ORIGINAL ARTICLES

Partial remission and early stages of pediatric type 1 diabetes display immunoregulatory changes. A pilot study

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Type 1 diabetes (T1D) is a chronic metabolic disease of unknown etiology that results from β-cell destruction. The onset of the disease, which arises after a long asymptomatic period of autoimmune attack, may be followed by a relapsing and remitting progression, a phenomenon that is most evident during the partial remission phase (PR). This stage lasts for a few months, shows minor requirements of exogenous insulin and could be explained by a recovery of immunological tolerance. This study aims to identify new biomarkers at early stages of pediatric T1D that reflect immunoregulatory changes. To that end, pediatric patients with T1D (*n* = 52) and age-related control subjects (*n* = 30) were recruited. Immune response-related molecules and lymphocyte subsets were determined starting at T1D onset and until the second year of progression. Results showed that circulating TGF-β levels decreased during PR, and that betatrophin concentration was increased in all the considered stages without differing among studied checkpoints. Moreover, an increase of regulatory T, B and NK subsets was found during T1D progression, probably reflecting an attempt to restore self-tolerance. By contrast, a reduction in monocyte levels was observed at the early stages of diabetes. The results reveal significant changes in immunological parameters during the different early stages of T1D in children, which could ultimately serve as potential biomarkers to characterize the progression of T1D. (Translational Research 2019; 210:8–25)

Abbreviations: aTreg = activated regulatory T; BLMS = B lymphocyte maturation stages; BMI = body mass index; Breg = regulatory B; CM = central memory; DP = double-positive;

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EM = effector memory; HbA1c = glycated hemoglobin; IDDAA1c = insulin dose-adjusted HbA1c; mTreg = memory regulatory T; NKreg = regulatory Natural Killer; PR = partial remission; PBMCs = peripheral blood mononuclear cells; Treg = regulatory T; TLMS = T lymphocyte maturation stages; T1D = type 1 diabetes

AT A GLANCE COMMENTARY

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Background

This study provides new candidate biomarkers – betatrophin, TGF-β, and regulatory T, B and NK subsets – that reflect the immunoregulatory attempts during early progression of pediatric type 1 diabetes, including partial remission stage.

Translational Significance

These findings suggest that regulatory lymphocytes as well as betatrophin and TGF-β molecules may be related to partial remission and progression of type 1 diabetes in children. Moreover, and because partial remission may be a good stage for immunointervention, these candidate biomarkers can be considered for clinical trials.

INTRODUCTION

Type 1 diabetes (T1D) is a metabolic disease caused by the autoimmune destruction of insulin-producing β-cells. After the clinical onset, and probably due to the beginning of insulin therapy, between 50% and 80% of pediatric patients with T1D undergo a partial remission (PR) phase, also known as honeymoon, starting at 6 months of disease progression and lasting up to 2 years.[1] This transient period is defined by low requirements of insulin and diminished glycated hemoglobin[2,3] (HbA1c) and could be an optimal phase for immune intervention and other therapeutic strategies. Clinical trials using immunotherapies have failed to provide long-term β-cell protection, partly due to the stage chosen for immunointervention.[4] Hence, it is necessary to identify new biomarkers which will enable the monitoring of the PR and the early stages of T1D.

A reliable biomarker has to be robust and allow for the distinction of control and patients, or in this case, patients at different stages of disease. For T1D, it should fulfil several requirements[4]: (1) To reflect the variations of the autoimmune response or β-cell regeneration[5]; (2) To be easy to obtain with minimum discomfort or risk for the patient; (3) To display variations over the progression of the disease; (4) To allow the monitoring of the patient's response to immunotherapies; (5) To have available a sensitive, specific, and highly reproducible detection method. At present, most used biomarkers in T1D are metabolic (HbA1c and C-peptide), but they do not reflect the pathophisiology of T1D. HbA1c is strongly dependent on daily glycemia and C-peptide levels are usually not detectable after the clinical onset.[6] Autoantibodies against β-cell antigens are immunological biomarkers for T1D but they do not always predict the development of the disease or the severity of secondary complications. Specific autoreactive T cells were also proposed as biomarkers but the reproducibility of experiments involving antigen-specific T cells has been questioned.[7]

Several candidate biomarker have arised in the last years. Betatrophin is one of these candidates, since its circulating levels in plasma were found increased in adult patients with long-standing T1D.[8] Another candidate was TGF-β, due to its role in immunoregulation and the alterations found in the pancreases from patients.[9] Moreover, plasma levels of TGF-β are increased in adult patients at the onset, indicating that TGF-β plays a role in chronic inflammation in T1D.[10] Other candidates are molecules with altered expression in the pancreases from patients at different disease stages,[11] and circulating leukocyte subsets,[12,13] that can be easily obtained from peripheral blood of pediatric subjects and be identified with a high resolution in an automated manner.[14] Our study aims at unravelling for the first time the characterization of the changes in these markers/subsets during the early stages of pediatric T1D.

MATERIAL AND METHODS

Participants. Pediatric patients with T1D ($n = 52$) and age-related control subjects ($n = 30$) were included in this study (Table 1). All patients fulfilled the classification criteria for T1D. Inclusion criteria were 1–18 years of age, and normal body mass index (BMI) according to the Spanish BMI pediatric cohort growth chart.[15] Exclusion criteria were being under immunosuppressive or anti-inflammatory treatment, the presence of other autoimmune diseases, type 2 diabetes, pregnancy, compromised kidney function, or liver diseases. All patients with T1D were positive for autoantibodies to GAD65 and/or IA-2 at the onset, and were negative for other autoantibodies (antithyroglobulin, anti-transglutaminase, and antithyroid peroxidase). Twenty-two out of the total 52 patients and 14 out of 30

Table 1. Clinical data for pediatric control subjects and patients with T1D at onset, and at 6, 12 and 18 months of disease progression

Variable	Control subjects ($n = 14$)	Patients at onset ($n = 22$)	Patients at 6 mo ($n = 15$)	Patients at 12 mo ($n = 14$)	Patients at 18 mo ($n = 13$)
Age (years)	8.1 ± 3.6	9.1 ± 4.5	9.6 ± 4.6	10.1 ± 4.6	10.6 ± 4.6
Gender (M/F)	3/11	11/11	8/7	8/6	9/5
BMI (kg/m^2)	19.4 ± 2.4	17.3 ± 3.4	18.3 ± 3.4	18.3 ± 3.6	17.8 ± 1.8
HbA1c (%)	ND	11.4 ± 2.5	7.2 ± 1.1	7.7± 1.04	7.6 ± 1.04
HbA1c (mmol/mol)	ND	101 ± 27.3	55 ± 11.6	61 ± 11.4	61 ± 11.4
Insulin dose (UI/kg/day)	ND	0.8 ± 0.4	0.53 ± 0.2	0.79 ± 0.7	0.92 ± 0.72
IDDAA1C (%)	ND	14.1 ± 3.4	9.4 ± 1.3	8.9 ± 3.5	10.2 ± 2.7

All values are mean ± SD. No statistical differences were found in age between groups. Statistical differences were found in: insulin dose (onset vs 6 mo: ¥, 6 mo vs 12 mo: *, 6 mo vs 18 mo: §, 12 mo vs 18 mo: #); glycated hemoglobin (HbA1c) (onset vs 6 mo: §, onset vs 12 mo: #, onset vs 18 mo: #); insulin dose-adjusted HbA1c (IDDAA1c) (onset vs 6 mo: ¥, onset vs 12 mo: ¥ and onset vs 18 mo: §). Statistics: Mann-Whitney test (*$P < 0.05$, #$P < 0.01$, §$P < 0.001$, ¥$P < 0.0001$). BMI, body mass index; HbA1c, glycated hemoglobin; IDDAA1c, insulin dose-adjusted HbA1c; ND, not determined.

control subjects were selected to be included in the ELISA experiments, whereas the remaining 30 patients and 16 control subjects were included in the leukocyte subsets' analysis. Patients were considered to be in PR when they fulfilled one or both of the two accepted criteria. PR was defined as <0.5 UI/kg/d and <7% HbA1c, or <9 insulin dose-adjusted HbA1c[3] (IDDAA1c). All the participants gave informed consent, and the study was approved by the Committee on the Ethics of Research of the Germans Trias i Pujol Research Institute and Hospital.

ELISA. ELISA method was used to determine plasmatic concentrations of TGF-β (ThermoFisher Scientific, Waltham, Massachusetts), betatrophin (Wuhan Eiaab Science, Wuhan, China), and HLA-G (sHLA-G, Enzo Life Sciences, Farmingdale, New York) in plasma samples obtained from control subjects and patients with T1D at the onset and at 6, 12, and 18 months of progression of the disease. Basic analytical characteristics of the methods were: for betatrophin, detection range: 78−5000 pg/mL and sensitivity: >0.051 pg/mL; for TGF-β, detection range: 8−1000 pg/mL and sensitivity: 8.6 pg/mL; for sHLA-G, detection range: 3.91−125 U/mL, and sensitivity: 0.6 U/mL.

Flow cytometry. For the analysis of cellular subsets, fresh venous blood samples were collected in ethylene diamine tetra acetic acid tubes from control subjects and patients with T1D at the onset and at 6, 12, and 18 months of disease progression. Samples of 1 mL of whole blood were washed and erythrocytes were lysed (Lysing Buffer, BD Biosciences, San Jose, California). A sample of 100 μL was then marked with a panel of different monoclonal antibodies (BD Biosciences) at room temperature and protected from light for 20 minutes. The panels, detailed in Table 2, were built as follows: (1) Th17 lymphocytes panel[16]: CD4 V450, CCR6 PE, CCR7 PECy7, and CCR4 AF647; (2) Treg panel[17]: CD45 FITC, CD3 V450, CD4 PerCPCy5.5, CD25 PE, CCR4 PECy7, CD127 AF647, CD45RO APCH7, HLA-DR V500; (3) T lymphocyte maturation stages panel[16]: CD3 V500, CD4 PerCPCy5.5, CD8 APCH7, CD45RA FITC, PTK7 PE, CCR7 PECy7, CD31 AF647, CD27 BV421; (4) Transitional B cell panel[16]: CD19 PerCPCy5.5, CD24 FITC, CD38 PE, CD27 APC; (5) B lymphocyte maturation stages panel[16]: CD3 V450, CD19 V500, CD27 APC, CD21 PE, IgD FITC, IgM PerCPCy5.5; (6) γδ T cell panel[16]: CD3 PerCP, γδ TCR PE, αβ TCR FITC, CD8 APCH7, CD4 V450 and (7) DC/Monocytes/NK[17]: CD45 AF700, CD3 APCH7, CD19 APCH7, CD14 V450, CD16 APC, CD11c PECy7, CD123 PerCPCy5.5, CD56 PE, HLA-DR V500, Slan FITC. At least 10,000 leukocyte events per sample were acquired using FACS Canto II and LSR Fortessa Flow Cytometers (BD Biosciences). Corresponding fluorescence minus one staining was used as control. The gating strategy to analyze specific leukocyte subsets was based on international consensus.[18] Absolute counts (cells/μL) were analyzed for all subsets using Perfect Count Microspheres (Cytognos SL, Salamanca, Spain). Data were analyzed using FACSDiva software (BD Biosciences).

Quantitative RT-PCR. RNA was isolated using RNeasy Micro Kit (QIAGEN) and reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). cDNA synthesis was carried out using random hexamers (0.5 mg/mL, BioTools, Valle de Tobalina, Madrid, Spain) and reverse transcriptase Moloney murine Leukemia virus (200 U/mL, Promega, Madison, Wisconsin). Quantitative RT-PCR assays were performed on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using SYBR Green I Master mix (Roche Diagnostics) and specific primers for *HLA-E, HLA-F, TLR3, LYZ, CD36, IL-8,* and *CXCL1* (Invitrogen, Life Technologies, Gaithersburg,

Table 2. Lymphocyte subsets analyzed in pediatric patients with T1D and their markers according to international consensus[18]

Lymphocyte subset	Phenotype	Reference subset
aTreg	$CD3^+CD4^+CD127^{low}CD25^+$ $CCR4^+$ $CD45RO^+HLADR^+$	mTreg
mTreg	$CD3^+CD4^+CD127^{low}CD25^+$ $CCR4^+$ $CD45RO^+$	$CD3^+CD4^+$
Th17	$CD4^+$ $CCR7^-$ $CCR4^+$ $CCR6^+$	$CD4^+$
Breg	$CD19^+CD27^+CD24^{hi}$	$CD19^+$
NKreg	$CD3^-CD19^-CD56^+CD14^-CD56^{br}CD16^-$	NK cells
Monocytes	$CD3^-CD19^-CD14^+$	$CD3^-CD19^-$
$CD14^+CD16^-$	$CD3^-CD19^-CD14^+CD16^-$	Monocytes
Total $CD27^+$	$CD19^+CD27^+$	$CD19^+$
Total B transitional	$CD19^+CD27^-CD24^{hi}CD38^+$	$CD19^+$
B transitional high	$CD19^+CD27^-CD24^{hi}CD38^{hi}$	$CD19^+$
B transitional low	$CD19^+CD27^-CD24^{hi}CD38^{lo}$	$CD19^+$
αβ	$CD3^+$ $\alpha\beta^+$ $\gamma\delta^-$	$CD3^+$
$CD3^+$ DP	$CD3^+CD4^+CD8^+$	$CD3^+$
γδ $CD8^+$	$CD3^+$ $\alpha\beta^-$ $\gamma\delta^+CD8^+$	Total γδ
$CD3^+CD4^+$	$CD3^+CD4^+$	Total lymphocytes
Recent thymic emigrants	$CD3^+CD4^+CCR7^+CD45RA^+CD31^+PTK7^+$	$CD4^+$ Naive
$CD4^+$ CM	$CD3^+CD4^+CCR7^+CD45RA^-CD27^+$	$CD3^+CD4^+$
$CD4^+$ EM	$CD3^+CD4^+CCR7^-CD45RA^-$	$CD3^+CD4^+$
$CD4^+$ EM $CD27^+$	$CD3^+CD4^+CD27^+CCR7^-CD45RA^-$	$CD3^+CD4^+$
$CD4^+$ EMRA	$CD3^+CD4^+CCR7^-CD45RA^+$	$CD3^+CD4^+$
$CD4^+$ EMRA $CD27^+$	$CD3^+CD4^+CD27^+CCR7^-CD45RA^+$	$CD3^+CD4^+$
$CD4^+$ EMRA $CD27^-$	$CD3^+CD4^+CD27^-CCR7^-CD45RA^+$	$CD3^+CD4^+$
$CD3^+CD8^+$	$CD3^+CD8^+$	Total lymphocytes
$CD8^+$Naive	$CD3^+CD8^+CCR7^+CD45RA^+CD27^+$	$CD3^+CD8^+$
$CD8^+$ CM	$CD3^+CD8^+CCR7^+CD45RA^-CD27^+$	$CD3^+CD8^+$
$CD8^+$ EMRA	$CD3^+CD8^+CCR7^-CD45RA^+$	$CD3^+CD8^+$
$CD8^+$ EMRA $CD27^+$	$CD3^+CD8^+CD27^+CCR7^-CD45RA^+$	$CD3^+CD8^+$

aTreg, activated regulatory T cell; mTreg, memory regulatory T; Breg, regulatory B; NKreg, regulatory Natural Killer; DP, double positive; CM, central memory; EM, effector memory; EMRA, $CD45^+$ effector memory.

Maryland) (Supplementary Table 1). Relative quantification was performed by normalizing the expression of each gene of interest to that of the housekeeping *HPRT* gene following the 2-DDCt method.[19]

Statistical analysis. The statistical analysis was performed using Prism 7.0. Software (GraphPad software Inc., San Diego, California). For comparisons of paired data, a nonparametric Wilcoxon test was used, whereas a nonparametric Mann-Whitney test was used for comparisons of unpaired data. To find statistically significant correlations between parameters, Spearman's test was used. A P value of <0.05 was considered significant.

RESULTS

Clinical features of pediatric patients with T1D throughout the study. No statistical differences between groups were found in age and BMI; however, they were found in terms of insulin dose, HbA1c values and in IDDAA1c values (Table 1).

Circulating TGF-β levels decrease during PR stage. Circulating TGF-β concentration was determined in 10 control subjects and in 22 patients with T1D. Samples from these patients were obtained at 3 checkpoints: clinical onset ($n = 14$), 6 months ($n = 13$), and 12 months ($n = 13$) of progression of the disease. TGF-β concentration was significantly decreased at the checkpoint of first year when compared to the onset (Fig 1, A). Then, the possible correlation between this alteration and PR was evaluated. In fact, there is a significant decrease in circulating levels of TGF-β in patients with PR at 6 and 12 months of disease progression (Fig 1, B). In addition, patients at onset were divided in 2 groups (PR or not PR) and TGF-β levels were compared to understand whether those with PR have a less aggressive T1D onset or rather have a real different evolution of their diabetes. The results showed that both groups have similar circulating TGF-β concentration at the onset, independenlty from T1D progression. These results show a differential pattern of circulating TGF-β that discriminates between patients with or without spontaneous PR. Patients with PR do not have a less aggressive T1D onset, but a real different evolution of their diabetes.

Plasmatic concentration of betatrophin is increased in pediatric patients with T1D. Plasma betatrophin levels (pg/mL) were significantly increased in patients with T1D at

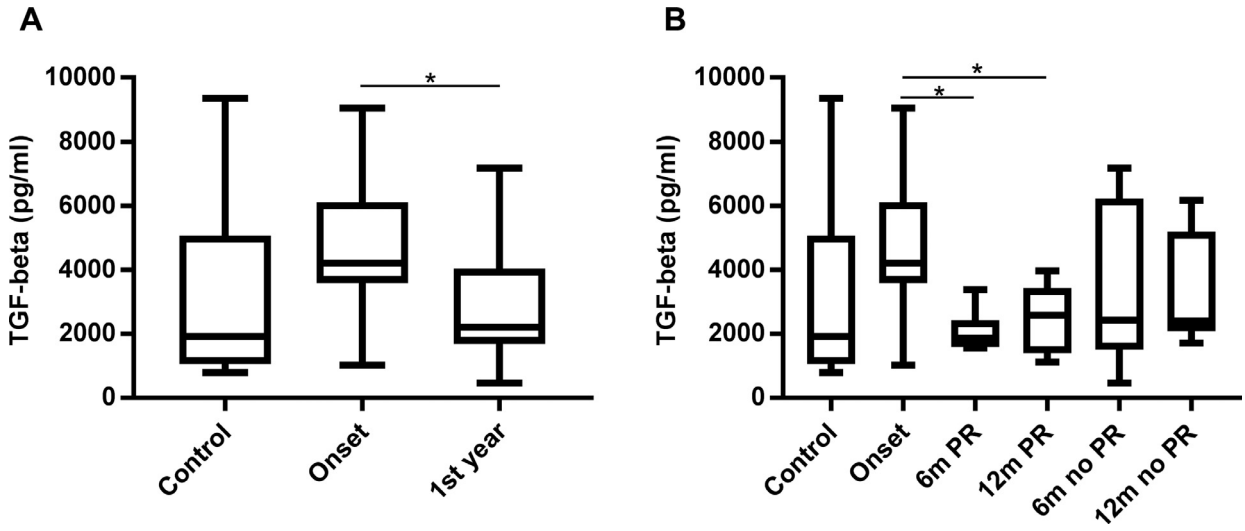

Fig 1. Plasma levels of TGF-β in pediatric patients with T1D. *A*, Concentration of TGF-β (pg/mL) in plasma of control subjects and patients with T1D at different checkpoints (onset and first year of progression). Data presented as plot box and whiskers (Tukey). (*$P < 0.05$, Mann-Whitney test). *B*, Circulating TGF-β concentration (pg/mL) in plasma of control subjects and patients with T1D at different checkpoints (onset, 6, and 12 months) grouped with and without spontaneous partial remission (PR) development at 6 months. Data presented as plot box and whiskers (Tukey). (*$P < 0.05$, Mann-Whitney test).

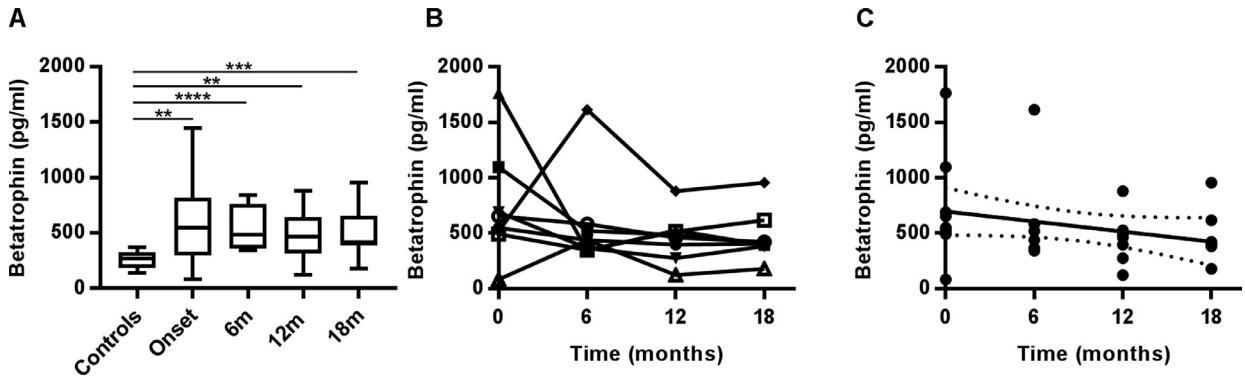

Fig 2. Plasma levels of betatrophin in pediatric patients with T1D. *A*, Circulating betatrophin concentration (pg/mL) in plasma of control subjects and patients with T1D at different checkpoints (onset, 6, 12, and 18 months of progression). Data presented as plot box and whiskers (Tukey). Statistical differences were found between patients and controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, Mann-Whitney test). *B*, Follow-up of the circulating plasma betatrophin concentration (pg/mL) of the patients with T1D in partial remission stage. *C*, Linear regression of the individual values of plasma betatrophin concentration (mg/dL) (Spearman $r = -0.3118$, P value = 0.0823).

the 4 different checkpoints (Fig 2, A) when compared to control subjects, whereas no statistical differences were found between patients at different disease stages. However, 12 months after the onset of the disease a trend to decrease betatrophin concentration was observed ($P = 0.057$). No statistical differences were found when grouping separately patients with and without spontaneous PR at 6 months.

To determine betatrophin levels in spontaneous PR, 8 patients with T1D showing PR criteria at 6 months were selected from the initial group of 22 patients (Table 3) and follow-up was performed during 18 months after diagnosis. No statistical differences were found in BMI between groups, although they were observed in insulin dose, HbA1c and in IDDAA1c. No statistical differences were found in circulating betatrophin levels in plasma between the 4 different checkpoints in PR patients (Fig 2, B), although a tendency for betatrophin to decrease was observed during disease progression ($P = 0.0823$) (Fig 2, C).

The correlation between circulating betatrophin levels with BMI was explored both in general T1D and

Table 3. Clinical data for pediatric patients with T1D at disease onset, and at 6, 12, and 18 mo of disease progression included in the follow-up measurements of plasma betatrophin

	Onset	6 mo	12 mo	18 mo
n	8	8	8	8
Gender (M/F)	5/3	5/3	5/3	5/3
Age (years, mean ± SD)	9.63 ± 3.78	10.13 ± 3.78	10.63 ± 3.78	11.13 ± 3.78
BMI (kg/m^2)	17.06 ± 4.59	18.27 ± 2.33	17.57 ± 1.2	17.67 ± 1.41
Insulin dose (UI/kg/day)	0.72 ± 0.58	0.43 ± 0.25	0.66 ± 0.22	0.75 ± 0.31
HbA1c (%)	10.26 ± 1.66	6.84 ± 0.90	7.86 ± 1.10	8.23 ± 1.04
HbA1c (mmol/mol)	89 ± 18.1	51 ± 9.8	62 ± 12	66 ± 11.4
IDDAA1C (%)	13.15 ± 1.93	8.15 ± 1.19	10.58 ± 1.47	11.33 ± 1.05

Data presented as mean ± SD. No statistical differences were found in age between groups. Statistical differences were found in insulin (onset vs 6 mo: #, 6 mo vs 12 mo: *, 6 mo vs 18 mo: *); glycated hemoglobin (HbA1c) (onset vs 6 mo: #, onset vs 12 mo: *, onset vs 18 mo: *, 6 mo vs 12 mo: #, 12 mo vs 18 mo: #); and in insulin dose-adjusted HbA1c (IDDAA1c) (onset vs 6 mo: §, onset vs 12 mo: * and 6 mo vs 18 mo: §). Statistical differences calculated from Mann-Whitney test (*$P < 0.05$, #$P < 0.01$, §$P < 0.001$, ¥$P < 0.0001$). BMI, body mass index; HbA1c, glycated hemoglobin; IDDAA1c, insulin dose-adjusted HbA1c.

PR patients as well as in control subjects (Fig 3). No correlation was found between BMI-betatrophin levels at the onset stage in T1D patients or in PR patients. Finally, At 18 months, a statistically significant positive correlation was found between BMI-betatrophin levels both in general and in PR patients ($P = 0.0047$ and 0.0480, respectively).

Immune response-related molecules are altered at the onset of T1D. Different molecules involved in immune response were analyzed as potential biomarkers. These molecules were selected in basis of the altered expression in the pancreases from patients with T1D.[11] One of these molecules is HLA-G, considered an immunomodulatory molecule involved in tolerance and proposed as a pancreatic regulator.[20] However, circulating sHLA-G did not reveal significant differences between control subjects and patients at different stages (Fig S1, A). To gain further knowledge within the nonclassical HLA network, the expression of these molecules was also measured in peripheral blood mononuclear cells (PBMCs) from patients with T1D. Overexpressed mRNA levels of HLA-E were found in PBMCs from patients at the onset (Fig S1, B) when compared to control subjects. In contrast, mRNA levels of HLA-F were not altered in PBMCs from patients at the onset when compared to control subjects (Fig S1, C). The expression of other altered molecules such as IL-8, TLR3, and CXCL1 was increased in PBMCs from newly diagnosed patients (Fig S2). A positive correlation was found between the expression of 2 chemokines involved in leukocyte recruitment, IL-8 and CXCL1 (Fig S3), being moderately positive correlated in control subjects ($R^2 = 0.8615$) and strongly positive correlated ($R^2 = 0.9498$) in patients at the onset of T1D. The expression of other genes, such as CD36 and LYZ, in PBMCs was not significantly different than that of control subjects (Fig S2).

Regulatory leukocyte subsets are biomarkers of T1D onset and progression stages. No statistically significant differences were found in age and BMI between groups (Table 4). Lymphocyte subsets involved in different immunoregulation strategies were studied, including regulatory T cells (Treg), both activated (aTreg) and memory (mTreg), Th17 cells (which contain a regulatory Th17 subset[21]), regulatory B cells (Breg), and regulatory Natural Killer cells (NKreg) (Fig 4). No correlation was found between these subsets (% and numbers) and the age of the subjects in the different groups, ie, controls, onset, first year, and second year.

Different percentages of the Treg subset including both aTreg and mTreg were observed at each T1D stage (Fig 4, A–D). The percentage of aTreg was increased during the first year of disease in comparison to the second year and control subjects (Fig 4, B), whereas the percentage of mTreg was decreased only at the onset when compared with control subjects (Fig 4, D). In both cases the concentration was maintained along the different groups (Fig 4, A and 4, C). The Th17 cell subset, which includes a specific and minority Th17 regulatory subset, showed an increased percentage during the first year of progression when compared to the second year (Fig 4, F). The corresponding cell concentration was maintained during T1D progression, at levels similar to those found in control subjects (Fig 4, E). The Breg cell subset was found increased both in concentration and in percentage during the first year of disease (Fig 4, G and H). Finally, the percentage of NKreg cells was increased during the second year when compared to the first year of disease (Fig 4, I and J). In summary, an increase in regulatory T, B and NK subsets was found during T1D progression, reflecting the attempts to restore tolerance to self during these early stages.

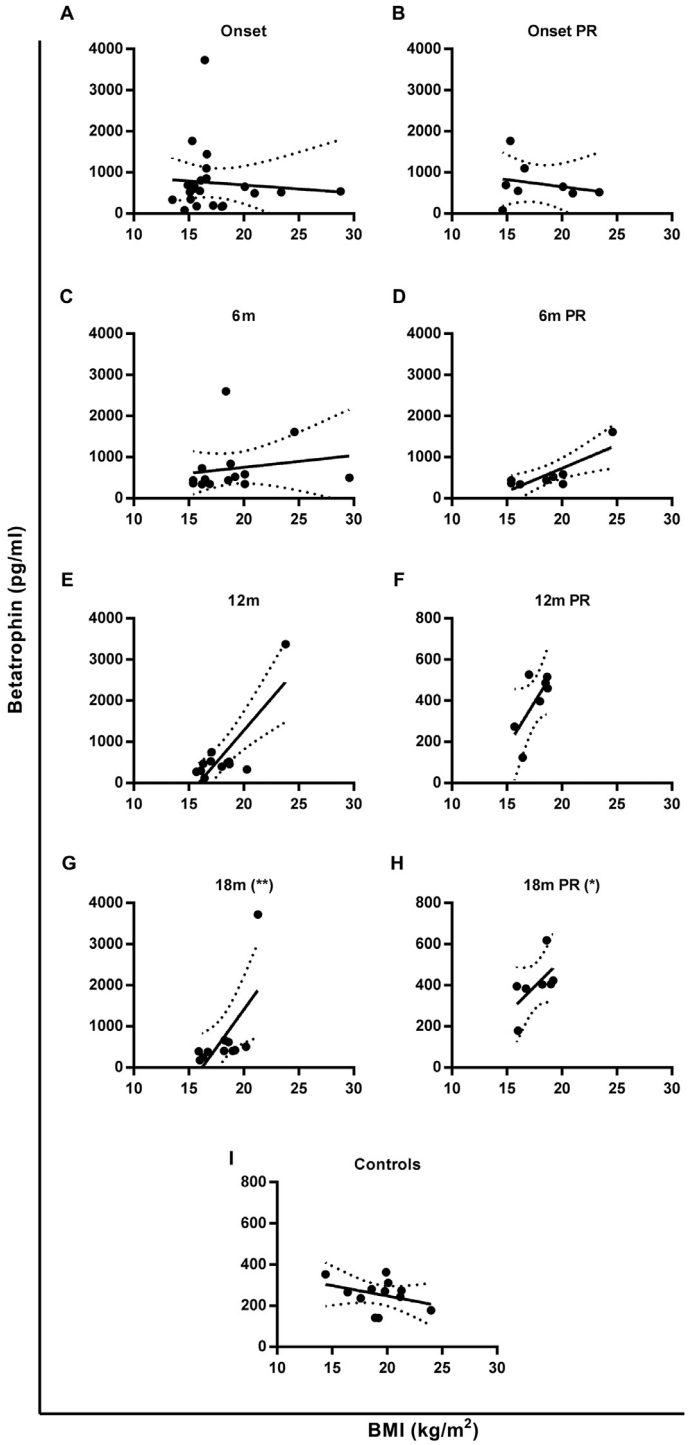

Fig 3. Correlation of betatrophin levels with BMI in pediatric patients with T1D at different stages of the disease. Linear regression of circulating plasma betatrophin concentration (pg/mL) vs BMI (kg/m^2) in patients with T1D and control subjects. *A*, Patients with T1D at the onset (Spearman $r = -0.00226$, P value = 0.9999). *B*, Patients who underwent partial remission (PR), at the onset of T1D (Spearman $r = -0.1429$, P value = 0.7520). *C* and *D*, Patients at 6 months of disease progression (Spearman $r = 0.634$, P value = 0.1705) and those who underwent PR (Spearman $r = 0.3877$, P value = 0.1009). *E* and *F*, Patients at 12 months of disease progression (Spearman $r = 0.4895$, P value = 0.1098), and those who underwent PR (Spearman $r = 0.5$, P value = 0.2667). *G* and *H*, Patients at 18 months of disease progression (Spearman $r = 0.8$, P value = 0.0047, **) and PR group (Spearman $r = 0.7857$, P value = 0.0480, *). *I*, Control group (Spearman $r = -0.07692$, P value = 0.8171).

Table 4. Clinical data for controls, and patients at onset, first and second year of T1D progression included in lymphocyte subsets analysis

	Control	Onset	1 y	2 y
N	16	11	12	7
Gender (M/F)	9/7	6/5	3/9	1/6
Age (years, mean ± SD)	8.58 ± 4.42	8.69 ± 5.16	10.20 ± 3.87	10.01 ± 2.78
BMI (kg/m^2)	19.43 ± 1.26	18.24 ± 3.10	17.53 ± 2.67	18.51 ± 2.13
Insulin dose (UI/kg/day)	ND	0.69 ± 0.27	0.62 ± 0.20	0.83 ± 0.23
HbA1c (%)	ND	8.87 ± 1.98	7.86 ± 1.17	7.82 ± 1.68
HbA1c (mmol/mol)		73 ±21.58	62 ±12.75	62 ±18.3
IDDAA1C (%)	ND	10.11 ± 13.21	8.93 ± 11.79	7.09 ± 15.25

Data presented as mean±SD. Statistical differences were found in BMI (control subjects vs patients with T1D during the first year: (*)). Statistical differences calculated using the Mann-Whitney test (*$P < 0.05$). ND, not determined.

Low circulating monocyte count is associated with T1D onset and second year of progression. Alterations in monocyte counts were found at the early stages of T1D (Fig 5). Total monocyte count showed a trend to be lower at the onset of the disease when compared to control subjects, but at the second year of T1D progression differences were significant (Fig 5, A and B). Within this monocyte family, the numbers of classical monocytes ($CD14^+CD16^-$) confirmed these results and also showed a decrease at the onset of the disease (Fig 5, C), but this was not reflected in the percentage (Fig 5, D). The nonclassical monocyte subset ($CD14^+CD16^+$) showed no statistical differences or tendencies between the different groups (data not shown). In summary, the concentration of total monocytes and its major subpopulation, the classical $CD14^+CD16^-$ monocyte subset, was diminished at the early stages of diabetes. Since monocytes are precursors of antigen-presenting cells, their decrease in periphery at the second year of disease progression suggests an active extravasation to target tissues, probably to contribute to the chronification of the autoimmune response.

B lymphocyte subsets are quantitatively altered at the onset and at early stages of T1D progression. The analysis of B lymphocytes at different maturation stages, memory/activation subsets, and B transitional lymphocytes, revealed quantitative alterations during the first 2 years of disease progression (Fig 6). The concentration of $CD27^+$ B cells was increased at the first year of disease when compared to the onset and to the second year of disease. However, it was not reflected in the percentage (Fig 6, A and B). The total B transitional subset also showed an increase in concentration during the first year of disease when compared to the second year and to control subjects, but this alteration was not observed in the percentage (Fig 6, C and D). Within the B transitional population, alterations were found both in B transitional high and low subsets (Fig 6, E−H), previously referred as T1 and T2 in the literature. A decrease in B transitional high subset counts was found at the second year when compared to control subjects and to the first year of disease (Fig 6, E), but this change was not reflected in the percentage (Fig 6, F). Regarding B transitional low subpopulation, the amount of these cells was lower at the second year of disease when compared to the first year (Fig 6, G and H). This subset includes functional regulatory B cells and despite its role is not completely understood, their increase may reflect the attempts to restore self-tolerance during T1D early stages.

T lymphocyte subsets are quantitatively altered at the onset and at early stages of diabetes progression. Because T cells are the final effector cells in T1D, both αβ and γδ T cell populations were screened (Fig 7). The concentration and percentage of αβ T cell subpopulation (Fig 7, A and B) were decreased at the second year of disease when compared to control and other disease stages. Among different αβ T cells subsets, only double positive T lymphocytes were decreased in counts at the second year when compared to the first year of disease (Fig 7, C) but not in percentage (Fig 7, D). Regarding the γδ $CD4^+$ T cell subset, no differences were found among different groups (data not shown). The $CD8^+$ γδ subset was decreased in concentration at the first year of progression when compared to the second year, and the percentage tended to decrease during disease progression (Fig 7, E and F). In summary, alterations in total αβ and γδ T cell subpopulations were observed during 2 years of T1D progression.

The different T lymphocyte maturation stages were determined and showed alterations at the onset and at the different stages of T1D (Figs 8−10). Only the total $CD3^+$ $CD4^+$ T lymphocyte population concentration was found decreased at the onset of T1D when compared to control subjects and the first year of disease (Fig 8, A and B). Within this population, the central memory (CM) $CD27^+$ subset revealed alterations both in concentration and percentage. There was an increase in concentration in the second year when compared to

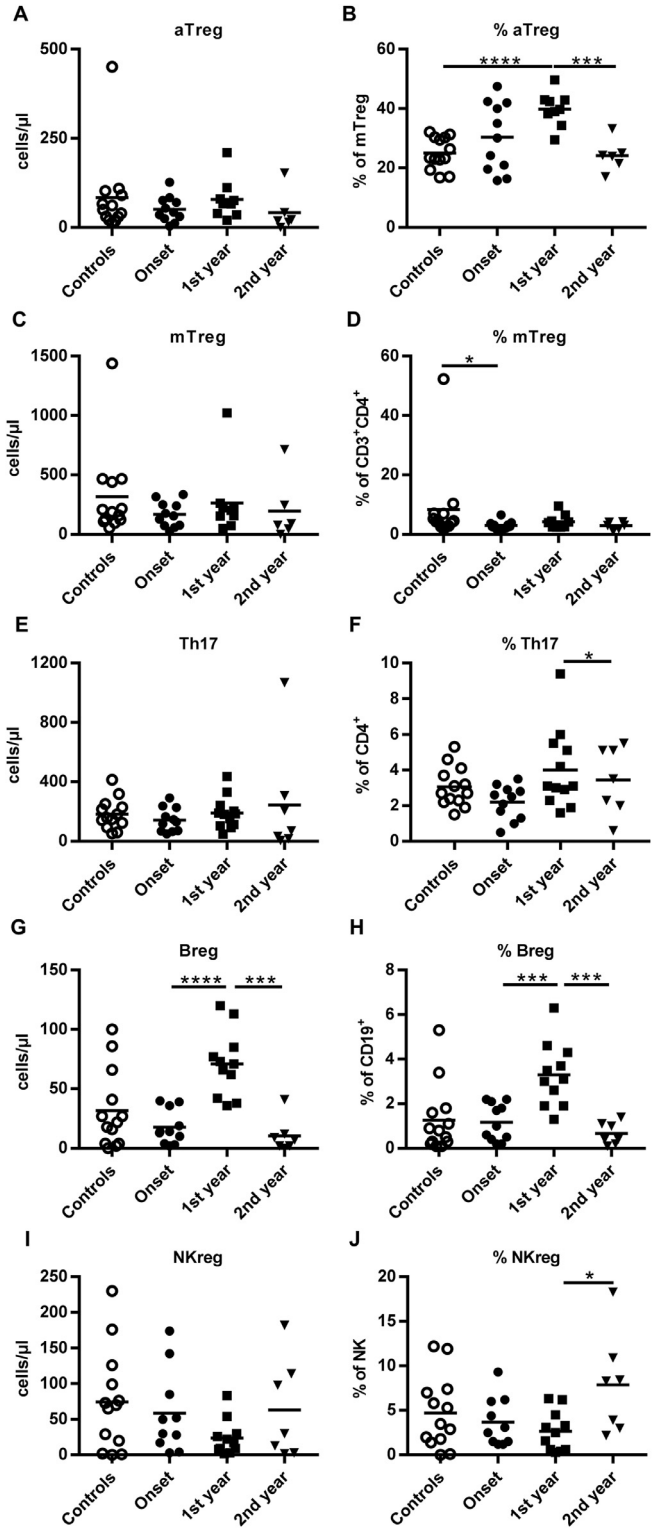

Fig 4. Concentration and percentage of regulatory lymphocyte subsets in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/µL) and percentage (%) of *A* and *B*, activated T regulatory cells (aTreg), *C* and *D*, memory T regulatory cells (mTreg), *E* and *F*, Th17 cells, *G* and *H*, B regulatory cells (Breg), and *I* and *J*, Natural Killer regulatory cells (NKreg). White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, Mann-Whitney test).

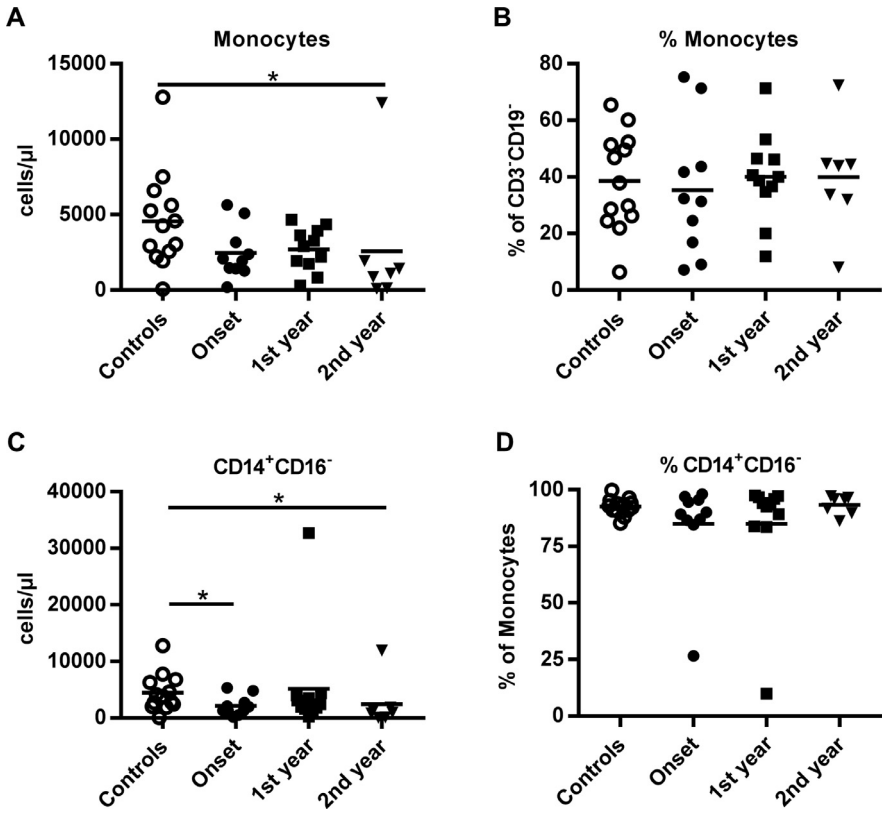

Fig 5. Concentration and percentage of monocytes in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/µL) and percentage (%) of *A* and *B*, total monocytes, and *C* and *D*, its major subset, classical monocytes. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, Mann-Whitney test).

the first year of disease (Fig 8, C). Also, there was an increase in percentage at disease onset when compared to control subjects, with a tendency to decrease with progression of diabetes until a rate similar to control group at second year (Fig 8, D). The PTK7$^+$ subpopulation, included in the CD4$^+$ naive subset, revealed an increase in percentage during the second year of disease (Fig 8, F).

The CD4$^+$ T lymphocyte effector memory subsets were analyzed (Fig 9). Total CD4$^+$ effector cells expressing the marker CD45RA (EMRA) subsets showed a remarkable decrease in both concentration (Fig 9, A) and percentage at the onset of the disease (Fig 9, B). Furthermore, this decrease in concentration was also observed in both CD4$^+$ EMRA subsets: CD27$^+$ (Fig 9, C) and CD27$^-$ (Fig 9, E), but not in their percentage (Fig 9, D and Fig 9, F, respectively). In addition, the CD4$^+$ EM cell subset revealed lower counts at disease onset when compared to the first year of disease (Fig 9, G). This is due to alterations in the CD4$^+$ EM CD27$^+$ subset (Fig 9, I). This effect was not reflected in percentage (Fig 9, H and Fig 9, J, respectively).

Besides the CD4$^+$ population, CM and EM in the CD8$^+$ subsets were also altered (Fig 10). Total counts of CD3$^+$ CD8$^+$ T cells were decreased at the onset of the disease when compared to control subjects, and normalized after diagnosis (Fig 10, A and B). Regarding the CM subset, a decrease in counts and percentage, was observed during the second year of progression (Fig 10, C and D). The analysis of the CD8$^+$ naïve cells subset showed a decrease in cell number at the onset and during the first year of disease (Fig 10, E), in agreement with the data of CD3$^+$ CD8$^+$ population (Fig 10, A). Moreover, a decrease in EMRA CD8$^+$ T cell counts (Fig 10, G) was observed, but not in percentage (Fig 10, H). This decrease in concentration was observed at T1D onset in the EMRA CD27$^+$ subset (Fig 10, I), whereas in percentage, a decrease was found at the first year of the disease (Fig 10, J). In summary, alterations in the CM and EM lineages of T lymphocytes and their maturation stages have been detected at early T1D progression. All these cell subsets are commonly found in the spleen and in the lymph nodes, and the decrease in peripheral blood

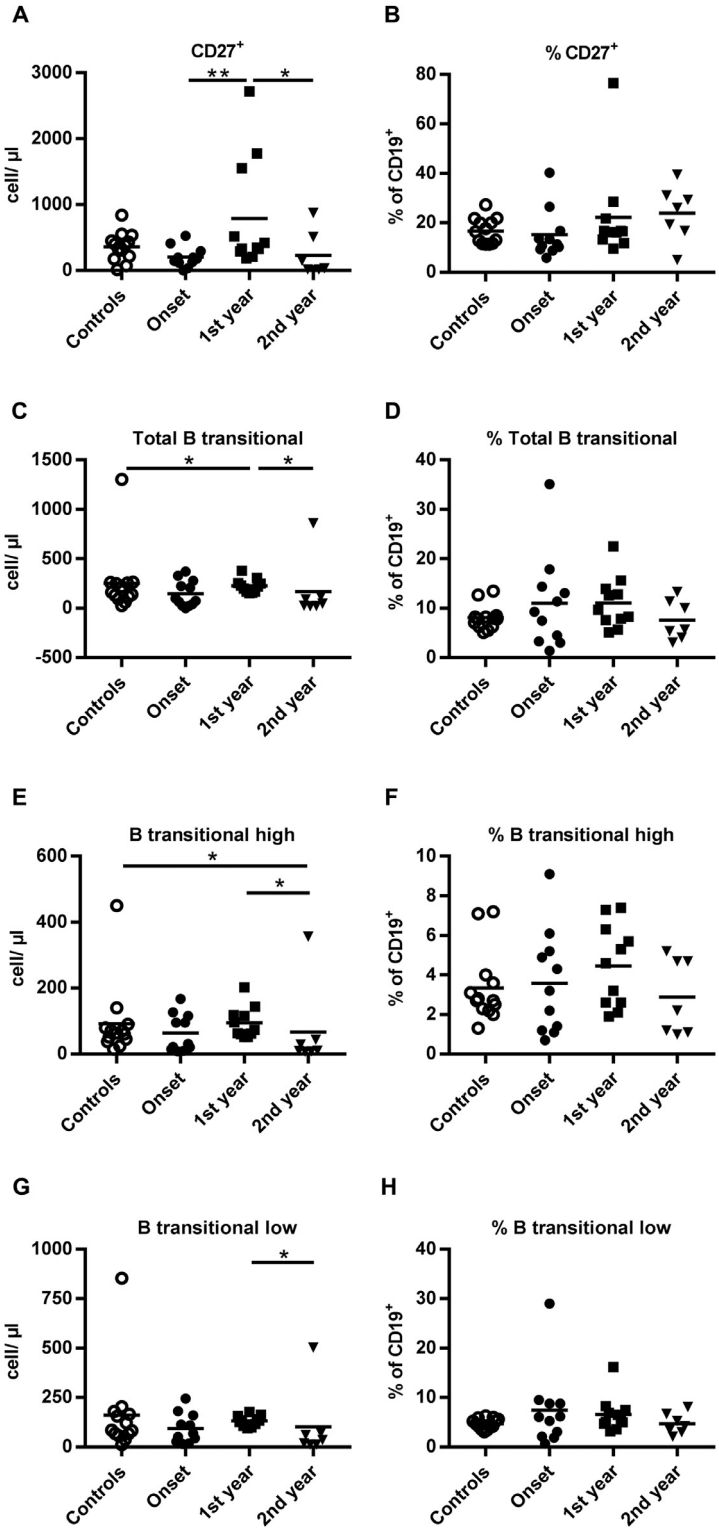

Fig 6. Concentration and percentage of B lymphocytes in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/µL) and percentage (%) of *A* and *B*, total CD27$^+$ peripheral blood mononuclear cells (PBMCs), *C* and *D*, total B transitional lymphocytes, *E* and *F*, B transitional high, and *G* and *H*, B transitional low. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, **$P < 0.01$, Mann-Whitney test).

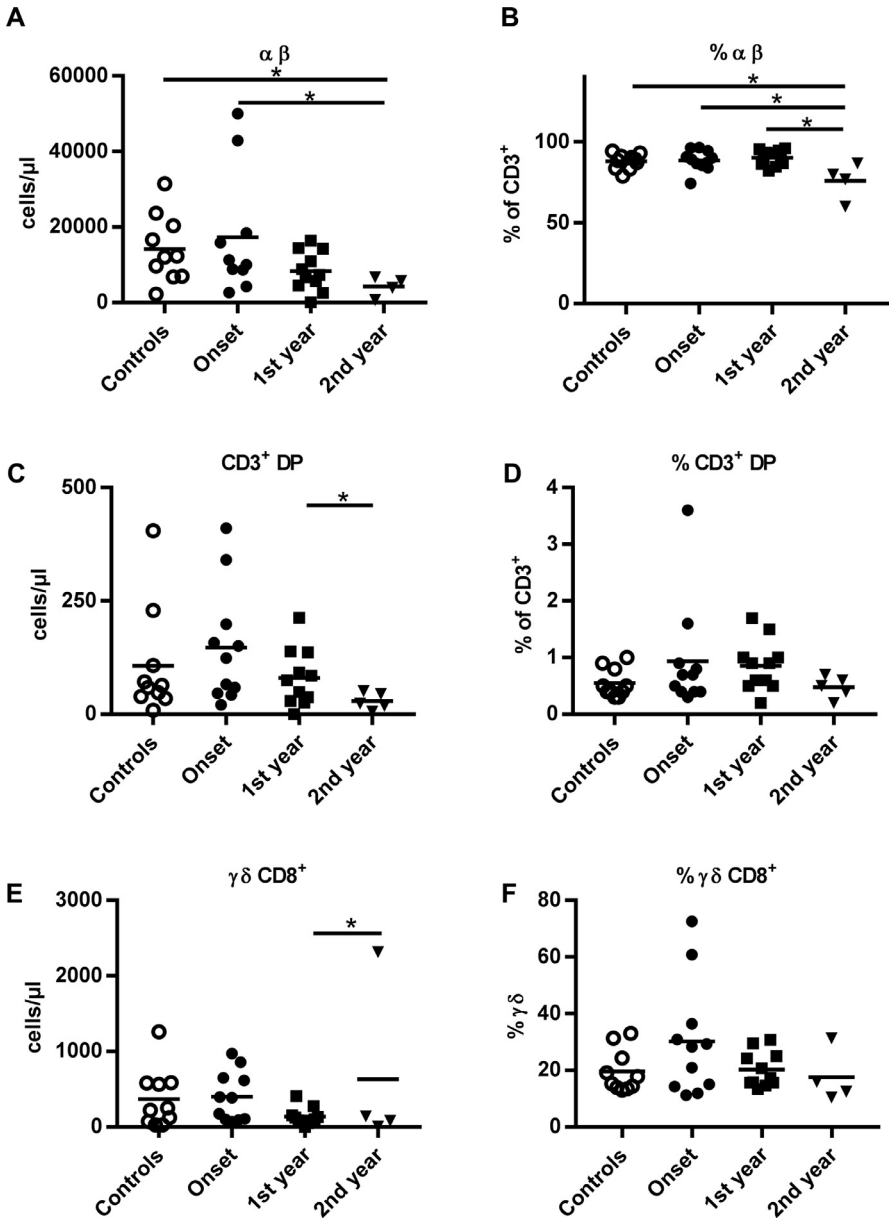

Fig 7. Concentration and percentage of αβ and γδ T lymphocytes in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/μL) and percentage (%) of *A* and *B*, total αβ, *C* and *D*, its subset $CD3^+$ double positive (DP), and *E* and *F*, γδ $CD8^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, Mann-Whitney test).

of EM and EMRA T cells both in the $CD4^+$ and the $CD8^+$ T cell subsets at the onset could reflect the need for a compensatory mechanism, due to an active migration to target tissue.

DISCUSSION

It is known that the lack of biomarkers of T1D stages hinders the proper stratification patients, the prediction of secondary complications, and the discovery of optimal checkpoints for potential therapies. Metabolic parameters are currently the most reliable biomarkers, and during disease progression, C-peptide levels are considered to reflect residual insulin storage. Although this is a good biomarker for endogenous insulin production and secretion, the sensitivity of the technique prevents the assessment of low values[22] and most pediatric patients show undetectable C-peptide levels at disease onset.[6] Thus, novel biomarkers for stratifying subjects and for

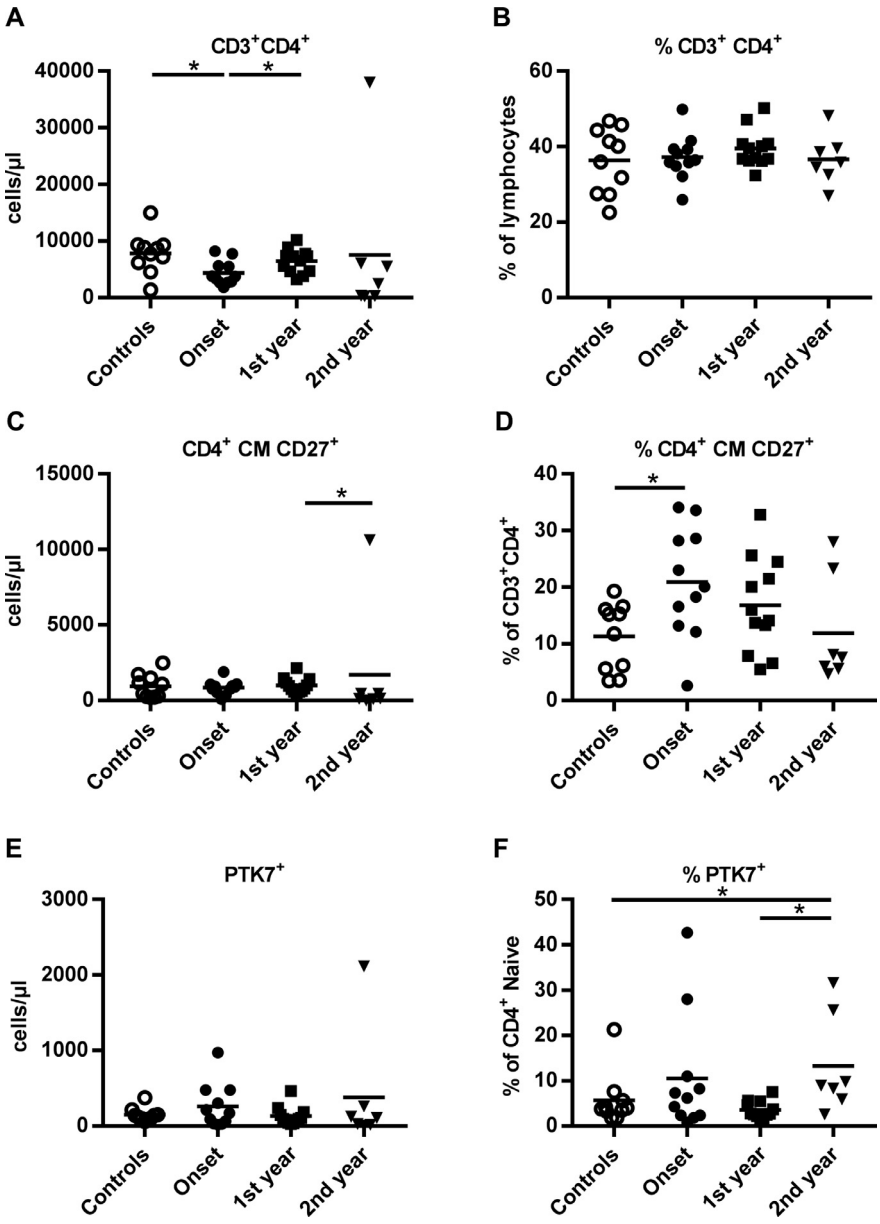

Fig 8. Concentration and percentage of $CD4^+$ T lymphocyte subsets. Concentration (cells/µL) and percentage (%) of *A* and *B*, total $CD3^+CD4^+$, *C* and *D*, $CD4^+$ CM $CD27^+$ and *E* and *F*, its major subset $PTK7^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis (*$P < 0.05$, Mann-Whitney test).

immunomonitoring are needed. This article presents a screening of candidate biomarkers in pediatric patients with T1D, with special interest in the early stages.

There are many and diverse immune biomarkers of T1D.[23] In this pilot study, aimed at determining differential molecular and cell patterns in children diagnosed with T1D in our geographic area, potential candidates have been considered. Betatrophin, a promising biomarker in long-standing adult patients with T1D,[8] showed a triplicated concentration in plasma at the onset of pediatric T1D. Furthermore, those increased levels remain steady during the early progression of the disease, including the PR stage. Thus, circulating betatrophin levels in plasma do not distinguish PR stage. Our results of the correlation between a metabolic parameter such as BMI and circulating betatrophin levels are in keeping with those described in T1D[8] and T2D[24] that suggest that hyperglycemia may be the pivotal point for betatrophin upregulation in diabetes.

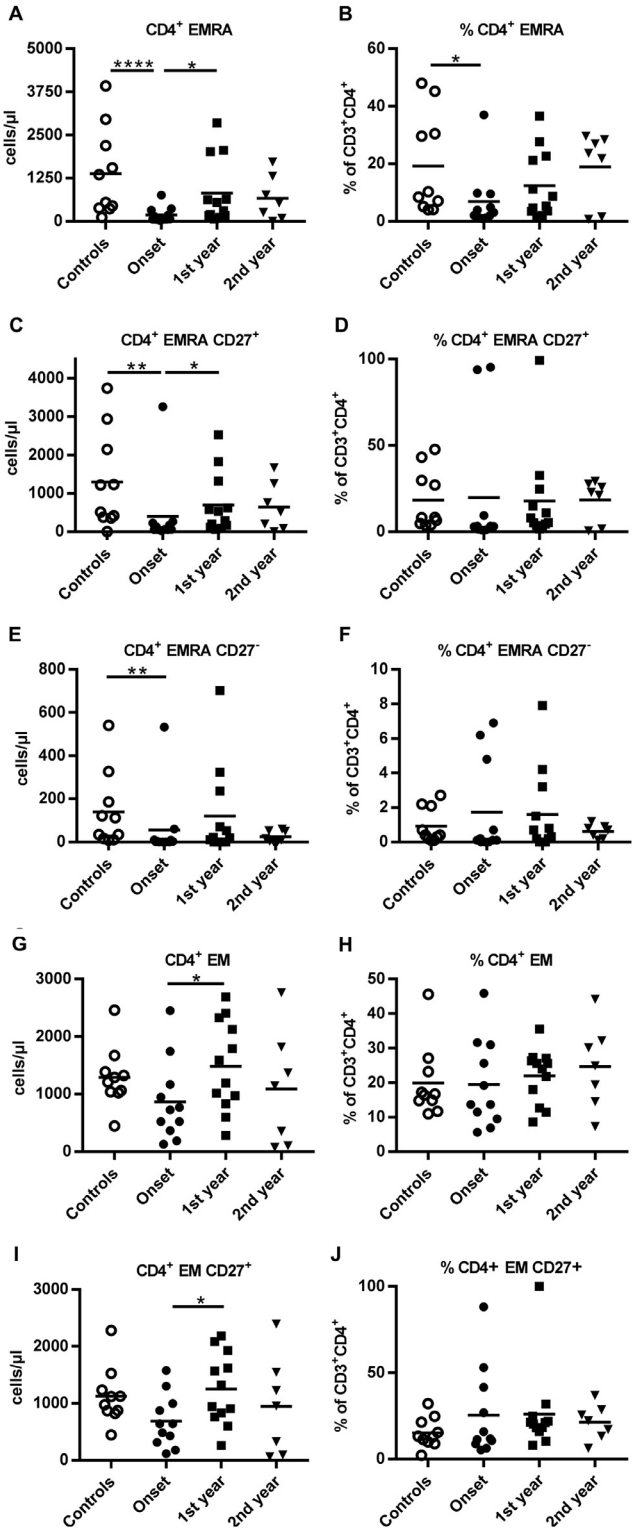

Fig 9. Concentration and percentage values of $CD4^+$ and its effector memory (EM) subset, included in the T lymphocytes maturation stages. Values in concentration (cells/µL) and percentage (%) of *A* and *B*, total EMRA $CD4^+$, *C* and *D*, $CD4^+$ EMRA $CD27^+$, *E* and *F*, $CD4^+$ EMRA $CD27^-$, *G* and *H*, total $CD4^+$ EM, and *I* and *J*, $CD4^+$ EM $CD27^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis ($^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, Mann-Whitney test).

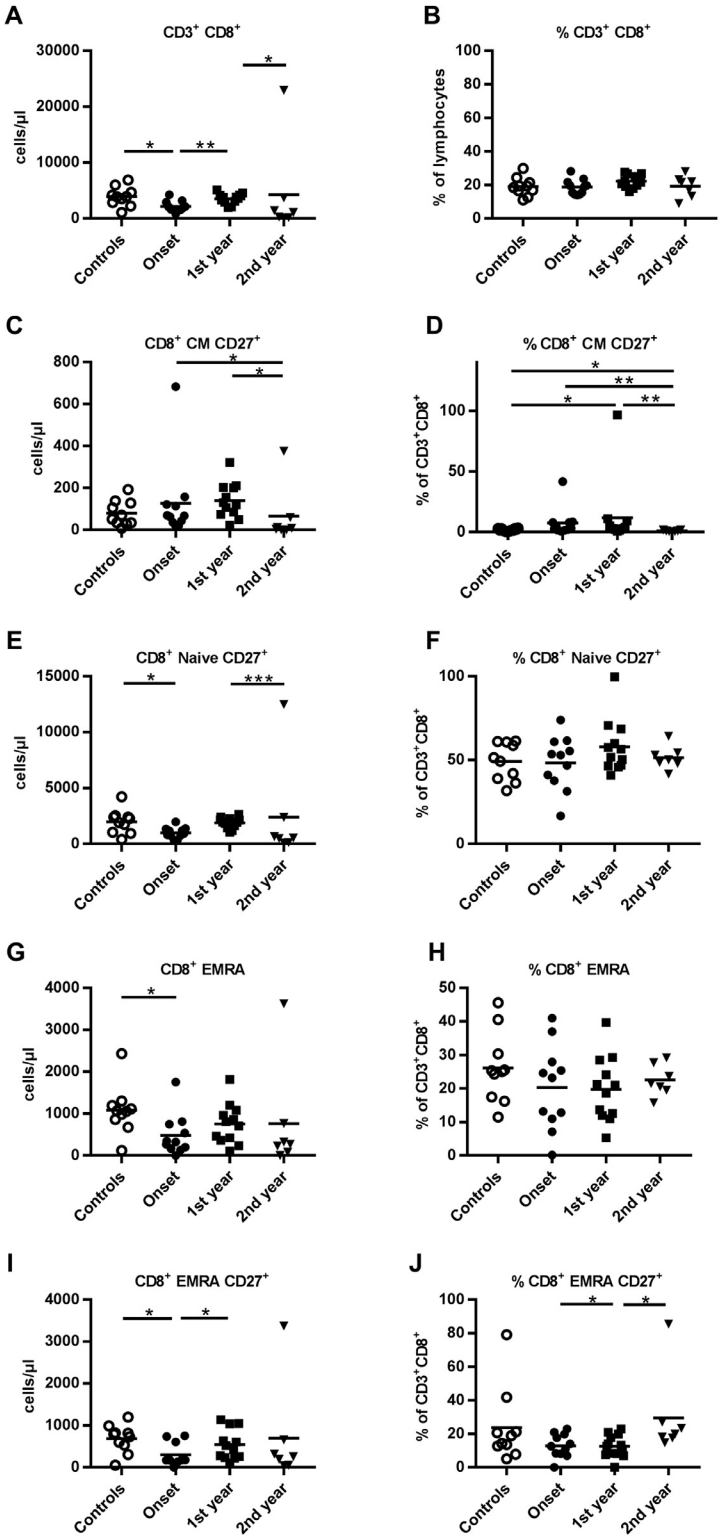

Fig 10. Concentration and percentage values of $CD8^+$ lymphocytes and their subsets, included in the T lymphocytes maturation stages. Values in concentration (cells/µL) and percentage (%) of *A* and *B*, total $CD3^+CD8^+$, *C* and *D*, $CD8^+$ CM $CD27^+$, *E* and *F*, $CD8^+$ Naïve CD27, *G* and *H*, total $CD8^+$ EMRA, and *I* and *J*, its subset $CD8^+$ EMRA $CD27^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Mann-Whitney test).

Table 5. Summary of candidate biomarkers altered during pediatric T1D early progression

Candidate biomarker	Source	Stage	Outcome
Betatrophin	Plasma	Onset, 6, 12, and 18 mo of progression	Increased
TGF-β	Plasma	Partial remission	Decreased
aTreg (%)	Blood	First year of progression	Increased
Breg (% and counts)	Blood	First year of progression	Increased
NKreg (%)	Blood	Second year of progression	Increased

Then, the potential of other immune mediators whose expression was strongly altered in the pancreases from patients with T1D[11] was analyzed. Here we demonstrate for the first time a decrease in circulating TGF-β during PR when compared to the onset. This candidate biomarker for PR distinguishes pediatric patients until 1 year after diagnosis. Low TGF-β levels in PR could reflect the transient recovery of self-tolerance proposed at this stage.[1] In fact, TGF-β is increased in patients at the onset of T1D[10] reflecting a response to autoimmunity activation and correlating with the fact that hyperglycemia stimulates TGF-β expression.[25] Because many cells can secrete TGF-β, a deeper understanding of the mechanisms involved in the decrease of plasma concentration during PR is needed. In this stage, the remaining β-cells are still able to produce enough insulin and, in some cases, transient insulin-independence can be achieved. The identification of reliable biomarkers would allow for the identification and monitorization of PR. Because there are no differences in TGF-β levels when comparing patients at PR and non PR stages at 6 and 12 months of evolution of the disease, at present it would be useful for the monitoring of PR for each particular patient.

Additionally, lymphocyte cell subsets have been used as biomarkers for several immune mediated diseases. One of the main advantages of using them is the amount of given information due to the multiple parameters that can be analyzed in the sample, and its ease of tracking during disease progression. Lymphocyte regulatory subsets have been partially characterized in T1D. Since they are crucial in self-tolerance and show phenotypic, functional and migration alterations in autoimmunity,[26,27] this study aimed at determining the changes in regulatory leukocyte subsets during the first 2 years of T1D in children. Alterations were found in all regulatory leukocyte subsets both in concentration and percentage, reflecting a robust significance. Moreover, the alterations observed in one variable can be due to compensatory mechanisms undergone by other cell lineage subsets to accommodate the first change. Since lymphocyte subsets can only be determined in peripheral blood at a time, peripheral subsets are tightly regulated by their generation in primary lymphoid organs and by their migration to the target tissue, ie, the pancreas in T1D. The decrease of the percentage of mTreg at the onset of the disease in children is accompanied by a strong increase in aTreg at the first year. Since Tregs are crucial in self-tolerance, these data reflect the autoimmune attack at T1D onset as mTregs are reduced. The increase in aTregs during the first year of disease, which is also when the classical appearance of PR occurs, may reveal the attempts to restore self-tolerance. In this sense, a mathematical model explains that T1D onset is the consequence of an increase in effector T lymphocytes over Treg and that the PR stage may be due to an inversion in the frequencies of these subsets.[28] As for Breg and NKreg cell subsets, their role in T1D is not completely understood. This is the first report showing an increase of Breg and NKreg in early stages of pediatric T1D. Future studies may provide insight into the role of these cells in PR and T1D.

We are well aware of the limitations of this study. Despite a relatively small sample size, the *P* value of the here proposed biomarkers – betatrophin, TGF-β and regulatory T, B, and NK subsets – determines statistical differences. Furthermore, these candidates were selected from previous data and in basis of their biological effect. Future analysis of "big data" obtained from an independent cohort would be beneficial for both validation of these candidate biomarkers and for the screening of new ones. Nevertheless, the strength of the study is the identification of immunologic alterations in early stages of the disease, with special interest in the PR, an unexplored – and often undetected – phase of T1D. In our cohort PR was evident at 6 months after diabetes onset. Thus, we plan to include this time checkpoint for all patients and variables to be investigated in future studies.

Overall, the results (summarized in Table 5) reveal the characterization of the changes in these parameters during the different early stages of T1D in children – betatrophin, TGF-β and regulatory T, B and NK subsets – that could be used in clinical practice to characterize the progression of T1D. Moreover, and because PR may be an ideal stage for immunointervention, these candidate biomarkers can be considered for clinical trials. This study constitutes an open door, not only

to explore the underlying pathophysiology of the early stages of T1D, but also to stratify the patients for future therapeutic strategies.

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AUTHOR CONTRIBUTIONS

AV, MF, MM, SRF, RMA, MBR, RP, JB, and MVP designed the experiments, AV, MF, SRF, RMA, MBR, and RP performed the experiments, AV, SRF, and MVP wrote the manuscript, MM, DPB, JB reviewed the manuscript, BQS and AT contributed to the discussion. All authors revised the manuscript and gave final approval of the current version.

CONFLICT OF INTEREST STATEMENT

The authors have read the journal's policy on disclosure of potential conflicts of interest and all authors have disclosed any financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2019.03.002.

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