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ORIGINAL ARTICLE

TGF-β1 gene polymorphism in renal transplant patients with and without gingival overgrowth

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BACKGROUND: The incidence of gingival overgrowth among renal transplant patients treated with cyclosporine A ranges from 13% to 84.6%, and the overgrowth is not only esthetic but also a medical problem. We studied the determination of association between TGF-β1 (*TGFB1*) gene polymorphism and gingival overgrowth in kidney transplant patients medicated with cyclosporin A.

METHODS: Eighty-four kidney transplant patients with gingival overgrowth and 140 control transplant patients without overgrowth were enrolled into the case control study. *TGFB1* polymorphism was determined using the PCR-RFLP assay for +869T>C in codon 10 and +915G>C in codon 25 as well as TaqMan real-time PCR assays for promoter –800G>A and –509C>T SNPs.

RESULTS: In kidney transplant patients suffering from gingival overgrowth, mean score of gingival overgrowth was 1.38 ± 0.60, whereas in control subjects it was 0.0. The patients with gingival overgrowth were characterized by similar distribution of *TGFB1* genotypes and allele in comparison to subjects without gingival overgrowth. Among 16 potentially possible haplotypes of *TGFB1* gene, only four were observed in the studied sample of kidney transplant patients: G_C_T_G, G_T_C_G, G_C_C_C, and A_C_T_G, with similar frequency in patients with and without gingival overgrowth.

CONCLUSION: No association between the *TGFB1* gene polymorphism and gingival overgrowth was revealed in kidney transplant patients administered cyclosporine A.

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Keywords: TGF-β1; gingival overgrowth; polymorphism

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Introduction

Gingival overgrowth frequently occurs in transplant patients receiving immunosuppressive drugs such as cyclosporine (INN ciclosporin), which has been widely used since 1970s. The incidence of gingival overgrowth among renal transplant patients treated with cyclosporine A ranges from 13% to 84.6% (Somacarrera *et al*, 1994; Margiotta *et al*, 1996; Pernu *et al*, 2001; Afonso *et al*, 2003). Gingival overgrowth induced by cyclosporine A is probably the consequence of alterations in the turnover of epithelial and connective tissues, resulting in an increase in structural proteins sometimes associated with epithelial thickening. Numerous studies have investigated potential risk factors in the development of the condition, including the extent of renal disease, interval since transplantation, duration of renal replacement therapy, dose of cyclosporine A, gingival inflammation, plaque indices, and recipient HLA-phenotype. Although some studies have suggested some associations between the incidence and severity of gingival overgrowth and gender, pretransplant diagnosis, age at transplantation, duration of therapy, and cyclosporine dosage, others have failed to find any significant drug-related risk factors for the development of the disease as previously reported (Seymour *et al*, 1996; Thomas *et al*, 2000, 2001; Afonso *et al*, 2003). However, it remains unclear why a proportion of patients are susceptible to gingival overgrowth, whereas others remain unaffected.

It was hypothesized that gingival overgrowth induced by cyclosporine results in a disturbance in the homeostatic balance, which is characterized by an increase in both the number of fibroblasts and an increase in the volume of the extracellular matrix. This loss of growth control results in an accumulation of redundant tissue of relatively normal composition (McGaw and Porter, 1988). There is considerable information supporting the role for transforming growth factor-β1 (TGF-β1) in mediation of the dysregulated fibroblast proliferation and extracellular matrix synthesis. Evidence from experimental studies demonstrates that gingival fibroblasts in culture exhibit a proliferative response to TGF-β1

(Anderson *et al*, 1998; James *et al*, 1998). It was also reported that TGF-β1 suppresses extracellular matrix degradation via downregulation of metalloproteinases (MMP) production and inducing tissue inhibitor of MMP synthesis (Edwards *et al*, 1987; Overall *et al*, 1989). Also, administration of TGF-β1 antisense oligonucleotide increases the expression of collagen degrading enzymes in human gingival fibroblasts (Cotrim *et al*, 2002). Experimental data also revealed that proliferation of rat gingival cells under cyclosporine A depended on TGF-β1 mRNA induction by the drug (Yoshida *et al*, 2005). Clinical studies demonstrated that circulating and gingival crevicular fluid levels of TGF-β1 were increased in cyclosporin-medicated transplant patients (Buduneli *et al*, 2001), and TGF-β1 was found to be an independent risk factor for gingival overgrowth in immunosuppressed patients, also medicated with cyclosporine A (Ellis *et al*, 2004; Gurkan *et al*, 2008). Recently, an accumulation of extracellular matrix related to TGF-β1 overexpression in cultured fibroblasts from patients medicated with cyclosporine A has been reported by Dreyfuss *et al* (2010).

It was reported that expression of TGF-β1 gene (*TGFB1*) may depend on genetic constitution, i.e. within the *TGFB1* some functional single nucleotide polymorphism (SNPs) were defined. Clinical studies in kidney transplant patients medicated with cyclosporine A suggested an association between *TGFB1* gene polymorphism located in coding regions (codon 10 - +869T > C Pro10Leu and codon 25 + 915G > C Arg25Pro) and gingival overgrowth (Linden *et al*, 2001; Radwan-Oczko *et al*, 2006). However, contradictory results in kidney transplant patients with respect to the same as aforementioned SNPs are also available (Radwan-Oczko *et al*, 2008).

Therefore, we investigated the role of four SNPs within *TGFB1* gene, previously associated with alterations in gene expression level, in gingival overgrowth in kidney transplant patients medicated with cyclosporine A. Two of the SNPs were previously evaluated, and lead to aminoacid substitution: +869T > C in codon 10 (rs1800470, Pro10Leu) and +915G > C in codon 25 (rs1800471, Arg25Pro). The other two SNPs are located in promoter region of *TGFB1*: −800G > A (rs1800468) and −509C > T (rs1800469), and thus, can affect promoter function and gene expression (Grigner *et al*, 1999; Grainger *et al*, 2000).

Materials and methods

The protocol of the study was approved by the ethics committee of the Pomeranian Medical University, Szczecin, Poland, i.e. the experiments were undertaken with the understanding and written consent of each subject and according to ethical principles, including the World Medical Association Declaration of Helsinki (version, 2002). Patients of Polish origin, Caucasians, hospitalized and then followed up at the Department of Nephrology, County Hospital, Szczecin, Poland were recruited for the study from 2002 to 2010, after giving informed consent. A total of 84 unrelated kidney transplant patients suffering from gingival overgrowth (61 male patients, 23 female patients) aged from 16 to 70 years (mean 42.2 ± 13.1 years) were enrolled in the study. All patients were examined by two independent consultant periodontal specialists 6 months after kidney transplantation. Periodontists were blinded to SNP status. The patients were assessed using a clinical scoring method according to Pernu *et al* (1992). The patients were ascribed a general whole-mouth score between 0 and 3: 0 = no overgrowth seen; 1 = mild gingival overgrowth (thickening of the marginal gingiva and/or lobular granulation of the gingival pocket as well as overgrowth covering the gingival third of the crown or less); 2 = moderate gingival overgrowth (overgrowth extending to the middle of the crown); 3 = severe gingival overgrowth (overgrowth covering two-thirds of the crown or affectation of the whole attached gingiva).

Control samples were from 140 kidney transplant patients (91 male patients, 49 female patients), aged from 17 to 66 years (mean 39.7 ± 11.4 years), who were free from gingival overgrowth signs at 6 months after transplantation, as evaluated by consultant periodontal specialists. Patients' characteristics are shown in Table 1.

During the study period, all subjects were administered cyclosporin A, azathioprine, prednisone, and one of two calcium channel blockers, i.e. diltiazem or verapamil; eight patients without gingival overgrowth were given atenolol or prazosine. The medication regimens administered in both groups of the study, including cyclosporin A dose and concentrations, dosages of verapamil, diltiazem, and prednisone evaluated at monthly intervals during 6 months were comparable, i.e. all calculated *P*-values were > 0.05 (Table 2). The serum concentrations of cyclosporin A were measured by fluorescence polarization immunoassay using TDx analyzer (Abbott) in all patients.

Genotyping

Four SNPs within *TGFB1* gene, previously associated with alterations in gene expression level were selected for the purpose of this study. Two of them are located in promoter region of *TGFB1*: −800G > A (rs1800468) and −509C > T (rs1800469), the others lead to aminoacid

Table 1 Patients' characteristics

Parameter	*Patients with gingival overgrowth (n = 84)*	*Patients without gingival overgrowth (n = 140)*	P
Gender distribution (F/M)	23/61	49/91	0.301
Age (years)*	42.2 ± 13.1	39.7 ± 11.4	0.233
Place of residence: city/country	44/40	83/57	0.332
Smoking	16	31	0.615
Diabetes	39	47	0.065
Acute rejection	3	20	0.011

All calculations performed by means of Fisher exact test, except *calculated by *t*-test.

substitution: +869T > C (rs1800470, Pro10Leu) and +915G > C (rs1800471, Arg25Pro). Genomic DNA was extracted from 200 μl of whole blood samples using GeneMATRIX Quick Blood DNA Purification Kit (EURx, Poland). Each individual was genotyped for a presence of four SNPs in *TGFB1* gene. The allelic discrimination TaqMan real-time polymerase chain reaction (PCR) assays (Assay IDs: C_8708474_20, C_8708473_10, and C_22272997_10, Applied Biosystems, USA) were used for detection of −800G > A,−509C > T, and +869T > C SNPs, respectively. Fluorescence data were captured using an ABI PRISM 7500 FAST Real-Time PCR System (Applied-Biosystems), after 40 cycles of PCR. For analysis of +915G > C polymorphism, a PCR-RFLP method was applied. Briefly, PCR was performed in 15μl of total volume, with a pair of primers: 5′-cgc tgc tgt ggc tac tgg t-3′, and 5′-ctc cgg ttc tgc act ctc c-3′, previously described by Pulleyn *et al*, 2001;. Subsequently, a 254 bp PCR product was digested with the *Cfr13*I endonuclease (Fermentas, Lithuania), yielding two DNA fragments (171 + 83 bp) in case of wild-type G allele. The +915G > C substitution creates additional restriction site recognized by *Cfr13*I, yielding three fragments (141 + 83 + 30 bp), which was visualized after electrophoresis in 3.5% agarose gels stained with ethidium bromide (Pulleyn *et al*, 2001).

Statistical analysis

The data were tested for Hardy–Weinberg equilibrium by calculating expected frequencies of genotypes and comparing them to the observed values using the Chi-squared test (Statistica 8.0, Statsoft Software, Warsaw, Poland). Associations between categorical variables were assessed by the Fisher exact test. The EH program (Jurg Ott, Rockefeller University, New York) was used to estimate haplotype frequencies. Linkage disequilibrium (LD) was measured as follows: the D' was calculated using 2LD software, and squared correlation coefficient (r^2) was evaluated. Odds ratios (OR) and 95% confidence interval (95% CI) were calculated using the Newcombe-Wilson method without the continuity correction. A *p*-level of less than 0.05 was considered statistically significant.

Results

Out of 84 patients with gingival overgrowth, 57 subjects were classified as score 1 of gingival overgrowth, 22 patients were ascribed score 2 and 5 subjects score 3. Mean score of gingival overgrowth was 1.38 ± 0.60 according to Pernu's scoring system (Pernu *et al*, 1992). Control transplant patients were characterized by healthy gingiva, i.e. were scored 0.

The genotype frequency distribution for all analyzed SNPs did not show a significant deviation from Hardy-Weinberg equilibrium in any of the study groups ($P > 0.1$). The distribution of *TGFB1* gene genotypes and alleles in kidney transplant patients is shown in Table 3. The patients with gingival overgrowth induced by immunosuppressive medication were characterized by similar distribution of *TGFB1* genotypes and allele to

Table 2 Characteristics of medication at monthly intervals after transplantation

	1 month	*2 months*	*3 months*	*4 months*	*5 months*	*6 months*
Patients with gingival overgrowth (n = 84)						
Cyclosporine concentration (ng/ml)	420.0 ± 234.2	380.1 ± 206.7	308.3 ± 170.2	306.3 ± 198.2	276.5 ± 193.3	280.8 ± 179.4
Cyclosporine dose (mg/day)	300.0 ± 84.1 (n = 84)	280.9 ± 78.8 (n = 84)	260.7 ± 79.3 (n = 84)	251.2 ± 76.9 (n = 84)	245.2 ± 68.6 (n = 84)	235.4 ± 65.4 (n = 84)
Diltiazem dose (mg/day)	173.8 ± 24.5 (n = 56)	173.8 ± 24.5 (n = 56)	173.8 ± 24.5 (n = 56)	173.8 ± 24.5 (n = 56)	173.8 ± 24.5 (n = 56)	173.8 ± 24.5 (n = 56)
Verapamil dose (mg/day)	174.5 ± 72.2 (n = 28)	174.5 ± 72.2 (n = 28)	174.5 ± 72.2 (n = 28)	174.5 ± 72.2 (n = 28)	174.5 ± 72.2 (n = 28)	174.5 ± 72.2 (n = 28)
Prednisone dose (mg/day)	18.8 ± 7.4 (n = 84)	16.3 ± 5.2 (n = 84)	15.1 ± 4.3 (n = 84)	12.4 ± 3.7 (n = 84)	10.6 ± 3.0 (n = 84)	9.5 ± 3.1 (n = 84)
Patients without gingival overgrowth (n = 140)						
Cyclosporine concentration (ng/ml)	413.2 ± 258.1	370.8 ± 288.2	326.8 ± 263.5	276.6 ± 159.8	296.9 ± 195.6	249.8 ± 172.2
Cyclosporine dose (mg/day)	282.4 ± 99.1 (n = 140)	274.4 ± 88.2 (n = 140)	256.5 ± 85.4 (n = 140)	254.9 ± 83.6 (n = 140)	246.2 ± 77.2 (n = 140)	238.4 ± 73.0 (n = 140)
Diltiazem dose (mg/day)	172.2 ± 30.4 (n = 104)	172.2 ± 30.4 (n = 104)	172.2 ± 30.4 (n = 104)	172.2 ± 30.4 (n = 104)	172.2 ± 30.4 (n = 104)	172.2 ± 30.4 (n = 104)
Verapamil dose (mg/day)	184.3 ± 60.5 (n = 36)	184.3 ± 60.5 (n = 36)	184.3 ± 60.5 (n = 36)	184.3 ± 60.5 (n = 36)	184.3 ± 60.5 (n = 36)	184.3 ± 60.5 (n = 36)
Prednisone dose (mg/day)	17.9 ± 4.5 (n = 140)	15.2 ± 3.0 (n = 140)	13.4 ± 3.4 (n = 140)	12.1 ± 2.5 (n = 140)	11.9 ± 2.6 (n = 140)	11.9 ± 2.6 (n = 140)

Table 3 Distribution of *TGFB1* alleles and genotypes among kidney transplant patients with and without gingival overgrowth

	Gingival Overgrowth (n = *84*) n *(%)*	*Healthy gingiva* (n = *140*) n *(%)*	P *value*	*OR (95%CI)*
Genotype −800G > A				
GG	78 (92.9)	120 (85.7)	–	–
GA	6 (7.1)	19 (13.6)	0.188	0.48 (0.18–1.27)
AA	0 (0.0)	1 (0.7)	1.000	–
Minor allele carriers (GA + AA)	6 (7.1)	20 (14.3)	0.132	0.46 (0.17–1.20)
Minor allele frequency	6 (3.6)	21 (7.5)	0.103	
Genotype −509C > T				
CC	43 (51.2)	61 (43.6)	–	–
CT	32 (38.1)	63 (45.0)	0.306	0.72 (0.40–1.28)
TT	9 (10.7)	16 (11.4)	0.657	0.80 (0.32–1.97)
Minor allele carriers (CT + TT)	41 (48.8)	79 (56.4)	0.272	0.74 (0.43–1.27)
minor allele frequency	50 (29.8)	95 (33.9)	0.404	
Genotype +869T > C				
TT	34 (40.5)	50 (35.7)	–	–
TC	38 (45.2)	65 (46.4)	0.652	0.86 (0.47–1.55)
CC	12 (14.3)	25 (17.9)	0.424	0.71 (0.31–1.55)
Minor allele carriers (TC + CC)	50 (59.5)	90 (64.3)	0.480	0.82 (0.47–1.42)
Minor allele frequency	62 (36.9)	115 (41.1)	0.404	
Genotype +915G > C				
GG	72 (85.7)	120 (85.7)	–	–
GC	12 (14.3)	20 (14.3)	1.000	1.00 (0.46–2.16)
CC	0 (0.0)	0 (0.0)	1.000	-
Minor allele carriers (GC + CC)	12 (14.3)	20 (14.3)	1.000	1.00 (0.46–2.16)
Minor allele frequency	12 (7.1)	20 (7.1)	1.000	

All calculations performed by means of Fisher exact test, using homozygotes for a major (more frequent) allele or major allele frequency as reference.

Table 4 Frequency of *TGFB1* haplotypes among kidney transplant patients with and without gingival overgrowth

	Gingival Overgrowth (2n = *168*) n *(%)*	*Healthy gingiva* (2n = *280*) n *(%)*	P *value*
G_C_T_G	100 (59.5)	144 (51.4)	*0.116
G_T_C_G	50 (33.9)	95 (29.8)	0.236
G_C_C_C	12 (7.2)	20 (7.1)	0.848
A_C_T_G	6 (7.5)	21 (3.6)	0.063

All calculations performed by means of Fisher exact test, using frequency of major haplotype as reference, except* - in relation to the sum of other haplotypes.
EH program (Jurg Ott, Rockefeller University, New York) was used to estimate haplotype frequencies based on results of typing for following SNPs: −800G > A, −509T > C, +869T > C, +915G > C. Among 16 potentially possible haplotypes derived from four SNPs, only four were observed in the studied sample of kidney transplant patients, with respective frequencies: G_C_T_G (0.545), G_T_C_G (0.324), G_C_C_C (0.071), and A_C_T_G (0.060).

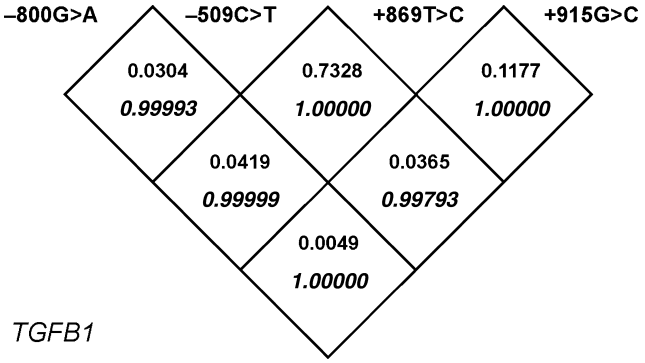

Figure 1 Pairwise LD between the studied *TGFB1* SNPs, estimated on the base of analysis of all study subjects (*n* = 224). Numbers represent r^2 (square correlation coefficient), and D' values (*italics*)

subjects without overgrowth, i.e. SNPs within the two coding regions: +869T > C in codon 10 (rs1800470, Pro10Leu) and +915G > C in codon 25 (rs1800471, Arg25Pro) as well as two promoter region SNPs: −800G > A (rs1800468), and −509C > T (rs1800469). In the next step of the study, we subjected for analysis haplotypes of *TGFB1* gene (Table 4). The results of linkage analysis showed that all analyzed SNPs are in strong linkage in a Polish population (Figure 1). The EH program (Jurg Ott, Rockefeller University, New York) was used to estimate haplotype frequencies based on results of typing for the following studied SNPs: −800G > A, −509T > C, +869T > C, +915G > C. Among 16 potentially possible haplotypes derived from four SNPs, only four were observed in the studied sample of kidney transplant patients, with respective frequencies: G_C_T_G (0.545), G_T_C_G (0.324), G_C_C_C (0.071), and A_C_T_G (0.060). Similar to distribution of the genotypes and allele, frequency of *TGFB1* haplotypes were comparable in kidney transplant patients with and without gingival overgrowth.

Discussion

TGF-β1 patophysiological data as well as reports on functional polymorphism of its gene – *TGFB1* suggest

potential role of the cytokine and polymorphism in the pathology of drug-induced including cyclosporin A, gingival overgrowth (Edwards *et al*, 1987; Overall *et al*, 1989; Anderson *et al*, 1998; James *et al*, 1998; Grigner *et al*, 1999; Grainger *et al*, 2000 Buduneli *et al*, 2001; Cotrim *et al*, 2002; Ellis *et al*, 2004; Yoshida *et al*, 2005; Gurkan *et al*, 2008). However, available data on the effects of *TGFB1* gene polymorphism on gingival overgrowth in kidney transplant patients medicated with cyclosporin A are controversial. Linden *et al* (2001) and Radwan-Oczko *et al* (2006) revealed that polymorphisms in *TGFB1* gene located in coding regions of the gene, i.e. codon 10 - +869T > C Pro10Leu and codon 25 + 915G > C Arg25Pro are genetic factors associated with risk of gingival overgrowth. A subsequent report from the latter group (Radwan-Oczko *et al*, 2008) showed that the aforementioned polymorphisms are not associated with gingival overgrowth observer in kidney transplant patients medicated with cyclosporin A.

Therefore, it was decided to evaluate *TGFB1* gene polymorphism in kidney transplant patients medicated with cyclosporine A. Former studies included polymorphisms in the coding regions of the gene resulting in aminoacid substitutions, namely in codon 10 - +869T > C Pro10Leu and codon 25 + 915G > C Arg25Pro (Linden *et al*, 2001; Radwan-Oczko *et al*, 2006, 2008). However, it is well known that polymorphisms located in promoter region of the gene can also be functional, affecting expression level of genes; hence in the present study, polymorphisms in the promoter of *TGFB1* gene were evaluated at positions −800G > A and −509C > T (Grigner *et al*, 1999; Grainger *et al*, 2000). The promoter polymorphisms, although functional, have not been studied in post-transplant patients with gingival overgrowth yet. Furthermore, we evaluated haplotypes of *TGFB1* gene as potential risk factors associated with gingival overgrowth in patients administered cyclosporine A.

Previous reports, similar to our report, included patients from Caucasian populations, but number of cases analysed was smaller than in the present study. Study by Linden *et al* (2001) involved 164 kidney transplant patients medicated with cyclosporin A (46 with gingival overgrowth), by Radwan-Oczko *et al*, 2006 comprised 92 subjects (50 with gingival overgrowth) and Radwan-Oczko *et al*, 2008; included 98 subjects (of them 54 with gingival overgrowth). The present study involved 224 patients, including 84 with gingival overgrowth. Having in mind the power of the study, the present report seems to be most reliable in comparison with the further studies. The results of the present study are in line with the report of Radwan-Oczko *et al* (2008) demonstrating no association of the coding region polymorphisms of *TGFB1* gene and gingival overgrowth, contrary to studies of Linden *et al*, 2001 and Radwan-Oczko *et al*, 2006. The present study was extended to functional polymorphisms located in promoter region of the *TGFB1* gene, and similarly to results from coding regions any associations of genetic factors with gingival overgrowth in kidney transplant patients were revealed. Finally, haplotype frequencies based on results of typing for the studied SNPs: −800G > A, −509T > C, +869T > C, +915G > C were analyzed. Similar to genotypes, the analyzed haplotypes did not discriminate the studied groups, i.e. kidney transplant patients medicated with cyclosporin A, with and without gingival overgrowth.

Interaction between simultaneously administered drugs affecting enlargement have been also reported. Cyclosporin A-treated patients are often comedicated with azathioprine and prednisolone, which can modify the severity of gingival overgrowth (Wilson *et al*, 1998). In contrast, patients on cyclosporin A who are also receiving calcium channel blockers present with a greater risk of the gingival lesions than patients treated with cyclosporine alone (Thomasson *et al*, 1997). However, in the present study, both groups, i.e. patients with and without gingival overgrowth were medicated similarly during observation period. So, the effect of treatment modality of gingival pathology could be neglected in the data analysis.

Another factor that could potentially influence the study conclusions is the number of evaluated cases. As in the present study, analyzed groups comprised of 84 and 140 patients. The conclusions drawn should be considered as preliminary data, and the results of the study should be confirmed by observations from other populations involving larger groups of patients.

Based on the results from the present study it can be concluded that there is no significant association between the *TGFB1* gene polymorphism and gingival overgrowth in kidney transplant patients administered with cyclosporine A as a principal immunosuppressive agent.

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Conflict of interest

There is no conflict of interest.

Author contributions

Dr Kozak and Dr Dziewanowski were responsible for patient evaluation and data analysis, Dr Wajda, Dr Lapczuk, and Dr Kurzawski were responsible for experiments, study design and manuscript preparation, Dr Lipski and Dr Drozdzik M were responsible for research design and manuscript revisions.

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