The Profile of Microorganisms Responsible for Port-Related Bacteremia in Pediatric Hemato-Oncological Patients

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Abstract
Patients with pediatric cancer face an increased risk of infections. In most cases, these infections are associated with the use of a long-term central venous catheter. This study describes the epidemiology of a port-associated bacteremia as well as a profile of microorganisms responsible for port-associated bloodstream infections (PABSI) in pediatric patients with cancer treated in a single center. The retrospective analysis included patients with cancer who had implanted a port, hospitalized between 2010 and 2015 at the Department of Pediatric Oncology, Hematology and Bone Marrow Transplantation, Poznan University of Medical Sciences. The medical records of patients were reviewed for demographic characteristics, diagnosis, port-related complications, and their management. Data were collected from patients’ electronic medical records containing complete information on medical examinations and supplementary tests, diagnosis, timing, and type of port-associated complications. In a study period, 277 ports were inserted to 241 patients. A total of 183,094 catheter days were analyzed. Sixteen patients had more than 1 insertion of a port. The commonest observed complication was PABSI (40.07%) and the incidence density was 0.6 per 1000 port-days. Staphylococcus was the most commonly isolated organisms from patients with PABSI. From all port-associated complications, bloodstream infections and mechanical complications were the most often observed complications. The commonest pathogens responsible for PABSI were coagulase-negative staphylococci. Pathogens resistant to standard antibiotic treatment play an important role in PABSI, with methicillin-resistant Staphylococcus epidermidis being the predominant pathogen. Port-associated bloodstream infections are a common reason for preterm removal of a port.

Keywords
bacteremia, central venous catheter, cancer

Introduction
Children with cancer are a specific group of patients—they are hospitalized for a long time and thus are already colonized by hospital flora, and their natural barriers are weakened by toxic effects of chemotherapy. Moreover, cancer itself can induce immunosuppression. Pediatric hemato-oncological patients face an increased risk of infections. In most cases, these infections are associated with the use of a long-term central venous catheter (CVC). It has been estimated that 14% to 51% of the CVCs implanted in children...
with malignancies may be complicated by bacteremia and that the incidence of infections is 1.4 to 1.9 episodes per 1000 CVC days. 1-5 Central venous catheters are inserted due to various conditions: to administer intravenous fluids, medications, blood products, and parenteral nutrition fluids, as well as to monitor hemodynamic status and to provide hemodialysis. Port or chemo port from all implantable venous access devices in children with cancer offers many advantages, also less risk of catheter-related sepsis in comparison to other central venous access devices.

There are several possible complications connected to port implantation such as pneumothorax, hemotherax, hematoma, arterial puncture, arrhythmias, wound dehiscence, hematoma, seroma, wound infection, catheter occlusion or misplacement, reservoir fracture, rotation or membrane disruption, drug extravasation, upper extremity deep vein thrombosis, venous thromboembolism, catheter embolization, pinchoff syndrome, local skin infection, and bloodstream infection (BSI). Of them, all BSIs are most important and can be life-threatening. Risk factors for catheter-related bloodstream infection (CRBSI) are well defined: extreme age, immunosuppression, coexisting diseases such as head and neck cancer, diabetes, malnutrition, neutropenia, and chronic steroid use. 1,4,6,7 Several types of CVC infections were defined according to Infectious Diseases Society of America (IDSA) guidelines: exit-site infection, tunnel and pocket infection, and BSI. 8 The last one depending on the type of CVC are called port-associated bloodstream infections (PABSIs) or CRBSI and catheter-associated bloodstream infections (CABSI). Confirmation of CRBSI or PABSI requires specific laboratory testing that identifies the catheter as the source of infection. According to IDSA definition, PABSI/CABSI are: at least 1 set of positive blood cultures of recognized pathogen, including Enterobacteriaceae or other Gram-negative bacilli, Staphylococcus aureus, and fungi, without other identifiable infection foci, or at least 2 sets of positive blood cultures of bacteria that were potential skin contaminants, including coagulase-negative staphylococci, Propionibacterium species, Bacillus species, or micrococci, together with clinical signs of active infection (fever over 38°C, chills, or leukocytosis). 8 The sudden onset of fever, chills, and the growth of inflammatory markers are indications for blood collection and blood culture diagnostic tests. According to the IDSA guidelines, blood cultures should not be routinely collected in children without the symptoms of infection. 8

After suspicion of PABSI/CABSI, treatment gives several options: first, empiric antibiotic treatment, then targeted antibiotic treatment, and the least advantageous option, which is catheter removal. 8-10 The treatment should be continued until resolution of the infection. In case of PABSI-related septic shock, the port should be removed immediately. 8,11

This study describes the epidemiology of a port-associated bacteremia as well as a profile of microorganisms responsible for PABSI in pediatric hemato-oncological patients treated in a single center.

### Material and Methods

The retrospective analysis included hemato-oncological patients who had implanted a port, hospitalized between 2010 and 2015 at the Department of Pediatric Oncology, Hematology and Bone Marrow Transplantation, Poznan University of Medical Sciences. The medical records of patients were reviewed for demographic characteristics, diagnosis, port-related complications, and their management. Data were collected from patients’ electronic medical records containing complete information on medical examinations and supplementary tests, diagnosis, timing, and type of port-associated complication. This retrospective analysis obtained ethical clearance from the Ethics Committee of Poznan University of Medical Sciences. An informed consent for study participation was obtained from all patients’ guardians.

Port insertion was performed by a pediatric surgeon in an operating room. To avoid the risk of bleeding and perioperative infection, the procedure for port insertion required platelets count not less than 50 000/mm³, absolute neutrophils count higher than 0.5 × 10⁹/L, and international normalized ratio not exceeding 1.5. Ports were cared for by trained nurses, including changing needle and the insertion site dressing once in 7 days. If the port was not used, the port was flushed every 4 weeks with 10 mL of 0.9% saline and locked with 4 mL heparinized saline (100 IU/mL). Blood cultures were taken in suspicion of BSIs, in the presence of fever, chills, or hypotension and increase of inflammatory markers, without an apparent source of infection. Blood cultures samples were collected before administration of the first dose of intravenous antibiotics.

Blood cultures were obtained from the port or peripheral veins by sterile technique. When the blood sample was obtained through a port, the catheter hub was cleaned with alcoholic chlorhexidine (>0.5%), allowing adequate drying time to mitigate blood culture contamination. Port cultures were always performed when a catheter was removed for suspected PABSI. Blood specimens were inoculated into BACTEC Peds Plus/F (Becton-Dickinson, Sparks, Maryland) culture bottles. Bacterial identification and antibiotic susceptibility testing, including extended-spectrum beta-lactamases, methicillin-resistant Staphylococcus epidermidis (MRSE), and vancomycin-resistant enterococci, were performed in a hospital laboratory. Bloodstream infection was referred to the growth of a bacterial pathogen in blood culture derived from a patient with fever or other signs of infection.

In the analysis, patients were divided into 2 groups, the first with BSI and the second one without bacteremia. Port-related complications and its frequency were compared between the 2 groups. The incidence of PABSI was estimated as PABSI episodes per 1000 port-days. Port-associated bloodstream infection–free survival was defined as the duration between port implementation and development of the first PABSI in “BSI group” or last follow-up date in the “nonbacteremia group.” In all patients, laboratory and microbiologic data of PABSI were collected. The data included timing of infection and time since port insertion; laboratory and microbiologic characteristics:
C-reactive protein level, procalcitonin level, bacterial identification, and antibiotic susceptibility tests; and management data: port removal, delay between the day the infection is detected and port removal.

Statistical analyses were conducted with GraphPad Prism 6 software (GraphPad, San Diego, California). Differences between patients’ groups were analyzed with Student t test or Mann-Whitney U test. Throughout the analysis, P value <.05 was considered statistically significant.

Results
In a period between January 2010 and January 2015 at the Department of Pediatric Oncology, Hematology and Bone Marrow Transplantation, 277 ports were inserted in 241 patients. A total of 183 094 catheter days were analyzed: 16 patients had more than 1 insertion of a port (11 children 2 times, 2 children 3 times, 1 child 4 times, and 2 children 5 times). The demographic features and underlying diagnosis are presented in Table 1. Most of the children (58.84%) were diagnosed with hematological malignancies (leukemia and lymphoma).

The prevalence of complications connected to the implantation or presence of the catheter was analyzed. The commonest observed complication was PABSI. The cumulative incidence rate of PABSI was 40.07%, and the incidence density was 0.6 per 1000 port-days. In 2 patients, time between port implantation and PABSI was shorter than 7 days; in 11 children, BSI was observed between 8 and 30 days after port implantation; and in the rest, 98 patients BSI appeared over 30 days after port implantation. The second most commonly observed complication was mechanical ones—in 30 of 277 patients. The cumulative incidence rate of mechanical complications was 10.83%, and the incidence density was 0.17 per 1000 port-days (details are presented in Table 2). There were no deaths directly related to port complications.

In 86 patients (31.05%), ports were removed before termination of the chemotherapy, due to complications related to the port. The mean duration of catheterization per patient was 660.99 days, ranging from 1 to 2088 days, and was 465.83 days in the BSI group and 791.49 days in the nonbacteremia group. In the BSI group, the need for removal of the catheter was more common in comparison with a nonbacteremia group. The difference was of statistical significance. In the BSI group, 67 (60.36%) of 111 patients had the catheter removed before termination of the therapy. Among them, 2 patients had catheters removed within 2 weeks after implantation, another 2 patients between 2 and 4 weeks, and the rest 63 over 30 days after port implantation. The delay between the onset of the infection and port removal was as follows: 58 ports were removed within 14 days the infection was detected, 6 ports between 2 and 4 weeks the BSI was found, and 3 over 4 weeks after the infection was proved. In patients without bacteremia, significantly less catheters were removed before termination of the treatment (19/166; 11.45%).

| Table 1. The Demographic Features and Underlying Diagnosis of Analyzed Patients. |
|---------------------------------------------------------------|
|                  | All patients (N = 277) | Patients With BSI (n = 111) | Patients Without Bacteremia (n = 166) |
|------------------|-----------------------|-----------------------------|--------------------------------------|
| **Age**          |                       |                             |                                      |
| Median           | 4.3                   | 2.8                         | 5.85                                 |
| Min              | 0.17                  | 0.17                        | 0.2                                  |
| Max              | 18                    | 18                          | 18                                   |
| **Gender**       |                       |                             |                                      |
| n                | n                     | n                           | n                                    |
| **F**            | 108                   | 43                          | 65                                   |
| **M**            | 169                   | 68                          | 101                                  |
| **Diagnosis**    |                       |                             |                                      |
| ALL              | 92                    | 49                          | 43                                   |
| AML              | 32                    | 17                          | 15                                   |
| Lymphoma         | 39                    | 19                          | 20                                   |
| Neuroblastoma    | 24                    | 10                          | 14                                   |
| CNS tumors       | 29                    | 11                          | 18                                   |
| Nephroblastoma   | 12                    | 4                           | 8                                    |
| Soft tissue sarcoma | 13                   | 6                           | 7                                    |
| LCH              | 8                     | 4                           | 4                                    |
| Germinal tumors  | 10                    | 2                           | 8                                    |
| Other            | 18                    | 11                          | 7                                    |
| **Median**       |                       |                             |                                      |
| Age              | 4.3                   | 2.8                         | 5.85                                 |
| Min              | 0.17                  | 0.17                        | 0.2                                  |
| Max              | 18                    | 18                          | 18                                   |
| **Gender**       |                       |                             |                                      |
| n                | n                     | n                           | n                                    |
| **F**            | 108                   | 43                          | 65                                   |
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| CNS tumors       | 29                    | 11                          | 18                                   |
| Nephroblastoma   | 12                    | 4                           | 8                                    |
| Soft tissue sarcoma | 13                   | 6                           | 7                                    |
| LCH              | 8                     | 4                           | 4                                    |
| Germinal tumors  | 10                    | 2                           | 8                                    |
| Other            | 18                    | 11                          | 7                                    |

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BSI, bloodstream infection; CNS, central nervous system; F, female; LCH, Langerhans cell histiocytosis; M, male.

Staphylococcus species were the most commonly isolated organisms from the patients with PABSI. Coagulase-negative staphylococci were the pathogens most commonly isolated (60/111; 54.05%), with Staphylococcus epidermidis present in 39.64% (44/111) of BSI patients. Gram-negative microorganisms were also prevalent (30/111; 27.03%). Candida was cultured from 5.4% of blood samples (6/111). Details about microorganism identification and antibiotic susceptibility tests are presented in Table 3. Bacteria resistant to standard antibiotic treatment were detected in 38.74% (43/111) of blood samples. The most prevalent resistant pathogen was MRSE—30.63% (34/111 positive samples). More than 1 pathogen was detected in 19 blood samples (17.12%).

Discussion
The presented retrospective analysis provided the description of PABSI, especially in relation to the incidence of bacteria resistant to standard antibiotic treatment in children treated for hematono-logical diseases. Although the port of all implantable venous access devices offers the lowest risk of catheter-related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port. Pektas et al. showed that BSI was also the commonest related sepsis compared to other central access venous devices, BSIs were the most common complication associated with the port.
The infection rate of the port has been reported in the literature, ranging between 0.09 and 2.8/1000 catheter-days and 0.8% to 7.5% in different pediatric oncology case series. However, there is still only a few studies being conducted in pediatric cancer populations, in comparison to adults. The majority of them concern nonhomogeneous groups in terms of different types of implantable venous access devices. Catheter-related bloodstream infection is defined as the presence of bacteremia originating from an intravenous catheter. The diagnosis of CRBSI requires that the same organism grows from the catheter tip or blood samples taken from the catheter and from the peripheral vein. Other situations are clearly stated in guidelines, but they all require several cultures collected on separate occasions. However, it should be stated

| Complications          | All (N = 277) | BSI Patients (n = 111) | Nonbacteremia Patients (n = 166) |
|------------------------|---------------|------------------------|----------------------------------|
|                        | Prevalence per 1000 Port-Days | n (%) | n (%) | n (%) |
| Periprocedurala        |               | 9 (3.25) | 3 (2.70) | 6 (3.61) |
| Local skin infection   |               | 26 (9.39) | 19 (17.12) | 7 (4.22) |
| Mechanical             |               | 30 (10.83) | 20 (18.02) | 10 (6.02) |
| Catheter occlusion     |               | 12 (4.33) | 5 (4.50) | 7 (4.22) |
| Drug extravasation     |               | 2 (0.72) | 2 (1.80) | 0 |
| BSI                    |               | 111 (40.07) | 111 (100%) | 0 |
| Reason for port removing |               | n (%) | n (%) | n (%) |
| Infection              |               | 54 (19.49) | 54 (48.65) | 0 |
| Termination of treatment |             | 169 (61.01) | 36 (32.43) | 133 (80.12) |
| Otherb                 |               | 54 (19.49) | 21 (18.92) | 33 (19.88) |

Abbreviation: BSI, bloodstream infection.
aPneumothorax, hemothorax, hematoma, arterial puncture, arrhythmias.
bCatheter mechanical complication (occlusion or misplacement, reservoir fracture, rotation, or membrane disruption), periprocedural complications, and drug extravasation.

| Pathogens Resistant to Standard Treatment, 43/111 (38.74%) | n (%) |
|-----------------------------------------------------------|-------|
| Staphylococcus epidermidis                               | 6/111 (5.40%) |
| Staphylococcus haemolytic                                | 19/111 (17.12%) |
| MRSE (34/43; 79.07%)                                      |       |
| Staphylococcus epidermidis                               | 28 (65.12) |
| Staphylococcus haemolytic                                | 3 (0.07) |
| Staphylococcus warneri                                   | 1 (0.02) |
| Staphylococcus aureus                                    | 2 (0.05) |
| VRE (1/43; 2.32%)                                         |       |
| Enterococcus faecium                                     | 1 (0.02) |
| ESBL (7/43; 16.28%)                                       |       |
| Klebsiella pneumonia                                     | 4 (0.09) |
| Enterobacter cloacae                                     | 2 (0.05) |
| Pseudomonas aeruginosa                                   | 1 (0.02) |

Abbreviations: ESBL, extended-spectrum $\beta$-lactamases; MRSE, methicillin-resistant *Staphylococcus epidermidis*; VRE, vancomycin-resistant enterococci.
that the proposed definitions are more important for monitoring and reporting purposes than for clinical use. It is difficult to obtain reliable blood cultures in children. In the case of such a special population as children undergoing chemotherapy, the symptoms of infection without a reliable source and a positive blood culture test are sufficient to start empirical treatment.\textsuperscript{8,15} Any additional intravenous blood collection causes unnecessary harm to children, especially for oncology patients undergoing so many painful procedures. Ammann et al. proved that taking simultaneous central and peripheral cultures was not beneficial, comparing to one sample, in febrile neutropenic patients.\textsuperscript{1} Sensitivity of blood culture is also variable. Positive rates are low in patients who are already on antimicrobial treatment, making a reliable diagnosis of BSI even more difficult. In our analysis, not all children meet IDSA criteria for BSI diagnosis, but positive blood cultures were accompanied by clinical signs of infection.\textsuperscript{8,16,17} In the case of BSI in a child with symptoms of infection without a defined other source, the introduction of treatment is in the best interest of the patient.

From the risk factors for BSIs, young age is one of the most important factors. In some studies, BSI predominated in children younger than 2 years of age.\textsuperscript{10} It was a preponderance of BSI also in our patients. The mean age in the BSI group was significantly lower in comparison to the group without infection (5.22 vs 7.68 years). It can be explained by hygienic aspects. In younger children, using diapers brings the risk of contamination with fecal flora. In older children, it is easier to isolate the body area around the catheter, and the care over this area is taken by nurses. In small children, parents are more involved in hygienic procedures. The immature immune system can also increase the risk of infection.\textsuperscript{18}

Coagulase-negative staphylococci, among all BSI cases, were the most frequently detected pathogens in our BSI patients. The profile of microorganisms and rates of infection may vary depending on the health-care center and the characteristics of the patient population.\textsuperscript{19-22} Risk factors in oncological children are different from most of other patients. Neutropenia and chronic steroid use were associated with higher rate of PABSI caused by Gram-negative and Gram-positive bacteria.\textsuperscript{23} The organisms associated with CRBSI represent mostly the normal resident flora of the skin at the insertion site. Some of the infections can be caused by inappropriate technique of implantation, causing infection from the skin. But the long time span between implantation and infection (in our group, most infections occurred more than 30 days after the implementation) excludes such probability. Ammann et al. postulate that in patients with severe chemotherapy-induced mucositis, pathogens, such as Escherichia coli or Streptococcus viridans, can cause secondary BSI.\textsuperscript{2} Finding coagulase-negative staphylococci in only one blood culture can be considered as skin contamination, and in the absence of clinical features of infection, patient should not be diagnosed with BSI. According to the Clinical Practice Guidelines for the Diagnosis and Management of Intravascular Catheter-Related Infection, about 34% of CRBSI in children are caused by coagulase-negative staphylococci and 25% by Staphylococcus aureus.\textsuperscript{8} Children with cancer are a special group of patients; thus, they are difficult to compare with others. In a study by Viana Taveira et al published in 2017, during 77 541 catheter-days, 50% of patients developed central line-associated bloodstream infections (CLABSIs).\textsuperscript{4} The commonest isolated pathogens were coagulase-negative staphylococci, but Gram-negative microorganisms (46.8%) were also prevalent. In a study by Tsai et al on 146 pediatrics patients, 32.9% of all complications were infections.\textsuperscript{13} The CLABSI incidence was 4.49/1000 catheter-days. Enterobacteriaceae (40.2%) and coagulase-negative staphylococci (20.7%) were the predominant pathogens. Amman et al. in a prospective multicenter study done in oncology pediatrics patients found BSI in 70% of patients, coagulase-negative staphylococci bacterial sepsis in 27%, and candidemia in 2%.\textsuperscript{1} In a study conducted on a population similar to ours published in 2015 by Pektas et al, coagulase-negative staphylococci were also dominant pathogens.

In our BSI patients, a significant percentage (close to 39%) of found pathogens were resistant to antibiotics. Proportion of multidrug-resistant bacterial pathogens was high, probably due to broad-spectrum antibiotics often and repeatedly used in pediatric oncology patients. Bacteria resistant to standard antibiotic treatment are important causative agents of bacteremia, with MRSE being the predominant pathogen. The presence of multidrug-resistant bacteria is of special importance, with the first priority of multidrug-resistant bacteria Acinetobacter, Pseudomonas aeruginosa, and Enterobacteriaceae resistant to carbapenem. Resistance to antibiotic is a growing problem, especially in hospital-acquired infections. The awareness of that problem is necessary for planning the treatment.

Invasive Candida infection is an important cause of morbidity in children with cancer. In our study, Candida was cultured from 5.4% of blood samples. In a retrospective 4-year study performed by Gokcebay, the incidence of candidemia in children with hematological diseases was 5.2 per 1000 hospital-days.\textsuperscript{24} Indwelling CVC antimicrobial therapy lasting longer than 3 days have been identified to be a risk factor for candidemia in patients with malignancy.\textsuperscript{24,25} All these risk factors were identified in our patients. The guidelines for the treatment of candidemia recommend the removal of CVC in the presence of BSIs.

In our patients with bacteremia, early removal of the port was a common practice (in almost 49% of patients). In most of the cases, the catheter was removed within 14 days from the diagnosis of BSI. Removal of the central catheter is a last choice, often associated with discontinuation of antinecancer treatment and the need for CVC reimplantation. Therefore, the decision to remove the port in this group of patients is particularly difficult, but it is required in BSI with resistant pathogens or fungal infections. Shim et al proved that for Staphylococcus and Candida species, infections removal is always necessary, while the CVC can be successfully maintained for infection with coagulase-negative Staphylococcus, Corynebacterium jeikeium, or P aeruginosa.\textsuperscript{26} Ji et al indicated that port must be removed immediately when chemotherapy was terminated, and thrombosis, occlusion, skin necrosis, or
BSI from *Staphylococcus*, *Candida*, *Pseudomonas*, *Bacillus*, and *C. jeikeium* were observed.\(^1\)\(^2\)\(^7\) If a patient presents with fever and a negative culture study, the decision for removing the port device is difficult. In that situation, we can talk about probable catheter-related infection with negative blood culture drawn from a port and from a peripheral vein with clinical signs of sepsis and without other focus of infection.

Venous thromboembolism, bleeding, and mechanical complication occurred rarely 0.2 per 1000 catheter-days. In our study, the prevalence of catheter occlusion due to thromboembolism was 0.06 per 1000 catheter-days and due to mechanical complications 0.17 per 1000 catheter-days.

There are limitations of our study such as retrospective and single-centered study. The incidence of infection could have been underestimated if there was the clinically undetected port-related bloodstream infection.

Children undergoing chemotherapy are at high risk for invasive infections. A feverish child with features of an infection with no established source, such as an inflammatory reaction around the catheter, an inflammatory response suggesting a bacterial infection, and positive blood culture from the port, requires immediate treatment. Even if positive bacterial culture is the colonization of the central catheter, it must be taken seriously and treated with appropriate antibiotics. Bacteriological results obtained from blood culture collected from the port are a very valuable clue. To rely on blood cultures taken from the port, they should be collected only in symptomatic patients suspected of infection. Routine blood collection by the central catheter for prophylactic purposes is not recommended. It can lead to positive cultures in a patient without infection.

**Conclusions**

From all port-associated complications, BSIs and mechanical complications were the most often observed complications. The commonest pathogens responsible for PABSI were coagulase-negative staphylococci. Pathogens resistant to standard antibiotic treatment play an important role in PABSI, with MRSE being the predominant pathogen. Port-associated bloodstream infections are a common reason for preterm removal of a port.

**Authors’ Note**

Ewelina Gowin designed the study, collected data, and wrote the article. Bogna Sztwiątek-Kościelna collected data and wrote the article. Przemysław Mańkowski collected data and wrote the article. and Danuta Januszkiewicz-Lewandowska conceived the presented idea, provided critical feedback, helped shape the research, and also wrote the article. All authors discussed the results and contributed to the final article. 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.

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