Received: 9 March 2016 | Revised: 19 June 2016 | Accepted: 19 July 2016

DOI 10.1111/pedi.12429

WILEY ISPAD International Society for Pediatric and Adolescent Diabetes

ORIGINAL ARTICLE

Coincidence of *PTPN22* c.1858CC and *FCRL3* -169CC genotypes as a biomarker of preserved residual β-cell function in children with type 1 diabetes[†,‡]

Małgorzata Pawłowicz[1,2] | Rafał Filipów[3] | Grzegorz Krzykowski[4] | Anna Stanisławska-Sachadyn[5] | Lucyna Morzuch[5] | Julia Kulczycka[6] | Anna Balcerska[1] | Janusz Limon[5]

[1]Department of Paediatrics, Haematology, Oncology and Endocrinology, Medical University of Gdańsk, Gdańsk, Poland

[2]Department of Developmental Neurology, Medical University of Gdańsk, Gdańsk, Poland

[3]Institute of Mathematics, Faculty of Mathematics, Physics and Informatics, University of Gdańsk, Gdańsk, Poland

[4]Institute of Informatics, Faculty of Mathematics, Physics and Informatics, University of Gdańsk, Gdańsk, Poland

[5]Department of Biology and Genetics, Medical University of Gdańsk, Gdańsk, Poland

[6]Laboratory of Immunology and Clinic Transplantology, University Clinical Centre of Gdańsk, Gdańsk, Poland

[†]The study part associated with *PTPN22* c.1858T>C polymorphism evaluation was awarded in the 2nd edition of the *InnoDoktorant* scholarship contest (to M.P.) organized by the Pomorskie Voivodeship and European Union as particularly important for Pomeranian region development.

[‡]Preliminary results from first year of follow up were presented on Fifth Meeting of the EASD Study Group on Genetics of Diabetes (EASD-SGGD) (Poster Session), 7-9 May 2015, Krakow, Poland.

Funding Information: This study was supported by the Grant Number N N407 184137 (Decision Number 1841/B/P01/2009/37 to M. P.) and N N407 339239 (Decision Number 3392/B/P01/2010/39 to A. B.) from the Ministry of Science and Higher Education of Poland/National Science Centre of Poland.

Corresponding Author: Małgorzata Pawłowicz MD, PhD, Department of Developmental Neurology, Medical University of Gdańsk, Dębinki 7, 80-952 Gdańsk, Poland (malgorzatapawlowicz@gumed.edu.pl)

Background: Genotype-phenotype studies in type 1 diabetes (T1DM) patients are needed for further development of therapy strategies.

Objective: Our aims were to investigate the distribution of selected *PTPN22* and *FCRL3* gene polymorphisms and their associations with clinical course of disease in children with newly diagnosed T1DM from the Pomeranian region of Poland.

Subjects/methods: The prospective, longitudinal study of 147 children with newly diagnosed T1DM—autoimmune subtype was conducted. The *PTPN22* c.1858T>C (rs2476601) and *FCRL3* -169C>T (rs7528684) polymorphisms were analyzed using polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) and DNA sequencing. The frequencies of genotypes were compared between the study and population-matched control group (327 random anonymous samples from the Pomeranian region). Selected patients underwent a 24-monthly follow up [periodic re-evaluation of fasting C-peptide concentration (FCP) and hemoglobin A1c (HbA1c) level].

Results: A significantly lower coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes was found in the study group compared with controls ($P = 0.04$). The *PTPN22* c.1858CC and *FCRL3* -169CC genotype combination, restricted to female patients only, was associated with well-preserved residual β-cell function throughout the entire follow up (prolonged FCP level increase up to the sixth month of disease, with further very stable dynamics—FCP median level ≥0.67 ng/mL without significant decrease up to the 24th month). HbA1c levels in this subgroup also remained the lowest during the observation period.

Conclusions/interpretation: Ascertained phenomenon could be explained by an interacting mechanism of the two polymorphisms through estrogen-regulated nuclear factor kappa B signaling in regulatory T (T_{reg}) lymphocytes. This hypothesis, if confirmed, may lead to further development of T_{reg} administration-based therapies.

KEYWORDS

biomarkers; type 1 diabetes mellitus; FCRL3 protein, human; protein tyrosine phosphatase, non-receptor type 22; sex distribution

1 | INTRODUCTION

Our research focused on selected *PTPN22* (protein tyrosine phosphatase non-receptor type 22) and *FCRL3* (Fc receptor-like 3) gene polymorphisms, namely, *PTPN22* c.1858T>C (rs2476601) and *FCRL3* -169C>T (rs7528684), for which significant associations with type 1 diabetes mellitus (T1DM) incidence and/or its autoimmune traits were observed in some, but not all previous studies.[1–3]

The mechanisms of autoimmune response control by the products of both variants appear to be correlated during the activation processes of nuclear factor kappa B (NF-kB) in T-cell/B-cell receptor (TCR/BCR) signal transduction and could mainly affect the suppressive function of regulatory T (T_{reg}) lymphocytes.[4–6]

The *PTPN22* gene encodes lymphoid tyrosine phosphatase (LYP)—the main negative regulator of proximal TCR/BCR signaling molecules.[4,7] The *PTPN22* c.1858T allele, resulting in amino substitution (Trp → Arg) at residue 620, is associated with a higher capacity of LYP to dephosphorylate molecules involved in TCR/BCR signal transduction[4,7]. Genetically determined TCR resistance in T_{reg} subpopulation could decrease its suppressive activity by disturbances in TCR-dependent regulatory functions or interleukin 2 (IL-2) synthesis, mainly associated with NF-kB activation[8,9]. Recently, an additional perinuclear and nuclear LYP pools were detected[7,10]. Nevertheless, their function remains unknown[7,10].

The FCRL3 molecule is mainly expressed in $CD19^+$ B, natural-killer (NK) cells, and at lower levels in T_{reg} lymphocytes[5,6]. The presence of ITAMs and ITIMs (immunoceptor tyrosine-based activation/inhibitory motifs) in its structure indicates a potential regulatory function in TCR/BCR signal transduction, probably via tyrosine protein kinases pathways[5]. The *FCRL3* -169C>T polymorphism is located in the promoter region and functionally modifies NF-kB binding site activity[1,5]. Its higher affinity for NF-kB is associated with the *FCRL3* -169C allele and leads to promoter activity and *FCRL3* gene expression enhancement[5].

The phenomenon of grouping opposing risk genotypes was observed during the joint evaluation of the *PTPN22* c.1858T>C and *FCRL3* -169C>T polymorphisms in rheumatoid arthritis patients[11]. So far, no similar studies in T1DM subjects have been performed.

Taking into account the above-mentioned gene association studies and proposed molecular mechanisms, we conducted a longitudinal study of the selected *PTPN22* and *FCRL3* sequence variants and their influence on residual β-cell function and metabolic control of disease in pediatric patients with newly diagnosed T1DM.

2 | RESEARCH DESIGN AND METHODS

2.1 | Subjects and study criteria

A total of 147 consecutive children (73 boys and 74 girls, mean age: 8.91 years, range: 1.077-17.899 years) with newly diagnosed T1DM—autoimmune subtype[12], residing in the Pomeranian region of Poland were included in this prospective, longitudinal study. The qualification procedure was conducted between October 2008 and December 2011. T1DM diagnosis was based on the World Health Organization (WHO) and International Society for Pediatric and Adolescent Diabetes (ISPAD) criteria with the disease onset date established as the first insulin injection date[12,13]. Only patients with one or more positive autoantibody titres (GADA, glutamic acid decarboxylase autoantibodies; IAA, insulin autoantibodies, or ICA, islet cell autoantibodies) were included in the study.

In line with the Immunology of Diabetes Society (IDS) guidelines[14], 29 patients were excluded from our study: 20 patients with idiopathic T1DM (negative titre for GADA and IAA and ICA) or type 2 and other specific types of diabetes, one child aged under 12 months, two patients residing outside of Pomerania, two subjects who underwent intervention therapies modifying β-cell function (i.e., recombined humanized anti-CD3 antibody or $CD4^+CD25^+CD127^-$ T_{reg} cell therapy), and four persons without informed consent.

2.2 | Study stages

At diagnosis, all participants underwent diagnostic evaluation consisting of (1) clinical evaluation [diabetic ketoacidosis (DKA) or non-DKA presentation defined according to the American Diabetes Association (ADA)[15] and ISPAD guidelines[16]], (2) biochemical and serologic assays including examination of residual β-cell function, specific autoantibody titres, and HbA1c level (evaluated 4-6 days after the correction of disturbances in acid-base balance), (3) blood sample collection for genetic studies (storage at −20°C before use, no longer than 6 months). After study inclusion patients were followed up for 24 months (from October 2008 to March 2014). Periodic re-evaluation of residual β-cell function (fasting C-peptide concentration—FCP) and HbA1c level was established with follow-up visits every 3 months in the first year and every 6 months in the second year of disease.

Dried blood specimens from 327 consecutively screened anonymous neonates representative for the Pomeranian region of Poland were used as a population-matched control group for genetic studies (16-20 samples from each of 19 Pomeranian towns, analyses performed from March 2013 to October 2015). The ethical limitation of using a pediatric control group for genetic analyses, especially if disease development prevention is unavailable, was the rationale behind the above-presented control group structure.

2.3 | Genotyping

Genomic DNA was extracted from participants' blood samples using Genomic Midi AX Kits (A&A Biotechnology, Gdynia, Poland). The *PTPN22* c.1858T>C (rs2476601; localization: chromosome 1p13.2) and the *FCRL3* -169C>T (rs7528684; localization: chromosome 1q21-q22) polymorphisms were analyzed in the study and control groups using polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) and blood-direct PCR-RFLP methods, respectively. The *PTPN22* c.1858T>C polymorphic site was amplified using primers 5′-CACGTTAGCCAGGATGGTTT-3′ and 5′-GGCCTCAATGAACTCCTCAA-3′. PCR products (547 bp) were digested with the restriction enzyme *RsaI* (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA), yielding RFLP fragments of 44 bp, 115 bp, 160 bp and 228 bp for the C allele and 115 bp, 160 bp and 272 bp

for the T allele (115 and 160 bp fragments associated with the double restriction site). The *FCRL3* -169C>T polymorphic site was amplified using primers 5′-TCAAAGGGGCAAGAATTGAC-3′ and 5′-GCTT AATGAGCGTGGTGTGA-3′. PCR products (660 bp) were digested with the restriction enzyme *FaqI* (Fermentas, Thermo Fisher Scientific Inc.), producing RFLP fragments of 135 bp, 212 bp and 313 bp for the T allele and 212 bp and 448 bp for the C allele (212 bp fragment associated with double restriction site). PCR-RFLP results (control and every 10th sample) were validated by direct DNA sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kits and ABI Prism 310 Sequencer (Life Technologies Corporation, Foster City, CA, USA).

2.4 | C-peptide assays

FCP level measurements (chemiluminescence assay LIAISON C-Peptid, DiaSorin S.p.A., Saluggia, Italy) were used for the evaluation of residual β-cell function. The detection threshold of the method was 0.01 ng/mL (normal range 0.8-4.2 ng/mL). For further analyses, only the assays that fulfilled IDS guidelines[14,17] were chosen (Table S1, Supporting Information): fasting blood glucose range 72-200 mg/dL during serum sample collection and appropriate patient's preparation (in the case of multiple subcutaneous insulin injections the last insulin administration was on the evening prior to testing; in the case of continuous subcutaneous insulin infusion, no morning bolus was given, and the basal rate was stopped 15 minutes before blood sample collection). Body mass index (BMI) Z-score and daily insulin requirement data were included in FCP level dynamics analyses.

2.5 | HbA1c level assays

For HbA1c level assays a high-performance liquid chromatography (HPLC) certificated by the National Glycohemoglobin Standardization Program (NGSP) (TOSOH G8 Variant HLC-723G8 chromatograph, TOSOH, Tokyo, Japan) was used. All measurements were performed according to IDF (International Diabetes Federation)/ISPAD guidelines[18] and the consensus statement on the worldwide standardization of HbA1c assays[19].

2.6 | Autoantibody assays

Qualitative and quantitative assessment of GADA, IAA, and ICA titres were performed using commercial enzyme-linked immunosorbent assay (ELISA) kits (GAD Autoantibody ELISA kit, RSR Ltd., Cardiff, UK; Anti-Insulin ELISA test, DiaMetra, Perugia, Italy) and an indirect immunofluorescence test (IFT Autoantibodies against islet cells, Euroimmune, Luebeck, Germany). The sensitivity and specificity of the measurements were 92% and 98%—for GADA, respectively, 60% and 90%—for IAA, respectively, and 100% (both)—for ICA. The detection thresholds were established as 0.57 U/mL—for GADA, 0.08 U/mL—for IAA and 1:10 (50 JDFU—Juvenille Diabetes Foundation Units)—for ICA. The intra- and inter-assay coefficient of variation range was 7.3%-8.5% and 5.2%-5.7% for GADA, respectively, below 9% and 10% for IAA, respectively. All measurements were performed using sera that had been stored in aliquots at −20°C no longer than 3 months.

2.7 | Masking of samples

Masking of blood samples was performed before genetic, biochemical, and serologic analyses. The coding system was completely different for each type of analysis. The samples were decoded after study termination to minimize the influence of the researcher and lab assistant[14].

2.8 | Ethical considerations

The study was approved by the Local Ethics Committee on Clinical Investigation of the Medical University of Gdansk (NKEBN/27/2008, NKEBN/27-15/2010). Written informed consent was obtained from all participants' parents/guardians and also from subjects when applicable, in accordance with the Helsinki Declaration.

2.9 | Statistical analysis

Statistical analyses were performed using the R program version 2.15.2 (The R Foundation for Statistical Computing, Austria).[20] The Kolmogorow-Smirnow and χ^2 tests were used to evaluate the data for normal distribution and to compare observed and expected frequencies in each category. Because the data were not normally distributed, a number of non-parametric tests, in particular the Wilcoxon test for matched or non-matched parameters, were used. Data are presented as median and 25-75th interquartile range. The Fisher's exact test was performed for analysis of contingency tables. A *P* value of less than 0.05 was considered to indicate statistical significance. The record "<0.01" was used in the case of a *P* value less than 0.01. Missing values were handled by case-wise deletion. FCP slopes across time of follow up were estimated on the basis of individual mean slope between subsequent points of observation[21].

3 | RESULTS

3.1 | Completeness of data

A total of 135 participants were retained in the study through the first year of follow up (91.84%, complete follow up 88.44%) and 119 patients through the next year (80.95%; complete follow up 78.23%) (flow diagram—Figure S1). The required attendance within the established time frame through the whole study-period was achieved in 72.53% of patients (first year of follow up, 2-week time frame: 80.92%; second year, 4-week time frame: 65.99%).

3.2 | Comparison of the distribution of the *PTPN22* c.1858T>C and *FCRL3* -169C>T polymorphisms in the study and control groups

The distributions of the *PTPN22* c.1858T>C and *FCRL3* -169C>T genotypes were consistent with Hardy-Weinberg equilibrium in the study and control groups.

A significant difference in the distribution of the *PTPN22* c.1858T>C genotypes was observed between the two groups ($P = 0.03$), with a substantial increase in homozygosity for the T allele

in the study group (*P* = 0.011) (Table 1). The *FCRL3* -169C>T genotype and allele distribution were comparable in the study and control group (Table 1).

Joint analysis of both polymorphisms showed markedly lower coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes in the study group than in controls (*P* = 0.04) (Figure 1A,B). The above subgroup consisted of female patients only. No gender-associated variation was noticed in the distribution of single genotypes.

3.3 | General trends in residual β-cell function and HbA1c level dynamics in the study group

3.3.1 | The dynamics of residual β-cell function

In the entire study group, the FCP concentration peak was observed at the third month from diagnosis (diagnosis: 0.275 ng/mL, 0.186-0.436 ng/mL; third month of diabetes: 0.538 ng/mL, 0.348-0.8 ng/mL) (*P* < 0.01), with a significant loss of residual β-cell function in the subsequent months (*P* < 0.01) (Figure 2). The decline in the slope of FCP had a biphasic character with stable dynamics between the 3rd and 18th months of disease (−0.028 to −0.015 ng/mL/month, −0.093 - 0.033 to −0.037 - −0.004 ng/mL/month) (*P* > 0.05) and a further substantial slope reduction after the 18th month of follow up (−0.008 ng/mL/month, −0.031 - 0.001 ng/mL/month) (*P* = 0.02). Simultaneously, the median FCP level, comparable with its concentration at the onset of disease, was observed at the 18th month of diabetes (0.233 ng/mL, 0.092-0.488 ng/mL) (*P* = 0.3) (Figure 2).

Significant gender-specific differences in FCP levels only at disease onset were noticed (girls: 0.32 ng/mL, 0.22-0.445 ng/mL; boys: 0.206 ng/mL, 0.162-0.417 ng/mL) (*P* = 0.03). The observed significant reduction in the decline in the slope of FCP after the 18th month was restricted to girls (−0.003 ng/mL/month, −0.016 - 0.001 ng/mL/month) (*P* = 0.03).

TABLE 1 Allele and genotypes distribution of selected polymorphisms in the study and control groups

Distribution of selected polymorphic variants	Study group (*n* = 147)		Control group (*n* = 327)		Statistical analysis
	Number of samples	Percentage (%)	Number of samples	Percentage (%)	*P* value
***PTPN22* c.1858T>C polymorphism**					
Allele distribution					
T	68	23.1	138	21.1	0.54
C	226	76.9	516	78.9	
Genotypes distribution					
TT	12	8.2	10	3.1	0.03
TC	44	29.9	118	36.1	
CC	91	61.9	199	60.8	
***FCRL3* -169C>T polymorphism**					
Allele distribution					
C	99	33.7	236	36.1	0.52
T	195	66.3	418	63.9	
Genotypes distribution					
CC	16	10.9	42	12.8	0.76
CT	67	45.6	152	46.5	
TT	64	43.5	133	40.7	

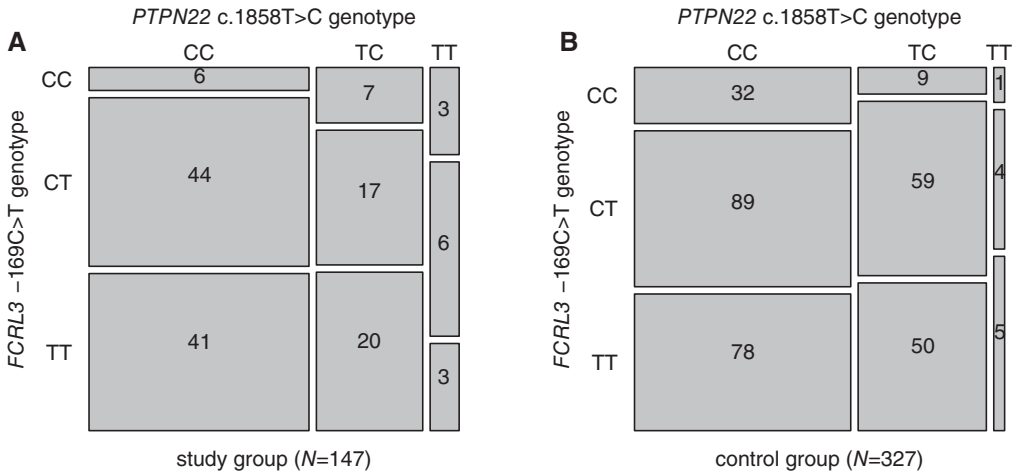

FIGURE 1 Comparative analysis of the coincidence of the *PTPN22* c.1858T>C and *FCRL3* -169C>T genotypes in the study (A) and control (B) group.

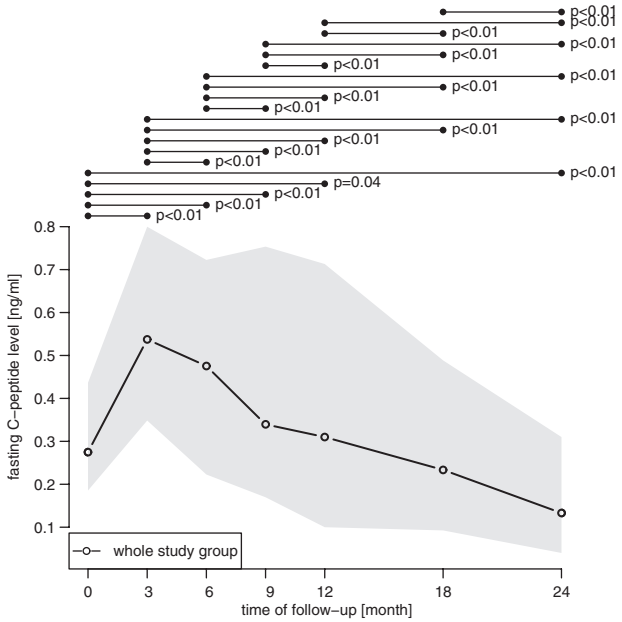

FIGURE 2 Dynamics of residual β-cell function loss in the study group over follow-up time. Black line = median; gray pool = 25th–75th interquartile range.

The trends in FCP dynamics observed in the study group throughout the entire follow up were independent of patients' BMI Z-scores and daily required insulin dose.

3.3.2 | The dynamics of HbA1c level

HbA1c level dynamic trends in the study group were inversely proportional to the loss of residual β-cell function. The lowest median value of HbA1c level was observed in the third month (6.93%, 6.365%-7.425%, or 52.24 mmol/mol, 46.06-57.65 mmol/mol), followed by a significant, stable increase on follow up (increase in each follow-up period between the 3rd and 24th months: 0.15%-0.215%; $P < 0.05$). No gender-specific correlations were noted.

3.4 | Clinical effects of coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes

Clinical characteristics of patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes in comparison with the reminder of the study group are presented in Table 2.

3.4.1 | Dynamics of residual β-cell function

In patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes, the beneficial dynamics of residual β-cell function loss was observed throughout the entire follow up (Figure 3A). In this subgroup, after the typical FCP increase in the third month of disease, residual insulin secretion up to the 24th month remained very stable (FCP median level ≥0.67 ng/mL), without any significant differences between the subsequent observation periods (Figure 3A). Median FCP levels observed in this subgroup in the 9th and after the 18th month were significantly higher than in the remainder of the study group (9th month: $P = 0.04$; 18th month: $P = 0.02$; 24th month: $P = 0.02$) (Figure 3A). Further female-limited comparisons due to the exclusively female representation in the analyzed subgroup also confirmed the above significant differences (9th month: 0.789 ng/mL, 0.475-1.13 ng/mL vs 0.3 ng/mL, 0.169-0.616 ng/mL, $P = 0.03$; 18th month: 0.67 ng/mL, 0.58-0.82 ng/mL vs 0.15 ng/mL, 0.09-0.3 ng/mL, $P = 0.01$; 24th month: 0.7 ng/mL, 0.318-1.215 ng/mL vs 0.095 ng/mL, 0.03-0.239 ng/mL, $P = 0.01$).

Analysis of FCP level slopes over observation time in patients with both *PTPN22* c.1858CC and *FCRL3* -169CC genotypes revealed a prolonged increase of residual insulin secretion in the first 6 months of follow up, which was significantly different from the trends seen in patients with other genotypes ($P = 0.02$), in whom a substantial FCP slope was observed ($P < 0.01$) (Figure 3B). After the first 6 months, the FCP slopes in the *PTPN22* c.1858CC and *FCRL3* -169CC carriers did not differ significantly from those in the rest of the studied group. However, the dynamics of β-cell loss after the 18th month of disease were superior to those observed in the remaining patients, as there was a tendency for FCP to increase and for no further decline to occur, as in other patients (Figure 3B).

3.4.2 | Comparison the specificities of *PTPN22* c.1858CC and *FCRL3* -169CC genotype co-influences with *PTPN22* c.1858CC or *FCRL3* -169CC single-genotype effects

Comparative analysis of FCP changes in the case of the *PTPN22* c.1858CC or *FCRL3* -169CC single-genotype incidence with the dynamics observed in the *PTPN22* c.1858CC and *FCRL3* -169CC cocarriers suggests that the *FCRL3* -169CC genotype influences could be dominant in the stabilization of residual insulin secretion throughout the entire follow up (Figure 4B). The *PTPN22* c.1858CC genotype

TABLE 2 Clinical characteristics of patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes in comparison with the reminder of the study group

	Coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes		Statistical analysis
Clinical parameters	**Present (*n* = 6)**	**Absent (*n* = 141)**	***P* value**
Gender (boys/girls)	0/6	73/68	0.03
Mean age at diagnosis (years)	10.08 ± 4.73	8.87 ± 4.52	0.5
Tanner stage at diagnosis (prepubertal/pubertal)	3/3	84/57	0.69
Patient's condition at diagnosis (without disorders in acid-base status/diabetic ketoacidosis)	6/0	82/56	0.08
Mean BMI Z-score at diagnosis	0.23 ± 0.37	0.34 ± 0.3	0.27
Family history of type 1 diabetes	1	23	1.0

Abbreviation: BMI, body mass index.

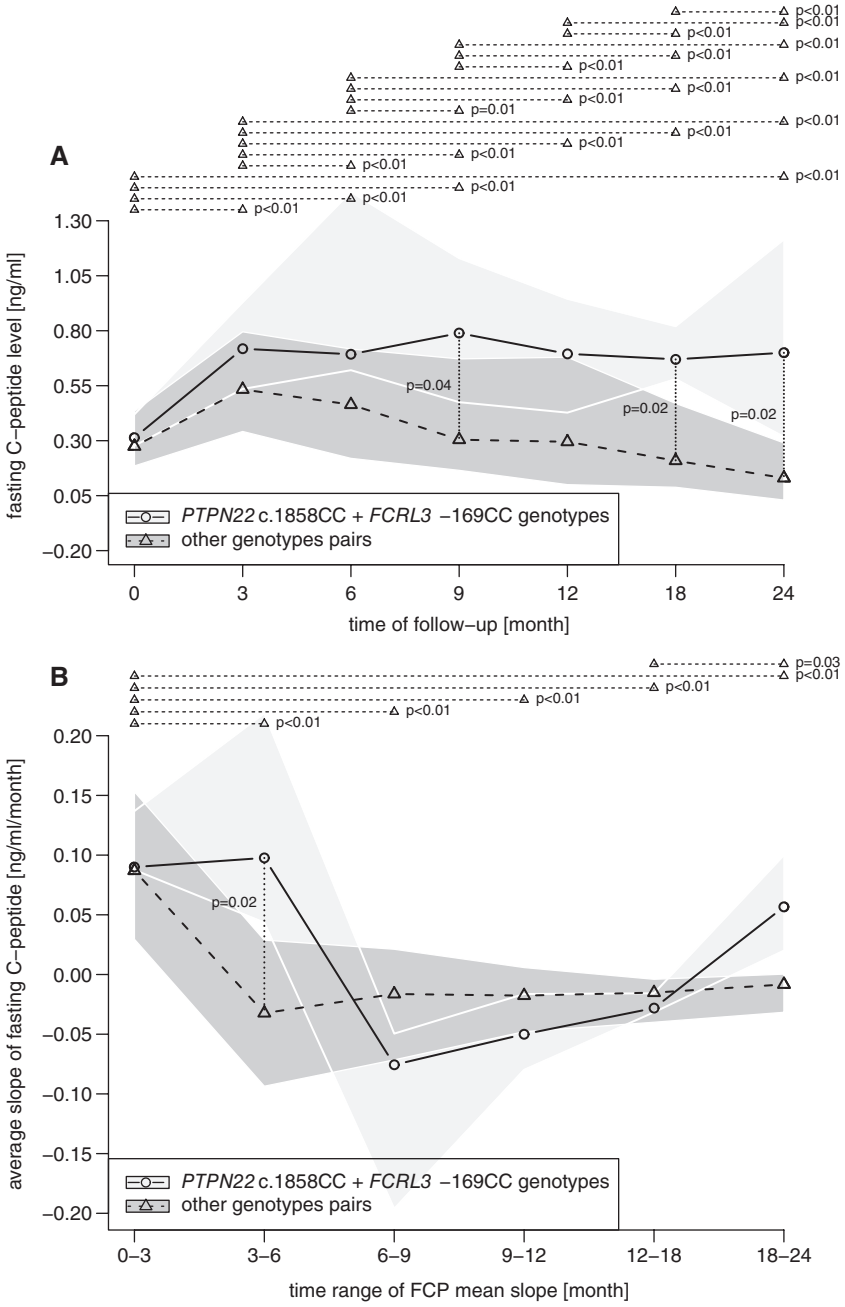

FIGURE 3 Comparative analysis of residual β-cell function loss dynamics in patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes relative to subgroups with other genotype combinations: (A) trends over time of fasting C-peptide level changes; (B) trends over time of average fasting C-peptide level slopes. Black lines = median; gray pools = 25th–75th interquartile range.

could be associated with a minor effect on FCP stabilization at the sixth month of disease (Figure 4A).

3.4.3 | Dynamics of HbA1c level

HbA1c levels in the *PTPN22* c.1858CC and *FCRL3* -169CC cocarriers were inversely proportional to FCP concentrations at all time points of follow up and remained lower than in patients with other genotypes, although this trend did not reach statistical significance (Figure 5).

4 | DISCUSSION

To the best of our knowledge, this study is one of the first to evaluate the joint effects of the *PTPN22* c.1858T>C and *FCRL3* -169C>T polymorphisms on residual insulin secretion in pediatric patients with T1DM.

In brief, our results can be summarized as follows: significantly less frequent coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes in T1DM patients was associated with well-preserved residual β-cell function throughout the first 24 months of disease. This phenomenon concerned female patients only.

The relatively small size of the evaluated groups (147 T1DM subjects and 327 controls) should be considered as one of the study limitations. However, data completeness at 24 months of observation exceeded 78%. The precision of the study was further improved by the application of numerous unified procedures, that is, data categorization according to the current guidelines of scientific societies, sample masking, certified laboratory tests, and PCR-RFLP analysis with double restriction sites localized within the analyzed amplicon. Another advantage of the study was its restriction to T1DM patients

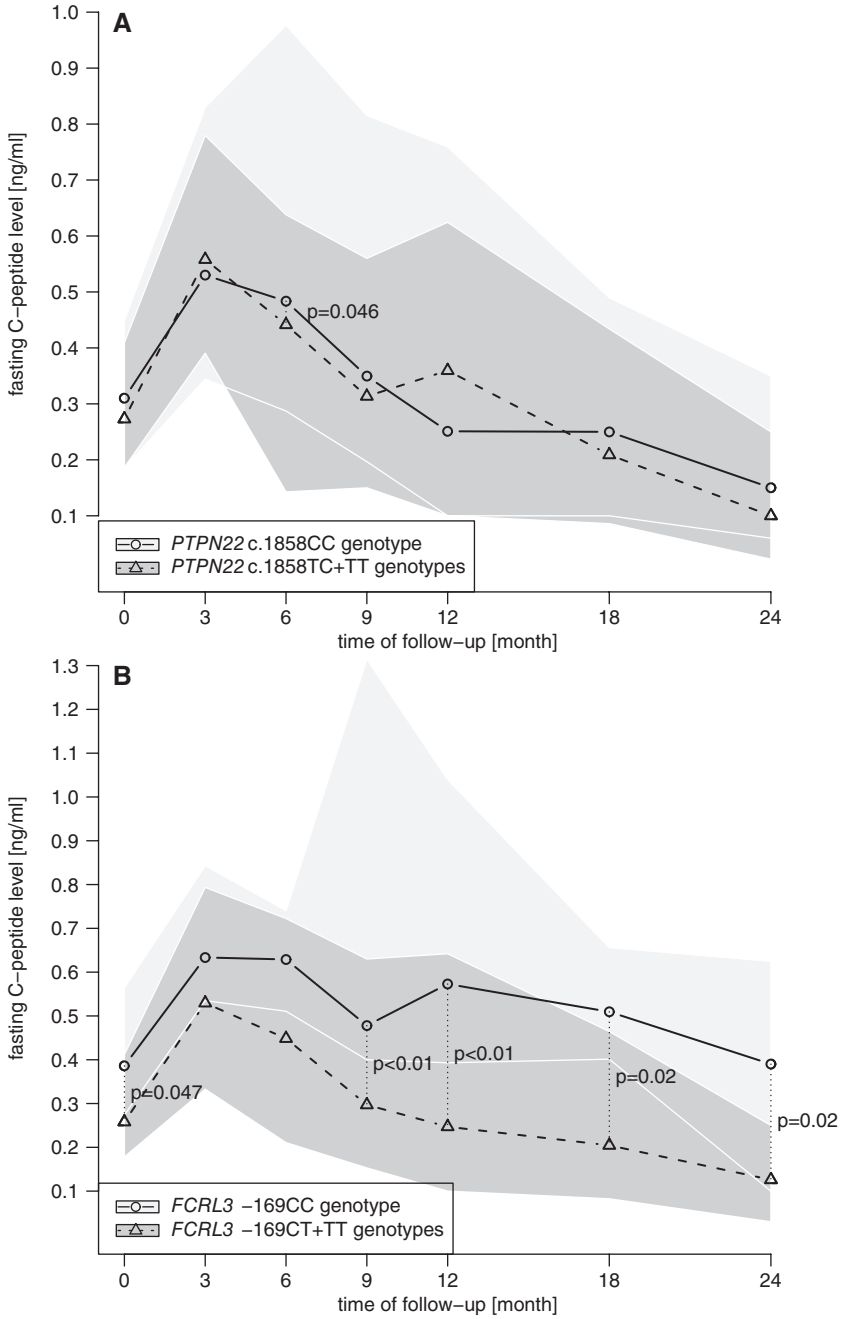

FIGURE 4 Comparative analysis of residual β-cell function loss dynamics: (A) in patients with the *PTPN22* c.1858CC genotype relative to carriers of other genotypes; (B) in patients with the *FCRL3* -169CC genotype relative to carriers of other genotypes. Black lines = median; gray pools = 25th–75th interquartile range.

with the confirmed autoimmune subtype of the disease. The selection of autoimmune activity markers used in the study qualification procedure was based on their high frequency in patients with newly diagnosed T1DM (GADA, ICA) and their high specificity for the pediatric population (IAA)[22]. It was limited, however, by the unavailability of standardized laboratory tests for ZnT8 (zinc transporter 8) autoantibodies at the beginning of the study.

The significantly lower coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes in the study group in comparison with controls may indicate a special protective function of this genetic conformation in T1DM patients. This conformation could be considered as a new quality because of the grouping of genotypes described so far as associated with contrary autoimmune diseases risk (the *PTPN22* c.1858CC with low risk and the *FCRL3* -169CC with high risk)[2,23,24]. However, the stratification of *FCRL3* -169C>T genotypes according to the risk of autoimmune response development is still discussed. A recent meta-analysis by Yang et al. suggests that above correlation could be allele-specific for each autoimmune disease and in the case of T1DM the T allele should be considered as a risk factor[25], contrary to the previous reports[1,23].

The spread of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes pairs observed in our study could be population dependent, especially when the *PTPN22* c.1858T allele distribution in European countries with north-east to south-west gradients is considered[26]. The frequency of the *PTPN22* c.1858T allele in our study group (23.1%) is comparable with the highest T allele frequencies reported in European T1DM subjects, especially with the neighboring Finnish (23.81%) and Estonian (25.65%) populations[2]. Also the T allele frequency determined in our control group (21.1%) is more than doubled as compared with the averaged data concerning Caucasians (9.8%)[2].

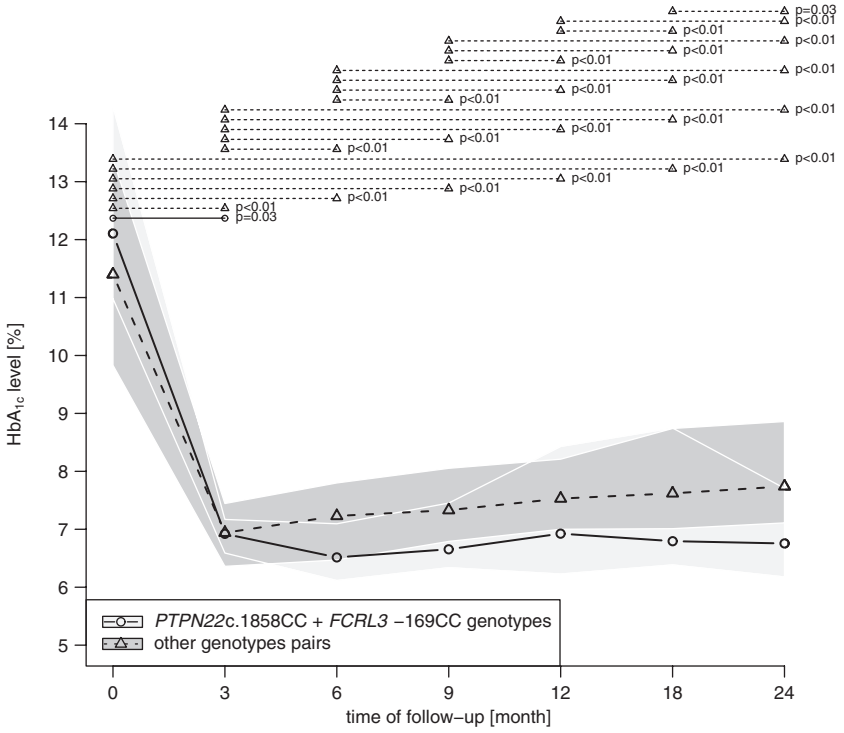

FIGURE 5 Comparative analysis of HbA1c level dynamics in patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes relative to subgroups with other genotype combinations. Black lines = median; gray pools = 25th–75th interquartile range.

Our results need to be validated by further studies in larger and diversified populations.

Clinically, the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes was associated with a particularly beneficial functional effect on residual insulin secretion and HbA1c level dynamics throughout 24-month follow up. The dynamics of residual β-cell function loss differed markedly between these patients and the remaining subjects from the study group. The trend was associated with three key points: (1) prolonged increase in residual insulin secretion within the first 6 months; (2) subsequent non-significant FCP level slopes after the sixth month of disease and (3) a moderate increase instead of a further decline after 18 months of observation.

Separate analysis of the influence of each polymorphic variant indicates that beneficial FCP changes in patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes could be associated with a minor effect of the *PTPN22* c.1858CC genotype on FCP stabilization at the sixth month of disease and a more effective role of the *FCRL3* -169CC genotype throughout the entire follow up. The latter effect is unexpected in the context of the previously described *FCRL3* -169CC genotype association with a more aggressive autoimmune response in other diseases, such as rheumatoid arthritis and Graves' disease[1,11]. However, our observation involving the *FCRL3* -169CC genotype effects could confirm indirectly the C allele association with a lower T1DM risk ascertained by Yang et al.[25]

The effects of *PTPN22* c.1858T>C polymorphism on the residual β-cell function in T1DM patients were studied extensively; however, without reaching a univocal conclusion (Table 3). The results of this study and that of Petrone et al.[24] indicated more beneficial trends in residual insulin secretion in at least one of the C allele carriers. Non-significant differences in the stimulated C-peptide levels between the opposite allele carriers observed by Nielsen and Pörksen[27] were explained by the comparatively preserved capability to hyperglycaemia compensation; however, associated with an increased proinsulin level (marker of β-cell stress) in the T allele subgroup. The contrary

TABLE 3 Comparison of studies analyzed the correlation between the *PTPN22* c.1858T>C polymorphism and clinical course of T1DM in the view of presented research results

Study	Patients (*n*)	Phase of T1DM	Follow up (month)	Frequency of CC genotype (*n*)	Dynamics of residual β-cell function loss in patients according to respective polymorphic variants
Present study	147	Clinical onset	24	91 (61.9%)	The *PTPN22* c.1858CC genotype carriers: significantly higher fasting C-peptide level at 6th month of disease
Petrone et al.[24]	120	Clinical onset	12	88 (73.3%)	The *PTPN22* c.1858C allele carriers: significantly higher fasting C-peptide levels through the whole follow up
Nielsen et al.[27]	257	Clinical onset	12	186 (72.4%)	The *PTPN22* c.1858T allele carriers: non-significantly lower stimulated C-peptide levels with increased proinsulin level (the marker of stressed residual β-cell function)
Blasetti et al.[28]	113	Clinical onset	6	93 (82.3%)	The *PTPN22* c.1858T allele carriers: significantly higher C-peptide levels at T1DM onset
Andersen et al.[39]	129	Clinical onset	12	N/A	The *PTPN22* c.1858C and T allele carriers: no significant association with stimulated C-peptide levels throughout the first year of T1DM

T1DM, type 1 diabetes mellitus.

results of the study by Blasetti et al.[28] could reflect an impaired compensatory response of T-cell to hyperglycaemia and DKA in the T allele carriers leading to more aggressive β-cells destruction and an excessive release of C-peptide[29,30].

The unique trend of insulin secretion observed in patients with the coincidence of the *PTPN22* c.1858CC and *FCRL3* -169CC genotypes resulted in a tendency for these individuals to have the lowest HbA1c levels over the observation time in comparison with carriers of other genotype pairs. This indicates a better long-term prognosis and lower risk of chronic diabetes complications, as was shown in the Diabetes Control and Complications Trial (DCCT)[31].

The coincidence of the *PTPN22* c.1858CC and the *FCRL3* -169CC genotypes in our study was exclusive to females. This phenomenon may indicate some gender-specific mechanisms modifying the effect of genetic factors. Among these, the NF-kB signaling pathway is one of strongest candidates. Activity of the NF-kB signaling pathway in T cells could already be upregulated from the prepubertal stage by physiologically higher estrogen levels in girls[32] via direct association of α/β oestradiol receptors with NF-kB in the nucleus[33]. Some other studies, performed following the gender-specific correlation of the *PTPN22* c.1858T>C polymorphism with T1DM incidence[2,34], however, found no nuclear estrogen receptor in the *PTPN22* gene promoter[35]. Altogether, these results may indicate the probable regulatory role of the perinuclear/nuclear PTPN22 isoform in the T cell NF-kB signaling pathway, associated with the effects of estrogen. A simultaneous impaired proliferative response of T_{reg} $FCRL^+$ cells to TCR/IL-2 stimulation and TCR-independent expression of the FCRL3 molecule observed by Nagata et al.[6] could be further indicative of the regulatory action of the perinuclear/nuclear PTPN22 isoform, mainly in the T_{reg} subpopulation—the only T cell subset expressing both PTPN22 and FCRL3 molecules[5–7,10]. Moreover, the functional phenotype of T_{reg} $FCRL^+$ cells[25] corresponds to previously described deficiencies of T_{reg} mediated functions in T1DM (decreased IL-2 response and IL-10 production)[36,37].

The proposed mechanism for the interaction of the two polymorphisms through perinuclear/nuclear PTPN22-estrogen-regulated NF-kB signaling pathway needs to be confirmed by functional studies for supporting further development of therapies based on T_{reg} administration[38].

ACKNOWLEDGMENTS

M. P. had complete access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

We thank all the subjects with T1DM and their families for the participations in the study, nurses Elżbieta Cypukow, Alina Borowska, Aleksandra Grabowska, Dorota Morawska, Małgorzata Ostrowska, Edyta Serocka, Joanna Warda (Department of Paediatrics, Haematology, Oncology and Endocrinology, Medical University of Gdańsk, Poland) for sample collection. We also thank Małgorzata Młodzianowska and Jolanta Ornatowska (Laboratory of Immunology and Clinic Transplantology, University Clinical Centre of Gdańsk, Poland) for helping with ELISA measurements.

Conflict of interest

There are no potential sources of conflict of interest.

How to cite this article: Pawłowicz M, Filipów R, Krzykowski G, Stanisławska-Sachadyn A, Morzuch L, Kulczycka J, Balcerska A, Limon J. Coincidence of *PTPN22* c.1858CC and *FCRL3* -169CC genotypes as a biomarker of preserved residual β-cell function in children with type 1 diabetes, *Pediatr Diabetes* 2016. DOI:10.1111/pedi.12429

REFERENCES

1. Chistiakov DA, Chistiakov AP. Is FCRL3 a new general autoimmunity gene? *Hum Immunol*. 2007;68:375–383.
2. Xuan C, Lun LM, Zhao JX, et al. PTPN22 gene polymorphism (C1858T) is associated with susceptibility to type 1 diabetes: A meta-analysis of 19,495 cases and 25,341 controls. *Ann Hum Genet*. 2013;77:191–203.
3. Plagnol V, Howson JM, Smyth DJ, et al. Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. *PLoS Genet*. 2011;7:e1002216.
4. Vang T, Congia M, Macis MD, et al. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet*. 2005;37:1317–1319.
5. Swainson LA, Mold JE, Bajpai UD, McCune JM. Expression of the autoimmune susceptibility gene FcRL3 on human regulatory T cells is associated with dysfunction and high levels of programmed cell death-1. *J Immunol*. 2010;184:3639–3647.
6. Nagata S, Ise T, Pastan I. Fc receptor-like 3 protein expressed on IL-2 nonresponsive subset of human regulatory T cells. *J Immunol*. 2009;182:7518–7526.
7. Bottini N, Vang T, Cucca F, Mustelin T. Role of PTPN22 in type 1 diabetes and other autoimmune diseases. *Semin Immunol*. 2006;18:207–213.
8. Gregersen PK, Lee HS, Batliwalla F, Begovich AB. PTPN22: Setting thresholds for autoimmunity. *Semin Immunol*. 2006;18:214–223.
9. Gregersen PK, Behrens TW. Genetics of autoimmune diseases—disorders of immune homeostasis. *Nat Rev Genet*. 2006;7:917–928.
10. Vang T, Miletic AV, Bottini N, Mustelin T. Protein tyrosine phosphatase PTPN22 in human autoimmunity. *Autoimmunity*. 2007;40:453–461.
11. Newman WG, Zhang Q, Liu X, et al. Rheumatoid arthritis association with the FCRL3 -169C polymorphism is restricted to PTPN22 1858T-homozygous individuals in a Canadian population. *Arthritis Rheum*. 2006;54:3820–3827.
12. Craig ME, Hattersley A, Donaghue KC. Definition, epidemiology and classification of diabetes in children and adolescents. *Pediatr Diabetes*. 2009;10:3–12.
13. World Health Organization. *Definition, Diagnosis and Classification of Diabetes Mellitus and Its Complications. Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus*. Geneva, Switzerland: World Health Organization; 1999:2–30.
14. Greenbaum CJ, Harrison LC. Guidelines for intervention trials in subjects with newly diagnosed type 1 diabetes. *Diabetes*. 2003;52:1059–1065.
15. Wolfsdorf J, Glaser N, Sperling MA. Diabetic ketoacidosis in infants, children, and adolescents: A consensus statement from the American Diabetes Association. *Diabetes Care*. 2006;29:1150–1159.
16. Wolfsdorf J, Craig ME, Daneman D, et al. Diabetic ketoacidosis in children and adolescents with diabetes. *Pediatr Diabetes*. 2009;10:118–133.
17. Palmer JP, Fleming GA, Greenbaum CJ, et al. C-peptide is the appropriate outcome measure for type 1 diabetes clinical trials to preserve beta-cell function: Report of an ADA workshop, 21–22 October 2001. *Diabetes*. 2004;53:250–264.
18. International Diabetes Federation/International Society for Pediatric and Adolescent Diabetes. *Global IDF/ISPAD Guideline for Diabetes in Childhood and Adolescence, [Online]*. Brussels, Belgium: International

Diabetes Federation; 2011. https://www.idf.org/global-idfispad-guideline-diabetes-childhood-and-adolescence (Accessed January 3, 2012).

19. Hanas R, John G. 2010 consensus statement on the worldwide standardization of the hemoglobin A1c measurement. *Pediatr Diabetes*. 2010;11:209–211.
20. Altman DG. *Practical Statistics for Medical Research*. 2nd ed. Boca Raton, FL: CRC Press LLC; 2006.
21. Greenbaum CJ, Beam CA, Boulware D, et al. Fall in C-peptide during first 2 years from diagnosis: Evidence of at least two distinct phases from composite Type 1 Diabetes TrialNet data. *Diabetes*. 2012;61:2066–2073.
22. Pihoker C, Gilliam LK, Hampe CS, Lernmark Å. Autoantibodies in diabetes. *Diabetes*. 2005;54:S52–S61.
23. Duchatelet S, Caillat-Zucman S, Dubois-Laforgue D, Blanc H, Timsit J, Julier C. FCRL3 -169CT functional polymorphism in type 1 diabetes and autoimmunity traits. *Biomed Pharmacother*. 2008;62:153–157.
24. Petrone A, Spoletini M, Zampetti S, et al. The PTPN22 1858T gene variant in type 1 diabetes is associated with reduced residual beta-cell function and worse metabolic control. *Diabetes Care*. 2008;31:1214–1218.
25. Yang Y, Su X, Zhang K, Zhou R. The Fc receptor-like 3 gene polymorphisms and susceptibility to autoimmune diseases: An updated meta-analysis. *Autoimmunity*. 2013;46:547–558.
26. Burn GL, Svensson L, Sanchez-Blanco C, Saini M, Cope AP. Why is PTPN22 a good candidate susceptibility gene for autoimmune disease? *FEBS Lett*. 2011;585:3689–3698.
27. Nielsen LB, Pörksen S, Andersen MLM, et al. The PTPN22 C1858T gene variant is associated with proinsulin in new-onset type 1 diabetes. *BMC Med Genet*. 2011;12:41.
28. Blasetti A, Di Giulio C, Tumini S, et al. Role of the C1858T polymorphism of protein tyrosine phosphatase non-receptor type 22 (PTPN22) in children and adolescents with type 1 diabetes. *Pharmacogenomics J*. in press, DOI: 10.1038/tpj.2016.6.
29. Kitabchi AE, Stentz FB, Umpierrez GE. Diabetic ketoacidosis induces in vivo activation of human T-lymphocytes. *Biochem Biophys Res Commun*. 2004;315:404–407.
30. Stentz FB, Kitabchi AE. Hyperglycemia-induced activation of human T-lymphocytes with de novo emergence of insulin receptors and generation of reactive oxygen species. *Biochem Biophys Res Commun*. 2005;335:491–495.
31. Diabetes Control and Complications Trial Research Group. Effect of intensive therapy on residual beta-cell function in patients with type 1 diabetes in the diabetes control and complications trial. A randomized, controlled trial. *Ann Intern Med*. 1998;128:517–523.
32. Paris F, Servant N, Térouanne B, Balaguer P, Nicolas JC, Sultan C. A new recombinant cell bioassay for ultrasensitive determination of serum estrogenic bioactivity in children. *J Clin Endocrinol Metab*. 2002;87:791–797.
33. Hirano S, Furutama D, Hanafusa T. Physiologically high concentrations of 17beta-estradiol enhance NF-kappaB activity in human T cells. *Am J Physiol Regul Integr Comp Physiol*. 2007;292: R1465–R1471.
34. Nielsen C, Hansen D, Husby S, Lillevang ST. Sex-specific association of the human PTPN22 1858T-allele with type 1 diabetes. *Int J Immunogenet*. 2007;34:469–473.
35. Nielsen C, Barington T, Husby S, Lillevang ST. Expression of human PTPN22 alleles. *Genes Immun*. 2007;8:131–137.
36. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes*. 2005;54:92–99.
37. Long SA, Buckner JH. CD4+FOXP3+ T regulatory cells in human autoimmunity: More than a numbers game. *J Immunol*. 2011;187:2061–2066.
38. Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, et al. Administration of CD4(+)CD25(high)CD127(−) regulatory T cells preserves β-cell function in type 1 diabetes in children. *Diabetes Care*. 2012;35:1817–1820.
39. Andersen MLM, Rasmussen MA, Pörksen S, et al. Complex multiblock analysis identifies new immunologic and genetic disease progression patterns associated with the residual β-cell function 1 year after diagnosis of type 1 diabetes. *PLoS One*. 2013;8:e64632.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.