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Inflammatory Immune Markers Associated With Thyroid Peroxidase Autoantibodies in Children Diagnosed With Both Type 1 Diabetes and Celiac Disease

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ABSTRACT

Autoimmune thyroid disease (AITD) is associated with other autoimmune endocrine diseases such as type 1 diabetes (T1D) and celiac disease (CeD). Thyroid peroxidase autoantibodies (TPOA) are biomarkers of AITD but may also occur in patients with other autoimmune diseases. We examined cross-sectional correlations between TPOA and an array of immune markers in a cohort of 90 children with exclusively T1D ($n = 27$), CeD ($n = 16$) or a combination of these two diseases ($n = 18$), compared to a reference group of children without these diagnoses ($n = 29$). Children with exclusively T1D or T1D in combination with CeD had higher levels of TPOA with an overrepresentation among girls. The correlations between immune markers and TPOA were distinctly different between all study groups. In children with T1D, TPOA correlated mainly with the T helper 1 associated IFN-γ and pro-inflammatory IL-1β. In contrast, in children with combined diagnoses, TPOA was correlated with pro-inflammatory MCP-1, the acute phase proteins ferritin, fibrinogen, and serum albumin A, and adipocytokines resistin and visfatin. Children with exclusively CeD did not show the same strong association between immune markers and TPOA. In conclusion, TPOA positivity was mainly detected in patients with T1D and female sex. Several inflammatory markers correlated with TPOA, indicating a relation to autoimmune parameters in children with T1D, CeD or both, but preceding symptoms AITD.

1 | Introduction

Autoimmune thyroid disease (AITD) encompasses conditions leading to both hypo- and hyperthyroidism, including Graves' disease and Hashimoto's thyroiditis. AITD is often seen alongside type 1 diabetes (T1D) as well as celiac disease (CeD) [1]. Ingested gluten triggers an autoimmune process in individuals with CeD, and the prevalence of AITD is four times higher compared to controls [2]. T1D is caused by the destruction of insulin-producing β-cells resulting in impaired metabolic regulation and hyperglycaemia, which is associated with an increased prevalence of CeD [3]. About 10% of individuals with T1D have CeD, and 25%–30% have AITD [4, 5]. AITD includes several different conditions in the thyroid organ with unique clinical manifestations and pathological levels of certain autoantibodies. In clinical practice, the measurement of thyroid peroxidase autoantibodies (TPOA) is commonly used when investigating patients with AITD; high levels are often

indicative of Hashimoto's thyroiditis [1]. This disease results in the destruction of the thyroid gland, with subsequent inability to produce thyroid hormones. Another common AITD is Graves' disease, where TSH receptor autoantibodies stimulate thyroid hormone secretion, leading to high levels of thyroid hormones in the blood [6]. The associations between different autoimmune conditions can partially be explained by shared pathophysiological mechanisms in immune regulation, as these diseases are all associated with immune cell activity and increased levels of circulating immune markers [7, 8]. Currently, there is no curative treatment for either T1D, CeD or AITD Hashimoto's thyroiditis, and this motivates further studies on shared signalling pathways underlying these diseases [9].

AITDs, as well as T1D and CeD, are all organ-specific autoimmune diseases defined by their activation of several different subsets of immune cells and secretion of several different immune markers. T1D is traditionally associated with a T helper (Th)-1 and Th17-like pro-inflammatory profile, both during the prediabetic phase and following the clinical onset of the disease. T helper-1-like subtype cells secreting predominantly interferon (IFN)-γ and tumour necrosis factor (TNF)-β are important for the destruction of the insulin-producing β-cells [10]. In children with CeD, pro-inflammatory cytokines, especially TNF-α, interleukin (IL)-1β, -6 and -8, chemokines like macrophage inflammatory protein (MIP), but also Th-17-like lymphocytes secreting IL-17A, -21, -22 and IL-33, are involved in the immune response in the gut [11–13]. Acute-phase proteins (APPs) like procalcitonin (PTC), ferritin, tissue plasminogen activator (tPA), fibrinogen, and serum amyloid A (SAA) have both pro- and anti-inflammatory effects on different immune cells and are also of importance in these autoimmune diseases [14]. Alterations in cytokine and chemokine levels are observed in children diagnosed with T1D and/or CeD [12, 13, 15], with a suppressed immune profile of Th17 cytokines, chemokines, APPs, adipocytokines like visfatin and resistin, and matrix metalloproteinases (MMP) [16]. Resistin and visfatin are important adipocytokines with pro-inflammatory, regulatory, and immunomodulating properties [17, 18]. Visfatin stimulates secretion of the pro-inflammatory interleukins (IL)-1β, -1Ra and -6, and TNF-α as well as the T regulatory (Treg) associated IL-10 [17]. Further, visfatin can cause endothelial dysfunction [19] and plays a role in matrix degradation [20]. Resistin has a regulatory role in several inflammatory processes and, possibly, inflammatory disorders and is mediated by the capacity to stimulate pro-inflammatory TNF-α, IL-1β and IL-6 [18]. MMP are generally involved in pathological processes characterized by either degradation of tissue matrices or inflammatory responses [21, 22]. Dysregulation of MMPs is observed in T1D [23, 24] and CeD [25–28]. MMP −1, −2 and −3 can regulate TNF-α which plays an important pathogenic role in both T1D and CeD [22, 29]. IFN-γ can stimulate the secretion and activation of MMPs, thereby contributing to the degradation of the extracellular matrix and tissue remodeling [25, 30].

Activation of different subsets of immune cells and secretion of different immune markers is also seen in children with hypothyroidism, for example, via increased release of IL-1β [31]. Acute phase proteins have also been associated with these conditions. Circulating ferritin changes with thyroid hormone balance in AITD [32]. Fibrinogen and SAA are increased by the low-grade inflammatory process in Hashimoto's thyroiditis [33]. Certain adipocytokines have also been associated with autoimmune diseases. Resistin may be altered in Graves' disease but does not appear to change with Hashimoto's thyroiditis [34]. Visfatin increases in autoimmune hypothyroidism [35], while it is also known to be lower in children with CeD and T1D [16].

Studies examining the immunological pattern in children are few in the context of combined autoimmune diseases. A deeper understanding of how different immune markers correlate with autoantibodies may open up better understanding of possible underlying pathophysiological pathways. Here, we examine TPOA in children diagnosed with T1D, CeD or both, but without clinical symptoms of AITD, and their association with an array of immune markers associated with autoimmune diseases.

2 | Materials and Methods

2.1 | Study Cohort

2.1.1 | Diagnostic Criteria and Examination Procedures

This is a multi-center study that includes children with T1D, CeD, or both, or those with neither disease, collected at Linköping University Hospital, Linköping, Sweden and Ryhov County Hospital, Jönköping, Sweden, as previously described [16].

The cohort of this study included a total of 90 children: 18 children diagnosed with both T1D and CeD (T1D + CeD), 27 children with T1D exclusively, 16 children with CeD exclusively, and 29 reference children without these diseases (REF). In some parts of the study, children with exclusively T1D are combined with children with both diagnoses to make a total of 45 children. Information regarding age, duration of disease (T1D and/or CeD) and sex (data on sex are missing for two children with combined diagnosis) is summarised in Table 1.

T1D was diagnosed according to the International Society for Paediatric and Adolescent Diabetes (ISPAD) guidelines [36]. Symptoms of diabetes plus casual plasma glucose concentration ≥ 11.1 mmol/L (200 mg/dL) or fasting plasma glucose ≥ 7.0 mmol/L (≥ 126 mg/dL) or 2-h post-load glucose ≥ 11.1 mmol/L (≥ 200 mg/dL) during an OGTT (Oral Glucose Tolerance Test). Duration of T1D was calculated from the date of diagnosis until the date of sample collection.

CeD was diagnosed according to the modified version of The European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) criteria [37]. The duration of CeD was calculated from the date of biopsy-confirmed diagnosis until the date of sample collection.

All children in the combined diagnosis group were diagnosed with T1D before being diagnosed with CeD.

The control (reference) group consisted of healthy children, and neither the reference children nor their first-degree relatives

TABLE 1 | Characteristics of participants; children with type 1 diabetes (T1D), celiac disease (CeD), T1D and CeD (T1D + CeD) and reference children (REF).

	T1D	CeD	T1D + CeD	REF
n	27 (30%)	16 (18%)	18 (20%)	29 (32%)
Girls/Boys (*n*)	14/13	10/6	7/9 (2[a])	15/14
Age (years)	13.2 (4.6)	10.3 (3.8)	10.8 (5.2)	11.9 (4.3)
Girls	12.7 (3.9)	12.6 (4.9)	8.2 (4.3)	11.3 (5.8)
Boys	13.7 (4.9)	10.0 (2.3)	12.0 (4.4)	12.0 (4.8)
Duration T1D (years)	4.2 (4.5)		3.5 (3.3)	
Girls	5.7 (4.5)		2.6 (3.0)	
Boys	3.3 (4.5)		3.6 (5.6)	
Duration CeD (years)		5.9 (7.6)	2.1 (3.6)	
Girls		6.6 (5.2)	4.5 (4.1)	
Boys		1.0 (3.8)	1.9 (1.9)	

Note: Data for continuous variables are presented as median (IQR), and for categorical variables as *n* (%).
[a]Age, sex and disease duration data not available.

displayed any clinical signs of T1D, CeD, AITD or other autoimmune diseases. The participants in the different groups were matched on age and sex as far as possible.

The general inclusion criteria for participating in the study were that the children in all study groups should not show any signs of allergy, colds, or other infections at the time of sample collection.

2.1.2 | Sample Collection

Blood samples (approximately 5 mL) were collected in Vacutainer tubes without anticoagulant (BD Biosciences, San Jose, CA, USA) in connection with a routine visit at the paediatric clinic. One hour prior to blood sampling, a local anaesthetic ointment was placed on the arm. Thirty minutes after sampling, sera were separated by centrifugation of the whole blood samples at 2000×*g* for 10 min. Thereafter, sera were aliquoted and stored at −80°C until analysis. Multiple freeze–thaw cycles were avoided.

2.2 | Analysis of Soluble Immune Markers

Twenty-eight immune markers, including cytokines/chemokines, adipocytokines, MMPs and acute phase proteins, were analysed in sera of 90 children (Table 1) using multiplex fluorochrome sandwich immunoassays based on Luminex xMAP technology (Luminex, Bio-Rad Laboratories, Hercules, CA, USA) Bio-Plex assays on the Bio-Plex 200 system according to the manufacturer's instructions (Bio-Rad Laboratories). The median fluorescence intensity (MFI) for each sample was registered and analysed with Bio-Plex Manager Software 5.0 (Bio-Rad Laboratories). The analyte concentrations were estimated using a five-parameter logistic model standard curve. Immune markers were sub-grouped, and the cut-off values for minimum detectable concentrations are presented in Table 2.

Quality controls (recombinant protein supplied by the manufacturer) for each immune marker were included in each experiment to monitor assay performance. All quality controls were within the expected range (determined by the manufacturer). To evaluate the reproducibility of the assay, intra- and inter-assay coefficients of variation (CV, presented as percentage) were calculated for the quality controls assayed in duplicate. The inter-assay CV was 1.3% for IL-6 and 2.7% for IL-10.

2.3 | Analysis of Thyroid Peroxidase Autoantibodies

TPOA were detected with a novel agglutination-PCR (ADAP) assay [38] according to previous protocols for T1D and CeD-associated autoantibodies [39–41]. Reagents were obtained from Enable BioSciences, San Francisco, CA, and the analysis was run at Lund University, accordingly. The ADAP assay includes antibody agglutination of two complementary DNA-barcoded antigen proteins. Positioning of these compatible DNA conjugates in close proximity with the addition of an oligo bridge enables quantification by PCR. The analysis was run on the fully automated Hamilton MicroLab STAR liquid handling platform [40, 42] (Hamilton, Bonaduz, Switzerland), and SYBR green-based qPCR (RT-qPCR) was used for quantification in 40 cycles (i.e., first part 95°C for 10 min, second part 95°C for 15 s and 56°C for 1 min in 39 cycles) using the Roche Lightcycler 480 System II (Roche Diagnostics International AG, Rotkreuz, Switzerland). TPOA levels were expressed in ΔCt, following subtraction of the PCR cycle threshold of the patient sample from that of the average cycle threshold of 8 negative control samples (Phosphate-Buffered Saline and Triton-X). A strong qPCR signal, defined by a lower cycle threshold, was proportional to a larger count of amplifiable DNA conjugates, and in turn proportional to a larger count of autoantibody binding interactions to these specific protein-DNA conjugates. The cut-off for TPOA positivity was 7 ΔCt, as determined previously [39].

TABLE 2 | Cut-off values for the analyzed soluble immune markers.

Soluble immune markers	Cut-off value
Cytokines and chemokines	
Th1 associated	
IFN-γ	19.30 pg/mL
Th2 associated	
IL-5	0.80 pg/mL
IL-9	0.70 pg/mL
IL-13	2.10 pg/mL
Th17 associated	
IL-17A	0.49 pg/mL
IL-22	0.30 pg/mL
IL-25	0.07 pg/mL
IL-33	0.58 pg/mL
Treg associated	
IL-10	0.90 pg/mL
Pro-inflammatory	
G-CSF	1.10 pg/mL
IL-1β	0.80 pg/mL
IL-6	1.10 pg/mL
IL-8	0.50 pg/mL
IL-15	4.20 pg/mL
MCP-1	6.70 pg/mL
MIP-1α	2.40 pg/mL
MIP-1β	1.10 pg/mL
TNF-α	3.00 pg/mL
Acute phase proteins (*APPs*)	
Ferritin	1.30 pg/mL
Fibrinogen	2.80 ng/mL
PCT	11.30 pg/mL
SAA	1.10 ng/mL
tPA	6.15 pg/mL
Adipo-cytokines	
Resistin	1.00 pg/mL
Visfatin	8.00 pg/mL
Matrix metalloproteinases (*MMPs*)	
MMP-1	33.70 pg/mL
MMP-2	39.70 pg/mL
MMP-3	28.50 pg/mL

Abbreviations: G-CSF, Granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinases; PCT, procalcitonin; SAA, serum amyloid A; Th, T helper cells; TNF, tumour necrosis factor; tPA, tissue plasminogen activator; Treg, T regulatory cells.

2.4 | Analysis of T1D and CeD Related Autoantibodies

To estimate the total antibody burdens, the T1D and CeD related autoantibodies against glutamic acid decarboxylase 65 (GADA), islet antigen-2 (IA-2A), insulin (IAA), ZnT8 transporter (ZnT8A) and tissue transglutaminase (TGA) were analysed. The analysis was performed with the same agglutination-PCR (ADAP) assay as described above for the detection of TPO-antibodies. The cut-off for positivity of the autoantibodies (GADA, IA-2A, IAA, ZnT8A and TGA) was determined according to the manufacturer's instructions [39].

2.5 | Analysis of TSH and T4

The levels of circulating Thyroid Stimulating Hormone (TSH) and Tyroxine4 (T4) were analysed using CMIA technology. The chemiluminescence microparticle assay was performed on the Architect System (Abbott Laboratories, Wiesbaden, Germany). CMIA is a two-step sandwich immunoassay. In the first step, the analyte (TSH or T4) from the sample binds to antibody-coated paramagnetic microparticles. After washing under a magnetic field, acridinium-labelled conjugated secondary antibodies are added to the reaction mixture. The resulting chemiluminescent reaction was measured as relative light units (RLUs). The concentration of TSH present in the sample is directly related to the detected RLUs. The concentration of T4 present in the sample is indirectly related to the detected RLUs. The concentration (mU/L) of TSH and T4 was calculated out of the established calibration curve according to the manufacturer's instructions.

2.6 | Statistical Analysis

All statistical analyses were conducted with GraphPad Prism 10.0 (GraphPad, San Diego, CA, USA). Since the sample size was limited and data did not follow a Gaussian distribution, non-parametric tests were used throughout. Spearman's rank correlation test was used to test correlations between variables, stratified by diagnose group. Composite scores were compiled by the sum of several z-transformed variables. Z-transformation was done by dividing the concentration of each marker with its standard deviation. The correlation of composite scores of immune markers with TPOA was analysed using Spearman's rank correlation test. Mann-Whitney *U*-test was used to compare differences between two independent groups. Differences were considered statistically significant for $p < 0.05$.

Exploratory analysis of the relationships between the immune markers in the different diagnostic groups within the study cohort was performed. The purpose was to graphically show the relationship (similarities/differences) between the immune profiles in children with combined diagnosis and children with single diagnosis, and children without these diagnoses (i.e., reference children), respectively. The results of the exploratory analysis are presented as correlograms of the relationship between the different variables based on Pearson's correlation analysis of non-normalised data. The analysis was performed using the software package Hierarchical Clustering Explorer

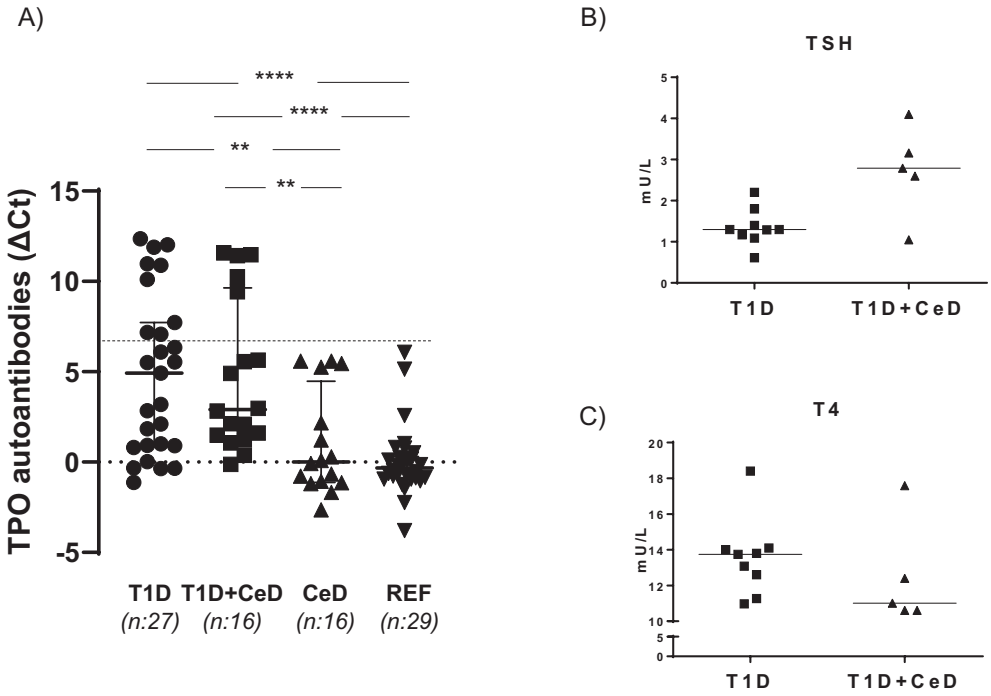

FIGURE 1 | Increased TPOA in children with type 1 diabetes (T1D) or in combination with celiac disease (CeD). Increased levels of thyroid peroxidase antibodies (TPOA) in children with exclusively T1D and in combination with celiac disease (T1D + CeD) compared to children with exclusively CeD or none of these diseases (reference, REF) (A). Normal levels of Thyroid Stimulating Hormone (TSH) (B) and Tyroxine4 (T4) (C) were observed in TPOA-positive children with either exclusively T1D (A) or children with combined T1D + CeD. Cut-off for positivity is 7.0 ΔCt. $^{**}p < 0.01$, $^{****}p < 0.0001$.

version 3.5 (Human-Computer Interaction Lab, University of Maryland, College Park, MD, USA).

2.7 | Ethics

This study was conducted in accordance with the Helsinki Declaration and approved by the Research Ethics Committee of the Faculty of Health Sciences, Linköping University, Linköping, Sweden, and by the Regional Ethics Committee for Human Research, Linköping (approval number: Dnr 2006/M89-06 and complementary Dnr: 2012/27–32 and 2017/230–12). Informed consent was received from all parents or responsible guardians and adolescents. All children received oral and written information adapted for their age prior to any blood sampling.

3 | Results

3.1 | Thyroid Peroxidase Autoantibodies in Children With T1D Only and in Combination With Celiac Disease

Autoantibodies against thyroid peroxidase (TPOA) differed in levels between the four groups (Figure 1). In children diagnosed with exclusively T1D, the TPOA levels were higher compared to children with exclusively CeD ($p < 0.01$) and reference children ($p < 0.0001$) (Figure 1A). Children with a combined diagnosis of T1D and CeD had increased TPOA levels compared to children with exclusively CeD ($p < 0.01$) and reference children ($p < 0.0001$) (Figure 1A). There was no significant difference in TPOA levels between children with exclusively T1D and children with T1D in combination with CeD. Children diagnosed exclusively with CeD and reference children without a diagnosis of either T1D or CeD had very low or no detectable levels of autoantibodies against TPOA, and none had TPOA levels above the cut-off for positivity. All children who were positive for TPOA had normal levels of TSH and T4, ruling out an undiagnosed and manifest thyroid disease (Figure 1B,C).

3.2 | Higher Levels of Thyroid Peroxidase Autoantibodies in Girls

In children with exclusively T1D, nine out of 27 children were TPOA positive; six of these nine children were girls (Figure 2A). In children with a combined diagnosis of T1D and CeD, five out of 16 children were TPOApositive and four of these children were girls ($p < 0.0001$, Figure 2B). Neither girls nor boys with a diagnosis of CeD exclusively nor children without these diseases had TPOA levels above the cut-off for positivity (Figure 2C,D, respectively).

3.3 | Thyroid Peroxidase Autoantibodies in Children With Type 1 Diabetes Only and in Combination With Celiac Disease Are Related to T-Helper and Pro-Inflammatory Immune Response

Serum levels of all soluble immune markers analysed in the four groups are listed in Supplement—Appendix S1, and correlations are presented in Table 3.

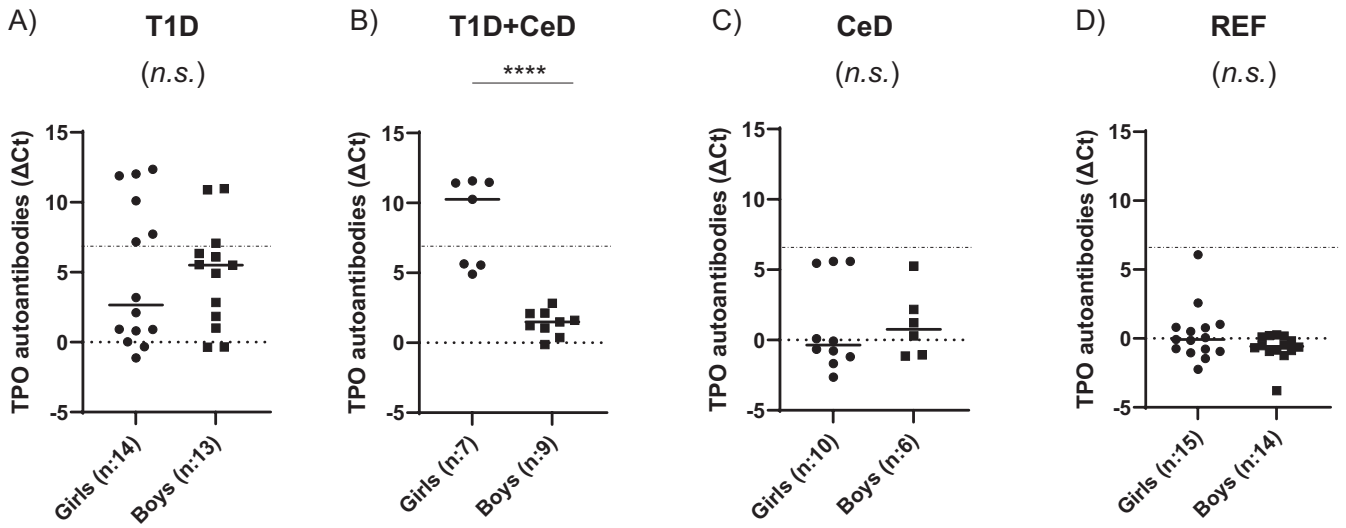

FIGURE 2 | Higher production of TPOA in girls with exclusively type 1 diabetes (T1D) or in combination with celiac disease (CeD). Higher production of thyroid peroxidase antibodies (TPOA) in children with exclusively T1D (A) and in children with combined T1D and celiac disease (T1D + CeD (B)), especially in girls, compared to children with exclusively CeD (C) or none of these diseases (reference, REF (D)). Cut-off for positivity is 7.0 ΔCt. ****$p < 0.0001$, n.s, not significant.

In children with exclusively T1D and also in T1D children combined with CeD, TPOA was positively correlated to Th1 associated IFN-γ ($r = 0.43$, $p < 0.05$, Figure 3A, and $r = 0.30$, $p < 0.05$, Figure 3B, respectively) and the pro-inflammatory immune marker IL-1β ($r = 0.56$, $p < 0.01$, Figure 3B and $r = 0.35$, $p < 0.05$, Figure 3D, respectively).

In contrast, TPOA was negatively correlated to the chemokine MIP-1β ($r = -0.45$, $p < 0.05$, Table 3), and those T1D children with TPOA above the cut-off for positivity had a lower secretion of MIP-1β compared to the TPOA-negative children with T1D ($p < 0.05$, Figure 3E).

The Th2-associated cytokine IL-13 was inversely correlated with TPOA in children with exclusively T1D ($r = -0.38$, $p < 0.05$, Table 3), whereas IL-5 was increased in T1D children with TPOA above the cut-off for positivity ($p < 0.01$, Figure 3F).

3.4 | Thyroid Peroxidase Autoantibodies in Children With Combined Type 1 Diabetes and Celiac Disease Are Related to Adipocytokines, Pro-Inflammatory, and Acute Phase Proteins

In children diagnosed with both T1D and CeD, TPOA were related to several immune markers associated with pro-inflammation, acute phase proteins, and adipocytokines. TPOA in children with a combination of T1D and CeD correlated with the adipocytokines resistin ($r = 0.54$, $p < 0.05$, Figure 4A) and visfatin ($r = 0.50$, $p < 0.05$, Figure 4B). TPOA in the same group of children were also correlated to the pro-inflammatory monocyte chemoattractant protein (MCP)-1 ($r = 0.49$, $p < 0.05$, Figure 4C). The acute phase proteins ferritin ($r = 0.47$, $p < 0.05$, Figure 4D), fibrinogen ($r = 0.56$, $p < 0.05$, Figure 4E) and SAA ($r = 0.70$, $p < 0.01$, Figure 4F) were all related to TPOA in children with a combined T1D and CeD diagnosis.

Children with a combined diagnosis of T1D and CeD and with levels of TPOA above the cut-off for positivity also had higher levels of SAA ($p < 0.01$, Figure 5A) and MCP-1 ($p < 0.05$, Figure 5B) and tended to have higher levels of MMP-3 ($p = 0.063$) and MIP-1α ($p = 0.12$) compared to children without TPOA. Clustering of SAA, MCP-1, MMP-3 and MIP-1α by Z-transformation led to a higher composite score in children with levels of TPOA above the cut-off for positivity compared to children with low or no detectable TPOA levels ($p < 0.01$, Figure 5C).

3.5 | Differences in Immunological Pattern Between Children With or Without Thyroid Peroxidase Autoantibodies

Analysis of correlation matrices (presented as correlograms) revealed distinct differences in how circulating inflammatory markers correlate, depending on TPOA status in children with T1D and both T1D and CeD. Children positive for TPOA with T1D exclusively (Figure 6A) and in children with T1D or both T1D and CeD (Figure 6B) showed similar immunological patterns for both positive and negative correlations between the immune markers studied. A different immunological pattern was observed in TPOA-negative children with T1D exclusively (Figure 6C) and children with T1D or both T1D and CeD (Figure 6D). These children demonstrated weaker or reversed correlations for G-CSF, IL-8, MIP-1β, PCT, SAA, tPA, visfatin and MMP-2 with other markers, compared to the TPOA-positive children. Meanwhile, the TPOA-negative reference children had the most distinct correlation matrix, with most markers being either consistently positively correlated or inversely correlated to other markers (Figure 6E). Collectively, stratifying children with T1D, with or without CeD, by TPOA status reveals different patterns of correlations for circulating inflammatory markers.

TABLE 3 | TPO autoantibodies (TPOA) in relation to immune markers in type 1 diabetes (T1D) and/or celiac disease (CeD) versus reference (REF) children.

TPO autoantibodies versus immune markers	T1D $n=27$		CeD $n=16$		T1D + CeD $n=18$		REF $n=29$	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Cytokines and chemokines								
Th1 associated								
IFN-γ	**0.43**	**0.025**	0.11	0.69	0.13	0.60	**−0.47**	**0.010**
Th2 associated								
IL-5	0.23	0.25	−0.15	0.57	0.07	0.78	−0.15	0.43
IL-9	−0.04	0.83	−0.22	0.42	0.14	0.57	0.29	0.13
IL-13	**−0.38**	**0.049**	−0.27	0.31	0.25	0.31	0.10	0.60
Th17 associated								
IL-17	−0.27	0.17	−0.27	0.33	0.16	0.52	−0.09	0.66
IL-22	−0.13	0.52	−0.25	0.36	−0.11	0.65	0.16	0.40
IL-25	−0.04	0.84	−0.19	0.48	0.04	0.88	0.04	0.85
IL-33	−0.15	0.45	**−0.60**	**0.015**	0.37	0.13	0.27	0.16
Treg associated								
IL-10	0.28	0.016	−0.18	0.50	−0.13	0.62	−0.04	0.83
Pro-inflammatory								
IL-1β	**0.56**	**0.003**	0.21	0.44	−0.02	0.94	**−0.40**	**0.30**
IL-6	0.17	0.41	−0.40	0.13	−0.31	0.22	−0.07	0.71
IL-8	0.16	0.43	0.24	0.38	0.05	0.85	−0.13	0.49
IL-15	−0.05	0.80	−0.18	0.51	**0.48**	**0.043**	0.11	0.56
G-CSF	0.16	0.43	−0.34	0.20	−0.05	0.84	−0.21	0.26
MCP-1	−0.24	0.17	−0.04	0.88	**0.49**	**0.040**	0.15	0.42
MIP-1α	−0.27	0.17	−0.24	0.36	0.15	0.56	−0.21	0.27
MIP-1β	*−0.45*	*0.019*	0.13	0.63	0.17	0.50	−0.26	0.17
TNF-α	0.10	0.63	0.17	0.52	−0.30	0.23	−0.33	0.077
Acute phase proteins								
Ferritin	−0.13	0.53	−0.02	0.93	**0.47**	**0.047**	−0.24	0.22
Fibrinogen	−0.16	0.41	−0.09	0.75	**0.56**	**0.015**	0.05	0.81
PCT	−0.35	0.075	**−0.51**	**0.047**	0.20	0.43	0.14	0.46
SAA	−0.34	0.09	−0.17	0.54	**0.70**	**0.0012**	−0.22	0.25
tPA	−0.14	0.49	−0.15	0.58	0.34	0.17	0.15	0.44
Adipocytokines								
Resistin	−0.01	0.95	−0.04	0.88	**0.54**	**0.020**	−0.15	0.43
Visfatin	0.18	0.38	−0.30	0.25	**0.50**	**0.036**	0.01	0.95
Matrix metalloproteinases								
MMP-1	−0.30	0.13	−0.04	0.89	0.12	0.64	0.006	0.98
MMP-2	0.21	0.29	−0.21	0.44	−0.03	0.89	−0.10	0.61
MMP-3	0.10	0.62	−0.36	0.17	0.48	0.052	0.29	0.12

Note: Spearman's ranked correlation test between TPO autoantibody levels (TPOA) and different immune markers T helper (Th) associated, pro-inflammatory, acute phase proteins, adipocytokines and matrix metalloproteinases in type 1 diabetic (T1D) and/or celiac disease (CeD) versus reference (REF) children.
Abbreviations: G-CSF, Granulocyte colony-stimulating factor; IFN, interferon, IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinases; PCT, procalcitonin; SAA, serum amyloid A; TNF, tumour necrosis factor; tPA, tissue plasminogen activator.
Note: Bold style represent significant values.

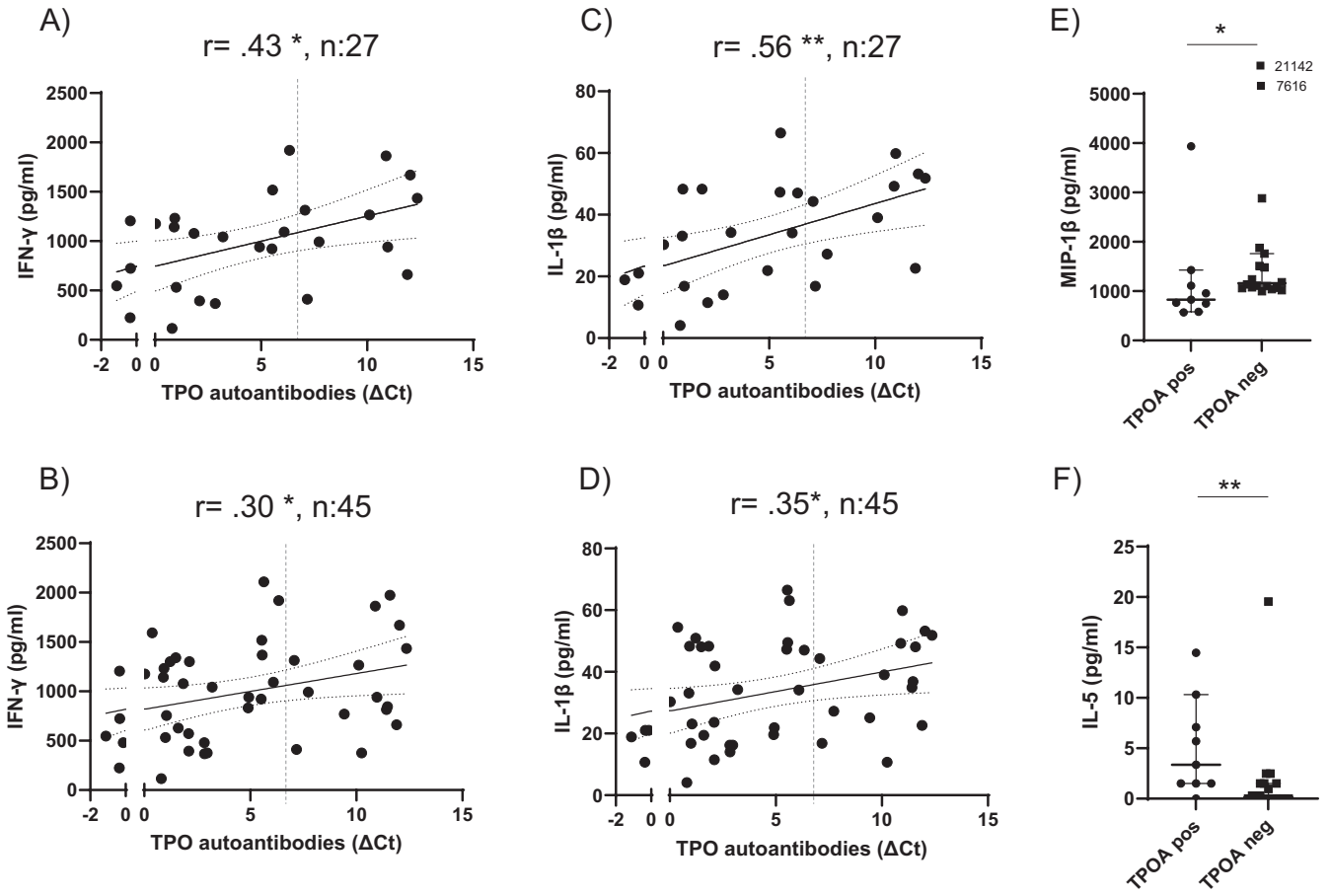

FIGURE 3 | TPOA is related to the Th1/pro-inflammatory immune response in type 1 diabetes (T1D) and in combination with celiac disease (CeD). Thyroid peroxidase antibodies (TPOA) are related to a T helper (Th)1/pro-inflammatory immune response, as seen by correlation to interferon (IFN)-γ in children with T1D ($n = 27$) (A) and in children with T1D and celiac disease (T1D and T1D + CeD, $n = 45$) (B). Thyroid peroxidase antibodies are also related to a T helper (Th)1/pro-inflammatory immune interleukin (IL)-1β in children with type 1 diabetes (T1D, $n = 27$) (C) and in children with T1D and celiac disease (T1D and T1D + CeD, $n = 45$) (D). A lower level of pro-inflammatory Macrophage Inflammatory Protein (MIP-1β) (E) but a higher level of Th2-associated IL-5 (F) was observed in T1D children above the cut-off for positivity for TPOA (9 out of 27). Cut-off for positivity is 7.0 ΔCt. $^*p < 0.05$, $^{**}p < 0.01$.

3.6 | Total Antibody Burden Among Children Positive for TPOA

All children positive for TPOA also presented increased levels of other autoantibodies related to T1D or CeD (Table 4). All TPOA positive children were also positive for ZnT8, and four of these children were positive for all four T1D related autoantibodies (GAD, IA-2A, IAA, ZnT8). The other 10 TPOA-positive children presented inconsistent outcomes between the different autoantibodies. Even though the antibodies measured in this study were analysed at the time of study inclusion, this was after the actual diagnosis of the children. All TPOA-positive children with CeD were still positive for TGA.

4 | Discussion

We examined the presence of TPOA and their correlation with immune markers in a cohort of children with exclusively T1D, CeD, a combination of these two diseases, and a group of children without these diagnoses. We found an overrepresentation of TPOA-positive children in the two groups with T1D, especially in girls. The pattern of correlation between TPOA and circulating immune markers was distinctly different among all groups studied, and TPOA correlated mainly with T helper-associated and pro-inflammatory markers in children with T1D.

Our findings indicated that the diagnosis of T1D might be a more significant risk factor for AITD than other autoimmune diseases like CeD. In this study, all TPOA positive children were asymptomatic and had normal levels of TSH and T4. This means that while these children currently present an autoimmune predisposition to develop thyroid disease, they do not meet the diagnostic criteria currently pointed out. A previous study reports that approximately 20% of T1D children are TPOA-positive [5], not far from the one-third we observed in this study. In a previous study, including only five children with combined diagnoses of T1D and CeD, a higher ratio of TPOA-positive children was observed [5]. We, however, did not find any significant differences in TPOA levels between children with T1D exclusively and children with T1D in combination with CeD. It is possible that differences in sample size or sex explain the variations observed. We noticed that TPOA-positivity was mainly influenced by female sex, in line with previous observations in adults [7].

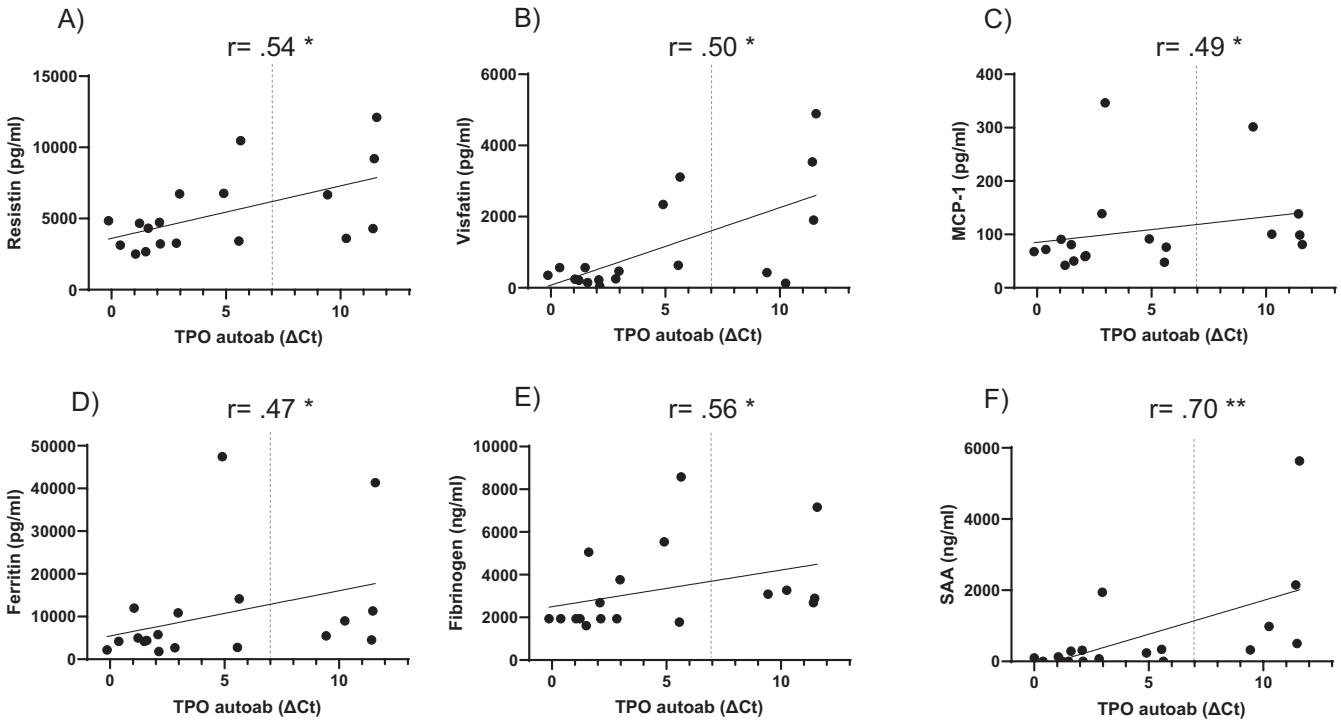

FIGURE 4 | TPOA in children with combined type 1 diabetes and celiac disease (T1D + CeD) is related to adipocytokine pro-inflammatory chemokine acute phase proteins. Thyroid peroxidase antibodies (TPOA) in children with combined type 1 diabetes and celiac disease (T1D + CeD) ($n = 18$) correlated with the adipocytokine resistin (A) and visfatin (B), the pro-inflammatory chemokine monocyte chemoattractant protein (MCP)-1 (C) and the acute phase proteins ferritin (D), fibrinogen (E) and serum amyloid A (SAA) (F). Cut-off for positivity is 7.0 ΔCt. *$p < 0.05$, **$p < 0.01$.

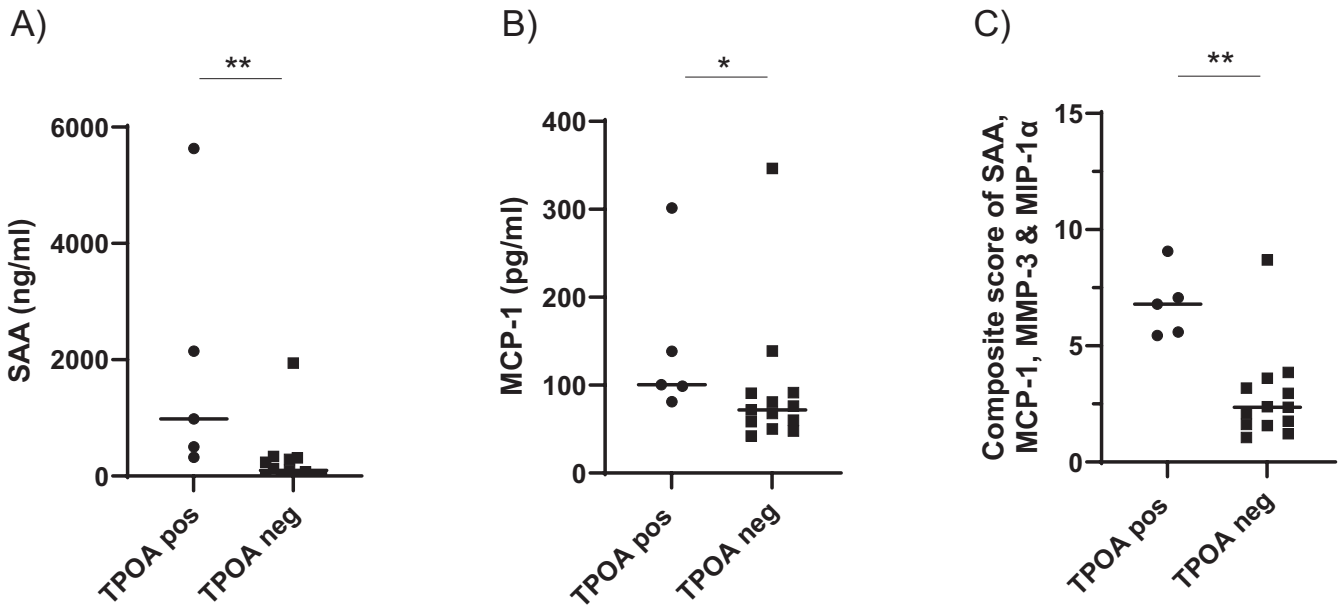

FIGURE 5 | Children with combined type 1 diabetes and celiac disease (T1D + CeD) with thyroid peroxidase antibodies (TPOA) above the cut-off for positivity had higher levels of serum amyloid A (SAA) (A) and monocyte chemoattractant protein (MCP)-1 (B). Clustering of SAA, MCP-1, matrix metalloproteinase (MMP)-3 and macrophage inflammatory protein (MIP)-1α led to a higher composite score in children with levels of TPOA above the cut-off for positivity compared to children with low levels or non-detectable TPOA (C). Cut-off for positivity is 7.0 ΔCt. *$p < 0.05$, **$p < 0.01$.

We found that girls with combined diagnoses of T1D and CeD had significantly higher levels of TPOA compared with boys. In children with T1D exclusively, there were no statistical differences in TPOA levels, but twice as many girls were TPOA-positive compared to boys in the same group.

T-helper-1 and pro-inflammatory immune markers, especially IFN-γ and IL-1β, correlated with high levels of TPOA in T1D children with or without CeD. It is well known that IL-1β and IFN-γ are recognised markers of the autoimmune process directed against the insulin-producing β-cells observed in T1D

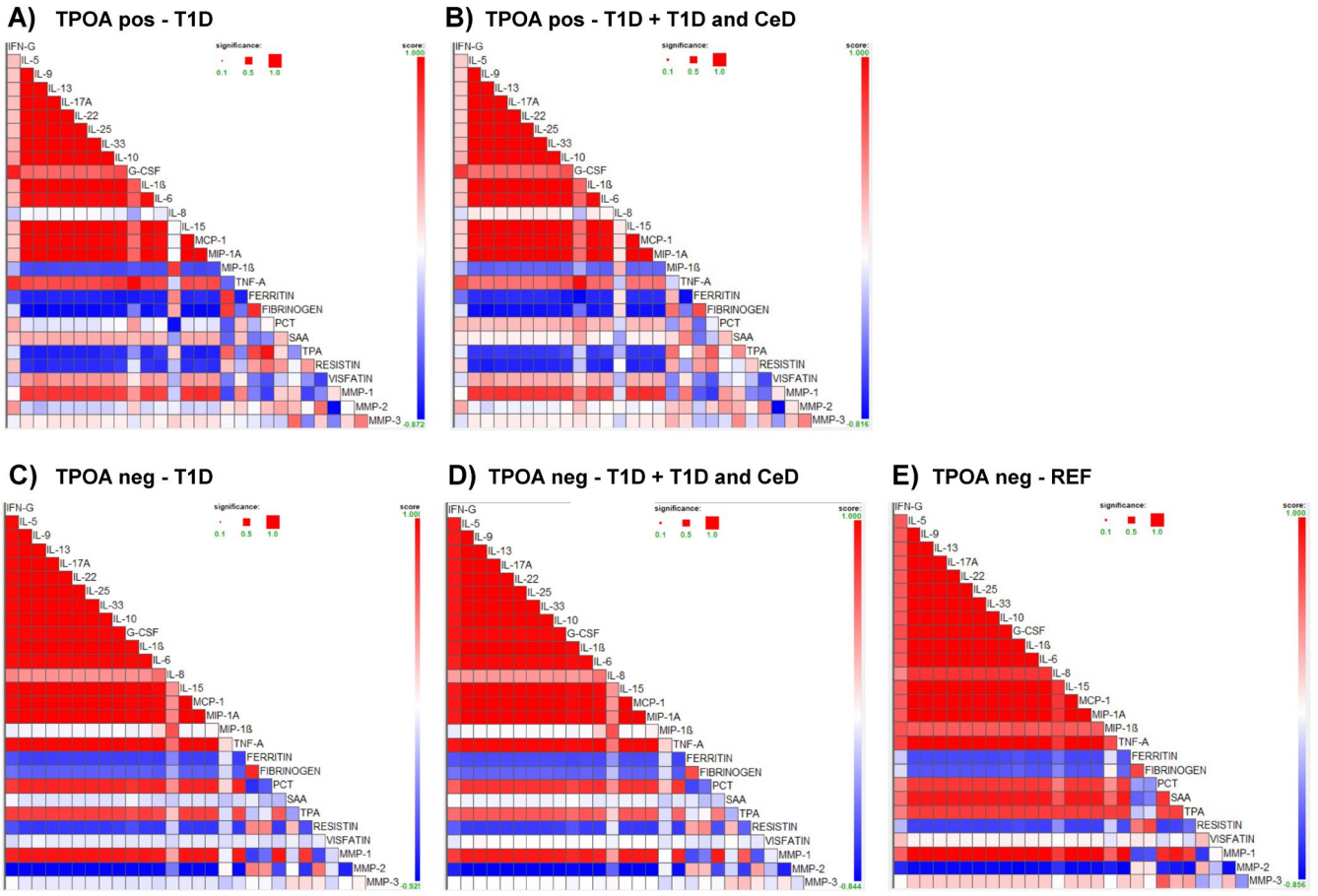

FIGURE 6 | Visualisation of a Pearson-correlation matrix by correlograms for inflammatory marker concentrations in the different subgroups: Type 1 diabetes (T1D) alone, combined with celiac disease (T1D and T1D + CeD) or reference (REF) and stratified by thyroid peroxidase antibodies (TPOA) status. Colours indicated the level of correlation (red—positive correlation, white—no correlation, blue—negative correlation). G-CSF, Granulocyte colony-stimulating factor; IFN, Interferon; IL, Interleukin; MCP, Monocyte chemoattractant protein; MIP, Macrophage Inflammatory Protein; MMP, matrix metalloproteinases; PCT, Procalcitonin; SAA, Serum amyloid A; TNF, Tumour necrosis factor; tPA, Tissue Plasminogen Activator.

[10, 43]. Also in AITD, autoreactive lymphocytes, such as Th1, Th2 and Th17 cells, infiltrate the targeted organ, that is, the thyroid gland [44]. Our observation indicates that higher levels of IFN-γ and IL-1β in children with T1D may increase the risk of concomitant AITD.

The Th2-associated cytokine IL-13 was instead inversely correlated to TPOA in children with exclusively T1D. Interleukin-5, another common Th2-associated cytokine, was found in T1D children with TPOA above the cut-off for positivity. Since Th2-associated cytokines are closely related to antibody production, this may explain the relation between Th2-associated cytokines and TPOA found in a few children with T1D.

Children with a combined diagnosis of T1D and CeD showed several significant positive correlations between TPOA and pro-inflammatory cytokines, adipocytokines, and acute phase proteins. Studies on the acute phase proteins ferritin, fibrinogen, and SAA in children with both T1D and CeD are few. These indicate that while levels of ferritin and fibrinogen might be decreased in these conditions, levels of SAA may be increased. In a study of children with T1D and CeD, ferritin was decreased in children with a persistent high level of transglutaminase autoantibodies, possibly as a sign of insufficiently treated CeD [45]. Children with combined T1D and CeD also present lower levels of fibrinogen than children with only T1D [16]. Conversely, a proteomic study on both children and adults with T1D demonstrated increased levels of SAA compared with healthy controls [46]. Pro-inflammatory IL-15 is associated with NK cell dysfunction in T1D, and high levels have been reported in children with concomitant T1D and CeD [47, 48]. Circulating MCP-1 is also altered in T1D and CeD, but reports are inconsistent concerning low or high concentrations in these conditions [16, 48, 49]. Our observed correlations between TPOA, pro-inflammatory cytokines, and acute phase proteins suggest that TPOA levels may be increased alongside an increased general inflammatory process. This is further emphasised by the correlations between TPOA and the adipocytokines resistin and visfatin. Previous studies have not found consistent alterations in circulating resistin in T1D or concomitant CeD, and visfatin is increased in T1D but decreased in T1D and CeD [16, 50]. Since TPOA were distinctly divergent between boys and girls with no overlap, as all girls presented higher TPOA levels compared with all boys, it cannot be ruled out that sex differences may influence the pattern observed between immune markers and TPOA.

TABLE 4 | Information on total autoantibody burden among children positive for TPO autoantibodies.

Child	Diagnosis	GADA	IA-2A	IAA	ZnT8	TGA
1	T1D	Pos		Pos	Pos	
2	T1D	Pos	Pos	Pos	Pos	Pos
3	T1D	Pos	Pos	Pos	Pos	
4	T1D			Pos	Pos	
5	T1D	Pos			Pos	
6	T1D	Pos	Pos	Pos	Pos	Pos
7	T1D		Pos	Pos	Pos	
8	T1D	Pos		Pos	Pos	Pos
9	T1D	Pos		Pos	Pos	Pos
10	CeD + T1D	Pos		Pos	Pos	
11	CeD + T1D	Pos	Pos	Pos	Pos	Pos
12	CeD + T1D			Pos	Pos	
13	CeD + T1D				Pos	
14	CeD + T1D	Pos		Pos	Pos	

Abbreviations: GADA, glutamic acid decarboxylase 65; IA-2A, islet antigen-2; IAA, insulin; TGA, tissue transglutaminase; ZnT8, ZnT8 transporter.

In contrast, children with CeD exclusively had a very low level of TPOA, and none of the children showed TPOA above the cut-off for positivity. The correlations observed between detectable TPOA and low IL-33 and procalcitonin were not clear. Interleukin-33 is several times higher in the blood of children and adolescents with active CeD compared to children without CeD [51]. It is possible that an ongoing local inflammation in the gut due to the disease process in CeD temporarily shifts the systemic immune activity, making a concomitant reaction in thyroid tissue less likely. Procalcitonin is secreted by C cells in the thyroid and may, to a degree, be reflective of total thyroid tissue mass [52]. Another possible explanation is the temporal differences in inflammatory activity between T1D and CeD. In T1D, the autoimmune process occurs over a longer timeframe, and children temporarily maintain some capacity to produce insulin. In contrast, children with CeD who are placed on a gluten-free diet soon lower their transglutaminase autoantibodies and reduce their inflammatory activity.

Correlation matrices for inflammatory markers stratified by TPOA status suggested that TPOA positivity was associated with a distinct immune pattern that was quite similar between children with T1D or a combined T1D and CeD diagnosis. This suggests an immunological shift in the development of AITD beyond that seen in T1D and CeD. The presence of autoantibodies other than TPOA was inconsistent among the children, and there was no clear pattern between the different autoantibodies. A few children were positive for all autoantibodies, but as the number was very small, a more detailed analysis of these children was not possible.

There are some limitations to consider in this study. The cross-sectional design prevents any causal conclusions regarding changes in the observed associations for different markers over time. Immune markers are known to vary between different phases during the pre- to post-diagnosis phases of both T1D and CeD. This could lead to various results in cross-sectional studies of children in different phases of the disease. However, we identified several interesting correlations between TPOA and inflammatory markers in these different study groups. These markers need to be evaluated in longitudinal studies to assess which implications they have in the development of AITD. All children were asked if they had any thyroid disease when entering the study and had normal levels of thyroid hormones (TSH and T4). The TPOA positivity of several children was, therefore, surprisingly high. It is known that the ADAP technique for measuring autoantibodies is more sensitive than traditional methods used in clinical settings, which may contribute to the high prevalence [38]. However, similar frequencies of TPOA positivity have been reported in previous studies on children with T1D that have used a traditional measuring technique, suggesting that our sample is representative [5, 53]. Future studies should determine the corresponding cut-offs for TPOA positivity measured with ADAP and clinically used methods. It has also been shown that levels of TPOA are higher in children with hypothyroid Hashimoto's thyroiditis and lower in euthyroid or treated children with Hashimoto's thyroiditis [1], suggesting that TPOA levels alone are an indicator of disease severity.

In conclusion, we found that TPOA levels were mainly influenced by T1D diagnosis and female sex, but not to the same extent in CeD, in a study of Swedish children with any combination of T1D and CeD or no diagnosis. We also identified several inflammatory markers which correlated with the level of TPOA, indicating a relation to autoimmune parameters in children with T1D, CeD, or both, but preceding symptoms of AITD. Future studies should evaluate the clinical potential of these immune markers in designated studies of T1D and CeD.

Author Contributions

Emanuel Fryk, Andrea Tompa, and Maria Faresjö: conceptualization. **Andrea Tompa and Maria Faresjö:** data curation and formal analysis. **Andrea Tompa, Alexander Lind, Rasmus Bennet, and Maria Faresjö:** methodology. **Emanuel Fryk, Andrea Tompa, and Maria Faresjö:** writing – original draft. **Emanuel Fryk, Andrea Tompa, Alexander Lind, Rasmus Bennet, and Maria Faresjö:** writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.