L2–L4 BMD [g/cm^2]	1.15 (0.12)	1.15 (0.16)	1.21 (0.13)	p = 0.14
L2–L4 T-score	−0.55 (1.09)	−0.52 (1.36)	−0.23 (0.78)	p = 0.41
L2–L4 Z-score	−0.21 (1.26)	−0.24 (1.32)	−0.03 (0.78)	p = 0.71
FN BMD [g/cm^2]	0.97 (0.14)	0.95 (0.16)	1.05 (0.12)	p = 0.02 [tt] vs [tt] p = 0.02 [tt] vs [tt] p = 0.08 [tt] vs [tt] p = 1.00
FN T-score	−0.36 (1.02)	−0.49 (1.30)	0.10 (0.94)	p = 0.09
FN Z-score	0.01 (0.99)	−0.06 (1.15)	0.37 (0.86)	p = 0.18
25 OHD [ng/mL]	23.60 (10.48)	20.97 (8.77)	21.63 (8.22)	p = 0.84
Parameter	Controls			
	TT n = 13	Tt n = 23	tt n = 3	p-value
L2–L4 BMD [g/cm^2]	1.25 (0.09)	1.20 (0.07)	1.31 (0.04)	p = 0.03 [tt] vs [tt] p = 0.08 [tt] vs [tt] p = 0.75 [tt] vs [tt] p = 0.21
L2–L4 T score	0.32 (0.66)	−0.13 (0.68)	0.80 (0.30)	p = 0.03 [tt] vs [tt] p = 0.07 [tt] vs [tt] p = 0.81 [tt] vs [tt] p = 0.15
L2–L4 Z score	0.34 (0.51)	−0.11 (0.70)	0.54 (0.52)	p = 0.03 [tt] vs [tt] p = 0.19 [tt] vs [tt] p = 1.00 [tt] vs [tt] p = 0.06
FN BMD [g/cm^2]	1.04 (0.12)	1.10 (0.18)	1.03 (0.14)	p = 0.58
FN T score	0.28 (0.76)	0.53 (1.19)	0.12 (0.81)	p = 0.77
FN Z score	0.31 (0.66)	0.46 (1.18)	0.10 (0.62)	p = 0.93
25 OHD [ng/mL]	21.44 (7.11)	22.04 (10.65)	18.34 (2.86)	p = 0.77

All results are presented as means with standard deviations (SD); BMD – bone mineral density; FN – femoral neck; ns – non-significant; SD – standard deviation.

in serum vitamin D concentrations between individuals carrying different polymorphic variants; moreover, our IBD patients did not presented any differences in serum 25(OH)D in comparison to controls. However, in the study conducted on a group of 308 patients with CD in New Zealand, a country with one of the highest incidence rates for this disease in the world, lower vitamin D concentrations were found in IBD patients compared to healthy individuals. These authors correlated low concentrations of 25(OH)D with age and low exposure to sunlight.[31]

Polymorphism analyzed in our study has been frequently studied with regard to its contribution to the development of osteoporosis in populations all over the world. The results remained equivocal or even contradictory. Molecular diagnostics based on analyzing allelic variants of candidate genes potentially associated with altered bone turnover in IBD patients would allow us to estimate the predisposition to developing osteoporosis long before the first symptoms appear. This would make it possible to apply prophylaxis or therapies, thus delaying osteoporosis development, which would considerably improve the quality of IBD patients' lives and reduce the cost of healthcare.

Conclusions

In conclusion, the *VDR* gene TaqI polymorphism may be related to BMD. We suggest that tt genotype has a protective effect on BMD particularly in UC patients.

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Biodegradable airway stents: Novel treatment of airway obstruction in children

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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. Tracheobronchomalacia is the most common cause of congenital airway obstruction in infants. An alternative for surgical approach (aortopexy) can be metallic airway stents. Usually, they are not considered as a first choice because of the relatively high risk of complications. Recent years have brought encouraging reports of biodegradable stents applied in children.

Objectives. The aim of this study was to report our experience in the treatment of airway malacia using biodegradable stents.

Material and methods. Six polydioxanone (PDS), self-expanding custom-made stents (ELLA-CS) were implanted in 2 children: 3 in the patient with left main bronchus occlusion due to postpnemonectomy syndrome and 3 stents in the baby with tracheomalacia.

Results. Airway collapse was always relieved after stent expansion. Both patients needed repeated stenting because of limited stent lifespan. All the stents were implanted without complications through a rigid bronchoscope. The baby with stented main bronchus died because of irreversible lung lesion.

Conclusions. This small study shows that biodegradable airway stents seem to be an attractive option in the treatment of tracheobronchomalacia in children. We consider this method to be safe, effective, repeatable, and reversible in small children with growing airways. As a time-buying procedure they can be especially useful in the treatment of tracheobronchomalacia.

Key words: biodegradable airway stents, tracheobronchomalacia, airway obstruction

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Introduction

Airway stenting in adults is a well-established therapeutic method for the treatment of benign and malignant tracheobronchial obstruction. Stents can be used in preventing restenosis after airway reconstruction, lung transplantation or in palliative therapy in non-resectable tumors.[1] In the long history of endoluminal airway support, the first stents to be invented were made of metal, later of plastic and then followed by mixed forms (hybrids).[2] Moreover, the last 2 decades have brought new technologies and materials that have led to the introduction of biodegradable materials in thoracic surgery. Initially, biodegradable stents were used in esophageal, intestinal, biliary, urethral, and vascular stenosis.[3–5] Finally, the idea was adapted for the treatment of airway obstruction.

In neonates and young children, we encounter 2 main types of pathologies: anatomical and dynamic airway obstructions. The former usually results from congenital tracheal stenosis or concomitant vascular abnormalities, and the later type is represented by dynamic obstructions with bronchial or tracheal wall collapse. Although surgical treatment is still the primary therapeutic option for patients with various types of congenital tracheal stenosis, in the second group with tracheobronchomalacia, stents could be an attractive alternative. To date, the use of metal stents in neonates has been limited due to numerous life-threatening local complications, such as tracheobronchial wall erosion, bleeding and severe mucosal tissue granulation with airway narrowing.[6,7] In addition, most metal stents are permanent once inserted and cannot be replaced, which greatly limits their use in the developing airways of children.[8]

The emergence of biodegradable airway stents seems to have opened a new chapter in the treatment of dynamic types of airway obstruction in newborns and small children. The use of polydioxanon (PDS) stents has been recently reported in a growing number of small patient series with encouraging clinical results.[9–12] Polydioxanon belongs to the family of biodegradable synthetic polymers and is widely used for the preparation of surgical sutures. As a monofilament fiber, it hardens binding parts of water, presents shape memory and degrades by random hydrolysis of its ester bonds with the mean biodegradation time between 12–15 weeks, proven in animal models.[13,14]

We report our preliminary experience with the use of bio-stents in children in the treatment of tracheobronchomalacia.

Material and methods

Six bio-stents were implanted in 2 infants. The present clinical trial was approved by our Institutional Ethics Committee. Parental informed consent was obtained before each procedure. Self-expanding PDS bio-stents were custom-made and delivered with the introducer by the manufacturer (ELLA-CS, Hradec Králové, Czech Republic). The decision regarding stenting was made by our multidisciplinary team: thoracic surgeons, pediatricians and anesthetists. The sizes of the ordered stents were decided after initial rigid bronchoscopy in which we measured the diameter of the airway and estimated the length of stents. Each stent was delivered expanded in an airtight container and with a separately packed introducer. Immediately before implantation, we manually pressed the stent and placed it on the introducer under a moveable sheath. All the bio-stents were implanted under general anesthesia through a rigid Karl Storz bronchoscope size 4.0. Expansion of the stent was controlled using a small-size optics (diameter 1.3 mm; length 30.6 cm), which is longer than the bronchoscope, inserted along together with the introducer and positioned laterally. After implantation, we used the same longer optics or a small-size bronchofiberoscope for insertion inside the expanded stent to check the results and control both ends of the stent.

Results

Patient 1

A female newborn, twin delivered by a Cesarean section because of life-threatening asphyxia after 34 weeks of gestation, with birth body mass of 1,770 g, presented respiratory compromise since birth. Chest x-ray and CT scan revealed congenital cystic adenomatoid malformation (CCAM) affecting the entire right lung. The patient needed an urgent right lobectomy in the neonatal period due to severe respiratory distress and mass effect. The postsurgical course was uneventful. Six months after surgery, dyspnea appeared along with recurrent respiratory infections because of postpneumonectomy syndrome (PPS) with a severe rightward mediastinal shift and rightward left lung expansion. Mechanical ventilatory support with intubation was mandatory. Bronchoscopy showed an almost complete collapse of the left main bronchus with bronchial tree rotation (Fig. 1).

Postnatally, the baby had extensive cerebral infarction, so the therapeutic team together with the parents abandoned the idea of possible surgical correction with cardiopulmonary bypass and decided to use a bio-stent. A PDS stent 5 × 20 mm was implanted. After 2 days, the patient was extubated and control bronchofiberoscopy confirmed proper stent positioning and open bronchus. The subsequent 2 stents were implanted after the dissolution of the previous one, increasing the diameter to 6 mm and length to 25 mm, which corresponded to the increasing diameter of the airway. After implantation of the first 2 stents, the patient stayed home in good general condition, gaining weight properly. After the 3rd stenting, the parents refused further therapy. Upon their request, the baby was placed in the hospice, where she died 4 months after the last stent implantation.

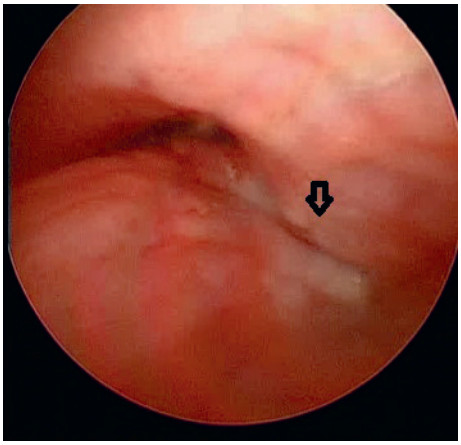

Fig. 1. Completely collapsed left main bronchus. Arrowhead points the place with stamp of the right main bronchus

Patient 2

A male newborn twin delivered after 36 weeks of gestation with a birth body mass of 2,100 g was operated on through left thoracotomy in the 1st day of life because of esophageal atresia.

After surgery, the patient could not be weaned from ventilatory support for the first 5 months and later needed continuous positive airway pressure (CPAP). The child was discharged home, but subsequently needed emergency hospitalization due to an episode of life-threatening asphyxia. Possible concomitant abnormalities, such as vascular ring, sling and complete tracheal rings had been previously excluded using CT scan in the referring hospital. The baby was referred to our center at the age of 6 months. On admission, bronchoscopy demonstrated severe tracheomalacia in the middle segment of the trachea, surprisingly situated not in the lower segment, close to the previous fistula (Fig. 2).

We decided to implant a PDS stent 5 × 25 mm, achieving dramatic resolution of the symptoms. The baby was extubated after the procedure and discharged home early. Subsequently, 2 other stents 1 mm larger in width were placed at intervals of 12–14 weeks with excellent tolerance. At present, the patient stays at home without respiratory problems. The baby is subject to follow-up bronchoscopy 12 weeks after the last stent implantation with possible consecutive stent implantation.

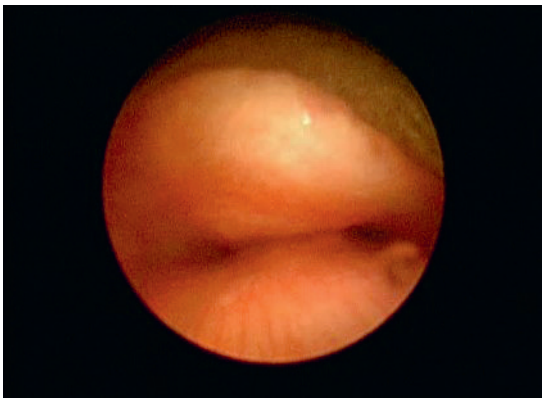

Fig. 2. Severe tracheomalacia in the middle portion of the trachea

Discussion

In the treatment of airway obstruction in small children, there are still no clearly defined indications for stenting and their use is considered individually for each patient.[2,12] Difficulty in stenting is related to the small diameter of the respiratory tract, to its growth and the possible need for stent replacement. The commonly used metal stents are considered by the majority of authors as "placed once and for good and bad" and potential replacement usually requires surgical intervention. In addition, the potential benefits of a particular stent group associated with their structure generate significant complications. For example, the risk of migration for metal stents is low, but their hard structure strongly impacts the mucosa, resulting in a high risk of granulation tissue formation and mucosal erosion with bleeding. On the other hand, softer plastic or silicone stents with a low rate of local complications present a considerable tendency for migration.[2]

In order to avoid the abovedescribed complications, the attention of researches has been focused on biodegradable materials. After encouraging experimental medical tests, the first bio-stents have been used with good results in adults after lung transplants.[15] In the last 8 years, an increasing number of reports addressing the use of bio-stents in children have been published.[2,9–11,16] So far, PDS stents have been used mostly in the treatment of tracheal and bronchial compression after cardiovascular procedures,[9] after a failed aortopexy,[10] after tracheoplasty due to congenital stenosis[11] or in the cases of severe tracheo- and bronchomalacia.[12,17,18]

We found the offered polydioxanon self-expanding stents CS-ELLA very suitable for the use in pediatric patients. The mechanism of self-expansion and thus self-fixing is one of the greatest advantages of PDS stents. It excludes the need for the balloon use to expand the stent and thereby reduces periods of full apnea. In our opinion, x-ray-controlled implantation with C-arm fluoroscopy is more logistically challenging and requires radiation, so we decided in favor of implantation under general vision. We first inserted the introducer with the stent to the bronchoscope 4.0 mm, having enough space to place laterally very thin optics (1.3 mm) longer than the bronchoscope. After a partial withdrawal of the rigid bronchoscope, at the critical area of the airway only the top part of the introducer was left along with thin optics with good visualization of the proximal stent border (Fig. 3A–D). Then, one of the operators withdrew the optics, the second released the mechanism of the sheath sliding and opening the stent. This maneuver allowed for the reduction of the apnea time to a few seconds only and was well

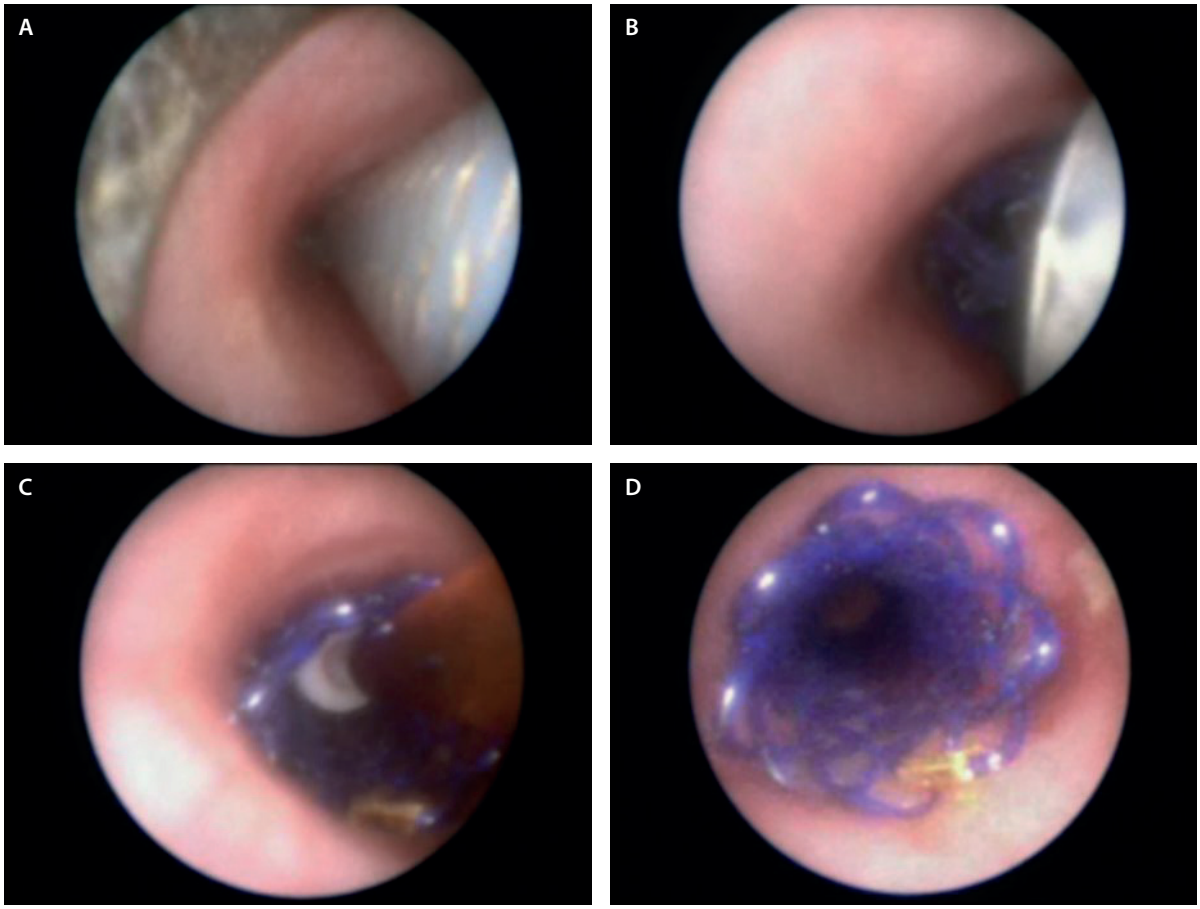

Fig. 3. A – laterally placed tiny optics shows apical portion of the introducer with a stent under the sheath; visible border of the rigid bronchoscope; B – sheath withdrawal and opening of the stent; C – final removal of the introducer; implantation completed; D – implanted and opened stent in the left main bronchus; visible golden marker at the proximal stent`s border

tolerated by the patients after preoxygenation. It also allowed us to abandon the C-arm fluoroscopy and offered more space and better comfort for the operative team. After 1 implantation, when the stent after expansion was located too distally, it was easy to relocate it upward using optical forceps. In both patients, the implanted stents kept the airway open with a round shape at the collapsing area without tendency for migration. Each stent maintained its integrity and hence stiffness for 12–15 weeks without noticeable morbidity.

A potential risk for the patient may be stent fragments in the final period of biodegradation that may narrow the airways.[18] Thus, in every case, starting at 12 weeks after implantation, we checked the stent condition every 2 weeks using bronchofiberoscopy.

In the treatment of tracheobronchomalacia, airway stenting should be considered as a time-buying procedure. A low tendency to form mucosal granulation tissue, biocompability and the possibility of implanting another stent with the diameter adequate to the dimensions of the growing airway naturally favor bio-stents. But, on the other hand, a limiting factor can be the rather short time of complete biodegredation and the need of frequent re-stenting.

Conclusions

Despite our limited experience with bio-stents, we consider PDS stents an effective, safe, repeatable, and reversible option in the treatment of dynamic airway obstruction in children. Further studies should be focused on extending the "working time" of the bio-stents and reducing the thickness of stents and the size of the surgical instruments.

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Isothiazolopyridine Mannich bases and their antibacterial effect

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None declared

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Abstract

Background. Infections caused by multidrug-resistant (MDR) strains, i.e., strains resistant to at least 1 antibiotic of the 3 groups of antibacterial agents, are among the most difficult to treat. New compounds with an antimicrobial action are being sought in order to avoid the complete resistance of bacteria to drugs and the spread of MDR strains.

Objectives. The aim of the research was to determine the antimicrobial activity of the new isothiazolopyridine derivatives.

Material and methods. All chemicals used were purchased from commercial suppliers. The ^{1}H NMR spectra were recorded on a Bruker 300 MHz NMR spectrometer. Infrared (IR) spectra were run on a Perkin-Elmer Spectrum Two UATR FT-IR spectrometer (Perkin–Elmer, Waltham, USA). Elemental analyses were carried out on a Carlo Erba NA 1500 analyzer (Carlo Erba Reagents SAS, Val de Reuil, France). Melting points were determined with a Mel-Temp II apparatus (Laboratory Devices, Holliston, USA). The bacteria panel, including *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 (MDR), *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, and multidrug-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) were cultured in Muller–Hinton broth (MHB) at 37°C overnight. Colistin, Polymyxin B, Vancomycin, and Daptomycin were used as controls of bacterial inhibitors. Inhibition of bacterial growth was determined visually and was recorded at 32 μg/mL; 100% inhibition was identified.

Results. The new dimethylisothiazolopyridines were prepared by the Mannich reaction. The structures of the isothiazolopyridines were determined based on spectral data analysis, such as IR and ^{1}H NMR. The antimicrobial screening of new compounds was performed. In the primary screen, 2 compounds showed antimicrobial activity (minimum inhibitory concentration (MIC) ≤32 μg/mL).

Conclusions. Taking into account the obtained results, it should be stated that the examined compounds did not exceed the activity of reference drugs and, therefore, further research should be carried out in the group of isothiazolopyridine derivatives.

Key words: synthesis, antibacterial agents, isothiazolopyridine

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Despite significant progress in the diagnosis and treatment of infectious diseases, bacterial infections continue to pose a serious threat to human life. According to a report published in 2011 by the World Health Organization, diseases caused by bacteria are one of the leading causes of death. Naturally occurring compounds and compounds obtained with chemical synthesis, which show bacterial growth inhibitory effect (bacteriostatic agents) or cause the death of bacteria (antimicrobial substances), are used to counter infections.

Unfortunately, the problem of drug resistance is growing along with the development of new antibacterial agents. This phenomenon no longer concerns only nosocomial pathogens, but increasingly also non-nosocomial pathogens. Infections caused by multidrug-resistant (MDR) strains, i.e., strains resistant to at least 1 antibiotic of the 3 groups of antibacterial agents, are among the most difficult to treat. In addition, extensively drug-resistant (XDR) strains have emerged, i.e., strains resistant to only 1 antibiotic of 1 or 2 groups of drugs used in the treatment of infections caused by this microorganism.

Some microorganisms are resistant to all available antibiotics and can only be countered with the use of experimental and often potentially toxic drugs – these are the so-called pandrug-resistant (PDR) strains.[1]

The limited possibility of effective treatment of infections results in increased morbidity and mortality due to bacterial infections. This is reflected by the data contained in a report prepared in 2009 by the European Medicines Agency (EMA) and the European Centre for Disease Prevention and Control (ECDC). They reveal that MDR strains cause approx. 25,000 deaths per year throughout the European Union.

The constantly increasing number of infections caused by MDR strains and a decrease in the number of new antibiotics introduced into medical practice may send us back to the "pre-antibiotics era".[2] New compounds with an antimicrobial action are being sought in order to avoid the complete resistance of bacteria to drugs and the spread of MDR strains.

The new antibacterial agents are developed by modifying existing antibacterial drugs or by creating a new class of drugs. Among a large number of new classes of compounds synthesized for this purpose, derivatives of bicyclic structures (benzoxazole, benzothiazole, benzisothiazole, quinolone, qinoxaline) exhibited promising results.[3–9]

In particular, benzisothiozol-3(2H)-one derivatives have a wide range of antimicrobial activity[10,11] and have been reported to react with thiol-containing proteins on target microorganisms, and are particularly potent against actively metabolizing cells.[12,13]

Taking into account the interesting antibacterial activity of benzisothiazoles, 4,6-dimethylisothiazolo[5,4-*b*]pyridine derivatives were synthesized. They can be treated as 7-aza analogues of benzisothiazoles. The new compounds were designed as N-Mannich bases with piperazine or piperidine residues in the side chain. Some authors suggest that such substituents may play the role of a pharmacophore for antibacterial activity.[14,15]

Material and methods

Chemistry

Chemical experimental section

All chemicals used were purchased from commercial suppliers. A dry solvent was obtained according to the standard procedure. The progress of the reaction was monitored by TLC on silica gel 60 F254-coated TLC plates (Fluka Chemie GmbH, Buchs, Switzerland) and visualized by UV light at 254 nm. The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker 300 MHz NMR spectrometer (Bruker, Billerica, USA) in *d*-chloroform ($CDCl_3$), while tetramethylsilane (TMS) was used as an internal reference. Chemical shifts are given in ppm units, and coupling constant values are given in Hz. Infrared (IR) spectra were run on a Perkin-Elmer Spectrum Two UATR FT-IR spectrometer UATR – universal attenuated total reflection; Perkin–Elmer, Waltham, USA), and frequencies are reported in cm^{-1}. The samples were applied as solids. Elemental analyses for carbon, nitrogen and hydrogen were carried out on a Carlo Erba NA 1500 analyzer (Carlo Erba Reagents SAS, Val-de-Reuil, France) and were within ±0.4% of the theoretical value. Melting points were determined with a Mel-Temp II apparatus (Laboratory Devices, Holliston, USA) and were uncorrected.

General procedure for the preparation of 6, 7, 9, 11, 12, 15, and 16

A mixture of 2.5 mmol of 4,6-dimethylisothiazolo[5,4-b]pyridin-3(2H)-one 1 with 0.5 mL of formaline (37% water solution) and 2.5 mmol of corresponding amine (piperazine or piperidine) derivatives in 30 mL of THF was refluxed with stirring for 10–11 h. After this time, the mixture was evaporated to dryness. The crude product was then purified by crystallization from appropriate solvents.

1. 4,6-dimethyl-2-[4-(4-iodophenyl)piperazin-1-yl-methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 6

Anal. $C_{19}H_{21}IN_4OS$ (m.w. 480.36); 55% yield, m.p. 172–174°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1680 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.60 (s, 3H, $CH_{3\text{-}pyridine}$), 2.74 (s, 3H,$CH_{3\text{-}pyridine}$), 2.84–2.88 (m, 4H, 2 × $CH_{2\text{-}piperazine}$), 3.17–3.21 (m, 4H, 2 × $CH_{2\text{-}piperazine}$) 4.71 (s, 2H, CH_2), 6.65–6.68 (m, 2H, PhH), 6.94 (s, 1H, $H_{\beta\text{-}pyridine}$), 7.49–7.52 (m, 2H, PhH)

2. 4,6-dimethyl-2-[4-(2-cyanophenyl)piperazin-1-yl-methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 7
Anal. $C_{20}H_{21}N_5OS$ (m.w. 379.47); 96% yield, m.p. 161–163°C (ethyl acetate), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 2220 (CN), 1670 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.61 (s, 3H, $CH_{3\text{-pyridine}}$), 2.74 (s, 3H,$CH_{3\text{-pyridine}}$), 2.93–2.97 (m, 4H, 2 × $CH_{2\text{-piperazine}}$), 3.23–3.27 (m, 4H, 2 × $CH_{2\text{-piperazine}}$) 4.74 (s, 2H, CH_2), 6.94 (s, 1H, $H_{\beta\text{-pyridine}}$), 6.98–7.03 (m, 2H, PhH), 7.45–7.57 (m, 2H, PhH)

3. 4,6-dimethyl-2-[4-ethyl-2,3-dioxopiperazin-1-yl-methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 9
Anal. $C_{15}H_{18}N_4O_3S$ (m.w. 334.39); 47% yield, m.p. 147–149°C (ethyl acetate), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1670 (C = O), ^{1}H NMR ($CDCl_3$) δ: 1.20 (t, 3H, CH_3, J = 9 Hz) 2.59 (s, 3H, $CH_{3\text{-pyridine}}$), 2.71 (s, 3H,$CH_{3\text{-pyridine}}$), 3.49–3.59 (m, 4H, 2 × $CH_{2\text{-piperazine}}$), 3.70–3.74 (m, 2H, CH_2) 4.90 (s, 2H, CH_2), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$)

4. 4,6-dimethyl-2-[4-(tetrahydrofuran-2-yl-carbonyl)piperazin-1-yl-methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 11
Anal. $C_{18}H_{24}N_4O_3S$ (m.w. 376.47); 42% yield, m.p. 126–128°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1680 (C = O), 1650 (C = O), ^{1}H NMR ($CDCl_3$) δ: 1.92–2.06 (m, 4H, CH_2), 2.59 (s, 3H, $CH_{3\text{-pyridine}}$), 2.68–2.72 (m, 7H, 2 × $CH_{2\text{-piperazine}}$ and $CH_{3\text{-pyridine}}$), 3.53–3.69 (m, 4H, 2 × $CH_{2\text{-piperazine}}$),3.77–3.94 (m, 2H, CH_2), 4.54–4.58 (m, 1H, CH) 4.68 (s, 2H, CH_2), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$)

5. 4,6-dimethyl-2-[(4-methyl-2-phenylpiperazin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 12
Anal. $C_{20}H_{24}N_4OS$ (m.w. 368.49); 27% yield, m.p. 108–112°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1675 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.27 (s, 3H, CH_3), 2.58 (s, 3H, $CH_{3\text{-pyridine}}$), 2.69 (s, 3H, $CH_{3\text{-pyridine}}$), 2.73–2.85 (m, 3H, CH and $CH_{2\text{-piperazine}}$), 3.14–3.18 (m, 2H, $CH_{2\text{-piperazine}}$), 3.74–3.78 (m, 2H, $CH_{2\text{-piperazine}}$), 4.67 (s, 2H, CH_2), 6.89 (s, 1H, $H_{\beta\text{-pyridine}}$), 7.31–7.40 (m, 3H, PhH), 7.49–7.52 (m, 2H, PhH)

6. 4,6-dimethyl-2-[((4-piperidin)piperidin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 15
Anal. $C_{19}H_{28}N_4OS$ (m.w. 360.51); 74% yield, m.p. 162–164°C (ethyl acetate), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1670 (C = O), ^{1}H NMR ($CDCl_3$) δ: 1.40–1.42 (m, 2H, $CH_{2\text{-piperidine}}$), 1.52–1.60 (m, 5H, 2 × $CH_{2\text{-piperidine}}$ and CH), 1.78–1.83 (m, 2H, $CH_{2\text{-piperidine}}$) 2.17–2.25 (m, 2H, $CH_{2\text{-piperidine}}$), 2.34–2.41 (m, 2H, $CH_{2\text{-piperidine}}$), 2.46–2.50 (m, 4H, $CH_{2\text{-piperidine}}$) 2.59 (s, 3H, $CH_{3\text{-pyridine}}$), 2.72 (s, 3H, $CH_{3\text{-pyridine}}$), 3.04–3.09 (m, 2H, $CH_{2\text{-piperidine}}$), 4.64 (s, 2H, CH_2), 6.91 (s, 1H, $H_{\beta\text{-pyridine}}$)

7. 4,6-dimethyl-2-[((4-benzyl-4-hydroxy)piperidin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 16
Anal. $C_{21}H_{25}N_3O_2S$ (m.w. 383.50); 63% yield, m.p. 106–108°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1660 (C = O), ^{1}H NMR ($CDCl_3$) δ: 1.52–1.57 (m, 2H, $CH_{2\text{-piperidine}}$), 1.69–1.79 (m, 2H, $CH_{2\text{-piperidine}}$) 2.59 (s, 3H, $CH_{3\text{-pyridine}}$), 2.67–2.72 (m, 5H, $CH_{3\text{-pyridine}}$ and $CH_{2\text{-piperidine}}$), 2.76 (s, 2H, CH_2), 2.78–2.82 (m, 2H, $CH_{2\text{-piperidine}}$), 4.65 (s, 2H, CH_2), 6.92 (s, 1H, $H_{\beta\text{-pyridine}}$), 7.18–7.21 (m, 2H, PhH), 7.24–7.33 (m, 3H, PhH)

General procedure for the preparation of 3, 4, 5, 10, 13 and 14

To a stirred mixture of 2.4 mmol of 2-hydroxymethyl-4,6-dimethylisothiazolo[5,4-b]pyridin-3(2H)-one 2 in 20 mL of ethanol, 2.4 mmol of appropriate amine was added and the stirring was continued for 24 h at room temperature. Then the precipitated crude product was filtered off and crystallized from the appropriate solvent.

1. 4,6-dimethyl-2-[(4-(2,3-dichlorophenyl)piperazin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 3
Anal. $C_{19}H_{20}Cl_2N_4OS$ (m.w. 423.36); 74% yield, m.p. 159–161°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1660 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.60 (s, 3H, $CH_{3\text{-pyridine}}$), 2.74 (s, 3H,$CH_{3\text{-pyridine}}$), 2.89–2.93 (m, 4H, 2 × $CH_{2\text{-piperazine}}$), 3.06–3.09 (m, 4H, 2 × $CH_{2\text{-piperazine}}$) 4.74 (s, 2H, CH_2), 6.91–6.98 (m, 2H, PhH and $H_{\beta\text{-pyridine}}$), 7.12–7.16 (m, 2H, PhH)

2. 4,6-dimethyl-2-[(4-(3,4-dichlorophenyl)piperazin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 4
Anal. $C_{19}H_{20}Cl_2N_4OS$ (m.w. 423.36); 65% yield, m.p. 146–148°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1660 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.60 (s, 3H, $CH_{3\text{-pyridine}}$), 2.74 (s, 3H,$CH_{3\text{-pyridine}}$), 2.84–2.87 (m, 4H, 2 × $CH_{2\text{-piperazine}}$), 3.17–3.20 (m, 4H, 2 × $CH_{2\text{-piperazine}}$) 4.71 (s, 2H, CH_2), 6.70–7.74 (m, 2H, PhH) 6.91–6.94 (m, 2H, PhH, and $H_{\beta\text{-pyridine}}$),

3. 4,6-dimethyl-2-[(4-(2,4-difluorophenyl)piperazin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 5
Anal. $C_{19}H_{20}F_2N_4OS$ (m.w. 390.45); 79% yield, m.p. 130–131°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1675 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.59 (s, 3H, $CH_{3\text{-pyridine}}$), 2.73 (s, 3H,$CH_{3\text{-pyridine}}$), 2.87–2.91 (m, 4H, 2 × $CH_{2\text{-piperazine}}$), 3.03–3.06 (m, 4H, 2 × $CH_{2\text{-piperazine}}$) 4.71 (s, 2H, CH_2), 6.74–6.82 (m, 3H, PhH), 6.85–6.91 (m, 2H, PhH), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$)

4. 4,6-dimethyl-2-[4-cyclohexylcarbonyl-piperazin-1-yl-methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 10
Anal. $C_{20}H_{28}N_4O_2S$ (m.w. 388.52); 39% yield, m.p. 125–128°C (cyclohexane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1680 (C = O), 1650 (C = O), ^{1}H NMR ($CDCl_3$) δ: 1.21–1.25 (m, 4H, CH_2), 1.43–1.51 (m, 2H, CH_2), 1.64–1.67 (m, 2H, CH_2), 1.75–1.78 (m, 2H, CH_2), 2.37–2.45 (m, 1H, CH) 2.59 (s, 3H, $CH_{3\text{-pyridine}}$), 2.67–2.70 (m, 4H, 2 × $CH_{2\text{-piperazine}}$) 2.72 (s, 3H, $CH_{3\text{-pyridine}}$), 3.49–3.51 (m, 2H, $CH_{2\text{-piperazine}}$), 3.62–3.65 (m, 2H, $CH_{2\text{-piperazine}}$), 4.68 (s, 2H, CH_2), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$)

5. 4,6-dimethyl-2-[(4-n-hexylpiperazin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 13
Anal. $C_{19}H_{30}N_4OS$ (m.w. 362.53); 74.6% yield, m.p. 78–80°C (n-heptane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1650 (C = O), ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, 3H, CH_3, J = 6 Hz), 1.21–1.29 (m, 6H, CH_2), 1.43–1.52 (m, 2H, CH_2), 2.29–2.35 (m, 2H, CH_2), 2.44–2.49 (m, 4H, $CH_{2\text{-piperazine}}$), 2.58 (s, 3H, $CH_{3\text{-pyridine}}$), 2.71 (s, 3H, $CH_{3\text{-pyridine}}$), 2.74–2.78 (m, 4H, $CH_{2\text{-piperazine}}$), 4.64 (s, 2H, CH_2), 6.91 (s, 1H, $H_{\beta\text{-pyridine}}$)

6. 4,6-dimethyl-2-[(4-phenylpiperidin-1-yl)methyl]isothiazolo[5,4-b]pyridin-3(2H)-one 14
Anal. $C_{20}H_{23}N_3OS$ (m.w. 353.48); 80% yield, m.p. 136–138°C (n-heptane), FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1670 (C = O), ^{1}H NMR ($CDCl_3$) δ: 1.80–1.86 (m, 5H, 2 × $CH_{2\text{-piperidine}}$ and CH), 2.43–2.51 (m, 2H, $CH_{2\text{-piperidine}}$) 2.61 (s, 3H, $CH_{3\text{-pyridine}}$), 2.75 (s, 3H, $CH_{3\text{-pyridine}}$), 3.12–3.18 (m, 2H, $CH_{2\text{-piperidine}}$), 4.71 (s, 2H, CH_2), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$), 7.19–7.23 (m, 2H, PhH), 7.27–7.32 (m, 3H, PhH)

General procedure for the preparation of 17 and 18

To a solution of 0.01 mol of 4,6-dimethylisothiazolo[5,4-b]pyridin-3(2*H*)-one 1 in 20 mL of ethanol, 0.8 mL of formaline (37% water solution) was added and the mixture was refluxed for 15 min. After this time, the mixture was cooled, and 0.005 mol of piperazine or ethylenediamine was added to the precipitated product 2. Next, the mixture was refluxed for 1 h. After cooling, the precipitated crude product 17 and 18 was filtered off and crystallized from the ethanol.

1. 1,4-bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo[5,4-b]pyridin-2-ylmethyl)piperazine 17
Anal. $C_{22}H_{26}N_6O_2S_2$ (m.w. 470.60); 44% yield, m.p. 233–235°C, FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 1680 (C = O), ^{1}H NMR ($CDCl_3$) *δ*: 2.59 (s, 6H, 2 × $CH_{3\text{-pyridine}}$), 2.72 (s, 6H, 2 × $CH_{3\text{-pyridine}}$), 2.76 (s, 8H, 4 × $CH_{2\text{-piperazine}}$), 4.65 (s, 4H, 2 × CH_2), 6.92 (s, 2H, 2 × $H_{\beta\text{-pyridine}}$)

2. 1,2-bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo[5,4-b]pyridin-2-ylmethyl)ethylenediamine 18
Anal. $C_{20}H_{24}N_6O_2S_2$ (m.w. 443.58); 42% yield, m.p. 148–150°C, FT-IR (UATR, selected lines) ν_{max}/cm^{-1}: 3450–3400 br(NH), 1670 (C = O), ^{1}H NMR ($CDCl_3$) δ: 2.55 (s, 6H, 2 × $CH_{3\text{-pyridine}}$), 2.67 (s, 6H, 2 × $CH_{3\text{-pyridine}}$), 3.04 (s, 4H, 2 × CH_2), 3.81 (s, 2H, 2 × NH), 4.74 (s, 4H, 2 × CH_2), 6.88 (s, 2H, 2 × $H_{\beta\text{-pyridine}}$)

Pharmacology

The antimicrobial screening of new compounds was performed by CO-ADD (Community for Open Antimicrobial Drug Discovery, Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia).[16] Isothiazolopyridines 3–18 were tested for bacterial growth inhibitory effect against a primary panel including *Escherichia coli*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and multidrug-resistant *Staphylococcus aureus* (MRSA) at a single point concentration of 32 μg/mL and MICs determined for the "hit" compounds from the primary screen. Colistin and Polymyxin B were used as positive inhibitor controls for Gram-negative bacteria. Vancomycin and Daptomycin were used as positive inhibitor controls for Gram-positive bacteria.

Single point bacterial inhibition assay

The primary bacteria panel, including *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 (MDR), *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 43300 (MRSA) were cultured in Muller–Hinton broth (MHB) at 37°C overnight. A sample of each culture was then diluted 40-fold in fresh MHB and incubated at 37°C for 1.5–3 h. The compounds were plated at a test concentration of 64 μg/mL. Colistin, Polymyxin B, Vancomycin, and Daptomycin were serially diluted twice across the wells, with compound concentrations ranging from 0.03 μg/mL to 0.64 μg/mL, as controls of bacterial inhibitors. The resultant mid-log phase cultures were diluted to the final concentration of 5×10^5 CFU/mL, then 50 μL was added to each well of the compound-containing 96-well plates (Corning; Cat. No. 3641, NBS), giving a final compound concentration range from 0.015 μg/mL to 32 μg/mL for control inhibitors and 32 μg/mL for test compounds. All the plates were covered and incubated at 37°C for 24 h.

Inhibition of bacterial growth was determined visually and was recorded at 32 μg/mL; 100% inhibition was identified.

MIC assay

The primary bacteria panel, including *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 (MDR), *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 43300 (MRSA) were cultured in MHB at 37°C overnight.

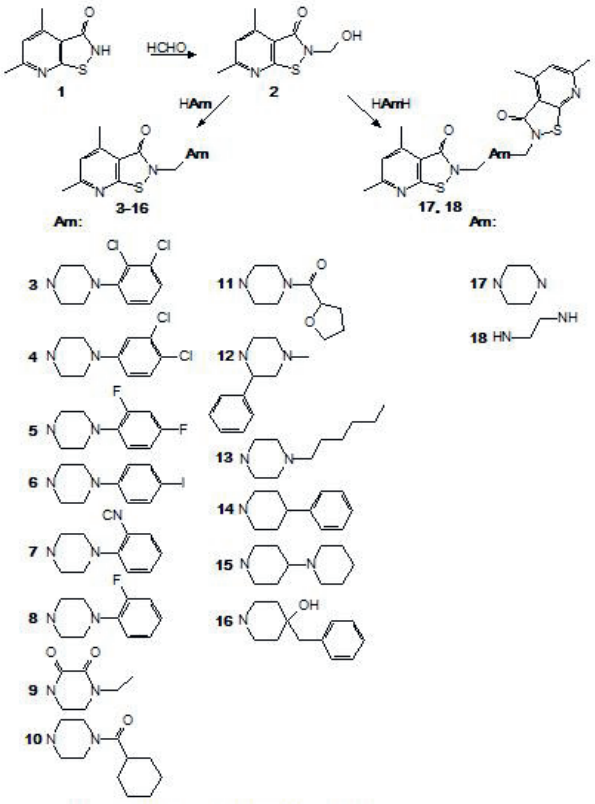

Fig. 1. Synthetic route of compounds 3-18

Table 1. Single point bacterial inhibition results

Compound	*E.coli* ATCC 25922	*K. pneumoniae* ATCC 700603 (MDR)	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 43300 (MRSA)
concentration [μg/mL]					
3	>32	>32	>32	>32	>32
4	>32	>32	>32	>32	>32
5	>32	>32	>32	>32	>32
6	>32	>32	>32	>32	>32
7	>32	>32	>32	>32	>32
8	>32	>32	>32	>32	>32
9	>32	>32	>32	>32	>32
10	>32	>32	>32	>32	>32
11	>32	>32	>32	>32	>32
12	>32	>32	32	>32	>32
13	>32	>32	32	>32	32
14	>32	>32	>32	>32	>32
15	>32	>32	>32	>32	>32
16	>32	>32	>32	>32	>32
17	>32	>32	>32	>32	>32
18	>32	>32	>32	>32	>32
Colistin	0.06	0.03	0.06	0.25	>32
Polymyxin B	0.06	0.03	0.03	0.25	>32
Vancomycin	>32	>32	>32	>32	1
Daptomycin	>32	>32	>32	>32	1

MDR = multidrug resistance; MRSA = methicillin resistant *Staphylococcus aureus*.

A sample of each culture was then diluted 40-fold in fresh MHB and incubated at 37°C for 1.5–3 h. The compounds were serially diluted twice across the wells of non-binding surface 96-well plates (Corning; Cat. No. 3641, NBS), with compound concentrations ranging from 0.03 μg/mL to 64 μg/mL, plated in duplicate. The resultant mid-log phase cultures were diluted to the final concentration of 5×10^5 CFU/mL, then 50 μL was added to each well of the compound-containing 96-well plates, giving a final compound concentration range from 0.015 μg/mL to 32 μg/mL. All the plates were covered and incubated at 37°C for 24 h.

Inhibition of bacterial growth was determined visually after 24 h, where the MIC is recorded as the lowest compound concentration with no visible growth.

Results

The target dimethylisothiazolopyridine derivatives 3–18 were prepared by the Mannich reaction according to the general procedure shown in Fig. 1. The key intermediates: 4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo[5,4-*b*]pyridine 1 and 2H-2-hydroxymethyl-4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo[5,4-*b*]pyridine 2, were synthesized according to the method described recently.[17] The preparation of the final isothiazolopyridines 3–18 involved the condensation of substrate 2 and the appropriate derivatives of secondary amine (piperazine, piperidine and ethylenediamine). Compound 8 was obtained earlier in another project.[18] The reactions were carried out under modified conditions in relation to those described earlier.[19] Under these conditions, most of the products were obtained with a high yield. The purity of the synthesized compounds was checked by elemental analyses. The structures of the isothiazolopyridines were determined based on spectral data analysis, such as IR and ^{1}H NMR.

The IR spectrum of compounds 3–18 showed a characteristic peak at 1650–1680 cm^{-1} due to the carbonyl function of isothiazolopyridine in position 3. In the case of compounds 9, 10 and 11, additional peaks of the carbonyl groups appeared.

The ^{1}H NMR spectra of compounds 3–16 displayed characteristic singlet signals derived from methyl groups of isothiazolopyridine appeared at 2.55–2.75 ppm integrating for 3 protons. In the case of compounds 17 and 18, these signals had twice the integration corresponding to 6 protons.

In the primary screen 2 compounds – 12 and 13 – showed antimicrobial activity that could be considered a "primary hit", where a "primary hit" is defined as an MIC ≤ 32 μg/mL. MIC values were determined for these compounds against the strains, where activity was seen in the primary screen. None of these compounds were considered suitable for further investigation following MIC confirmation, as the cut-off value was ≤16 μg/mL, which none of the compounds possessed in these assays. A full set of results is presented in Table 1.

Conclusions

In order to develop novel antimicrobial compounds, a series of isothiazolopyridine derivatives was synthesized and characterized by physiochemical and spectral means. The synthesized compounds were evaluated for their antimicrobial potentials. From all the compounds, only compounds 12 and 13 were found to be effective against *A. baumannii* and *S. aureus* strains. However, these compounds showed weaker activity compared to the reference drugs.

It should be added that the low antibacterial activity of the compounds may be the result of insufficient solubility of the compounds under the conditions in which the tests were carried out.

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Co-expression of the aryl hydrocarbon receptor and estrogen receptor in the developing teeth of rat offspring after rat mothers' exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the protective action of α-tocopherol and acetylsalicylic acid

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

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Conflict of interest
None declared

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Abstract

Background. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can cause adverse effects in many organs. Toxic effects are caused due to the formation of a TCDD complex with the cytoplasmatic aryl hydrocarbon receptor (AhR), whose mechanism of action is similar to that of the estrogen receptor (ER). Some substances, including α-tocopherol (E) and acetylsalicylic acid (ASA), can reduce the toxic effects of TCDD in offspring.

Objectives. The objective of this study was to evaluate the co-expression of AhR and ER in the incisors of rat offspring whose mothers were exposed to TCDD, using immunohistochemical and histological techniques. Moreover, the possible protective role of E and ASA was investigated.

Material and methods. Four groups of 2-day-old rat offspring, whose mothers were intoxicated by TCDD before mating, were established: control group (C), TCDD group, TCDD+E group and TCDD+ASA group.

Results. In the TCDD group, there was an increase in ER expression and a decrease in AhR expression in comparison with the C group. In the TCDD+E and TCDD+ASA groups, there was a weak or negative ER expression and slightly stronger expression of AhR than in the TCDD group.

Conclusions. The co-expression of AhR and ER during tooth development suggests the role of AhR and ER in the control of this process. Both receptors are also involved in the process of detoxification of TCDD. The increase in AhR in TCDD+E and TCDD+ASA groups indicate a preventive action of antioxidant and anti-inflammatory pharmaceutics, which may limit negative effects of TCDD.

Key words: rat, estrogen receptor, tooth, dioxin, aryl hydrocarbon receptor

Cite as
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Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the strongest dioxins, which is why exposure to it may induce severe malfunctions of many organs.[1,2] Our study and those of other authors showed that TCDD induces inflammatory processes and activates the processes of enzymatic digestion of connective tissue elements.[3,4] On the other hand, dioxins inhibit alkaline phosphatase in osteoblasts, thus disturbing the mineralization process.[5,6] Moreover, TCDD inhibits collagen I synthesis by altering gene expression and induces oxidative stress, leading to the activation of interleukins, which further activate osteoclastogenesis.[5,7] Disturbed fiber synthesis of type I collagen may result in a defective structure of dentin and cementum as well as the bone. The dioxin may influence the structure of teeth and the bone, especially during their development. In our previous studies, we found that there is a transmission of toxic effects of dioxins to the offspring of mothers who were exposed to TCDD.[4] Other authors reported that dioxins may alter calcium deposition in mineralized tissues by influencing the activity of some hormones (estrogens, corticosterone, T3).[8–11]

The aryl hydrocarbon receptor (AhR) plays a key role in the pathomechanism of TCDD-induced intoxication.[12–19] Apart from the classical response to xenobiotic exposure, this receptor is also involved in diverse endogenous processes, such as cell proliferation, T cell differentiation, hematopoietic stem cell expansion/differentiation, lung response to polycyclic aromatic hydrocarbons, and glucose tolerance via mechanisms that are not clearly defined for the most part.[18,19]

To prevent the expansion of the inflammation process caused by biotransformation of TCDD, numerous pharmaceutics have been used, i.e., α-tocopherol (E), a powerful antioxidant antagonist of AhR,[7,12,17,20–22] and acetylsalicylic acid (ASA).[8,9,21]

During development, estrogen controls numerous processes, including cell proliferation and differentiation via estrogen receptors (ER). Subsequently to hormone binding and transformation, receptor-ligand complexes interact with specific hormone response elements on target genes, which results in the regulation of transcription.[23]

Two classes of ER exist: nuclear estrogen receptors (ERα and ERβ), which are members of the nuclear receptor family of intracellular receptors, and membrane ERs, which are mostly G protein-coupled receptors. Estrogen receptors are regarded to be cytoplasmic receptors in their unliganded state; however, immunocytochemical research has shown that only a small fraction of ERs reside in the cytoplasm, while most ERs are found constitutively in the nucleus. Estrogen receptors have been reported to occur in fish, lizards and several mammals, including humans.[23]

Both ERs are widely expressed in different tissue types; however, there are several differences in their expression patterns. A wide distribution of ERα immunoreaction has been reported in fibroblasts, endothelial cells, mammary gland cells, ameloblasts, odontoblasts, chondrocytes, osteoblasts, and osteoclasts in addition to female reproductive tissues, which suggests that there is a diverse effect of estrogen on development, growth, and homeostasis.[23,24]

Estrogens play an important role during tooth development and its survivability in the surrounding tissues.[25,26] Alhodhodi et al. suggest that estrogens stimulate osteogenic differentiation and that this action is mediated mainly through the ERβ isoform. These findings may have profound consequences in terms of the investigation and treatment of oral pathologies, which are associated with imbalances in estrogen concentrations.[24,26] Nebel proved that estrogen affects chemokine expression in periodontal cells, which shows a complex pattern involving downregulation as well as upregulation of chemokines. Estrogen exerts both anti-inflammatory and proinflammatory effects through these mechanisms.[22,27] Therefore, the loss of the ERβ function during development may have an impact on periodontal diseases and tooth decay.[28]

Enamel formation and its subsequent maturation to become the hardest tissue in mammals are closely related to the differentiation of ameloblasts and morphological changes in them.[23,25] From the apical to the incisal end, rat incisor ameloblasts are classified regionally into presecretory, secretory, transitional, maturation, pigmentation, and reduced ameloblasts according to their maturation level.[23,25] Immunohistochemistry of the rat enamel organ revealed ERα expression as nuclear localization in ameloblasts as well as in mature and immature odontoblasts. The ERα pattern exhibited a periodic change at the maturation stage coherent with constant higher labeling in ruffle-ended ameloblasts than in smooth-ended ameloblasts. These findings suggest a possible role of ERα in ameloblast proliferation and differentiation.[23] In addition, Jukic et al. reported that several odontoblasts in humans exhibited ERα immunoreaction (1.0–1.3%), which suggests that tooth-forming cells are considered to be a target for estrogen action. To our knowledge, there has been no report on the expression of ERα in the cytoplasm in rat ameloblasts and associated structures.[29] There are no reports concerning TCDD-influence on tooth development and the co-expression of AhR and ER, and the protective role of chosen anti-inflammatory pharmaceutics.

The aim of this study was to identify AhR and ER co-expression in the developing incisors of rats, whose mothers were exposed to TCDD and treated with ASA or E. Additionally, it was evaluated whether there are possibilities of reducing potential post-dioxin defects in the structure of enamel and dentin in the intoxicated mothers' offspring by simultaneous application of α-tocopherol or ASA.

Material and methods

To conduct the experiment, 40 offspring from 24 female Buffalo rats (with body mass 130–150 g, at the age of 9–11 weeks) were used. The study was approved by the Local Ethical Committee on Animal Testing (permission No. 38/2009).

All female rats were kept in polystyrene cages (60 cm × 40 cm × 40 cm) with metal lids (6 animals per cage). The experiments were carried out under standard conditions. The rats were fed with standard Labofeed H feed and received water ad libitum.

The experimental animals were divided into 4 groups of 6 females, from which infants for investigation were obtained:

- control group (C)
- TCDD group – females, 3 weeks before mating, into which 5 μg/kg b.w. of TCDD (Greyhound Chromatography and Allied Chemicals, Birkenhead, UK) was injected intramuscularly.
- TCDD+E group – females, 3 weeks before mating, to which TCDD was administered as in the TCDD group and into which, additionally, α-tocopherol acetate (Hasco-Lek SA, Wrocław, Poland) at a dose of 30 mg/kg b.w. s.c. was injected every day for 3 weeks
- TCDD+ASA group – females, also 3 weeks before mating, in the case of which the TCDD injection pattern was the same as in the TCDD+E group and to which ASA (Bayer Polska, Warszawa, Poland) at a dose of 50 mg/kg b.w. p.o. was administrated instead of α-tocopherol.

The females from all groups were mated with randomly chosen males from the same strain that were not exposed to any chemical substances. After birth, 2-day old infants were euthanatized with Phenobarbital (Morbital®; Biowet, Puławy, Poland) and mandibles from 10 infant rats from each of the 4 groups were taken for analysis. Samples of incisors and the periodontal tissue were placed in a 4% buffered formalin solution for 48 h. Next, the material was rinsed in tap water and decalcified in ethylenediaminetetraacetic acid (EDTA), dehydrated in alcohol series, cleared in methyl benzoate, and embedded in paraffin.

For immunocytochemical analysis, serial sections 7–9 μm thick were cut, deparaffinized in xylene and rehydrated in alcohol series. For each of the antibodies, a minimum of 3 slides were used. Negative and positive probe was performed. Endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent (DAKO, Gdynia, Poland). Then, the sections were rinsed twice for 5 min in distilled water. Afterwards, the sections were digested with proteinase K (DAKO) and rinsed again twice for 5 min in distilled water. The sections were incubated with rabbit Anti Human AhR primary antibody (Serotec, Kidlington, UK) or Anti-Estrogen Receptor antibody, rabbit monoclonal antibody (Sigma–Aldrich, Dorset, UK) for 1 h in a dilution of 1:80; then, the sections were rinsed twice in phosphate-buffered saline solution (PBS) solution (pH 7.3) for 5 min. Later, the Novolink MinPolymer DS Novocastra (Leica Biosystems, Wetzlar, Germany) kit was used. The next stage involved staining with DAB+ substrate buffer and DAB+ chromagen (DAKO) visualization system; Mayer's hematoxylin (Merck, Darmstadt, Germany) was used to stain cell nuclei. The material was analyzed with a Nikon Eclipse 80i microscope (Nikon Corp., Minato, Tokyo, Japan).

In all subjects from each study group, the degree of expression of a given receptor was analyzed in 100 cells in at least 3 fields of view. Each individual cell received a numerical value 0–3 corresponding to the intensity of the reaction. Next, the average value for each of the studied groups was calculated. The statistical analysis of the obtained data of individual groups was performed with STATISTICA v. 12.0 (StatSoft Inc., Tulsa, USA) using the Student's test.

Results

In the C group, a differentiation process of the enamel organ was observed at the apex of the incisor. The cells are arranged in a columnar epithelium, the cells of which proliferate, differentiate and secrete enamel and dentin. At this stage, both enamel and dentin are not mineralized. The amount of the tooth matrix is slowly reduced towards the neck and root as well as the number of cells, which becomes undifferentiated (Fig. 1). The expression of the ER in the cytoplasm in odontoblasts, ameloblasts, cementoblasts, and osteoblasts is very weak or completely negative in the enamel organ (Fig. 1). A positive reaction for AhR was noted in the majority of cells of the developing teeth (Fig. 2). A clearly positive immunohistochemical reaction to AhR was noted in the inner enamel epithelium, whereas this reaction is weaker on the outer surface. A weaker reaction was noted in odontoblasts compared to ameloblasts. Aryl hydrocarbon receptor was expressed at different levels in the pulp, alveolar bone and connective tissue surrounding the teeth (Fig. 3).

In the TCDD group, the development of enamel and dentin was retarded. Ameloblasts were mostly cuboidal, rarely cylindrical. Odontoblasts were in close relation with mesenchymal cells of the pulp. The amount of synthesized dentin and enamel was twice as small as in the C group. An increased expression of the ER was mainly observed in odontoblasts and ameloblasts, but also in mesenchymal cells. The main localization of the ER was in the cytoplasm. This expression was noted in ameloblasts in the odontoblastic layer of the papilla (Fig. 1). In this group, a much weaker immunohistochemical reaction to AhR has been observed in ameloblasts (Fig. 2). In less differentiated ameloblasts, AhR is rarely present. The immunohistochemical reaction in the mesenchymal cells area is also weaker. The immunohistochemical reaction to AhR is primarily seen in cells of epithelial origin in the cytoplasm (Fig. 3).

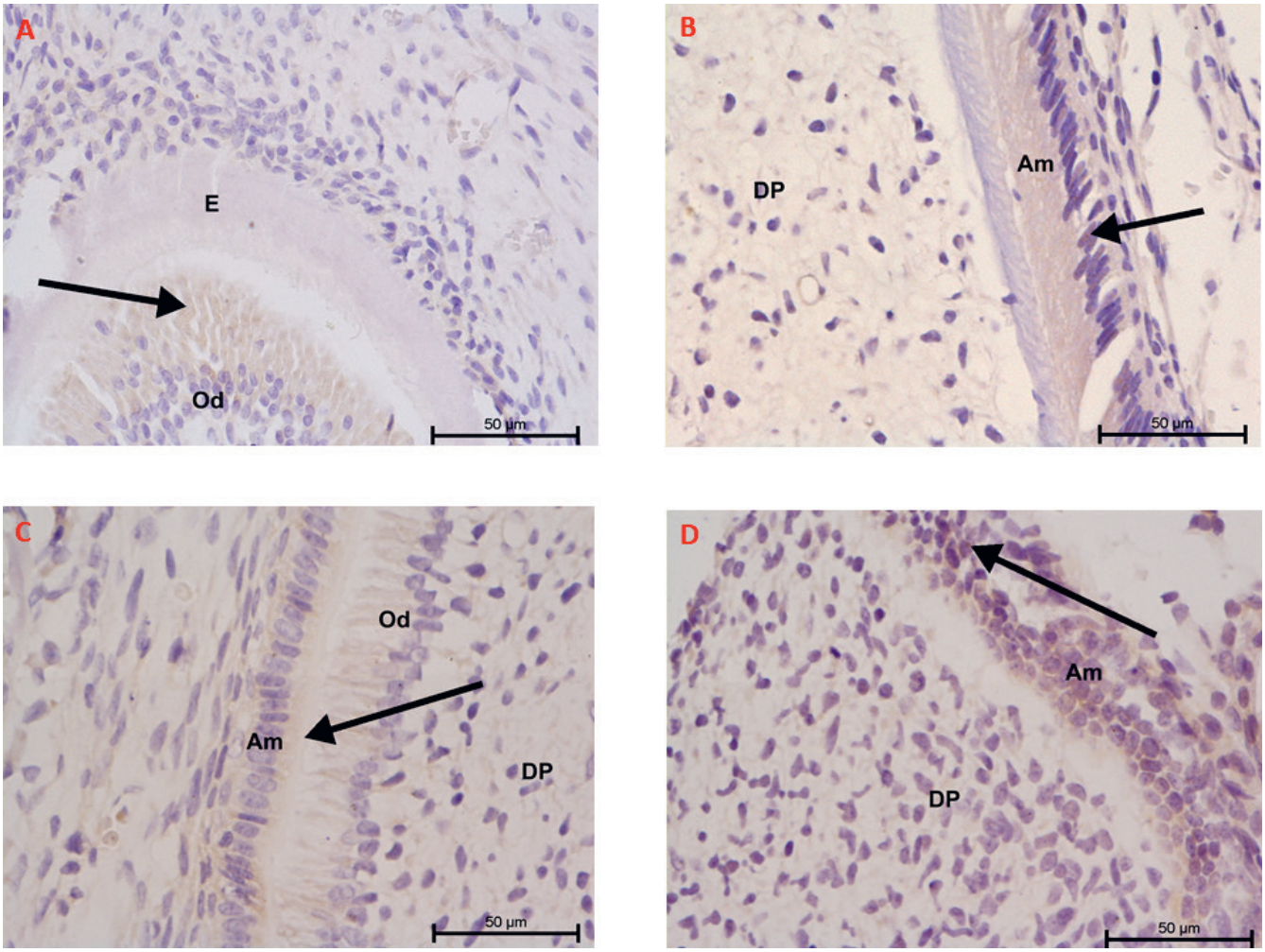

Fig. 1. Expression of ER in rat incisors in respective groups

A – control group; the apical portion of rat incisor; weak positive reaction to estrogen receptor (ER) (arrow) in odontoblasts (Od); note the lack of ameloblasts on enamel (letter "E") (×600 magnification); B – TCDD group; the lateral portion of the tooth; intensive synthesis of enamel by cylindrical, ER-positive in nucleus (arrow) ameloblasts (Am); dental pulp (DP) contains numerous capillary vessels (×600 magnification); C – TCDD+E group; very weak positive reaction to ER in cuboidal Am and cylindrical Od in area adjacent to the apical portion of the tooth; between layers of Am and Od cells thin layer on enamel (arrow) is visible (×600 magnification); D – TCDD+ASA group; numerous Am on the apical part of the tooth are ER-negative in cytoplasm; single cells in this region are ER-positive in nucleus (arrow); DP and surrounding mesenchyme are less developed than the C group (×600 magnification).

In the TCDD+E group, the histological image of developing incisors is similar to the image seen in the TCDD group. However, ameloblasts become mostly cylindrical. There are fewer cells at the differentiation stage (cuboidal) (Fig. 1). A weak or negative reaction to AhR in the enamel organ was observed (Fig. 2). In the apical and lateral area, the immunohistochemical reaction to the ER was very weak. No ER presence has been observed in the area of dental sac and alveolar bone in the cytoplasm. There was a weak positive reaction in the enamel organ; however, it was a little stronger than in the TCDD group (Fig. 1 and 3).

In the TCDD+ASA group, the effects of developmental disorders in comparison to the TCDD group could be observed. Ameloblasts are mostly cylindrical, but an increased proliferation of odontoblasts is observed. Moreover, numerous cells were polarized, which means that they were more mature than the incisors from C and TCDD groups (Fig. 1). In the enamel organ, the immunohistochemical reaction to AhR and ER was similar to that of the TCDD+E group and was noted in both cytoplasm and the nucleus. A weak immunohistochemical reaction to the ER was observed in odontoblasts and mesenchymal cells adjacent to the pulp (Fig. 1). A positive immunohistochemical reaction to AhR was observed in odontoblasts and mesenchymal cells in the cytoplasm and nucleus (Fig. 2,3).

Discussion

Expression of AhR at different tooth development stages in mice has been precisely described by Sahlberg et al. and Partanen et al.[15,30] In 14-day-old embryos, AhR expression in incisors, epithelium and osteoblasts was observed,

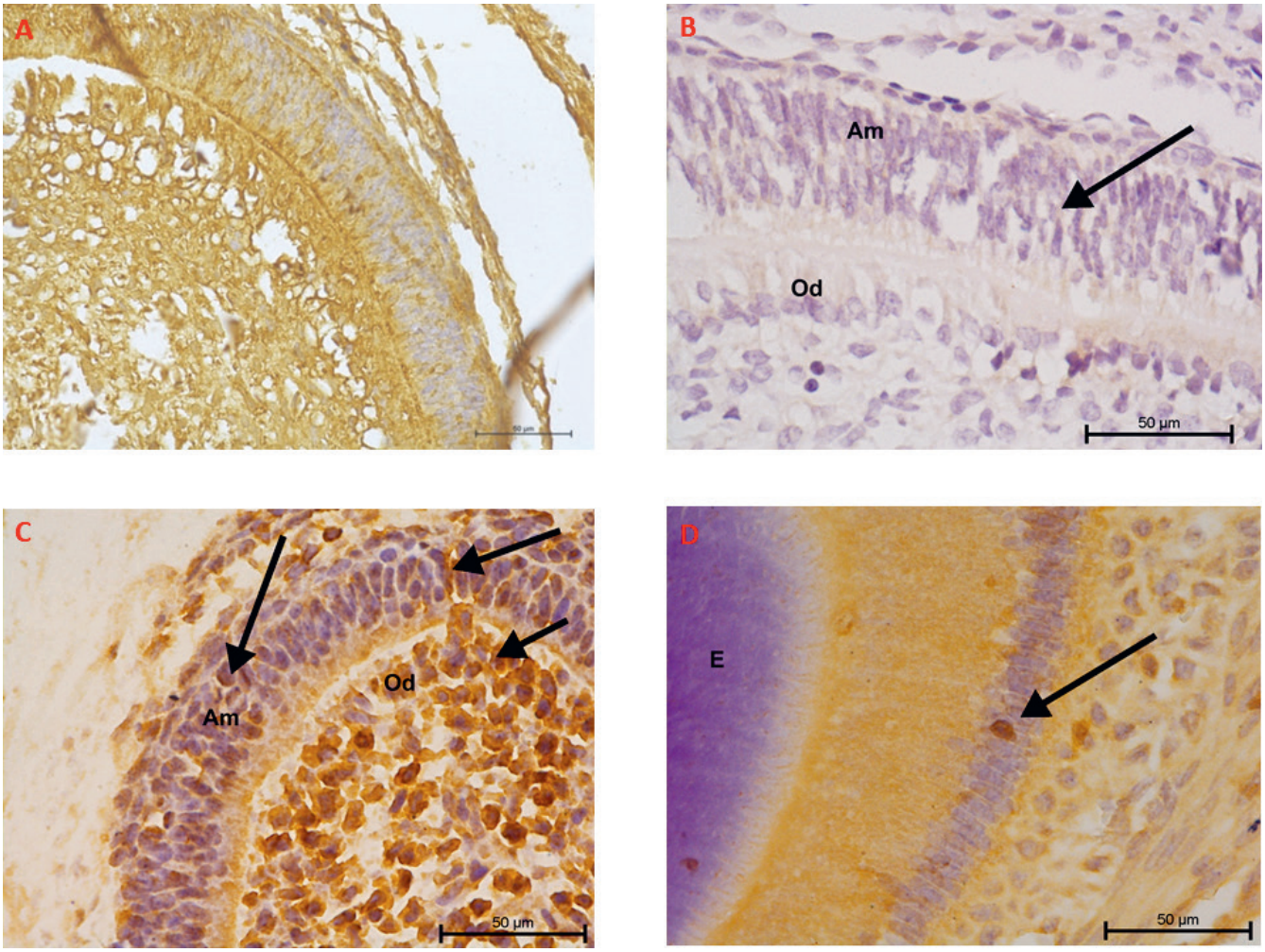

Fig. 2. AhR expression in rat incisors in respective groups

A – control group; the apical portion of rat incisor; strong positive reaction to aryl hydrocarbon receptor (AhR) in nucleus and cytoplasm of odontoblasts (Od) and ameloblasts (Am) (×600 magnification); B – TCDD group; the lateral portion of the tooth next to the apical part of the rat incisor; intensive proliferation of enamel by cylindrical, AhR negative (arrow) Am; Od show weak positive reaction to AhR (×600 magnification); C – TCDD+E group; positive reaction (arrow) to AhR in Am and Od in the apical portion of the tooth (×600 magnification); D – TCDD+ASA group; numerous AhR-positive in cytoplasm Am on the lateral part of the tooth; single cells in this region are AhR-positive in nucleus (arrow); enamel (letter "E") is already mineralized (×600 magnification).

and no AhR expression was observed in the buds of molar teeth. At the bell stage, on the 17th day of embryonic life, the expression of this receptor was visible in the inner enamel epithelium, whereas between the 19th day of embryonic life and the 12th day of ontogenesis – in line with odontoblastic differentiation and dentin formation – AhR expression was observed in preameloblasts and odontoblasts (only after terminating differentiation from mesenchymal tissue). Moreover, it has been found that AhR expression is more intense in ameloblasts and odontoblasts than in the intermediate stratum of the enamel organ or osteoblasts. This changeable expression of AhR in ameloblasts and odontoblasts, which depends on the stage of tooth development, was observed in healthy animals.[9]

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin stimulates inflammation and, thus, reduces the content of collagen fibers in tissues and as a result influences tooth development. Consequently, inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) are released; therefore, fibroblast activity is inhibited and osteoclasts are activated.[3,6,31]

During our own research, the inhibition of a negative impact of TCDD on the dental organ in offspring from the TCDD+E group have correlated with an increased AhR expression after administration of E, especially in the layer of odontoblastic and amelogenic cells compared to the offspring of mothers that were exposed to the dioxin only.[6,30] In rat newborns whose mothers were exposed to TCDD, a positive reaction to AhR was present mainly in epithelial cells but it was rare and of low intensity. This means that AhR, which is present in other areas of the dental organ, is blocked by trace amounts of TCDD in newborn organisms. On the basis of studies on mice genetically deprived of AhR, it has been proved that the lack of this

receptor significantly decreases the effects of dioxins, since the TCDD main pathway is through AhR.[22] According to Gao et al., the administration of TCDD to developing rat newborns reduced the reaction to AhR in ameloblasts to a larger extent than in differentiating dental papilla cells.[10] The observed range of AhR expression in ameloblasts and odontoblasts, demonstrated in our own and cited studies, may result from the difference in offspring age (2-day-old newborns – in case of our own studies, and 9- and 22-day-old in the study by Gao et al.[10]), as well as from the applied TCDD dose (5 μg/kg. b.w. – in case of our own studies and 50 μg/kg. b.w. and 1000 μg/kg b.w. – in the study by Gao et al.[10]). Moreover, it is possible that TCDD attached to AhR was transported into the nucleus. As a result, products of AhR-dependent genes suppress the expression of AhR.

A 3-week administration of E to the dams, from the moment of TCDD exposure to their pregnancy, was supposed to reduce the toxic activity of free radicals produced by the dioxin by inhibiting AhR in a direct and indirect way.[12,17,22,31] Other authors also demonstrated a positive influence of E on animals exposed to TCDD.[22] The administration of E to the dams decreases the concentration of TCDD in the serum.[31,32] Therefore, a smaller amount of this compound or end products is transferred to the fetus via the placenta and results in a higher expression of AhR in offspring in the TCDD+E group. The administration of ASA and E to the dams exposed to TCDD in our studies led to the observed immunohistochemical reaction to AhR present in TCDD+E and TCDD+ASA groups, which was stronger than in the TCDD group. Therefore, the supplementation with E or ASA administered to mothers intoxicated with dioxins before pregnancy could limit the number of defects in developing teeth in newborns.

Several developmental and experimental studies have shown the involvement of a steroid hormone including estrogen in dental tissues.[23,29,33] Osteoblastic activity has been reported to affect hard tissue formation via the activation of its specific ERs.[29] This evidence strongly

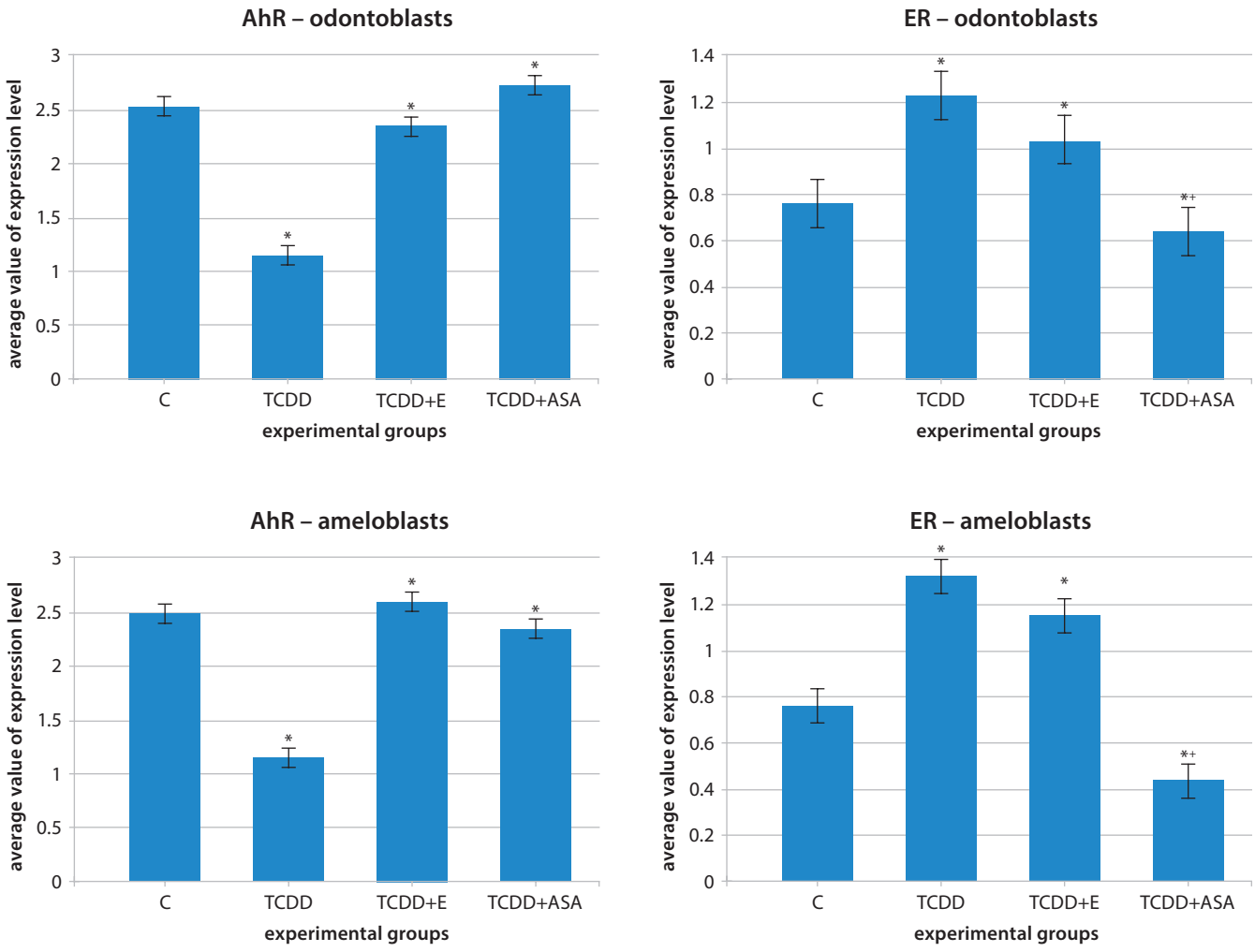

Fig. 3. Average value of aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) expression level in odontoblasts and ameloblasts

* – statistically significant difference at $p < 0.05$ to control group; + – statistically significant difference at $p < 0.05$ to TCDD group.

suggests that hard tissue-forming cells are under the control of estrogen.[23]

Ohtake et al. and Martin et al. studied a dioxin-estrogen-related action in cultured breast and uterine cancer cells.[34,35] The activated ERs dock to control regions of target genes and trigger their transcription, thus initiating a cascade of molecular and cellular events instead of AhR. The same estrogenic actions of dioxins were observed when researchers injected TCDD into ovariectomized mice, which had no circulating estrogens. The AhR-ligand complex was also found to repress the ER function when estrogens are present and bound to the receptors, thus providing an explanation for previous reports of anti-estrogenic activities of dioxins.[29,34]

In offspring, at the moment of birth, the level of estrogen is minimal regardless of the sex of individuals. Along with the age, the level of estrogen increases to approx. 25 pg/mL in the 8th week in females, whereas it shows the same pattern between birth and puberty in males as it does in female littermates.[29,36,37]

Therefore, in the absence of estrogens, an increased expression of ER within the cytoplasm in TCDD-treated animals may be explained by a permanent activation of ER synthesis by TCDD degradation end products or inhibition of its transfer to the nucleus.[36,37]

We have also found that the increased expression of ER was noted in the TCDD group, whereas the expression of AhR decreased. An increased level of ER, as an alternative pathway in the anti-inflammatory process, may be a part of the cellular self-defense against TCDD biotransformation products.[38]

Our findings demonstrate the localization and pattern of distribution of ER immunoreactions in rat ameloblasts and odontoblasts. Estrogen receptors and AhR were differently expressed in the C group and in the TCDD group. When considered together, these findings suggest a possible overlapping role of AhR and ER in the proliferation and differentiation of ameloblasts. However, a detailed mechanism of the involvement of estrogen in enamel formation including synthesis and secretion of an enamel protein remains unclear. Further research is required to clarify this issue.[23]

Conclusions

The observed co-expression of AhR and ER in the cell cytoplasm during development suggests the role of AhR and ER in the control of cell proliferation, differentiation and apoptosis. Both receptors are also involved in the process of detoxification of TCDD. The increase in AhR in TCDD+E and TCDD+ASA groups indicates a preventive action of antioxidant and anti-inflammatory pharmaceutics, which may limit negative effects caused by TCDD.

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin delays embryonic development as a consequence of suppressing the AhR synthesis and through the modulation of expression of estrogen-dependent genes. Moreover, in the TCDD group, ameloblasts and odontoblasts were at an early proliferative stage; therefore, ER was still present in the cytoplasm. The cells from TCDD+E and TCDD+ASA groups did not express ER.

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The role of toll-like receptors in multifactorial mechanisms of early and late renal allotransplant injury, with a focus on the TLR4 receptor and mononuclear cells

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Abstract

The innate immune system is activated before an adaptive immune response. An expression of a particular toll-like receptor (TLR) in a transplanted kidney depends on the localization of specific cells (e.g., endothelium, elements of the nephron structure), recent pathology and the time passed since transplantation. The TLR4 receptor is expressed on renal tubular epithelial (RTE) and endothelial cells, podocytes, blood and interstitial monocytes/macrophages, and dendritic cells. While circulating in blood, some monocytes are attracted and penetrate the transplanted organ, where they supplement the donor's resident macrophages. The intensity of migration depends on the local activation of inflammation in the graft and on the expression of specific receptors on kidney endothelial cells and monocytes/macrophages. The percentage of cells with shifted TLR4 expression usually increases in circulating monocytes. The TLR4 and the biochemical stimulation cascade derived from it in any type of cell, including monocytes, undergo multi-level regulation with feedback loops with other components of the primary system, and are also dependent on the action of immunosuppression. Toll-like receptor 4 senses stimuli that make monocytes contribute differently both to acute/chronic kidney injuries and to the development of tolerance. After kidney transplantation, TLR4 expression and related cytokine production capacity may vary depending on past diseases and oncoming problems. Since conventional immunosuppression does not prevent chronic allograft injury (CAI), peripheral blood monocytes and TLR4 constitute candidates for diagnostic and therapeutic targets. Considering the mutual communication among various elements of the primary immune system, future therapeutic intervention should be directed toward factors directly or indirectly regulating the expression or post-receptor signaling of the TLR4 receptor.

Key words: innate immunity, kidney transplant, toll-like receptor 4, monocyte, allograft injury

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Kidney transplantation is still the most promising therapeutic option for renal failure patients. Such unfavorable events as delayed graft function (DGF) or chronic allograft injury (CAI) limit kidney graft survival. Determining a set of easy-to-use immune markers for individual risk factors can help anticipate upcoming complications and extend median transplant survival. The best recognized sets of immune markers focus on elements of adaptive immunity: antibody- or T-cell mediated rejection.[1–4] The innate system is activated before an adaptive response. Cellular components of the system consist of phagocytic cells (e.g., monocytes/macrophages), antigen-presenting cells (e.g., monocyte-derived dendritic cells), natural killer cells, a subset of T and B cells participating in innate immunity, as well as epithelial and endothelial cells (natural barriers synthesizing cytokines and chemokines, recognizing danger signals).[5] Part of the activity of native immunity is based on pattern recognition receptors (PRRs), which are shared by various cellular compartments, and recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs)[5,6] sensing a "harmed or damaged self".[6,7]

Pattern recognition receptors can be divided either according to localization (into soluble, membrane-bound and intracellular) or according to their functional properties/structural homology (into NOD-like receptors, C-type lectin receptors, retinoic acid-inducible gene-like receptors, and toll-like receptors (TLRs)).[8–10] Activation of PRRs is usually induced by phagocytosis, inflammation and maturation of antigen-presenting cells (e.g., macrophages and dendritic cells). Phagocytosis (induced by the cluster of differentiation (CD36) scavenger receptor recognizing microbial diacylglycerides) is a necessary step for the activation of some other intracellular PRRs (e.g., TLRs).[5] Toll-like receptors are shared in different proportions both by non-immune cells (NIC, e.g., renal epithelial and endothelial cells) and by immune cells.[10] In the kidney, TLR1–4, TLR6 and TLR9 are widely expressed on renal tubular epithelial (RTE) cells. TLR2 and TLR4 are also found on endothelial cells and on podocytes.[7,8] Within the immune system, TLRs are expressed by cells of both the adaptive and innate compartments. Cells of the innate part that express TLRs include granulocytes (Grs), monocytes/macrophages (Mcs/Mfs) and dendritic cells (DCs).[6,7] Granulocytes, mastocytes and Mcs/Mfs are considered key players in early innate allo-immune response, especially in transplantation.[11,12] Part of the peripheral blood monocyte (PBMC) population penetrates the tissues,[6,13] where they undergo transformation into macrophages in ways that are specific to particular organs, e.g., Kupffer cells in the liver or mesangial cells in the kidney. Monocytes are characterized by the surface expression of CD14, CD11b as well as MAC1/3 and CD68.[7] For clinical purposes, monocytes are divided into 2 classes: M1 and M2.[14–16] The M1 class represents activated or pro-inflammatory macrophages and can be induced by interferon γ (IFNγ) or lipopolysaccharide (LPS). Upon stimulation, they may change phenotype to DCs.[14,16] The M2 class monocytes are wound-healing and pro-fibrotic.[14,16] The M2 cells are believed to be hyporesponsive to most TLRs and complement-mediated signals.[15] Renal epithelial cells are normally surrounded by macrophages and DCs, which create a dense network. Macrophages have diverse functions, including traditional inflammatory phagocytic function and trophic roles. They are believed to participate in the maintenance of tissue integrity.[5] All immune cells, including Mcs/Mfs and DCs, have a higher expression of TLR4 than non-immune cells.[12]

This article focuses on particular elements of the innate immune system: TLRs, especially TLR4, mononuclear Mc/Mf cells and their leading role in renal transplant injury.

General consequences of TLR4 stimulation

Like endogenous TLR4 ligands, DAMPs activate monocytes/macrophages cells at high concentrations, which contrasts with activation produced by low concentrations of a conserved lipid A.[17]

Stimulatory ligands – high-mobility group box (HMGB) proteins, fibrinogen (Fb) and uric acid – are among the most recognized DAMPs.[18] HMGB1 is a ligand of TLR4 (i.e., of endothelial cells), which is passively released from injured renal cells.[15,19,20] High local concentrations of HMGB1 can activate both TLR4 and TLR9.[18] Fibrinogen also causes early activation of TLR4. In cultured podocytes, Fb induces a release of chemokines.[21] Negative regulators of TLRs include splice variants of signaling molecules, soluble TLRs, cleavage enzymes, as well as receptors of the TLR/interleukin (IL)-1R family member TIR8, also known as single interleukin-1 receptor-1 (IL-1R1), SIGIRR.[22]

The main factors triggering TLR4 include upregulation of cytokines, chemokines or co-stimulatory molecules.[23] The PAMP/DAMP recognition leading to activation of TLR4 is a sequential process, requiring the cooperation and aggregation of several accessory molecules. Lipopolysaccharide-induced TLR4 activation begins with the transfer of LPS monomers to LPS-binding protein (LBP) and subsequently to cluster of differentiation 14 (CD14, cell membrane-linked or soluble). Finally, the LPS+CD14 complex is transferred to myeloid differentiation factor 2 (MD2), which associates with TLR4.[12,24] Ligand binding to MD2 favors the dimerization of 2 TLR4+MD2 complexes to the form of an activated heterodimer $(LPS.MD2+TLR4)_2$ on the plasma membrane.[12] The co-receptor CD14 is engaged in the initiation of endocytosis of $(TLR4.MD2.LPS)_2$ heterodimers.[5,12,17,19] When TLRs form hetero- or homodimers (e.g., TLR2/TLR6, TLR4/TLR4), an increase in TLR sensing efficiency is gained and subsequent dimerization of the cytoplasmic toll/IL-1 receptor domain

(TIR) activates intracellular signaling cascades.[5,6,12,25] The TLR4 is also the only TLR that activates both the cytosolic adapter protein myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent signaling cascade pathways.[5,12,17,19] Activation of MyD88 to the NF-κB pathway (in cooperation with complement C3 and C5 factors) leads to the production of pro-inflammatory cytokines such as TNF-α and IL-6 through the pro-inflammatory response.[18] The MyD88-independent pathway triggers the activation of interferon regulatory factor 3 (IRF3), leading to the production of type I IFN.[5,12,17,19] There is also crosstalk between TLR4 and TLR3.[26] Due to the crosstalk, different ligands can utilize components of TLR-dependent pathways to various degrees.[19] TLR4-MyD88-NF-κB signaling seems to influence TLR3 function, which explains the synergistic effect between TLR4 and TLR3 triggering in macrophages that has been observed in acute lung injury.[26] As a result of TLR4 stimulation of the dependent biochemical cascades, the generation of various short-chain RNA molecules (miRNA) becomes activated. For example, miRNA-511 positively regulates TLR4 signaling, but miRNA-200c silences MyD88 and impairs the LPS-induced expression of pro-inflammatory cytokines like IL-6, CXCL9 and TNF-α.[27]

The TLR4 senses stimuli which contribute differently to acute kidney injury (AKI) or to CAI, and also to the development of tolerance in either NIC or bone marrow-derived cells (BMDC).[23,28]

Innate immunity: from brain death to reperfusion injury

In brain-dead donors (BDD), the kidney is exposed to an excess of reactive oxygen species (ROS) and DAMPs.[29] Both contribute to maintaining the local inflammatory response, indirectly stimulate a local increase in E-selectin expression, intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1, and favor infiltration of the kidney by donor T cells and macrophages.[18] The transfer of a kidney from the BDD to the recipient includes several unfavorable stages. Endothelial cells, especially RTE cells, residing in the oxygen-sensitive region of the outer stripe of the medulla, are highly susceptible to ischemia. They undergo swelling and upregulate adhesion molecules (P- and E-selectins and ICAM-1).[18] After blood perfusion of the kidney is restored, humoral and cellular elements of innate/adaptive immunity flow in and out of the graft.[30,31] An inevitable consequence of the transplant procedure is renal ischemic and reperfusion injury (IRI),[5,30] which is known to amplify sterile inflammation[8,31] and increases the general immunogenicity of the transplanted organ.[32,33] The release of DAMPs and ROS induced during explantation, storage, surgery, and microbial contamination accelerates[19,31] and continues the activation of innate immunity.[7,18] Brain-dead donors also differ from living donors (LDs) in terms of DAMP generation, e.g., by more intensive release of HMGB1 by renal tubular cells[6,25] and of IL1β, TNF-α, IL12, and IL6 by local monocytes/macrophages and mature dendritic cells (mDCs) located inside the kidney.[7,15]

Several factors may contribute to damage of endothelia, e.g., anti-human leukocyte antibodies (HLA),[34] non-HLA antibodies[4,35] or shear stress causing shedding of glycocalyces with a subsequent increase in permeability.[8,31] Cellular stress induces an increase in complement activation with C3a (and C5a) generation[15] by resident cells and infiltrating leukocytes.[7,30] Experimental local inhibition of complement formation reduces TLR4 gene expression, while experimental TLR4 blockage decreases C3 and B factor expression.[30] Crosstalk between the complement system and TLRs has been well described. Signals mediated through decay accelerating factor (DAF)[9] or through the C3a receptor (C3aR) and C5a receptor (C5aR) expressed on macrophages and DCs are known to regulate the TLR4-mediated cascade.[15,30] Mannose binding lectin (MBL) is also considered a putative ligand for TLR4.[9] Activated complement further upregulates endothelial adhesion molecules with subsequent excessive accumulation of inflammatory cells.[8,30]

The TLR2 and TLR4 are at the center of these inflammatory actions of IRI.[9,18,19] In the kidney, the majority of the constitutive expression of TLR4 (and TLR2) is found in glomerular endothelial cells, in podocytes and RTE cells.[25] During reperfusion, changes in TLR expression may occur non-concurrently on all kidney cell types. The expression is increased both on endothelial and RTE cells following injury. Endothelia of the vasa recta of the inner stripe of the outer medulla exhibited TLR4 upregulation at 4 h following reperfusion compared with 24 h for RTE cells.[8,25] After TLR4 stimulation, the main upregulated cytokines are IL-6, IL-1β and TNF-α. This is accompanied by increased expression of macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1). Both are involved in the recruitment of trafficking Grs and monocytes.[8,11,19]

Following extravasation in response to TLR2, TLR4 and TLR9 stimulation, monocytes continue the release of pro-inflammatory cytokines and chemokines implicated in neutrophil recruitment.[6,13] Neutrophils often cluster around graft-infiltrating monocytes.[6] Monocytes recruited into inflamed tissue can differentiate into macrophages or myeloid dendritic cells (MDCs), depending on the local microenvironment.[12] Under the influence of different regulatory factors (e.g., specific miRNA or TLR ligands), MDCs can stop their development at the stage of immature cells or progress to mature MDCs (mMDCs).[27] In inflammatory surroundings, mMDCs express a high density of class II major histocompatibility complex (MHC) molecules and co-stimulatory receptors. They become potent inducers of type 1 alloimmunity after transplantation.[7,36] In human MDCs, TLR4 stimulation increases their

glucose consumption and lactate production (required for the secretion of cytokines) through higher expression of co-stimulatory molecule CD86 and ATP production.[12] TLR4 stimulation of macrophages from human kidney grafts induces nitric oxide (NO) secretion and decreases the activity of arginase-1.[37] A suboptimal response (due to low expression) or low-level TLR4 stimulation prevent MDCs from maturing. Immature MDC (iMDCs) express low levels of MHC class II and co-stimulatory receptors.[5] If iMDCs present alloantigens, they do not initiate T-cell responses, but favor the differentiation of regulatory T cells (the "tolerogenic" state).[36] In monocytes, macrophages or MDCs, TLR4-induced production of inflammatory cytokines (e.g., by LPS) leads to negative feedback mechanisms switching on with a short delay, resulting in the downregulation of TLR4 receptors and signaling cascades (from minutes to hours). This makes monocytes and MDCs hyporesponsive for as long as several days,[36] and may explain the low DGF rate in patients with recurrent Gram-negative infections. In cases of Gram-negative bacterial infection, this hyporesponsiveness is reversible through antibiotic therapy.[5]

It is still a matter of debate how much activated infiltrating leukocytes, as opposed to intrinsic renal cells, contribute to a high general level of TLR4 expression found in kidneys with IRI. Opinions range from equal contributions to a more significant role of TLR4 on renal cells.[19,38] Neutrophils and PBMCs that have abundant TLR4 expression are readily attracted to inflammatory sites,[7] so the proportion of cells with low TLR4 and MyD88 expression may increase in monocytes that remain in circulation.[11] In clinical conditions, this phenomenon can be observed in kidney transplant recipients in whom the average TLR4 expression of PBMCs is low within the first 24 h after transplantation. These patients often have DGF, the duration of which is in proportion to the early decrease in TLR4.[11,32] Data from one of our studies indicates that the low-level TLR4 expression of PBMCs may persist for a long time in patients with a history of DGF.[32]

Acute kidney injury

Before renal transplantation, candidates with an increased predisposition to acute kidney rejection may be singled out from patients with undisturbed adaptive resistance parameters. These candidates may have higher TLR4 expression on $CD14^+$ PBMCs and a greater capacity to induce tumor necrosis factor α (TNF-α) and IL-6 after LPS stimulation.[21,25,36] A timeline of PBMC TLR4 expression including an acute allograft rejection in early post-transplant follow-up follows a different pattern than the line of a non-rejecting patient. The "rejector's line" takes on a characteristic U shape with initial progressive downregulation of TLR4 expression which then returns toward the baseline. The downregulation of TLR4 expression in rejecting patients is associated with reduced TLR4-dependent production of TNF-α by stimulated PBMCs. Although it is not enough to conclude that excessive TLR4 signaling is an independent risk factor for acute rejection (AR), early signaling through TLR4 may represent a rate-limiting step in the rejection cascade. In contrast, the "non-rejector's" TLR4 expression timeline begins at a lower point than the U curve and remains more or less flat.[36] The long-term impact of AR on PBMC TLR4 expression does not seem to be permanent. In our study, we found no difference in long-term PBMC TLR2/TLR4 expression between DGF+AR(–) and DGF+AR(+).[32] A steady-state suboptimal TLR4 response may protect the organ recipient from rejection by minimizing the signaling required for both dendritic cell maturation and subsequent T-cell activation. This may underlie the low incidence of rejection in some patients with either lower levels of TLR4 expression or reduced TLR4 ligand responsiveness before a transplant, as well as the link between TLR4 polymorphisms and organ rejection rates.[36] The effects of known TLR polymorphisms (e.g., the TLR4/C14 complex) on acute kidney rejection rates are not uniform. Analyses of CD14 allele and genotype distribution indicate a lower incidence of AR in homozygous wild-type CD14*C/C, but without statistical significance.[38] Most TLR4 polymorphisms (e.g., A299G/T399I) observed in renal transplant recipients have been connected with a decrease in TLR4 expression in monocytes and neutrophils. The A299G/T399I polymorphism presents a lower risk of atherosclerosis and AR.[6,19,24,36,39] The findings of studies concerning the influence of the A299G/T399I polymorphism on the infection rate are discrepant. Some of them have reported more frequent severe bacterial infections (e.g., urinary) and cytomegalovirus infections,[24,39] but others have found no difference.[38] Corticosteroid pulse therapy is also associated with a major decrease in TLR4 expression.[38]

Early AR after kidney transplantation can be expected when neither recovery nor rapid deterioration of renal function is observed. Both parenchymal cells and a wide array of immune cells (monocytes/macrophages, T and B lymphocytes) are involved in AR, along with humoral factors.[21] Vigorous co-activation of TLR4 and CD14 increases HLA molecules with co-stimulatory receptor expression on antigen-presenting cells and induces pro-inflammatory cytokine production, which may finally lead to an adaptive immune response.[38] It is believed that AR begins when mMDCs and macrophages effectively present the donor's antigens to the recipient's T cells and activate T cells, leading to their further differentiation. Mature MDCs support T helper type 1 development.[40] The triggering mechanism is multifactorial. One of the proposed theories considers the delayed effects of IRI-dependent cellular stress, which induces the production of C3a (and C5a) and the release of DAMPs within the donor's organ.[15] In allogenic (not syngenic) transplants, there is an upregulation of TLR4 receptor expression on tubular cells and within

glomeruli – on intrinsic renal cells (e.g., mesangial cells, podocytes and vascular endothelial cells).[21] One consequence of vascular endothelial activation inside the kidney is upregulation of cytokines (TNF-α, MIP-2 and MCP-1) and adhesion molecules (leukocyte rolling is mediated by E-selectin, P-selectin and L-selectin).[15] Experimental data show that Ly6C^{low} monocytes patrol along the endothelium and scavenge microparticles that are attached to the endothelial cells.[13] Up to 30 min after the initiation of TLR3/4-mediated vascular inflammation, there is a mobilization of neutrophils[19] and shortly thereafter a change of patrolling Ly6C^{low} monocytes occurs. Subsequent accumulation of Ly6C^{low} monocytes to endothelia depends on the dominating stimulation of a particular TLR type. Agonists for TLR2 and TLR9 recruit crawling monocytes within 30 min; agonists for TLR3 and TLR4 first induce the arrival of granulocytes, followed by an influx of patrolling monocytes (which takes up to 2.5 h).[7,13,19] Following the leukocyte rolling, firm adhesion is mediated by the binding of ICAM-1 on endothelia to lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) present on leukocytes. This facilitates diapedesis and trafficking into the renal interstitium.[19] The proportion of Mcs (from a BDD) to Mfs (from the recipient) in the graft interstitium changes gradually after transplantation. In an experimental model of early post-transplant rejection, most CD11b^{+} cells and DCs expressed donor but not recipient MHC-2. Most DCs are formed from precursors that reside in the kidney, whereas few DCs are formed from recipient precursors recruited upon local upregulation of MIP-2 and MCP-1.[22]

TLR4 activation is only one element of a complex mechanism for stimulating the release of pro-inflammatory cytokines, e.g., IL-1. Generating of IL-1β by monocytes/macrophages requires the conversion of pro-IL-1β to the active form by caspase-1 activation through an inflammasome complex (e.g., NLRP3). NLRP3 assembly requires coordinated stimulation provided by TLR4 and an increase in the concentration of a number of substances present in the cell microenvironment: extracellular glucose, hyaluronan, uric acid, monosodium urate crystals, and ATP.[15] The cytokines released by Mcs/Mfs and MDCs also depend to a great extent on crosstalk between complement factors (C3aR and C5aR) and TLR4. C3a/C3aR engagement in human Mcs/Mfs increases the release of TLR4-mediated cytokines, leading to enhanced Th17 responses[15] and has been found to play a role in both cellular and humoral rejection.[18] In transplant biopsy specimens of kidneys with AR, TLR4 induction correlates with local chemokine synthesis (CCL2 and CCL20).[21]

The magnitude of TLR-stimulated inflammation (i.e., caused by monocytes/macrophages) depends on the feedback control mechanisms. The absence of a negative regulator of TLR responses, e.g., the inhibitory receptor toll IL-1R 8 (toll-IL-1R8), enhances IRI and DC maturation after kidney transplantation.[6] Toll-IL-1R8 mediates the suppression of TLR/IL-1R signaling on epithelial cells, monocytes, DC precursors, and NK cells. Toll-IL-1R8 is expressed at the highest levels in the kidney. Experimental deletion of toll-IL-1R8 in kidney allografts favors maturation of resident DC precursors, expansion of allo-reactive T cells, as well as inhibition of Treg generation and AR to a greater degree than deletion of toll-IL-1R8 in the kidney recipient.[22]

Chronic graft injuries

Chronic allograft injury remains a great challenge for transplant clinicians. It is characterized by arteriopathy, peritubular capillaropathy, interstitial fibrosis, and tubular atrophy.[25] The precise cause of CAI remains unclear, but appears to be related to pathological changes due to organ preservation, IRI prior to and after organ implantation,[17] drug-related nephrotoxicity (e.g., calcineurin inhibitors), and chronic immune injury[25] with, e.g., anti-endothelial cell antibodies, anti-angiotensin II type 1 receptor (anti-AT1R) antibodies, anti-endothelin receptor antibodies (anti-ETAR),[4] non-HLA antibodies,[4,35] or anti-HLA antibodies (in our observation, about 50% patients with anti-HLA antibodies return to dialysis).[35] A transplanted kidney undergoing CAI releases DAMPs of varying intensity. Several molecules are implicated in this process, including fragments of nucleic acids, extracellular matrices (hyaluronic acid, fibronectin, heparin sulfate), HMGB1, fibrinogen, uric acid crystals, heat shock proteins, and fibrin.[37,41] Time-varying chronic DAMP delivery may cause accumulation of more TLR4 mRNA in grafts displaying CAI lesions as well as in those with apparently normal histology.[42] On the other hand, evidence for TLR/DAMP signaling as a mediator of CAI in humans is only indirect and associative.[25]

In CAI, monocytes are recruited from the blood into the transplanted kidney with an intensity depending on the time and type of graft pathology causing its malfunction. This means that there is a functional balance between 2 groups of cells: those passing through the endothelium and those remaining in the blood.[32] Stressed renal epithelia,[37] tissue-resident monocytes and monocytes infiltrating injured tissue are danger sentinels and respond quickly and with a low signal threshold to the presence of PAMP/DAMPs with the secretion of proinflammatory cytokines[7] and chemokines (e.g., MCP-1 or transforming growth factor (TGF)-β).[37] Monocytes infiltrating the graft are considered a major cell-damaging component in CAI,[37] linking chronic endothelial dysfunction with TLR4 expression.[43] Maturation of resident DCs can be fostered by both HMGB1 and hyaluronan fragments via TLR2 and TLR4.[25] Over time after transplantation, the microenvironment at tissue sites induces a phenotypic change of monocyte into more macrophage-like cells, producing less IL-1β and IL-6 but sustaining low-grade chronic inflammatory

processes, thus contributing to transplant vasculopathy and fibrosis.[7] This inflammatory milieu guides proliferation and collagen synthesis by myofibroblasts and fibroblasts. In experiments, TLR deficiency reduces the number of tissue macrophages, DCs and primed T cells, leading to reduced cytokine release and stimulation of myofibroblasts, and reduced activity of infiltrating TLR-deficient fibroblasts. In this way, TLR inhibition can ameliorate fibrosis.[15,37] These findings indicate the possible contribution of the TLR4-dependent signaling cascade of resident renal cells to the development of chronic injury.[6,8,25] A study which focused on a comparison between circulating blood monocytes of patients with CAI (deteriorating renal grafts with chronic immune-mediated graft damage under standard immunosuppression) and patients with well-functioning grafts (in the absence of immunosuppression) found contrasting expression of MyD88 and TLR4 between the 2 groups. In the CAI group, the percentage of monocytes with raised TLR4/MyD88 expression was higher at both the transcriptional and protein levels. There was no difference in the absolute count of monocytes detectable in the blood. Thus, PBMC TLR4 expression patterns may give an idea of local graft TLR4 expression.[25,41,42] Effective treatment of AR of a kidney graft in the past seems to have a negligible effect on TLR4 or MyD88 expression in both the kidney and PBMC population in the future.[11,32]

Graft recipients with stable renal function and with known TLR4 polymorphisms have a lower expression of TLR4 in monocytes and neutrophils.[41] Loss-of-function TLR4 mutations (D299G and T399I) have beneficial impact on both acute rejection and atherosclerotic events for the possible price of higher susceptibility to post-transplant infections.[25] D299G polymorphism may occur more frequently in patients surviving 15 years after transplantation, and TLR4T399I seems to be present only in those recipients who did not manifest CAI, although the difference was not statistically significant.[19] Experimental "polymorphic" animals have been found to be protected from CAI, with declining effects downstream from TLR2/4 deficiency, through MyD88 deficiency, to TRIF deficiency.[37] Eritoran is a structural analog of the lipid A portion of LPS. In some studies, it has proven to be an antagonist of the pathophysiological effects of LPS by blocking translocation of NF-κB and decreasing expression of inflammatory cytokines.[17] However, contrary to expectations, clinical trials failed to demonstrate benefits from eritoran therapy.[5]

Almost all transplant patients receive immunosuppressive therapy. It has been found that cyclosporine A (CsA) influences TLR signaling. Cyclosporine A induces endoplasmic reticulum stress, which triggers unfolded protein response (UPR), which in turn activates c-Jun N-terminal kinases (JNKs) in several cell types. In renal epithelial cells, CsA upregulates TLR4 expression without increasing TLR4 gene expression. Cyclosporine A causes nuclear-to-cytoplasmic translocation of HMGB1, and its nephrotoxicity depends on an intact TLR4 signaling pathway. In macrophages, UPR promotes inflammatory cytokines following TLR4 activation.[20] In our study involving mycophenolate mofetil, in PBMC from patients between 1 and 6 months (but especially from 3 to 6 months) after transplantation, we found a negative correlation between doses of mycophenolate mofetil per kilogram of body weight and TLR4 expression.[32]

Summary

Expression of particular (TLRs in the kidney depends on the localization of specific cells (e.g., endothelium, elements of the nephron structure), recent pathology and time after transplantation. The TLR4 is widely expressed on RTE cells, endothelial cells and podocytes. It is also expressed on the majority of white blood cells involved in adaptive immunity (e.g., lymphocytes) and in native ones (monocytes/macrophages and dendritic cells). By remaining in a proportional numerical equilibrium with cells circulating in the blood, a portion of the monocyte population is attracted to and penetrates the tissues, where they supplement the donor's resident macrophage population. The percentage of cells with shifted TLR4 expression among circulating monocytes usually increases, sometimes becoming a mirror of intrarenal inflammation, especially in patients with a history of DGF. The TLR4 and its monocytes undergo multilevel crosstalk feedback control and immunosuppressive influences. It senses stimuli that make monocytes contribute differently both to acute/chronic kidney injury and to the development of tolerance. Following a kidney transplant, TLR4 expression and the associated cytokine production ability may be connected with different pathologies, depending on past disease and oncoming problems. Because conventional immunosuppression and diagnostics focusing on the adaptive immune response do not prevent chronic allograft damage, monocytes (PBMCs) and TLR4 are good candidates for the role of diagnostic and therapeutic targets.[37] Considering the mutual communication of various elements of the primary immune system, future therapeutic intervention should be directed toward factors directly or indirectly regulating the expression or post-receptor signaling of the TLR4 receptor.

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The role of MR volumetry in brain atrophy assessment in multiple sclerosis: A review of the literature

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Abstract

We review the current role of magnetic resonance (MR) volumetry as a meaningful indicator of neurodegeneration and clinical disease progression in multiple sclerosis (MS) patients. Based on a review of the current literature we summarize the mechanisms that contribute to brain atrophy. We present the newest magnetic resonance imaging (MRI)-based methods used in atrophy quantification. We also analyze important biological factors which can influence the accuracy of brain atrophy evaluation. Evidence shows that measures of brain volume (BV) have the potential to be an important determinant of disease progression to a greater extent than conventional lesion assessment. Finally, scientific reports concerning limitations of MRI-based volumetry that affect its implementation into routine clinical practice are also reviewed. The technical challenges that need to be overcome include creating a standardized protocol for image acquisition — a fully automated, accurate and reproducible method that allows comparison in either single-center or multicenter settings. In the near future, quantitative MR research will probably be the basic method used in neurology to monitor the rate of atrophic processes and clinical deterioration in MS patients, and to evaluate the results of treatment.

Key words: magnetic resonance imaging, multiple sclerosis (MS), brain atrophy, MR volumetry

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Multiple sclerosis (MS) is a chronic disabling disease. It is one of the earliest-known neurological diseases, described by Charcot in 1868. It is a progressive neurological disorder resulting in both physical and neurocognitive disability.[1] In the US, there are approx. 400,000 people currently diagnosed with MS but it is estimated that around 2.5 million people worldwide might suffer from it. The disease typically begins in early adulthood with variable prognoses; 50% of the patients will require aid in walking within 15 years. The process of diagnosing and understanding MS involves specialists from neurology, immunology, radiology, and many other disciplines.[2] In MS, the brain is attacked and destroyed by the body's own immune system, causing physical disability and cognitive impairment.[3] This results in cerebral changes that lead to brain atrophy at a much higher rate than when this process occurs with aging in healthy people.[4]

Magnetic resonance imaging (MRI) is a very useful tool not only in the process of initial diagnosis of MS, but also in monitoring disease progression and therapeutic response. Current MRI volumetric techniques can detect brain volume changes with an accuracy of 3 mL. This precise quantitative information has significant potential for evaluating disease activity and changes following therapy.[5–7] Magnetic resonance imaging volumetry became a clinically relevant component of disease assessment because of its high sensitivity and specificity in detecting volumetric changes of the brain.[8] It allows whole-brain volume to be measured, as well as the volume of particular brain lobes and gyri.[9] In general, whole brain atrophy is considered a good predictor of long-term clinical disability in all stages of MS.[10] Nevertheless, it should be always combined with assessments of the clinical state of a patient.

Within the last 10 years, numerous studies conducted on MS patients have reported accelerated brain atrophy rate in comparison with healthy subjects.[11,12] Hardmeier et al.[11] documented that gray matter (GM) volumes are lower in MS patients than in healthy age-matched controls. In addition, annual brain tissue loss occurred at a faster rate in MS patients (0.5–1% per year) than in the control group (0.1–0.3% per year). Global brain atrophy has been demonstrated in all subtypes of MS: in relapsing-remitting (RR), secondary progressive (SP) and primary progressive (PP) MS.[2,13,14] A considerable amount of data confirms that measurements of the percentage of brain tissue loss over time are among the best methods for quantifying neurodegeneration in MS and monitoring disease progression.[6]

In this paper, we review the data regarding MRI volumetric techniques and their clinical applications. We focus on the relationships between the severity of clinical symptoms, including disability, and the percentage of brain atrophy. We also present prospects for future uses of MRI metrics in the process of diagnosing and assessing brain volume (BV) changes in the course of MS.

The pathomorphological basis of atrophy in multiple sclerosis

Atrophy is seen at all clinical stages of MS and develops gradually. The loss of brain tissue is mainly due to myelin loss. However, there are also other tissue elements that contribute to whole BV loss, such as changes in tissue water content and the loss of glial cells, vascular elements and GM.

Furthermore, factors like protein catabolism, changes in electrolyte balance, vascular permeability, and dehydration may have an influence on brain atrophy. Changes caused by the inflammation process may also play a role. The main target for researchers has been to determine the pathologic process that leads to the development of plaques in MS. A lack of integrity in the blood–brain barrier is a mechanism that has received a great deal of attention; it allows the entrance of leukocytes into the normally immunologically privileged central nervous system (CNS) as a response to inflammation. The myelin sheath located in the CNS becomes the target of immunologic attack. This underlying pathology is present from the beginning of the disease, sometimes long before clinical symptoms are present, and continually progresses. The neurodegeneration translates to macroscopic brain atrophy that can be quantified in vivo through brain MRI.[8]

Multiple sclerosis has long been classified as a primary white matter (WM) disease of the CNS. Foci of demyelination in WM are readily visualized using MRI and remain a hallmark of the disease. Pathologic studies report that GM is also affected, and seems to be a large component of all the pathologies caused by MS. This damage includes widespread demyelination, neuron apoptosis, and atrophy affecting the cortex and deep GM structures.[9]

Nevertheless, inflammation also plays a protective role by expressing growth factors that promote remyelination and protect against axonal damage.[10] These neurotrophins are essential for normal oligodendrocyte development: they promote migration, differentiation and maturation of oligodendrocyte precursor cells.[11] These beneficial effects of inflammation have become an extremely promising therapeutic approach to MS.

The concept of neurodegeneration in multiple sclerosis

Although demyelination with hyperintense lesions on T2-weighted MRI remains the main diagnostic criterion of MS, there is growing evidence that the extent of clinical disability does not correlate with the number of foci. It has been reported that the extent of total axonal damage resembling neurodegeneration shows a strong correlation with clinical progression and strongly predicts irreversible disability.[8,12,15,16]

Reductions in N-actetylaspartate (NAA, a marker of axonal integrity) have also been observed in magnetic resonance spectroscopy (MRS) at a very early phase of the disease.[17] This finding indicates the neurodegenerative component of the MS background. A better understanding of the molecular mechanisms underlying MS progression will aid the development of new treatment strategies based on both anti-inflammatory and neuroprotective action.

Insight into atrophy measurements in multiple sclerosis

Conventional MRI techniques, using markers such as T2-weighted lesions and gadolinium-enhancing lesions, are now widely used in clinical practice in the diagnosis and follow-up of MS. However, the utility of these MRI measures in predicting disease progression in MS is limited. New areas of gadolinium enhancement are considered good markers for clinical relapses. Nevertheless, clinical deterioration is not clearly related to inflammatory lesions, but rather to a progressive and diffuse neuroatrophy.[18]

Recent evidence shows that axonal loss and dysfunction occur very early in the course of the disease and are apparent at all stages of the disease.[17] Atrophy is seen not only within the lesions but also in normal-appearing WM, where it is probably secondary to myelin loss and axonal damage caused by Wallerian degeneration.[8] For that reason in the past decade brain atrophy has become one of the most important indicators of neurodegeneration and clinical disease progression in MS patients. This probably reflects both inflammation-induced axonal loss and post-inflammatory neurodegeneration that may be due to inefficient remyelination.[19,20] Moreover, it has been shown that atrophy measures correlate with disability indices and cognitive performance in MS patients.[21,22]

Diffuse tissue loss in normal-appearing brain tissue can be monitored in a sensitive and reproducible manner using quantitative MR measurement. Magnetic resonance techniques provide an objective and direct assessment of the evolving pathology in MS.[8] This is a point of interest for trials of new agents aimed at preventing disability. Atrophy measurements should be included as an endpoint in trials of all disease-modifying agents to monitor treatment efficacy.

Factors affecting brain volume changes over time in multiple sclerosis

Many studies have demonstrated that there is no direct correlation between BV changes and patient's clinical state and rate of disability.[8,23] This suggests that there are other factors, unrelated to the disease, that influence BV changes in MS patients. This requires further investigation. For example, any inflammatory reaction can temporarily increase BV, causing vasogenic edema. In addition, processes like glial cell proliferation and gliosis can also contribute to false BV increase.[24]

There are other biological factors, including body fluid status, nutrition, addictions, menstrual cycle, genetic and environmental considerations, and gender- and age-related features which may contribute to BV changes. These can all cause physiologic variations in BV. Cerebral volume loss progresses with age but may be more pronounced when there are other risk factors such as alcohol abuse, smoking, dehydration, or concomitant diseases (e.g., diabetes, cardiovascular risk factors or Cushing's syndrome). All this may affect the accuracy of BV measurements.[25,26]

Duning et al.[27] showed that hydration status can significantly change BV: a lack of fluid intake for 16 h decreased BV by 0.55% (standard deviation (SD) = 0.69) and after rehydration total BV increased by 0.72% (SD = 0.21). Changes as high as 30–40 mL have also been observed after dialysis in patients with renal failure.[28] Dehydration can confound the assessment of brain atrophy.

For these reasons, in cases in which small changes of total BV are important diagnostic parameters – for example in MS and neurodegenerative diseases – comparative hydration status should be considered in longitudinal automated MR-based measurements of BV.[29]

Another important factor which should be considered is weight loss. Some studies on patients with anorexia nervosa (AN) have already revealed various effects of nutritional status on BV. Enlargement of the cortical sulci and cerebrospinal fluid spaces as well as pituitary gland atrophy in patients with AN have been reported.[25] Heinz et al. postulated that the mechanism responsible for the atrophic changes may be related to protein loss and fluid retention caused by hypercortisolism and loss of serum protein. This suggests that there is no simple correlation between cerebral atrophy and malnutrition. It should be emphasized that although in some cases of AN morphological brain changes have been reported, we cannot consider BV dependence on the nutritional state of the patient.[30,31]

It should be taken into consideration that the correlation between brain loss and progression of the disease is influenced by a phenomenon called brain plasticity. In patients with MS it is believed to be a compensatory mechanism based on activation of new brain areas in order to retain some brain functions. Neuroplasticity is a property of the nervous system to obtain functional goals. The increased functional recruitment of the cortex in MS patients might have an adaptive role in limiting the clinical impact of irreversible tissue damage. Cortical motor reorganization has been described in various diseases including tumors, vascular malformations and stroke.[32]

There is a growing body of evidence suggesting that functional cortical changes develop early in the disease and have a role in limiting the clinical impact of MS injury. Therefore, MS-related brain damage may occur undetected during the early phase of the disease. Moreover, MS may remain undiagnosed and untreated for a long time.[33] As adaptive cortical changes have the potential to limit the clinical impact of MS injury, the question arises of how strong the correlation is between MR volumetry and clinical outcomes in MS patients. This interrelation is still to be explored.[34]

The clinical value of brain atrophy measurements in MS necessitates future longitudinal follow-up studies conducted in a standardized manner on a large group of patients. Brain volume changes should be measured over at least a 1-year period and compared with changes in disability measured using disability status scales. The pathophysiological significance of altered brain activation patterns in MS patients and their influence on the clinical state of patients also needs further exploration.

There is also a strong need for validation of brain atrophy rates through longer-lasting trials in order to establish stable baselines. This will enable better interpretation of BV changes over time and allow introducing quantitive BV assesment into routine clinical practice.

The phenomenon of pseudoatrophy

Pseudoatrophy is a temporary phenomenon that represents accelerated water loss and reduced edema during the course of anti-inflammatory treatment. It is not associated with neuron or tissue damage. The mechanisms responsible for this effect include the induction of protein catabolism and the reduction of water volume in the brain as a result of decreased vascular permeability without real axonal loss.

Disease-modifying drugs at treatment initiation reduce edema, inflammation and extracellular water in the brain, leading to pseudoatrophy. It may initially confound real treatement effects in the first few months of therapy.[35] Many volumetric studies confirmed that changes in the volume of inflammatory cells, particularly glial cells, may be also relevant.[36] Several independent studies have reported that anti-inflammatory drugs transiently decrease BV within the first 6 months to 1 year of treatment.[11,37] An acute effect of intravenously administrated corticosteroids on BV has been shown by several researchers.[38–40] Hardmeier et al.[11] explored the kinetics of atrophy after treatment initiation. Their study included 802 patients with active RR MS disease. Among them, 189 subjects were randomized to 30 μg and 197 subjects to 60 μg of interferon beta-1a (IFN-1a IM) once a week. Brain parenchymal fraction (BPF), a normalized measure of whole-brain atrophy, and the volume of Gd-enhancing lesions (T1Gd) and T2 hyperintense lesions (T2LL) were evaluated. The BPF analysis showed a decrease of BV in the first year of treatment. Nearly 70% of BPF change occurred during the first 4 months and was accompanied by a drop in T1Gd volume. This nonlinear development of atrophy in the first phase of treatment is caused by edema resolution and the effect of IFN-1a on the blood–brain barrier, both imitates atrophy and can cause difficulties in assessing disease progression and the efficacy of immunomodulating and immunosuppressive therapies. The authors suggest that yearly measurement of BV loss after therapy may not be optimal for assessing atrophy changes. They have highlighted the importance of obtaining a "second baseline" after 4 or 6 months of treatment.

The same mechanism is also observed in patients with non-neurologic autoimmune diseases, for example Cushing's disease and other autoimmune diseases treated with prolonged daily use of corticosteroids over the mid- to long-term and showing significant BV loss.[31,41,42]

Another novel finding, presented in a study by Filippi et al., was the percentage of BV change (PBVC). The mean PBVC for patients on a placebo was –0.83% during the 1st year, –0.67% during the 2nd year and –1.68% during the entire study period. The respective values for treated MS patients were –0.62%, –0.61% and –1.18%. The explanation for these findings might be the ability of the treatment to reduce Gd-enhancing lesions and edema during the first phase of treatment, which in turn might result in pseudoatrophy.[43]

In contrast, focal edema in new demyelinating lesions may compensate true BV loss from tissue destruction, especially in WM, where inflammation is more pronounced. On the other hand, the process of remyelination within the lesion may mask atrophy.[29] This fact has important implications for the design of clinical trials. It is necessary to further investigate how much the pseudoatrophy in MS brains after the initiation of certain therapies may be related to the reduction of inflammation in order to distinguish it from true neurodegeneration.

Dynamic changes in BV in MS patients are therefore a combination of destructive and rebuilding processes of inflammation, neurodegeneration and correct, and the influence of immunomodulatory therapy. Therefore, it is very important to be aware of all these factors when interpreting whole-brain volume data because reductions in whole-brain volume may be associated not only with true atrophy.[44]

There is a strong need to establish normative values for BV changes – both for healthy subjects and for patients with MS – that take into account these confounding factors. These studies will enable the reliable use of whole BV measurements to prognose and predict therapeutic response.

Gray matter atrophy in multiple sclerosis

Recent studies report that clinical disability in MS patients cannot be explained by changes in WM observed in conventional MRI sequences. This suggests that GM damage

and subsequent GM atrophy can be relevant to the development of disability.[45,46] Indeed, cortical and hippocampal lesions result in symptoms of cognitive and processing speed impairments.[47–49] The detection of GM alterations will play an important role in the understanding of both physical and neurocognitive disability observed in MS patients. Measurements of WM and GM atrophy remain among the current methods that can be used to quantify the grade of disease progression.[46]

There have been a number of studies that investigated which brain structures are affected by atrophy in different MS subtypes and how early this pathology begins. Scientists have also focused on determining which cortical areas are primarily affected in patients presenting with their first clinically isolated syndrome (CIS). Several cross-sectional studies have reported that diffuse cerebral atrophy in MS is not sensitive enough in case of first episode of neurologic symptoms. This indicates a need for a more accurate biomarker for patients after the CIS as well as those with a short disease duration.[50]

A number of histopathological and MRI studies have demonstrated that significant global cortical thinning is a diffuse and early phenomenon in MS.[4,51,52] It correlates with clinical disability and is partially independent from WM inflammatory pathology. A longitudinal study carried out by Dalton et al.[53] in patients with CIS found progressive cortical atrophy in those subjects who developed a confirmed diagnosis of MS within 3 years of the clinical onset of CIS. They reported that the mean decrease in GM fractional (GMF) volume was –3.3% while in CIS subjects who had not developed MS it was –1.1%. No decrease in WM fractional (WMF) volume was seen. Changes in GMF volume correlated only modestly with changes in the T2 lesion load. These results suggest that progressive GM atrophy is present in the earliest clinically observable stages of relapse-onset MS, and this is only moderately related to lesion accumulation.

Recent data based on a large cohort of patients has proven that cortical thinning is already present and diffuse in a very early MS phase, when WM damage appears to be only modest. Moreover, cortical thinning is correlated with clinical disability and cognitive impairment more strongly than other MRI measurements.[54,55] Among many software techniques that enable the in vivo MRI-derived quantitative measurement of cerebral cortex thickness, one of the most often used is FreeSurfer (Laboratory for Computational Neuroimaging, Charleston, USA; Fig. 1). These measurements provide novel insights into neuroanatomical abnormalities during the course of the disease. Gray matter appears to be less sensitive to pseudoatrophy than WM, and could be a potentially attractive additional measurement for neuroprotection trials.[24,51,56]

Calabrese et al.[57] carried out a 5-year prospective longitudinal study in order to evaluate the extent to which cortical lesion load is associated with longer-term physical and cognitive disability. Patients with high cortical lesion loads had higher Expanded Disability Status Scale increases (median = 1.5; range = 0–3) and showed a greater GM fractional loss during the study than both patients with low cortical lesion loads and without cortical lesions. Analyzing cortical pathology may help in the early identification of patients with a worse prognosis and provide relevant clinical and therapeutic implications. Nevertheless, the extent to which MRI parameters of cortical thinning may be used for clinical purposes needs to be confirmed in further prospective longitudinal studies.

Considerable evidence exists to support the hypothesis that there are also another GM structures which seem to be more predictive than others. According to many studies, out of all subcortical GM areas, the thalamus is the structure most vulnerable to atrophy. Thalamic tissue volume loss has been found in all MS subtypes.[58] Thalamic nuclei are GM structures that play a major role in cortical activation, relaying sensory information to the higher cortical centers that influence cognition, sensory and motor functions. Involvement of these structures is associated with a wide range of clinical manifestations, including cognitive impairments and motor deficits in patients with MS.[59] It might become an important biomarker of disease progression.

To summarize, thalamic atrophy and cortical thickness are 2 very promising GM MRI metrics. Still more studies and longitudinal follow-ups are needed to characterize atrophy progression over time and the clinical relevance at both the group and the individual level.

Overview of quantitative volumetric MRI techniques

Numerous methods are available for the measurement of global and regional BV.[60] The optimal method for measuring brain tissue loss must be sensitive to small changes, reproducible and stable over time. Techniques of MRI analysis, measuring the size of brain structures, have ranged from manual traces for estimates of diameters or sectional areas of structures to fully automated methods based on advanced computational algorithms (Fig. 2).

It is important to note that a wide variety of techniques available for measuring tissue damage are not directly comparable with each other, which makes it very difficult to compare the results of different studies based on different tools.

Although automated voxel-based methods are designed to be robust and reliable structural change detection methods, it is known that they can be influenced by physical, methodological and physiological factors such as the scan parameters, the type of coil, MRI scanner field strength, the type of applied pulse sequence, the level of patient's hydration, the phase of women's menstrual cycle, and other factors. The potential impact of the scanning parameters can be minimized with strict adherence to a specific MRI scanner and imaging protocol.

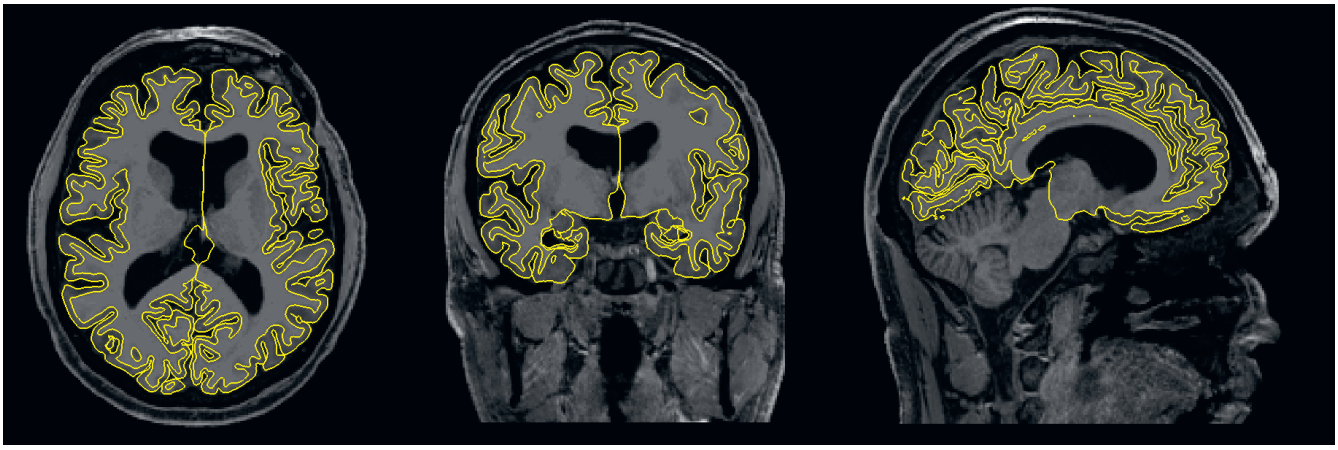

Fig. 1. Cortical thickness borders overlaid on an anatomical T1-weighted Freesurfer image

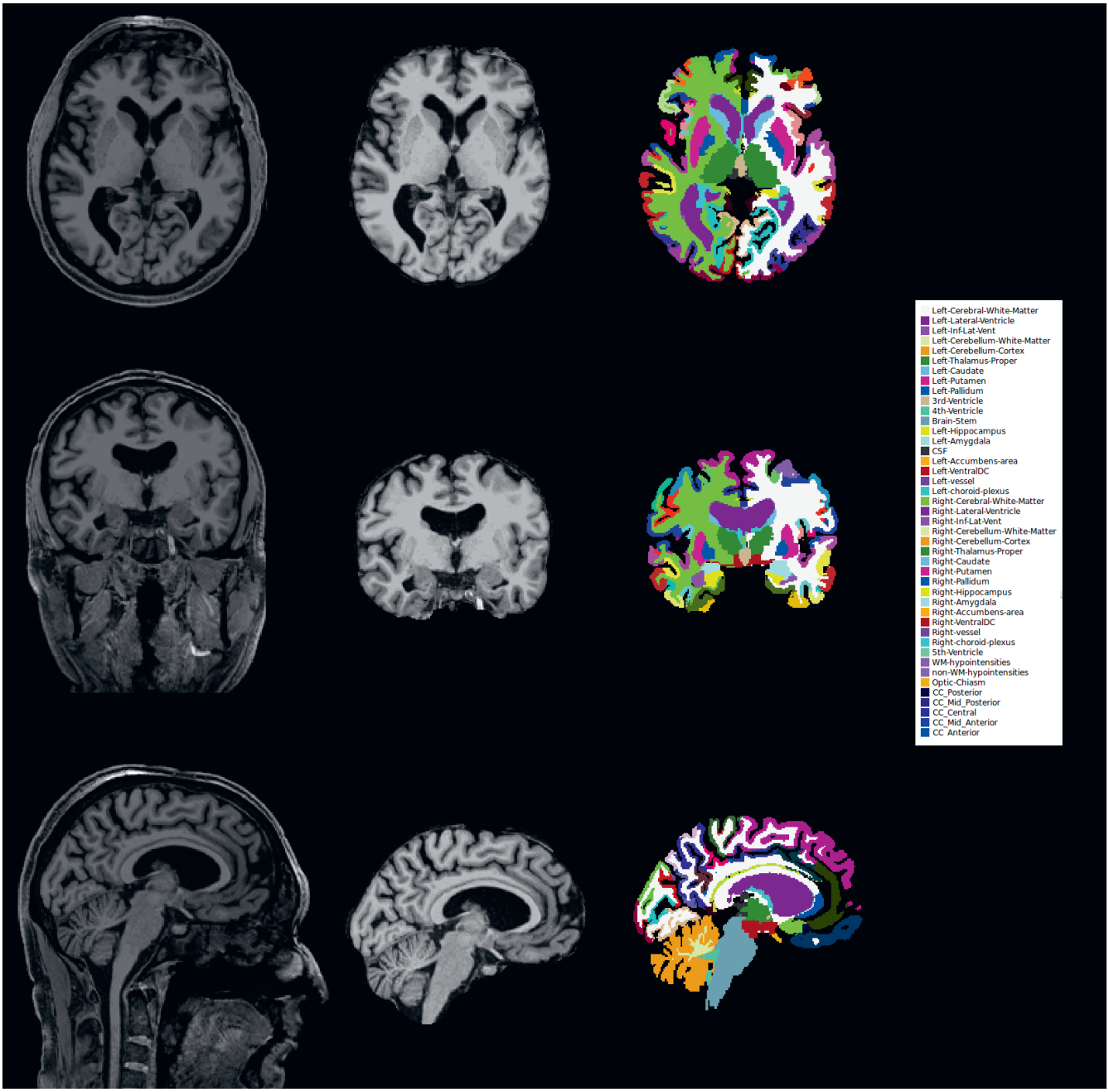

Fig. 2. Example of Brainsuite processing steps for volumetric assessment

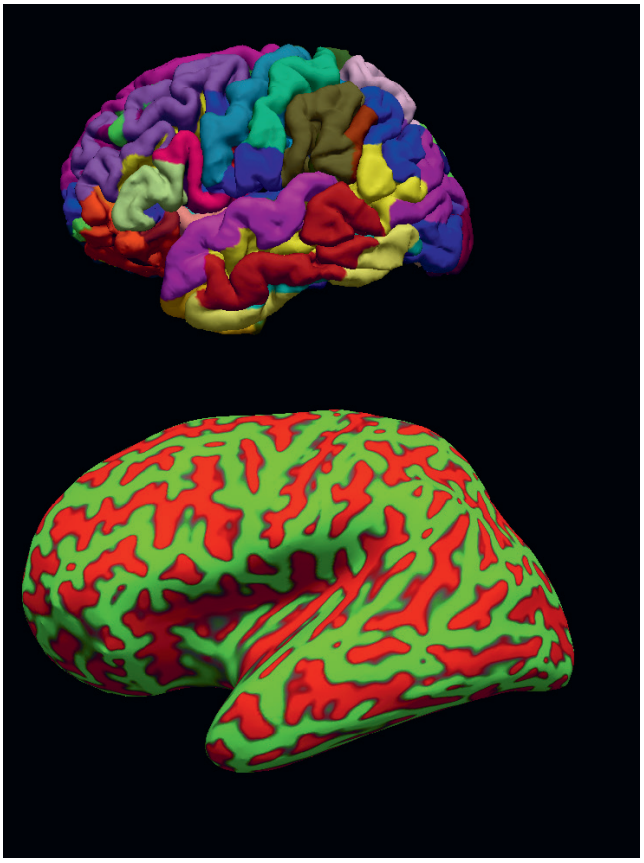

Fig. 3. Example of brain cortical and subcortical labeling using Freesurfer

Manual methods of volumetry are time-consuming and require great expertise in anatomy. The most common manual methods are the bicaudate ratio, brain width, the corpus callosum area, the midbrain to pons ratio, the lateral width, and the third ventricle width.[61]

A strong need for more objective, comparable and reproducible assessment of BV led to the creation of a variety of fully-automated tools such as SPM/VBM – Statistical Parametric Mapping (www.fil.ion.ucl.ac.uk/spm), Freesurfer, SIENAX, SIENA (Structural Image Evaluation using Normalisation, of Atrophy), FSL (FMRIB Software Library, Analysis Group, Oxford, UK), and BrainVISA (brainvisa.info).[62]

Among existing medical image segmentation techniques, one of the most popular is SIENA software, which allows measurement of whole and partial BV. The reproducibility of the measurements obtained is about 0.5%. To perform more specific volume measurements, one can use segmentation-based techniques that have the potential to automatically segment subcortical brain structures and can separately quantify the volume of GM and WM, as well as the volumes of particular gyri and lobes.[63] (Fig. 3). The performance of such methods depends on image registration accuracy and anatomical differences between the study subject and the atlas images (Fig. 4). This is why multi-atlas-based methods for subcortical structure segmentation have attracted a great deal of attention, partially because the spatial positions of anatomical structures are relatively stable among patients.[64]

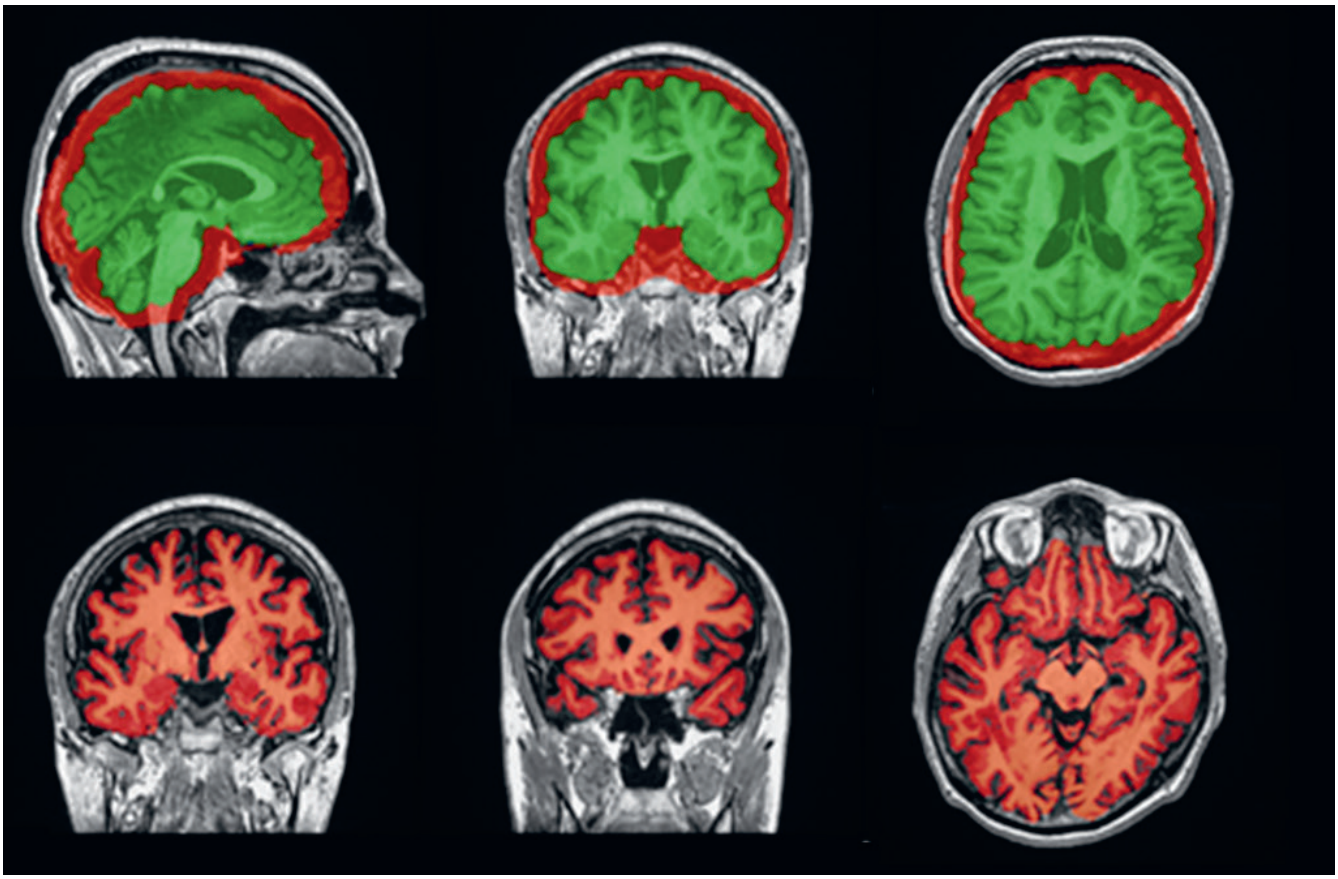

Fig. 4. Cortical thickness map (BrainVoyager QX cross-platform software)

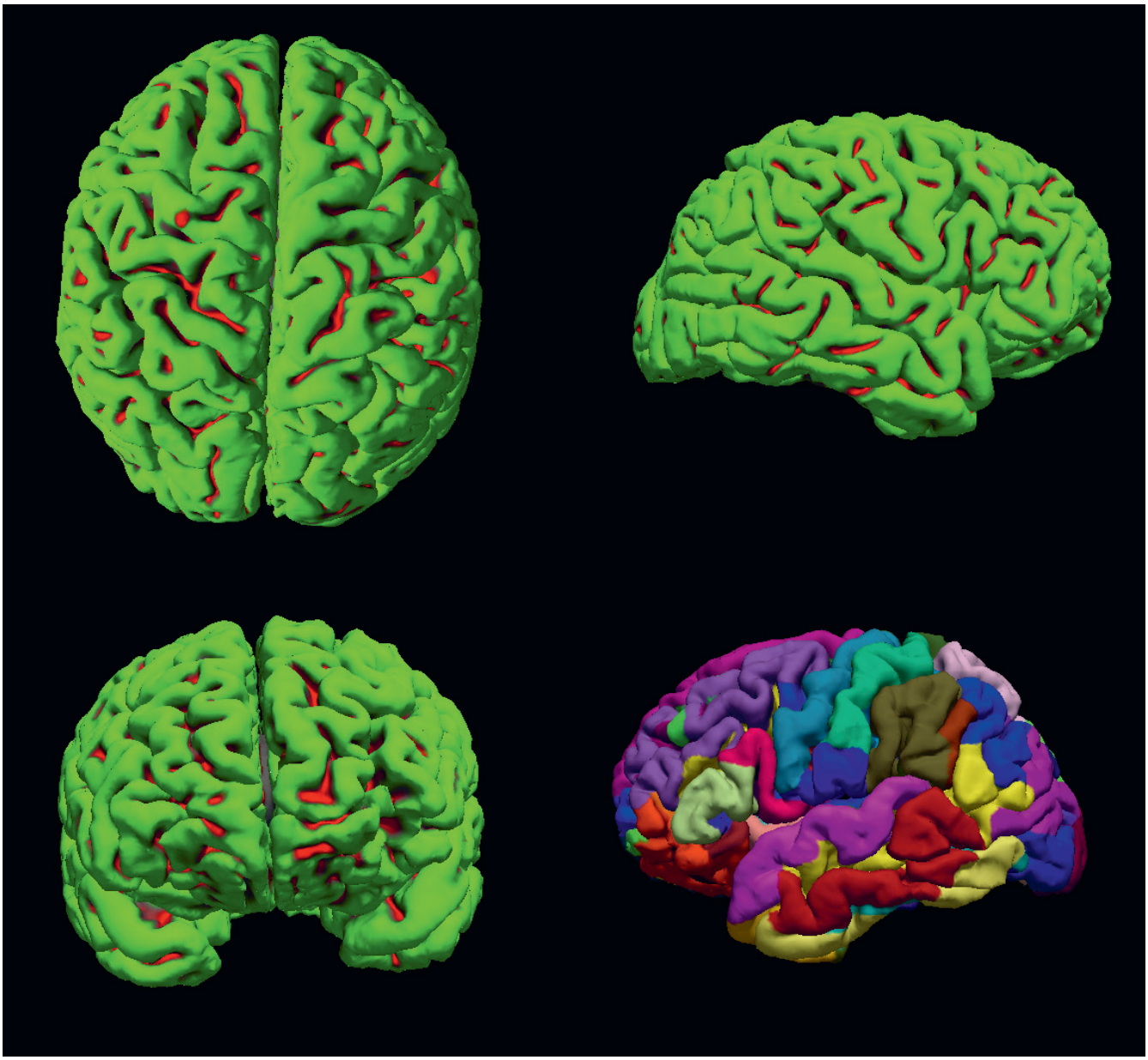

Fig. 5. Graphic representation of SIENAX field of view and standard space masking and whole brain segmentation

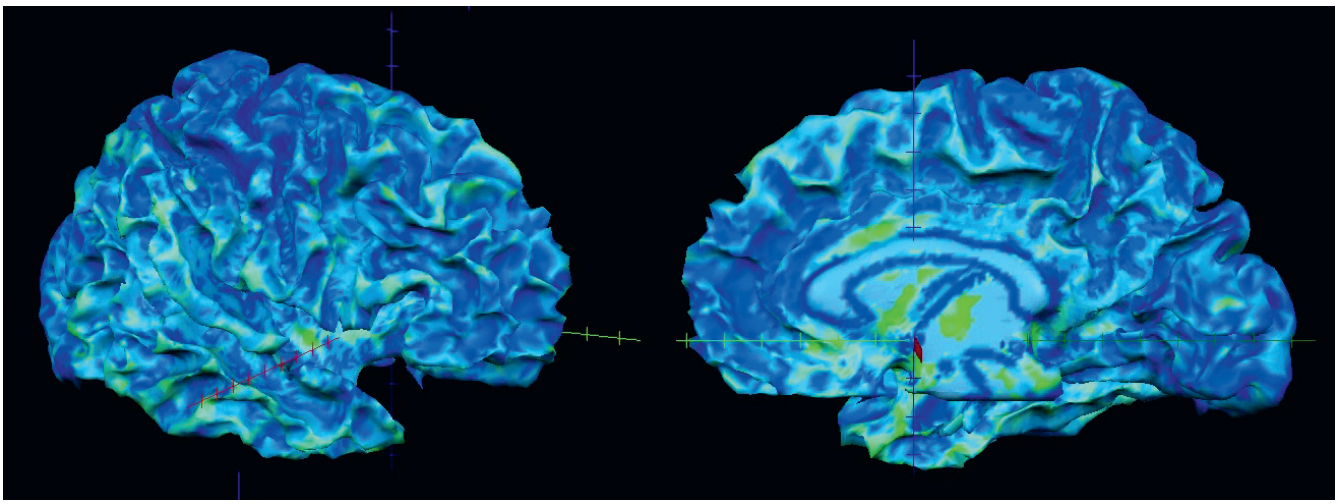

Fig. 6. 3D Pial surface (Freesurfer)

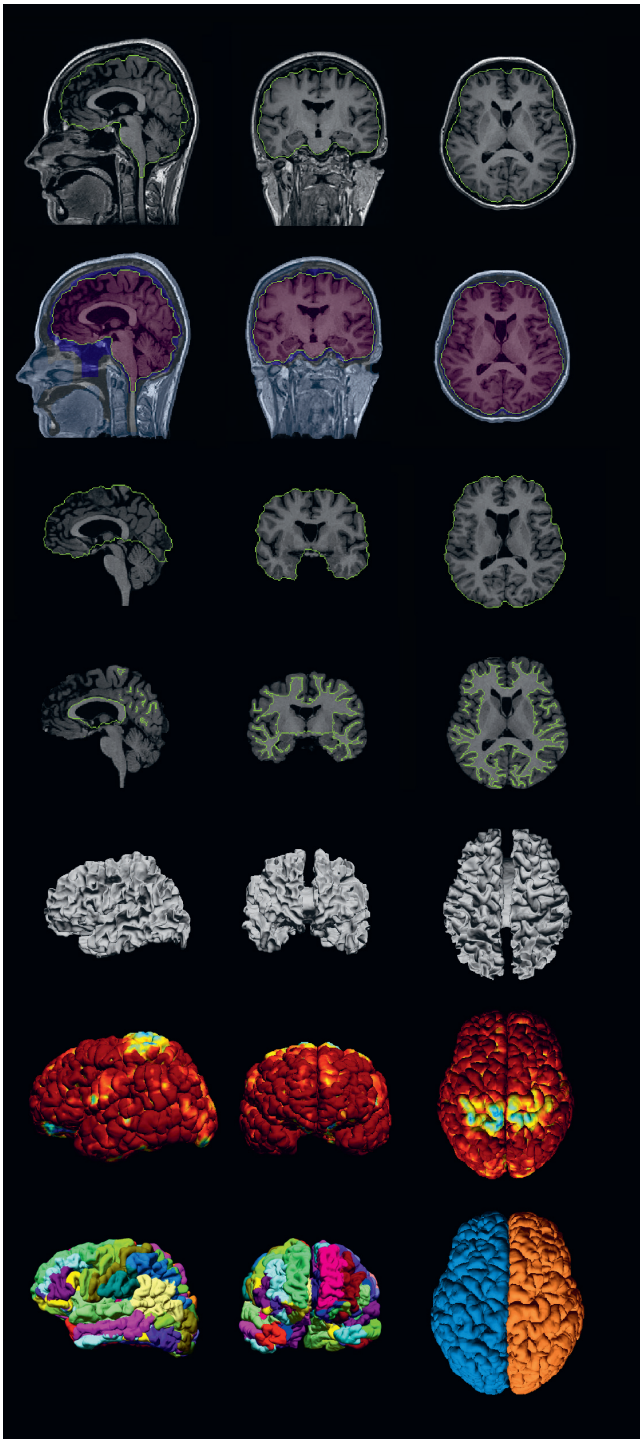

Fig. 7. WM/GM boundary surface reconstruction with overlaid cortical thickness map (BrainVoyager QX)

Most of this software, such as the FMRIB Software Library (FSL) package (FSL Analysis Group), BrainSuite (Ahmanson-Lovelace Brain Mapping Center/Biomedical Imaging Group, Los Angeles, USA) and FreeSurfer, are freely available and run online on a wide variety of hardware and software platforms (Fig. 5).[65,66] That is why these automatic tools have been used in numerous studies. All of these programs can create 3D models of the most macroscopically visible structures in the human brain (Fig. 6).

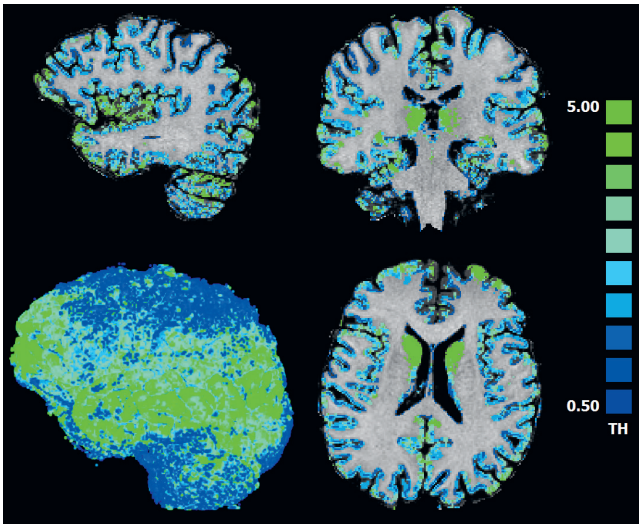

Fig. 8. Cortical Thickness Map – BrainVoyager

It is important to note that even when the voxel-based methods are used, due to the large variety of noise factors, the angulation of slices, the segmentation protocol, imaging protocol, patient-dependent factors, etc., there is a low probability of obtaining identical results of multiple studies performed during the same day on the same patient, even when using the same software.[35,67] Despite these limitations, fully automated computational methods have shortened analysis time and allowed the assessment of large datasets. Due to advances in computational technology, the number of measurement errors is limited, thus the results are mostly reproducible and reliable. That is why they are extensively used in a number of clinical studies, especially for volumetric assessment of the whole brain and other intracranial structures such as lateral ventricles, WM, GM, and the hippocampus.[2,68]

Apart from its potential to help in the follow-up of many neurological diseases such as MS, this online software can aid researchers and clinicians in developing new treatments and monitoring their effectiveness.

Conclusions

Brain atrophy rate might be successfully used as an adjunctive biomarker of disease severity in the course of MS. A considerable amount of data derived from the latest studies confirms that measuring percentages of brain tissue loss over time is one of the best methods for quantifying neurodegeneration in MS and monitoring the progression of the disease.

Evaluating GM atrophy over the course of MS and its relationship to disability remains an important issue in the MS field. It should be stressed that GM atrophy could be an attractive potential marker of tissue loss, as it correlates better with clinical disability than other MRI measures and appears to be less sensitive to pseudoatrophy factors.

New techniques need to be validated and MR protocols need to be standardized before they can be introduced into clinical practice. There is a need for a normative database, combined with important physiologic factors affecting estimations of brain atrophy. Obviously, efforts must be made to harness the potential of these measurements in assessing and monitoring pathologic evolution and treatment efficacy in MS.

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